THE ROLE OF L-TYPE VOLTAGE GATED CALCIUM CHANNELS AND PSYCHIATRIC RISK GENE CACNA1C IN ASSOCIATIVE LEARNING

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Do you ever look at someone and wonder, "What is going on inside their head?" *Joy – Inside out, Disney Pixar*

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ABBREVIATIONS

Anatomical

DG	Dentate Gyrus
CA1	Cornu Ammons 1
CA3	Cornu Ammons 3
mPFC	medial prefrontal cortex
PFC	prefrontal cortex

Behavioural and methods

CFC	Contextual Fear Conditioning
CFM	Conditioned Fear Memory
CS	Conditioned Stimulus
ISH	*in situ* hybridisation
LI	Latent Inhibition
LTM	Long-Term Memory
PCR	Polymerase Chain Reaction
RL	Reversal Learning
STM	Short-Term Memory
US	Unconditioned Stimulus

Chemical

ddH_2O	double distilled water
DTT	DL – Dithiothreitol
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
SSC	Saline sodium Citrate

Molecular

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

AMPAR	AMPA receptor
BDNF	Brain derived neurotrophic factor
IEG	Immediate early gene
LVGCCs	L-type voltage gated calcium channels
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor

Statistical

ANOVA	Analysis of variance
F	Variance ratio of ANOVA
P	Probability level of the sample statistic
SEM	Standard error of the mean

Measurements

bp	base-pairs
cm	centimetre
g	gram
hr	hour
kg	kilogram
min	minutes
rpm	rotations per minute
RT	room temperature
sec	seconds
nmol	nanomole
µg	microgram
µl	microlitre
µm	micrometre
pS	pico Siemens

SUMMARY

CACNA1C codes for the alpha-1 subunit of $Ca_v1.2$ L-type voltage gated calcium channels (LVGCCs). Variation in *CACNA1C* has been reliably implicated in psychiatric illness, including schizophrenia and bipolar disorder. Analyses have indicated a convergence of genetic risk for schizophrenia on abnormalities in the synapse and in behaviours including associative learning. LVGCCs play a role in synaptic plasticity and learning, partly through regulation of gene transcription. Their role in specific aspects of associative learning that are relevant for symptoms of psychiatric illness is yet to be fully elucidated.

A hippocampal-dependent fear conditioning paradigm was used to determine the role of *Cacna1c* and LVGCCs in specific aspects of associative learning in rats. Studies measured the activity-regulated expression of *Cacna1c* and the effect of inhibition of LVGCCs. A genetic *Cacna1c* knockdown rat model was used to investigate the effects of reduced expression on behaviour and the expression of brain derived neurotrophic factor (*BDNF)*. This model was additionally tested on reward-based reversal-learning. Analyses were translated to humans, to assess whether disease relevant variation in *CACNA1C* was associated with similar deficits in reversal learning and expression.

Inhibition of LVGCCs affected the consolidation, extinction and latent inhibition of contextual fear memory, whereas reduced expression of *Cacan1c* had a selective effect on latent inhibition. There were no effects on the acquisition of reward associations, but reversal learning was impaired. Similar deficits in reversal learning were associated with disease relevant variation in *CACNA1C* in humans. Reduced *CACNA1C* expression was found to be associated with changes in the expression of *BDNF* in both rats and humans.

Results indicate a role for *Cacna1c* and LVGCCs in the appropriate formation and update of aversive and reward associations. Impairments in these processes can underlie specific symptoms of disease including emotion dysregulation and delusions. The cross-species effects on *BDNF* require further investigation.

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1 INTRODUCTION

1.1 The association of *CACNA1C* and L-type voltage gated calcium channels (LVGCCs) with psychiatric disorders

The dysfunction of calcium channels, and calcium signalling more broadly, is robustly identified as playing a key role in the pathophysiology of multiple psychiatric illnesses including schizophrenia and bipolar disorder (Casamassima et al., 2010; Imbrici et al., 2013; Berger and Bartsch, 2014; Heyes et al., 2015).

Schizophrenia affects approximately 1 % of the general population (Sullivan et al., 2003). It is a severe, debilitating disorder characterised by a broad spectrum of positive, negative and cognitive symptoms. Positive symptoms include delusions, hallucinations and thought disorder and are considered to be the key symptoms of psychosis (Andreasen et al., 1990). Current treatments for schizophrenia primarily focus on the alleviation of these positive symptoms through the use of anti-psychotics, which primarily target the regulation of the dopamine system (Nordström et al., 1993; Gurevich et al., 1997). Negative symptoms tend to include dysregulation of emotion, flat affect and apathy and cognitive symptoms are evident by impairments in memory, executive function and attention (Andreasen, 1982; Andreasen et al., 1995). Onset of symptoms usually occurs in adolescence / early adulthood, though cognitive deficits can be observed in pre-psychotic high risk individuals (Lencz et al., 2006; Simon et al., 2007).

Twin studies and adoption studies have found that schizophrenia is highly heritable with estimates of heritability ranging from 65 - 80 % (Cardno and Gottesman, 2000; Sullivan et al., 2003). Similarly, high levels of heritability (approximately 85 %) have been reported for bipolar disorder (McGuffin et al 2003). Although symptom presentation differs, there is a significant overlap of genetic risk between the two disorders (International Schizophrenia Consortium et al., 2009; Lichtenstein et al.,

2009; Moskvina et al., 2009). Bipolar disorder is a mood disorder characterised by episodes of mania and depression with additional cognitive dysfunction. Mania is often accompanied by impulsivity, high risk behaviours and psychosis, (Bourne et al., 2013; Østergaard et al., 2013).

The strong genetic component of these and other related psychiatric disorders, has led to studies which aim to identify specific genetic variation which may underlie their etiology. Determining the function of the genes identified can contribute to the understanding of the neurobiological mechanisms which may be underlying illness. These disorders are highly complex, with interactions between multiple genetic and environmental factors (Sullivan et al., 2003). They are not associated with a single gene mutation, instead being highly polygenic, with multiple genetic variants making potentially small contributions to overall vulnerability to disease (International Schizophrenia Consortium et al., 2009). The advances in large scale genetic analyses over the last ten years, have led to the identification of over 100 genetic risk loci for schizophrenia (Ripke et al., 2014). Multiple methods have robustly identified risk associated variation in specific channel subunits of voltage gated calcium channels, as well as indicating a convergence of risk on calcium signalling more broadly.

1.1.1 Genome-wide association studies (GWAS)

GWAS rely on large sample sizes to detect variation within the whole genome that is associated with specific disorders or phenotypes. They are primarily utilised for identifying common variation with potentially small effect sizes (odds ratios of < 1.2). This is particularly useful when investigating complex polygenic disorders, such as bipolar or schizophrenia (Hirschhorn and Daly, 2005). One of the first and most robust findings of an association between LVGCCs and psychiatric illness, was between common variation in the *CACNA1C* gene and bipolar disorder. The single nucleotide polymorphism (SNP) rs1006737 was found to be significantly associated

with risk for bipolar disorder in a combined analysis of two GWAS (Wellcome Trust Case Control Consortium and STEP-UCL data), with the A allele conferring increased risk compared to the G allele (Sklar et al., 2008). This association was confirmed in a later study which included a new data set, increasing the sample size to 4,387 cases and 6,209 controls (Ferreira et al., 2008). The identified variant lies within a non-coding region, in intron 3 of the *CACNA1C* gene and is thought to be associated with altered gene expression (Gershon et al., 2014; Roussos et al., 2014; Yoshimizu et al., 2014), though results are inconclusive.

The investigation of association between rs1006737 and schizophrenia was subsequently conducted; given the mounting evidence supporting the overlap between genetic underpinnings of disorders involving psychotic phenotypes (Craddock et al., 2009). It was found that the same common variant was associated with increased risk for schizophrenia with the same effect size (Nyegaard et al., 2010).

Since the original association was identified between rs1006737 and both bipolar and schizophrenia, multiple studies have confirmed this in different population samples (e.g. Green et al., 2009; Lee et al., 2013; Ripke et al., 2013; He et al., 2014; Porcelli et al., 2015). The latest Psychiatric Genetics Consortium GWAS identifying 108 significant loci, found SNPs in multiple calcium channel subunits associated with increased risk of schizophrenia (Ripke et al., 2014). Including the replication of the association with *CACNA1C*, they found an association with variants in *CACNB2* and *CACNA1I.* The variation in *CACNA1C* was found to be strongest at rs2007044, which is also located in intron 3 of the gene.

Further studies have also identified other risk SNPs that are not in linkage disequilibrium (LD) with rs1006737, within the same intronic region of *CACNA1C.* Linkage disequilibrium refers to the non-random association between alleles at different loci (Reich et al., 2001). The identification of risk SNPs not in LD, indicates

that there may be independent signals within intron 3 of *CACNA1C* that confer risk for bipolar disorder and schizophrenia. For example, the risk SNP rs4765905 was found to be associated with schizophrenia in a new cohort including patients attending a clozapine clinic (CLOZUK)(Hamshere et al., 2013). The identified risk variation in intron 3 has been postulated to affect interactions with the *CACNA1C* promoter region and therefore alter its expression (Roussos et al., 2014).

Cross disorder association with rs1006737 and other risk SNPs suggests that common variation in *CACNA1C* is associated with symptom clusters that are shared between disorders, rather than a specific diagnosis. Variants in *CACNA1C* and *CACNB2* have been found to have shared effects across 5 disorders; attention deficit hyperactivity disorder (ADHD), autism spectrum disorder, bipolar disorder, major depressive disorder and schizophrenia (Smoller et al., 2013); though largest effects were observed for bipolar and schizophrenia. This highlights the value of understanding the functional relevance of common variation to specific symptoms, rather than within rigid diagnostic boundaries.

1.1.2 Rare deleterious mutations

In addition to common variation in individual calcium channel subunits, an enrichment in rare (less than 1 in 10,000 people) deleterious mutations has been found within voltage gated calcium channels associated with schizophrenia. Purcell et al., (2014) conducted a large scale exome sequencing study to investigate the polygenic burden of rare mutations in schizophrenia. They found that patients had a higher rate of rare disruptive mutations compared to controls, with a significant enrichment in a calcium ion channel gene-set. This enrichment was driven primarily by the pore-forming alpha-1 subunits and the alpha-2-delta auxiliary subunits of VGCCs. The alpha-1 subunits of LVGCCs were found to carry the most mutations in patients, with two deleterious mutations identified in the *CACNA1C* gene. These are found to be protein-truncating and hypothesised to lead to loss of protein function. Due to the

rarity of these variants, carriers are likely to have one functional copy of *CACNA1C,* indicating reduced rather than a loss of *CACNA1C* expression.

Rare mutations have also been identified in other calcium channel subunits in relation to autism spectrum disorder (For review see Schmunk and Gargus, 2013). *CACNA2D3* has been implicated through the identification of deleterious *de novo* mutations in a large exome sequencing study (Rubeis et al., 2014), as well as studies of copy number variants (Girirajan et al., 2013). Copy number variants refer to sections of the genome which may be duplicated or deleted and the number of copies varies between individuals. *CACNA2D3* codes for an alpha-2-delta auxiliary subunit of VGCCs and regulates their abundance at the synapse (Hoppa et al., 2012). In the same exome sequencing study conducted by Rubeis et al., (2014) five mutations were identified in *CACNA1D,* coding for the $Ca_v1.3$ channel subtype of LVGCCs. A summary of calcium channel associated risk for psychiatric illness is presented in Table 1.1.

1.1.3 Timothy syndrome

Single gene psychiatric disorders are fairly rare; however, mutations in the *CACNA1C* gene have been found to cause Timothy Syndrome. This is an autosomal dominant developmental disorder characterised by heart abnormalities, specifically long QT syndrome, which can cause arrhythmias and sudden death. Individuals with the disorder also suffer from webbing of the fingers and toes and approximately half of those diagnosed have severe facial dysmorphologies. It has been found that people who suffer with this disorder also present with symptoms of autism and autistic-like spectrum traits (Splawski et al., 2004). Children appear to have reduced socialisation skills as well as a developmental delay in language and speech, with evidence of learning disability and seizures.

There are different types of Timothy Syndrome, all known to be caused by gain of function mutations in *CACNA1C* (Splawski et al., 2004; Barrett and Tsien, 2008). Type 1, also known as the classic type, is caused by mutations in exon 8 of the gene, leading to prolonged depolarisation of the associated $Ca_v1.2$ channel and excessive calcium to enter the cell. Type 2, known as the atypical type, causes a more severe form of long QT syndrome and is caused by mutations in exon 8A. A novel mutation was later identified in exon 38, causing type 3 (Gillis et al., 2011). A rare case study in a patient with Timothy Syndrome that survived to adulthood, reported the development of bipolar disorder (Gershon et al., 2014). In the same study, it was found that the previously identified risk allele at rs1006737 was associated with decreased expression of *CACNA1C.* It was concluded that both increases and decreases in calcium influx in excitable cells may be related to bipolar disorder.

Table 1.1 Summary of evidence indicating variation in LVGCCs associated with psychiatric illness

Disorder	Gene	Mutation	Function	Reference
Schizophrenia; bipolar disorder; autism	*CACNA1C*	Common variants in intron 3 (including rs1006737)	**Loss** through effects on interaction with promoter regions	Roussos et al., (2012)
			Loss indicated by reduced expression in post-mortem brain	Gershon et al., (2014)
			Gain indicated by increased expression in induced human neurons	Yoshimizu et al., (2015)
Schizophrenia	*CACNA1C* *CACNA1S* *CACNB4* *CACNA2D1,2,4*	Rare non-synonymous or disruptive mutations	**Loss** through truncation and nonsense mediated decay	Purcell et al., (2014)
Autism	*CACNA1D*	Rare *de novo* mutations	**Gain** through loss of inactivation	Pinggera et al., (2015)
	CACNA2D3		**Loss** through truncation and nonsense mediated decay	De Rubeis et al., (2014)
	CACNB2		Inconclusive	Breitenkamp et al., (2014)
Timothy Syndrome (including autistic phenotypes)	*CACNA1C*	Rare missense mutations in exon 8, 8a and 38	**Gain** through loss of inactivation	Splawski et al., (2004)

1.1.4 Convergence on calcium signalling pathways

Further analysis across GWAS, in conjunction with gene expression data, supports the involvement of calcium signalling in schizophrenia and other psychiatric disorders. Hertzberg et al., (2015) integrated data from a large-scale genome wide expression dataset on post-mortem brain samples with GWAS data, to identify biological pathways and processes that may be implicated in schizophrenia. They reported significant enrichment in pathways which included ion channels and specifically calcium channel activity.

Other genes and biological processes have been identified in genetic analyses, which may not directly include LVGCCs, but do implicate calcium signalling dysregulation in psychiatric illness. There has been growing evidence of convergence of identified risk on the function of the synapse and its involvement in plasticity (For review see Hall et al., 2015). An exome sequencing study found that small *de novo* mutations in patients with schizophrenia were enriched in glutamatergic post synaptic proteins and those that are involved in the strengthening of synapses and plasticity (Fromer et al., 2014). Synaptic plasticity is known to require calcium influx through N-methyl-D-aspartate receptors (NMDAR) and VGCCs, resulting in the induction of new gene expression (West et al., 2001; Moosmang et al., 2005).

Two of the genes known to be regulated by calcium influx are activity regulated cytoskeletal-associated protein (ARC) and brain derived neurotrophic factor (BDNF) (Zheng et al., 2009, 2011). Pathway enrichment analyses have identified a role for neurotrophin signalling in schizophrenia (Chang et al., 2015), and the ARC complex was found to be associated in the de novo exome sequencing study mentioned above (Fromer et al., 2014). The post-synaptic density gene set identified in this study also included CAPN5, a gene for a calcium activated protease, which evidence has suggested can be activated by VGCCs in dendrites (Kanamori et al., 2013). Studies have also identified an association between both autism and schizophrenia and deletions in NRXN1, which encodes a synaptic adhesion protein (e.g. Kirov et al., 2009; Rujescu et al., 2009). This gene has been found to be important for the organisation of VGCCs in the synapse and their coupling to pre-synaptic machinery that is required for neurotransmitter release (Missler et al., 2003).

Variation in *CACNA1C* and dysfunction of LVGCCs more broadly, clearly has a robust association with risk for schizophrenia and related disorders. The involvement of calcium signalling in convergent areas of risk, highlights the need to investigate its role in specific biological processes implicated in psychiatric disorders and

interactions with wider networks of genes. The convergence on synaptic plasticity more specifically, further indicates the significance of investigating the role for *CACNA1C* and LVGCCs in behaviourally relevant phenotypes of learning and memory.

1.2 L-type voltage gated calcium channels (LVGCCs)

Voltage gated calcium channels mediate calcium influx into the cell in response to membrane depolarisation, which then regulates intracellular processes including: hormone secretion, pacemaker activity, neurotransmitter release and calcium-dependent gene transcription (Striessnig and Koschak, 2008). They function to transform an electrical signal into a molecular signal, which then communicates at the postsynaptic site with the nucleus through activation of specific secondary messengers and *de novo* proteins (Berridge et al., 2003).

LVGCCs are part of a wider family of VGCCs which include T-, P/Q-, N- and R-type channels, separately named to correspond to the type of calcium current they elicit (Randall and Tsien, 1995). These are sub-divided into three classes based on sequence homology; referred to as Ca_v1 (L-type), Ca_v2 (P/Q, N and R type) and Ca_v3 (T-type). The different types have distinct physiological properties and functions. Ca_v3 VGCCs are low voltage activated (LVA) channels and primarily function as pace-making and rebound bursting in neurons (Perez-Reyes, 2003). They have low unitary conductance (8 – 12 pS in 110 mM barium) and are rapidly inactivated over 20 – 50 ms. They do not appear to associate with any of the auxiliary subunits described below, functioning as monomers (Catterall et al., 2005). In contrast, Ca_v2 channels are high voltage activated (HVA) and are located at pre-synaptic nerve terminals. They have a unitary conductance of approximately 13 pS (in 110 mM barium) and show time dependent inactivation (50 – 110 ms) and voltage dependent inactivation. They have been identified to have a role in transmitter release and neuronal excitability and have been found to interact with β and $\alpha_2\delta$ and possibly with

γ subunits (Simms and Zamponi, 2014). A summary of the different subfamilies of VGCCs is presented in Table 1.2; for a full description of nomenclature and properties of Ca_v2 and Ca_v3 channels, see Catterall et al., (2005) and Catterall, (2015).

1.2.1 Structure and subunit composition

LVGCCs can be made up of multiple subunits which define voltage sensitivity, pharmacological sensitivity and trafficking of the channel. Each channel can be composed of up to 4 subunits to include: α_1, β, $\alpha_2\delta$ and γ subunits (Figure 1.1), which regulate trafficking and gating properties.

Alpha-1 (α_1) subunits

The α_1 subunit forms the physical channel pore through which calcium ions enter the cell. It defines the voltage sensitivity and pharmacological properties of the channel (Catterall et al., 2005). It is structurally comprised of four transmembrane domains (I – IV) separated by cytoplasmic linkers, which each consists of six membrane spanning helices (S1 – S6) (Simms and Zamponi, 2014). Each domain contains a voltage sensor and folds to form a quarter of the channel pore. In mammals there are four genes coding for different α_1 subunits of LVGCCs. These are, *CACNA1S, CACNA1C, CACNA1D* and *CACNA1F,* corresponding to the L-type channels, $Ca_v1.1$, $Ca_v1.2$, $Ca_v1.3$ and $Ca_v1.4$ respectively. There is greater than 70% amino acid sequence homology between LVGCC α_1 subunits, though less than 40% homology with other types of VGCCs (Catterall et al., 2005). The greatest sequence variation occurs in the cytoplasmic linkers and N and C termini, which are known to be important for protein-protein interactions and second messenger regulation (Dai et al., 2009). All genes undergo extensive alternative splicing, with over 40 known human transcripts of the *CACNA1C* gene (Tang et al., 2004a). There are three known and at least four predicted *CACNA1C* transcripts in the rat (*Ensembl genome browser; www.ensembl.org).*

Auxiliary subunits

There are three additional auxiliary subunits that may interact with the α_1 pore forming subunit; beta (β), alpha-2-delta ($\alpha_2\delta$) and gamma (γ) (Figure 1.1). β subunits bind to the intracellular loop between domains I and II of α_1 subunits (Pragnell et al., 1994) and function as chaperones by trafficking the α_1 to the cell membrane (Berrou et al., 2002, 2005). They regulate the gating properties of the channels and are important for channel modulation by protein kinases and G-proteins (Buraei and Yang, 2010). They have further been described as a membrane-associated guanylate kinase (MAGUK)-like intracellular scaffold protein, providing binding domains for additional intracellular signalling proteins (Catterall, 2015). For a detailed review of the β subunit of VGCCs see Dolphin, (2003) and Buraei and Yang, (2010).

$\alpha_2\delta$ subunits are coded by a single mRNA and are post-translationally cleaved, remaining linked by a disulphide bond. The δ part anchors the α_2 protein to the membrane via a glycophosphatidylinositol anchor and together they regulate the activation and inactivation kinetics of the channel (Klugbauer et al., 1999). Their main function is to increase calcium currents by promoting the trafficking of α_1 subunits to the cell membrane and improved synaptic targeting (Canti et al., 2003; Hoppa et al., 2012). For a detailed review of $\alpha_2\delta$ see Dolphin, (2012, 2013).

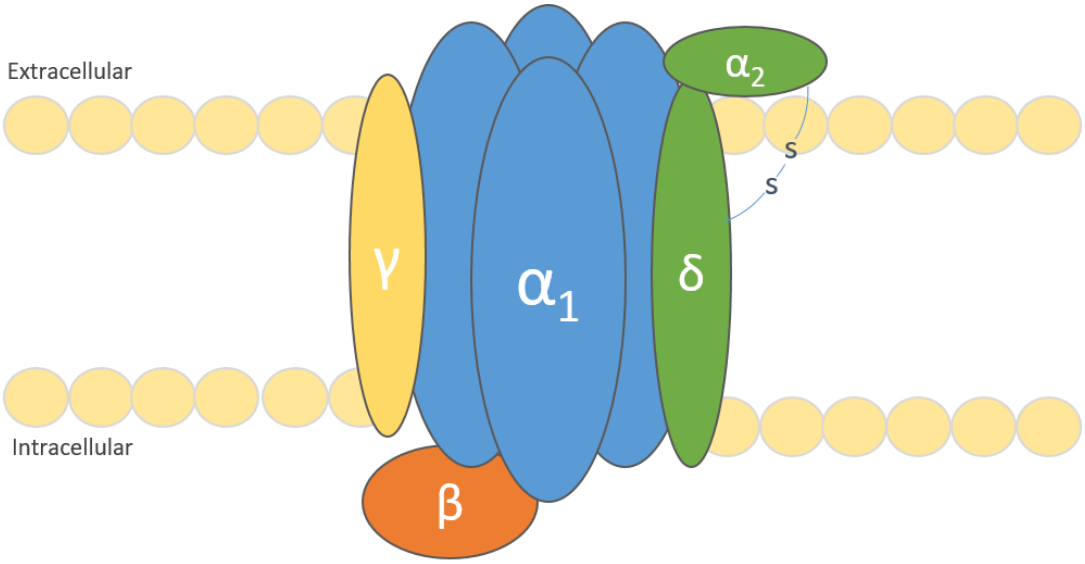

Figure 1.1 Example subunit composition of LVGCCs. Alpha-1 (α_1) subunits forms the channel pore and define voltage sensitivity and pharmacology of the channel. The auxiliary beta (β) and alpha-2-delta ($\alpha_2\delta$) subunits are involved in the trafficking of the α_1 subunits to the cell membrane and modulate the biophysical properties of the channel. Gamma (γ) subunits are also sometimes associated with LVGCCs though have diverse functions.

Less is known about the association of γ subunits with VGCCs than the other auxiliary subunits and appears to have only been confirmed in skeletal muscle calcium channels (Kuniyasu et al., 1992; Andronache et al., 2007). Seven γ subunits have been found to be expressed in the brain (Klugbauer et al., 2000). These neuronal expressed subunits have been found to regulate post synaptic glutamate receptors and are alternatively referred to as transmembrane AMPA receptor modulators (TARPs)(Nicoll et al., 2006). Although there is evidence of co-expression with VGCCs and potential effects on inactivation state (Andronache et al., 2007), functional association in the brain is not conclusive in vivo. For a review of the γ subunits see Black, (2003).

1.2.2 Physiological and pharmacological properties

LVGCCs generally require strong depolarisation for activation, are long-lasting and produce slow inward calcium currents (Ertel et al., 2000). They have been found to exhibit calcium-dependent inactivation, with little voltage-dependent inactivation, and

have relatively large single channel conductance (20 – 27 pS in 110 mM barium); though there is variation within sub-types of Ca_v1 channels (Lipscombe, 2004).

$Ca_v1.2$ channels are found to be activated by high voltages (> -35mV) and have slow inactivation, though subtle differences have been observed for different splice variants (Tang et al., 2004a). In contrast, it has been shown that $Ca_v1.3$ channels may be activated at subthreshold depolarisations (around – 55 mV) and may exhibit faster kinetics than the $Ca_v1.2$ L-type channel, having a role for pacemaking in the sinoatrial node (Zhang et al., 2002). Furthermore, it has been suggested that $Ca_v1.3$ channels may also contribute to R-type currents in neurons. These have been found to be involved in presynaptic transmitter release and synaptic plasticity in hippocampal pyramidal neurons (Yasuda et al., 2003).

The physiology of these channels has primarily been defined by utilising the selective affinity of LVGCCs to organic compounds that block calcium influx. This selectivity has been useful in determining the functional role for L-type currents in specific molecular processes and behaviours (Simms and Zamponi, 2014). The pore forming α_1 subunits of LVGCCs are specifically sensitive to a number of different organic agonists including dihydropyridines, benzothiazepines and phenylalkylamines, which are primarily used in the treatment of cardiac and hypertension conditions (Randall and Tsien, 1995; Triggle, 2006). These compounds have been found to be highly selective for the inhibition of calcium influx through LVGCCs, with a preference for the inactivated channel state (Welling et al., 1993), though phenylalkylamines have been found to affect T-type VGCCs, sodium and potassium channels at higher doses (Freeze et al., 2006).

Along with the physiological differences observed between $Ca_v1.2$ and $Ca_v1.3$ channels, there is evidence of varied affinity of these organic inhibitors to channel sub-type; though there are currently no inhibitors that can reliably distinguish between LVGCCs. $Ca_v1.2$ channels have been found to show greater sensitivity to inhibition

by the dihydropyridines isradipine and nimodipine compared to $Ca_v1.3$ (Koschak et al., 2001; Xu and Lipscombe, 2001). More recent studies have identified distinct properties and protein interactors between $Ca_v1.2$ and $Ca_v1.3$ channels which may be potentially useful in the future development of sub-type selective inhibitors (Zuccotti et al., 2011).

Table 1.2 Voltage gated calcium channel sub-types and distinguishing properties.

Current	Physiology	Channel	Gene	Subunit	Inhibitors	Localisation	Function
L	HVA 20 – 27 pS (in 110 mM Ba^{2+}) Long-lasting Calcium dependent inactivation	$Ca_v1.1$	*CACNA1S*	$\alpha_2\delta$, β, γ	Dihydropyridines Benzothiazepines Phenylalkylamines	Skeletal muscle, transverse tubules	Excitation contraction coupling
		$Ca_v1.2$	*CACNA1C*			Neuronal cell bodies and proximal dendrites; endocrine cells; cardiac/smooth muscle myocytes	Excitation contraction coupling; hormone release; gene transcription; synaptic integration
		$Ca_v1.3$	*CACNA1D*			Neuronal cell bodies and dendrites; cochlear hair cells; endocrine cells; cardiac atrial myocytes and pacemaker cells	Hormone release; gene transcription; synaptic regulation; hearing; neurotransmitter release (sensory cells)
		$Ca_v1.4$	*CACNA1F*			Retinal rod and bipolar cells; spinal cord; adrenal gland	Neurotransmitter release (photoreceptors)
P/Q	HVA 13 pS (in 110 mM Ba^{2+}) Voltage and time (50 – 110 ms) inactivation	$Ca_v2.1$	*CACNA1A*	$\alpha_2\delta$, β, possibly γ	ω – Agatoxin - IVA	Nerve terminals and dendrites; neuroendocrine cells	Neurotransmitter and hormone release; dendritic Ca^{2+} transients
N		$Ca_v2.2$	*CACNA1B*		ω – Conotoxin – GVIA	Nerve terminals and dendrites; neuroendocrine cells	
R		$Ca_v2.3$	*CACNA1E*		SNX - 482	Neuronal cell bodies and dendrites	Repetitive firing; dendritic Ca^{2+} transients
T	LVA 8 – 12 pS (in 110 mM Ba^{2+}) Rapid inactivation (20 – 50 ms)	$Ca_v3.1$	*CACNA1G*	none	Limited selective inhibitors	Neuronal cell bodies and dendrites; cardiac and smooth muscle myocytes	Pacemaking; repetitive firing
		$Ca_v3.2$	*CACNA1H*				
		$Ca_v3.3$	*CACNA1I*			Neuronal cell bodies and dendrites	

1.2.3 Expression profile of LVGCCs

Of the four LVGCCs, $Ca_v1.1$ and $Ca_v1.4$ are mainly restricted to skeletal muscle and retina respectively and are coded by *CACNA1S* and *CACNA1F.* For the purpose of the current work, the focus will subsequently be on the neuronal expression LVGCCs, $Ca_v1.2$, and $Ca_v1.3$, coded by *CACNA1C* and *CACNA1D.*

CACNA1C is located on chromosome 12p13 in humans and 4q42 in rats. *CACNA1D* is located on chromosome 3p21 and 16p16 in humans and rats respectively (*NCBI, www.ncbi.nlm.nih.gov)*. Broadly, they are both expressed in cardiac and smooth muscle, endocrine cells and neurons (Catterall et al., 2005). These genes are often co-expressed in many areas of the CNS, in many instances in the same cell (Striessnig et al., 2006), but they also have distinct regional as well as sub-cellular expression profiles. *CACNA1D* and $Ca_v1.3$ channels are found to be additionally expressed in sensory cells, including photoreceptors and inner hair cells.

$Ca_v1.2$ channels have been found to account for over 80% of all LVGCCs expressed in the brain, with $Ca_v1.3$ accounting for the remaining percentage (Clark et al., 2003; Sinnegger-Brauns et al., 2004; Sinnegger-Brauns and Huber, 2009). Both sub-types are found across regions of the brain, with differential expression observed in the amygdala, cortex, cerebellum, caudate putamen, hippocampus, nucleus accumbens and substantia nigra; though there is conflicting evidence over their relative abundance across species (Hell et al., 1993; Sinnegger-Brauns and Huber, 2009; Marschallinger et al., 2015). In mice, it was observed that $Ca_v1.2$ channels were more abundant than $Ca_v1.3$ in all regions of the brain, however, in rats there appeared to be similar levels of expression in some brain regions (Sinnegger-Brauns and Huber, 2009).

Within the hippocampus, expression of $Ca_v1.2$ is abundant in the dentate gyrus and CA3 regions throughout, with lower levels of expression observed in the pyramidal

cell bodies in the CA1 (Hell et al., 1993). $Ca_v1.3$ expression was found to be restricted to cell bodies and proximal dendrites, but with similar levels of expression observed across all sub-regions of the hippocampus (Hell et al., 1993).

The expression profiles of $Ca_v1.2$ and $Ca_v1.3$ have also been found to be regulated during development in the rat hippocampus. Kramer et al., (2012), found that steady-state expression levels of $Ca_v1.2$ was greatest during the early stage of the neonatal period, peaking at day 6 and decreasing by day 12. In contrast, $Ca_v1.3$ showed a gradual increase, reaching adult expression levels at approximately day 15. Expression was observed throughout the CA1 region of the hippocampus, as has been reported previously in mouse brain (Schlick et al., 2012). A subsequent study found a similar developmental profile of $Ca_v1.2$ in the CA3 region of the hippocampus in rats (Morton et al., 2013). The observed peak in $Ca_v1.2$ has been postulated to be involved in the maturation of the GABAergic circuitry, with a role in the shift from excitatory to inhibitory chloride currents observed in this period in the hippocampus (Ganguly et al., 2001; Kramer et al., 2012).

A more detailed investigation of sub-cellular expression profiles of LVGCCs has been conducted in the hippocampus (Tippens et al., 2008) and specifically in the CA1 region in vivo, using immunogold-labelling techniques and electron-microscopy (Leitch et al., 2009). $Ca_v1.2$ channels were found to be located in the soma and throughout dendritic processes, predominately found in post-synaptic regions, to include the post-synaptic density and extra synaptic sites. Although there was some evidence of pre-synaptic expression, it was extremely low relative to post synaptic expression observed. Expression was found to be significantly higher at the cell membrane compared to within the cytoplasm, especially in the smallest dendritic processes and spines. Localisation at more distal dendrites and within synaptic regions in spines indicates an appropriate positioning for the initiation of synapse to nucleus signalling (Leitch et al., 2009).

The concentrated expression of LVGCCs at cellular regions for initiating signalling cascades that may be important for gene expression and synaptic plasticity, further highlights the importance of fully understanding their role in related processes. Furthermore, the partially distinct expression profiles of $Ca_v1.2$ and $Ca_v1.3$ indicates the likelihood of separable functions, potentially through interactions with signalling molecules and localised regulators (Leitch et al., 2009).

1.2.4 Calcium signalling pathways and gene transcription

Calcium influx through LVGCCs is known to initiate downstream signalling cascades that regulate gene transcription and synaptic plasticity (Murphy et al., 1991; Striessnig et al., 2006; Calin-Jageman and Lee, 2008). Although blockade of LVGCCs appears to have a relatively minor effect on the increase in cytoplasmic calcium following induced synaptic activity, there appears to be a disproportionate effect on the transcription of activity-dependent and immediate early genes (IEGs) (Murphy et al., 1991; Dolmetsch et al., 2001; West et al., 2001). This highlights the importance of these channels in the initiation of plasticity related IEG expression.

Calcium influx through both $Ca_v1.2$ and $Ca_v1.3$ channels creates localised increases in intracellular calcium in close proximity to key signalling molecules. This allows rapid and efficient signalling to the nucleus, compared to other routes of calcium entry into the cell, for example Ca_v2 channels, which rely on a broader pooled increase in calcium to communicate across greater intracellular distances (Wheeler et al., 2006).

The calcium binding protein calmodulin (CaM) has been found to be bound to the C-terminal of $Ca_v1.2$ and $Ca_v1.3$ channels (Peterson et al., 1999), mediating both calcium dependent inactivation of the channel and activation of calcium/calmodulin dependent kinases (CaMK) and the Ras/mitogen-activated protein kinase (MAPK) pathway (West et al., 2001; Barbado et al., 2009). The MAPK pathway conveys the calcium signal from the mouth of the channel pore to the nucleus and initiates the

phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) at Ser^{133}. This allows the recruitment of CREB binding protein (CBP) and the initiation of gene transcription (Fields et al., 1997; Dolmetsch et al., 2001; Wiegert and Bading, 2011). Although phosphorylation of CREB can be initiated by calcium influx through other entry routes into the cell, including NMDA receptors (West et al., 2001), the sustained phosphorylation necessary for long term gene transcription is specifically induced by influx through LVGCCs (Dolmetsch et al., 2001).

The activation of these signalling cascades can differ due to different binding domains in the C-termini and intracellular loops of the α_1 subunits, which influences interactions with specific signalling molecules. For example, CaMKII interacts directly with the C-termini of $Ca_v1.2$ channels, however the interaction between $Ca_v1.3$ and CaMKII requires the protein densin (Hudmon et al., 2005). It is worth noting that CaMKII can also phosphorylate CREB at Ser^{142} which has been found to be inhibitory (Sun et al., 1994). Additionally, there has been found to be a C-terminus fragment of *CACNA1C* (Calcium channel associated transcription, CCAT), which can independently translocate to the nucleus and signal gene transcription (Gomez-Ospina et al., 2006). This suggests a potentially unique signalling ability of $Ca_v1.2$ channels to the nucleus. Conditional deletion of $Ca_v1.2$ channels in the cerebral cortex and hippocampus has also implied a specific role for these channels in the activation of the MAPK pathway in the hippocampus (Striessnig et al., 2014).

The gene transcription initiated by the phosphorylation of CREB includes brain derived neurotrophic factor (*BDNF)* (Tao et al., 1998, 2002). *BDNF* is a small neurotrophic factor which binds to tyrosine kinase TrkB receptors as well as p75 receptors (Binder and Scharfman, 2004). It is known to be important for neuronal survival and synaptic plasticity (Bramham and Messaoudi, 2005; Sakata et al., 2009; Waterhouse and Xu, 2009; Leal et al., 2014), and acts as a neurotransmitter at the

post synaptic membrane (Kafitz et al., 1999). Although the transcription of *BDNF* can be regulated by different routes of calcium influx into the cell, it has been found to be preferentially regulated by influx specifically through LVGCCs (Ghosh et al., 1994; West et al., 2001). The *BDNF* gene consists of several 5' non-coding exons (from I-VIII) and one 3' coding exon (IX). It contains nine distinct transcriptional initiation sites, as well as alternative splice donor sites, resulting in at least 22 predicted isoforms (Aid et al., 2007). Due to the presence of only one coding exon, all isoforms are translated into the same functional protein, albeit undergo distinct regulation by interactors with the different promotors regions (Tabuchi et al., 2000; Zheng et al., 2011). They are additionally found to have different temporal and spatial expression profiles (Pattabiraman et al., 2005; Chiaruttini et al., 2008; Baj et al., 2011). Multiple different variants may therefore be necessary to provide fine-tuned regulation of BDNF and allow it to function within multiple different cellular processes, depending on its route of activation. For example, acute versus transient increases in BDNF has been associated with different activation of TrkB receptors and distinct morphological changes in dendritic spines (Ji et al., 2010).

The route of calcium entry into the cell has been found to be important for transcript specific regulation of *BDNF*, specifically activity dependent isoforms *BDNFI* and *BDNFIV* (Zheng et al., 2012). In primary cultured cortical neurons, it was found that *BDNFI* was specifically activated by LVGCCs rather than NMDA receptors and that *BDNFIV* was differentially activated by both (Tabuchi et al., 2000). Calcium influx through LVGCCs has been found to regulate the transcription of specific promoters through the activation of a calcium-responsive transcription factor (CaRF), known to contain consensus phosphorylation sites for CaMKII and MAP kinase (Tao et al., 2002). Subsequent studies in primary cultures identified a combined regulation of *BDNFIV* transcription through calcium response elements (CaRE), CaRE1 and

CaRE3 by calcium influx through NMDAR and LVGCCs, with a unique activation of CaRF by LVGCCs (Zheng et al., 2011).

BDNF plays a role in many cellular functions including synaptic plasticity and long term memory (Pang et al., 2004; Bekinschtein et al., 2008; Lu et al., 2008). The specific and finely regulated expression of *BDNF* by calcium influx through LVGCCs, would suggest that LVGCCs have a role in a specific subset of these functions.

1.2.5 LVGCCs role in synaptic plasticity and learning

Synaptic plasticity is utilised as the primary model for investigation the molecular basis of learning and memory. Long-term potentiation (LTP) is a form of synaptic plasticity that leads to the increased efficiency of synaptic transmission, evident following pathway stimulation at certain frequencies (Bliss and Collingridge, 1993). Although there has not been conclusive evidence that LTP is the formation of memory, similar synchronised firing patterns is observed in the hippocampus during learning (Otto et al., 1991). As has already been alluded to, LVGCCs have a reliably implicated role in synaptic plasticity and learning and memory (Weisskopf et al., 1999; Moosmang et al., 2005; Striessnig et al., 2006; Seoane et al., 2009; Berger and Bartsch, 2014; Degoulet et al., 2015). Parallel to expression and signalling pathways, there is evidence of overlapping and distinct roles for $Ca_v1.2$ and $Ca_v1.3$ in these processes.

Freir and Herron, (2003), found that intra-peritoneal injection of LVGCC inhibitors verapamil or diltiazem reduced the induction of LTP in vivo in the CA1 region of the rat hippocampus. The results suggested a combined role for LVGCCs and NMDA receptor with low levels of LTP still observed when LVGCCs were inhibited. A separate study in rat brain slices, found that LTP in thalamic input synapses to the amygdala specifically depended on LVGCCs (Weisskopf et al., 1999). Utilisation of channel specific genetic knockdown models has identified a specific role for $Ca_v1.2$

channels in hippocampal L-LTP (Moosmang et al., 2005; Striessnig et al., 2006). Moosmang et al., (2005) found that conditional genetic deletion of $Ca_v1.2$ channels resulted in the loss of NMDA-independent Schaffer collateral/CA1 L-LTP. In contrast, disruption of $Ca_v1.3$ in mice has been found to have no effect on LTP in the CA1 (Clark et al., 2003).

Pharmacological block of LVGCCs has been found to facilitate working memory and spatial reference acquisition (Quartermain et al., 2001), and to reduce age-related decline of hippocampal-dependent learning in rodents and non-human primates (Sandin et al., 1990; Batuecas et al., 1998). The study in rodents utilised chronic dietary administration of the LVGCC antagonist nimodipine. Effects were linked with a small increase in synaptosomal calcium binding protein, specifically in the cerebral cortex, though no changes were observed in the hippocampus (Batuecas et al., 1998). In contrast, systemic and local perirhinal cortex infusion of different classes of LVGCC inhibitor, was found to impair the long-term acquisition of recognition memory, without affecting short term memory (STM)(Seoane et al., 2009). High doses of the inhibitor verapamil have also been found to impair retrieval during a passive avoidance task (Lashgari et al., 2006). These differential effects of inhibition highlight the potential for region specific roles of LVGCCs, as well as important considerations of differences in administration and timing of inhibition, when making conclusions about functionality.

The role for LVGCCs has been most extensively investigated in associative learning (Cain et al., 2002; Barad et al., 2004; McKinney and Murphy, 2006; Busquet et al., 2008; Mckinney et al., 2008; Davis and Bauer, 2012). Given the relevance to the current work and the potentially more complex aspects of learning involved in these processes, this will be discussed in the separate section below.

1.3 Associative learning

The formation of associations in everyday life is vital in informing subsequent behaviour. Prior associations allow predictions to be made about future experience and can provide an indication of when new learning may be required, when predictions are violated and associations need to be updated (McLaren and Mackintosh, 2000; Dickinson, 2012) . Approach and avoidance behaviour is primarily influenced by associations from prior experience; whether a particular behaviour received a reward or led to an aversive response will tend to determine the frequency of that behaviour in the future (Valins and Ray, 1967). Deficits in associative learning have been described as being a key feature of schizophrenia as early as 1911, when Bleuler emphasised the loosening of associations in relation to Dementia Praecox (Bleuler, 1950; Peralta and Cuesta, 2011).

The investigation of associative learning has evolved from Pavlov's classical conditioning paradigms (Pavlov and Anrep, 2012). Associative learning involves the formation of an association between an unconditioned stimulus (US) and a conditioned stimulus (CS), such that subsequent exposure to the CS in the absence of the US elicits a conditioned response (CR). The US may be rewarding or aversive in nature, eliciting an innate reactive response, such as salivation, freezing behaviour or avoidance.

Pavlovian fear conditioning is a single trial learning paradigm, involving the pairing of either an auditory cue or a context (CS) with an aversive stimulus (US); most often a footshock or burst of white noise. These paradigms are incredibly useful for studying the mechanisms of associative fear learning, due to learning being rapid, long-lasting and producing stereotypical, measurable behavioural outcomes in animals (LeDoux, 2003). The most common form of aversive associative learning studied in animals is cued/contextual fear conditioning, which is known to rely on regions including the

amygdala and hippocampus (Phillips and LeDoux, 1992; Daumas et al., 2005; Alvarez et al., 2008; Quirk and Mueller, 2008).

There are different stages of fear conditioning paradigms that can be, at least in part, separated into distinct psychological processes; acquisition of associations, their long-term consolidation and the recall/expression of associations during subsequent exposure to the CS. Additional manipulations of associative learning can be investigated by utilising paradigms that invoke extinction or latent inhibition.

The acquisition of CS-US associations is initiated by the presentation of the CS, in a way that it could feasibly predict the US. The likelihood or strength of an association formed can be influenced by multiple factors, including timing and relevance of the CS presentation to the US occurrence (Rescorla, 1988; Gallistel and Gibbon, 2000). Re-exposure to the CS is used to test the recall and expression of the associations, with fear response in the absence of the US indicating successful association formation. In the case of aversive conditioning in rodents, this is usually measured by context avoidance or freezing behaviour. Short-term acquisition is usually tested within the first four hours following training, with the consolidation of learning occurring over 24-48 hr period (Walker et al., 2003; Clopath, 2012).

The novelty of the CS is another important factor that can influence association formation and can be manipulated through latent inhibition. Pre-exposure to a CS before its co-occurrence with a US reduces the predictive value of the CS and therefore reduces the likelihood of it forming an association (Lubow, 1973). Latent inhibition is thought to involve the active inhibition of CS - US associations due to interfering information from prior experience through a CS – no US association (Escobar et al., 2002).

Similar to latent inhibition, extinction involves repeated or prolonged exposure of the CS in the absence of the US, though occurring after the initial association (Figure

1.2). This is postulated to lead to the formation of a competing CS-no US association, that reduces the response to the CS, rather than a degradation of the original association (Bouton, 2004; Bouton et al., 2006; Herry et al., 2010; Trent et al., 2015). Like the formation of the original CS-US association, there is evidence to suggest separable processes of acquisition and consolidation of extinction, supported to involve a neural circuit to include the amygdala, hippocampus and medial PFC (Corcoran and Quirk, 2007).

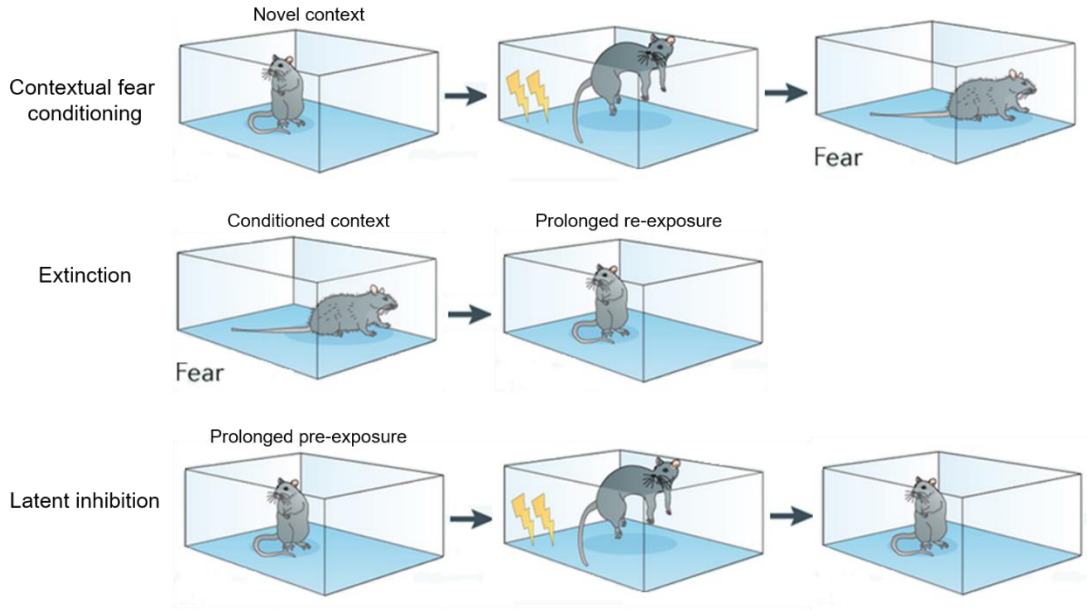

Figure 1.2 Standard paradigm for contextual fear conditioning in rodents. The novel context (CS) is associated the footshock (US), such that the CS then elicits fear related behaviour (freezing) in the absence of the US. Extinction of the association can be promoted by prolonged exposure to the conditioned context and the association between the CS and the US can be inhibited by prolonged pre-exposure to the to be conditioned context.

Reward based associative learning requires longer training paradigms than aversive conditioning and has been found to engage different neural circuits and mechanisms (Schultz et al., 1997). The activation of the 'reward circuit' is required, with a central role for the ventral striatum (nucleus accumbens in animals) and inputs from the PFC, amygdala and the midbrain (Haber, 2011). Although it is not as easy to distinguish

between potentially separate learning processes at a single trial or session level, it is possible to compare processes at different training phases. Similar to aversive conditioning, it is also possible to investigate the inhibition and update of reward based associations.

Pavlov's traditional reward association paradigm (Pavlov and Anrep, 2012) has been adapted to include reward associations with more complex and multiple stimuli. For example, studies of reversal learning require the learned discrimination between two stimuli for reward and the subsequent reversal of associations. They have been used to investigate the acquisition of reward associations, their inhibition when contingencies change, the ability to respond to a previously unrewarded stimuli and the extinction of associations when there is no longer an association with reward (e.g. Brigman et al., 2008; Ghahremani et al., 2010; Bussey et al., 2012).

These different behavioural paradigms have allowed the investigation of potentially distinct underlying molecular mechanisms for aspects of aversive and reward-based associative learning. Previous studies have identified roles for certain genes in distinct aspects of these tasks and have indicated selective deficits in psychiatric illness (Waltz and Gold, 2007b; Brigman et al., 2008; Jensen et al., 2008; Kirtley and Thomas, 2010).

1.3.1 Previous research into role of LVGCCs in associative learning

Previous studies have primarily used systemic injection of LVGCC inhibitors to investigate their role in specific aspects of cued fear conditioning. This approach has reported no effects of inhibition on the acquisition or consolidation of cued fear learning, however they impair extinction (Cain et al., 2002, 2005; Busquet et al., 2008) and an indication of impaired latent inhibition (Barad et al., 2004). Region specific investigation has utilised local inhibition of LVGCCs via intracerebral injection. Local infusion of LVGCC inhibitors into the basolateral amygdala has been found to impair

both consolidation and extinction of cued fear learning (Bauer et al., 2002; Davis and Bauer, 2012), though there appears to be different roles for $Ca_v1.2$ and $Ca_v1.3$ channels, with $Ca_v1.3$ implicated in consolidation (McKinney and Murphy, 2006; Busquet et al., 2008).

Studies of the role of LVGCCs in reward associations have focussed more specifically on addiction, which can be interpreted as a form of maladaptive reward learning (Everitt and Robbins, 2005; Isokawa, 2012). $Ca_v1.3$ channels have been found to be necessary for the development of addiction to cocaine and amphetamine, whereas $Ca_v1.2$ are implicated in its maintenance (Kuzmin et al., 1992; Licata et al., 2000). Research has indicated a role for LVGCCs in reward responsiveness, with an attenuation of consumption of rewarding liquids observed with block of LVGCCs (Calcagnetti and Schechter, 1992). Additionally, an association between risk variation in *CACNA1C* and reward responsiveness has been observed in healthy individuals (Lancaster et al., 2014). Functional investigations of these channels in normal processes involved in reward learning and its adaptation are lacking.

1.3.2 Associative learning deficits in psychiatric illness

There is growing evidence of a convergence of genetic risk for schizophrenia associated with synaptic function and synaptic plasticity, known to underlie long term memory formation (Harrison and Weinberger, 2005; Hall et al., 2015). It has also been hypothesised that the convergence of genetic risk on synaptic plasticity, as well as the mesolimbic dopamine system, may be understood by an associative learning theory (Hall et al., 2009). More recent analyses of copy number variants has indicated an enrichment in duplications and deletions in gene sets associated with abnormal associative learning, abnormal contextual conditioning and other related learning behaviour (Pocklington et al., 2015).

Evidence has shown that patients with schizophrenia exhibit deficits in specific aspects of associative learning across different tasks (Lubow et al., 1987; Escobar et al., 2002; Waltz and Gold, 2007b; Jensen et al., 2008; Holt et al., 2009, 2012). Patients appear to be able to acquire aversive associations equally as well as healthy controls, presenting with specific deficits in extinction. Holt et al., (2009) used measures of skin conductance response (SCR) to investigate Pavlovian fear conditioning in patients compared to healthy controls. They found that patients were able to successfully acquire and extinguish a conditioned fear response, however, when tested 24 hrs later, patients showed an excessive fear response by elevated SCR to the CS. This indicates a failure in patients to consolidate this extinction. In a subsequent study they found that these abnormalities were associated with differences in neural activation of the vmPFC and limbic areas, including the hippocampus and amygdala (Holt et al., 2012).

Studies have additionally observed deficits in latent inhibition at different stages of illness, indicative of processing of irrelevant stimuli (Baruch et al., 1988; Jones et al., 1992). Results from subsequent studies are mixed, with differences in latent inhibition appearing to be dependent on medication status and stage of illness (Swerdlow et al., 1996; Rascle et al., 2001; Vaitl et al., 2002).

Patients have additionally been found to have abnormal activation of the reward system in conjunction with deficits in associative learning behaviour. Abnormally strong activations of the ventral striatum were observed in patients, associated with inappropriate learning to neutral stimuli, suggesting deficits in appropriate association of motivational salience (Jensen et al., 2008). Specific deficits in discrimination learning and reversal learning have also been observed in first episode psychosis patients and patients with schizophrenia (Waltz and Gold, 2007b; Murray et al., 2008a), with errors in reversal learning additionally found to correlate with negative symptom severity (Murray et al., 2008a). These deficits have been more explicitly

linked with abnormal mesolimbic dopamine activity, through functional magnetic resonance imaging (fMRI) indicating physiological differences in the mid-brain, striatum and limbic system in relation to reward prediction error (Murray et al., 2008b).

It has been suggested that patients with schizophrenia do not necessarily have deficits in forming associations, but instead have a problem with retrieving, using and updating associative information (Escobar et al., 2002). Impairments in associative learning have been found to be shared across schizophrenia and bipolar disorder (Murray et al., 2008a; Brambilla et al., 2011) and deficits in latent inhibition have also been observed in “psychotic prone” healthy individuals (Baruch et al., 1988).

Associative learning deficits are only one aspect of cognition that has been found to be impaired in schizophrenia and related psychiatric illnesses (Galderisi et al., 2009; Dere et al., 2010). Cognitive deficits are key features of multiple psychiatric disorders, with impairments seen in multiple domains (Millan et al., 2012). The impact of these impairments can be equally, if not more, debilitating than positive or negative symptoms in everyday life (Rupp and Keith, 1993). Cognitive symptoms are found to be apparent long before onset of psychosis and the severity of impairments has been linked to outcome in patients with schizophrenia (Green et al., 2004; Galderisi et al., 2009).

Current treatments for schizophrenia do not specifically address cognitive impairments. Some efficacy has been observed with atypical antipsychotic drugs, including clozapine, olanzapine and risperidone, in the improvement of verbal learning and fluency, though they had no effect on visual learning and memory or attention (Meltzer and McGurk, 1999). It is evident that there is a need for new treatments which are specifically designed to alleviate cognitive symptoms of psychiatric illness. The recent progress in genetics has identified multiple genes and pathways which may contribute to cognitive dysfunction (Ripke et al., 2014). Furthermore, there has been significant advances in the measurement of specific

aspects of cognition as well as the development of translational behavioural assays (Bussey et al., 2012). In order to potentially identify valid novel treatment targets, it is necessary to further understand the molecular mechanisms which may link genetic risk for psychiatric illness with specific cognitive symptoms.

The current work aims to contribute to our understanding of the link between both LVGCCs and the psychiatric risk gene *CACNA1C* and specific aspects of associative learning. Calcium channels and *CACNA1C* specifically, have been reliably associated with risk for psychiatric illness. LVGCCs channels have a role in synaptic plasticity and regulate gene transcription. Previous studies have further suggested a role in distinct aspects of associative learning in select brain regions. Associative learning deficits are evident in patients with schizophrenia and other related psychiatric disorders. The role of LVGCCs in hippocampal-dependent fear conditioning and reward-based reversal learning, is yet to be fully understood.

1.4 Outline of experiments

The experiments detailed in this thesis utilise contextual fear conditioning and reversal learning paradigms to investigate the role of *Cacna1c* and LVGCCs more broadly in aspects of associative learning.

Chapter 3 presents the basal expression profile of *Cacna1c* in the rat brain and activity regulated expression changes during contextual fear conditioning. This paradigm is the simplest form of implicit associative fear memory, with rapid single-trial learning allowing the investigation of separable learning processes. Chapter 4 discusses the use of the selective LVGCC inhibitor diltiazem, to investigate the effect of inhibiting calcium influx through these channels on distinct aspects of contextual fear learning; consolidation, recall, extinction and latent inhibition.

To separate the potential roles for $Ca_v1.2$ and $Ca_v1.3$ channels and explore *Cacna1c* more specifically, we re-derived a chronic heterozygous knockout *Cacna1c* rat model. Chapter 5 details the basic molecular and behavioural characterisation of this model. Expression levels of *Cacna1c* and *Cacna1d* were measured to determine the validity of the model and investigate potential compensation effects. *BDNF* expression was additionally measured to determine whether there were effects of reduced *Cacna1c* on a relevant downstream signalling gene. Basic behavioural tests were conducted, including tests of locomotor activity, anxiety, startle response and pre-pulse inhibition.

This rat model was then used to determine the effects of reduced expression of *Cacna1c* on specific aspects of contextual fear conditioning, intended to complement the findings with inhibition of LVGCCs. The findings from these experiments are detailed in chapter 6. In chapter 7, the same rat model was then used to investigate the role of *Cacna1c* in reward based reversal learning, using a translational touch-screen paradigm.

In the final experimental chapter (chapter 8), analyses are translated to humans, to specifically investigate associations with risk variation in *Cacna1c*. The association between rs1006737/rs2007044 and expression of *Cacna1c* and different *BDNF* transcripts, was investigated utilising an online expression database (BRAINEAC). Behavioural and genetic data from a separate cohort of people that underwent a reversal learning task was analysed to determine whether there was an allelic association between risk variants in *Cacna1c* and reversal learning behaviour.

The overall findings of these experiments are discussed in relation to separable roles for LVGCCs in inhibitory learning and the convergence of effects on the synapse. The connection of these findings to current theories of schizophrenia and how these effects could manifest in symptoms of psychiatric illness is explored.

2 METHODS AND MATERIALS

2.1 Animals

2.1.1 Wild-type animals

Adult male Lister Hooded rats (250-275 g, Charles Rivers, Margate, UK) were housed in pairs on a 12:12 reverse light dark cycle (lights off at 10am), with ad libitum access to food and water unless where indicated. Experiments were conducted during the dark period. All animals were given a minimum of five days from arrival before being used in any experiments. Experiments were conducted under licence PPL 30/3135 and PIL IB00ACC93. At the end point of each experiment, all animals were sacrificed by schedule one, using a rising concentration of CO_2 in a home cage culling chamber (Clinipath Equipment Limited, Hull, UK).

2.1.2 Transgenic heterozygous *Cacna1c* knock-out animals

2.1.2.1 Generation of founders

Cacna1c heterozygous knock-out animals were obtained from cryo-preserved embryos (TGRA6930, Sage Research Labs, Pennsylvania, USA) on a Sprague Dawley background. Zinc Finger Nuclease (ZFN) technology was used to create the original line. This method utilises a site-specific endonuclease to bind and cleave DNA, causing a double stranded break at the targeted region (Figure 2.1 Genome editing using ZFN technology: Zinc finger binding domains guide the cleavage domain to specific sites in the genome. On forming a dimer the nuclease causes double stranded breaks in the DNA. Repair mechanisms can then lead to mutations and/or deletions in the genome. DNA repair can then occur through non-homologous end-joining, leading to mutations and/or deletions in the gene sequence (Bibikova et al., 2003). ZFNs consist of two domains, a binding domain and a cleavage domain. The binding domain can be constructed of various combinations of zinc fingers to form arrays that recognise specific sequences in the genome. The cleavage domain

must form a dimer to act as a nuclease, therefore requiring a pair of ZFNs. These properties improve the affinity and specificity of the genome editing that occurs (Pabo et al., 2001). A pair of 5-finger ZFNs, recognising a total of 30 base pairs were used to target a specific region of *Cacna1c.* The selected successful founders used for breeding had a 4bp deletion (location 460649bp-460652bp in genomic sequence) in exon 6, resulting in a frame shift and an early stop codon.

Cacna1c ZFN Target site: GTCCTGAACTCCATCatcaagGCCATGGTGCCCCTG

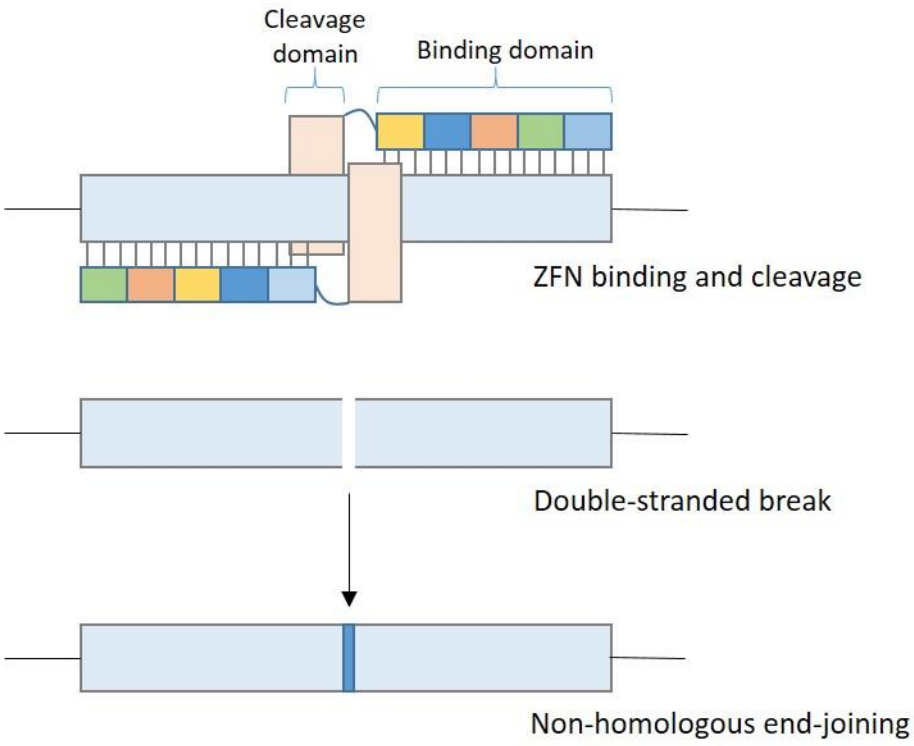

Figure 2.1 Genome editing using ZFN technology: Zinc finger binding domains guide the cleavage domain to specific sites in the genome. On forming a dimer the nuclease causes double stranded breaks in the DNA. Repair mechanisms can then lead to mutations and/or deletions in the genome.

Successful genetic manipulation of founders was confirmed by PCR and sequenced by Sage. A PCR reaction was created with 1 µl of DNA in a solution of 1 µM Forward primer (5'-GCTGCTGAGCCTTTTATTGG-3'), 1 µM Reverse Primer (5'-CCTCCTGGATAGCTGCTGAC-3'), 1 x JumpStart™ Taq ReadyMix™ (Sigma-

Aldrich, St Louis, USA) and 14 µl ddH_2O. The PCR reaction was run at 95˚C for 5 mins, followed by 35 cycles of 95˚C for 30 secs, 60˚C for 30 secs and 68˚C for 40 secs. The reaction was then held at 68˚C for 5 mins. Amplified products were treated with Exo-SAP (Affymetrix, Santa Clara, USA) to dephosphorylate any residual primers and dNTPs as well as degrade any single stranded molecules, then sent directly for sequencing. Sequencing data confirmed the site of the deletion in all founders (examples shown in Figure 2.2)

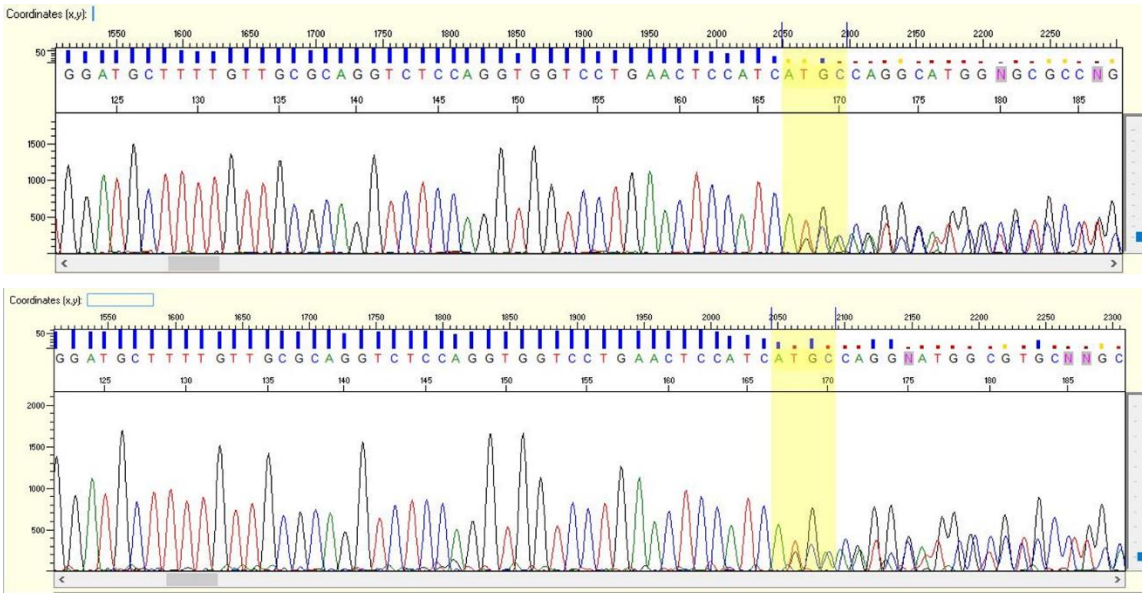

Figure 2.2 Example sequencing data from 2 separate Cacna1c$^{+/-}$ founders, displayed using Sequence Scanner Software 2 (Applied Biosystems). Site of 4 bp deletion is highlighted in yellow.

Homozygous knock-out of *Cacna1c* is embryonically lethal (Seisenberger et al., 2000); therefore breeding resulted in a Mendelian distribution of WT and HET pups, with litters of between 4 and 16. Selected heterozygous founders were shipped to Charles River (Margate, UK) and bred up for experimental colonies, with both heterozygous females and heterozygous males used for breeding. Cohorts of between 30 and 40 animals were transported to Cardiff University in litters at a minimum age of 12 weeks.

2.1.2.2 Housing

Animals were housed with littermates in groups of 1-4 dependent on weight in large rat cages (38 cm (W) x 56 cm (L) x 22 cm (H). Individual housing conditions are described separately for each experiment, including lighting and any water deprivation protocols.

2.1.2.3 Genotyping

2.1.2.3.1 DNA extraction

Ear punches taken from all weaned animals used experimentally were sent from Charles River for genotyping. A small section of tail tissue was taken from all experimental animals post-mortem to confirm genotyping results. Genomic DNA was extracted using Qiagen DNeasy Blood & Tissue kits (Qiagen, Manchester, UK) as per the standard protocol.

Approximately 0.6 cm of tail tissue or 1 single ear-punch was lysed overnight in 180 µl buffer ATL and 20 µl proteinase K at 56˚c. 200 µl of both buffer AL and 96-100% ethanol were added before putting through a spin column at 8000rpm for 1 min. Buffers AW1 and AW2 were added to the spin column one at a time, with a final spin of 14,000 rpm for 3 mins to dry the membrane. 200 µl of buffer AE was pipetted directly onto the membrane and incubated at room temperature for 1 min. DNA was eluted by centrifuging at 8000 rpm for 1 min and stored at -20˚c until required for PCR. The concentration of DNA and its purity was measured using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Delaware, USA). A 260/280 nm ratio of 1.8 or greater was deemed acceptable for DNA purity as per manufacturer's recommendation. Extraction resulted in yields of between 20 and 40 µg of DNA from 0.6 cm of tail tissue.

2.1.2.3.2 Polymerase Chain Reaction (PCR)

Two separate forward primers (24 bp) were designed to distinguish between WT and HET animals. The first primer targeted a region upstream of the 4bp deletion (Chr 4: 216724640 – 216724663) so that it would bind to DNA from all animals tested. The second HET specific forward primer spanned the deleted region and therefore would only bind if the 4 bp were absent (Chr 4: 216724462 – 216724485). The same reverse primer (22 bp) was used in both reactions (Chr 4: 216724019 – 216724040) (Table 2.1. Two simplex reactions were run for each sample to detect WT and HET bands separately. Master mixes were made up with a total of 24 µl per reaction. 1-2 µl (0.1 - 0.4 µg of DNA) was added from each sample to 17.25 µl distilled H_2O, 2.5 µl 10 x PCR Buffer, 1 µl of dNTPs, 0.25 µl HotStarTaq DNA polymerase (Qiagen, Manchester, UK) and 1 µl of each primer.

Samples were run on a BioRad Thermal Cycler (T100™ BioRad, Herts, UK). Conditions were 95˚C for 10 mins, followed by 40 cycles of 95˚C for 40 secs, 62˚C for 40 secs and 72˚C for 60 secs, with a fixed cycle of 72˚C for 5 mins. Samples were then cooled to 8˚C.

Table 2.1 Primer sequences for genotyping of Cacna1c HET and WT animals

FOR1	5’ – AGCCTTTTATTGGGATGTGTCTCC – 3’
FOR2 (mutant specific)	5’ – TCCTGAACTCCATCAGGCCA – 3’
REV	5’ – CTGATGAACGGTGGGTGCTTAC – 3’

2.1.2.3.3 PCR genotype analysis

A 1.5% agarose gel was made with 0.5 x TBE buffer and 3 µl ethidium bromide. 30 µl of the resulting PCR solution was loaded onto the gel and run at 100 mv for 30 mins. Gels were visualised on a BioRad Gel Doc XR imager (BioRad, Herts, UK) and bands were visually compared to distinguish WT and HET animals. The expected WT band was 645bp and the HET 463bp (Figure 2.3).

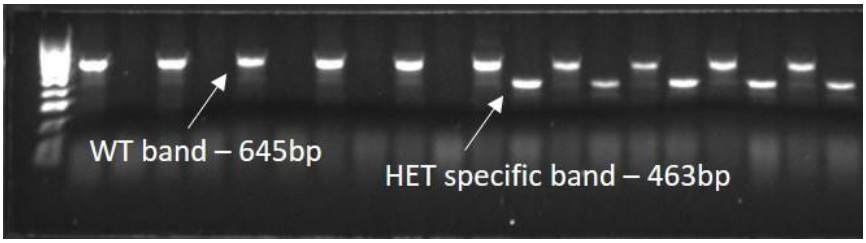

Figure 2.3 Example of genotyping results for Cacna1c WT and HET animals. The first 10 lanes show 5 WT samples with only the WT band (645bp) present. The last 10 lanes show HET samples, with bands at 645bp and 463bp clearly visible

2.2 Surgical procedures and infusions

All surgery was conducted on a stereotaxic frame (Bilaney, model 963, including Ultra Precise Micro Manipulator model 961, Kent, UK) to ensure accurate targeting of the dorsal hippocampus. Double guide cannula (Bilaney, Kent, UK) with projections extending 3 mm below pedestal were fitted onto the frame. All tools and materials that would come in contact with the animal were autoclaved prior to surgery. Rats were anaesthetised with 5% Isoflurane with an oxygen flow rate of 0.8 l/min and their heads were shaved to maintain a clear surgery site and clean wound. Before attachment of the rats to the frame, gas flow rates were set; 0.8 l/min oxygen, 0.4 nitrogen and Isoflurane at 3.5%. These levels were constantly monitored throughout surgery to ensure that they did not change, though anaesthetic levels were adjusted depending on the individual rat's weight and response at different points of the procedure.

Rats were attached to the frame using 18˚ tip ear bars and placed in a sterilisation bag on bubble wrap to keep them warm. The site of incision was cleaned with a Clinell alcohol disinfectant wipe and a single incision was made from approximately 4 mm anterior to bregma straight back to between the ears. Four curved Hemostats (InterFocus, Cambridge, UK) were used to maximise the area for cannula placement and the wound cleaned with sterile swabs so bregma was clearly visible. Co-ordinates were then taken with the cannula positioned above the skull so that the left hand guide was directly above bregma. The cannula was then moved + 0.19 cm in the coronal plane and - 0.35 cm in the sagittal plane to be accurately aimed bilaterally at the dorsal hippocampus (see Figure 2.4). Using a stereotaxic drill holder (Bilaney, Kent, UK) with drill bit (Bilaney, Kent, UK) two holes were drilled in the skull in line with the trajectory of the guide cannula prongs, just deep enough to penetrate the skull. Once the holes for the cannula were confirmed to be accurate, four screw holes were made using a Starrett pin vice (RS Components, Corby, UK); two holes in-line with bregma and two slightly anterior to lambda, as wide as possible to ensure the maximum spread of force on the cannula and therefore greater security. Using a quickwedge screwdriver (RS Components, Corby, UK), the four screws (1/16, Bilaney, Kent, UK) were screwed into place and then the cannula was lowered into the holes until flush against the skull. At this point, due to the reduced pressure on the skull, anaesthetic was typically lowered to 2.5% for the remainder of the surgery.

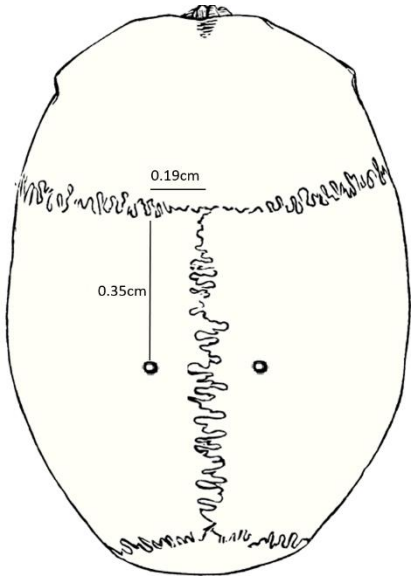

Figure 2.4 Placement of the guide cannula to target the dorsal hippocampus. Positioned +0.19 cm coronally and -0.35 cm sagittally from bregma

To secure the cannula to the skull Kemdent Simplex Rapid Powder and Fluid (Associated Dental Products Ltd, Swindon, UK) were used to create an

autopolymerising acrylic resin. Using a metal spatula applicator (Fine Science Tools, Heidelberg, Germany) a thin solution was added around the base of the guide cannula to seal it to the skull. A matrix of more viscous resin was then built up gradually to secure the cannula and spread to the four screws. Each layer was left to dry between applications. During this time analgesic was administered subcutaneously to each animal (Metacam 2 mg/kg) to alleviate post-surgery discomfort. Once the matrix was dry, the guide cannula was carefully detached from the frame holder and a dummy cannula (Bilaney, Kent, UK) was inserted, extending 1 mm beyond the guide cannula.

The resin was left for a further 5 min to ensure that it was sufficiently dry and then the wound was sutured up using Mersilk sutures (Henry Schein, Cardiff, UK). Two stitches were made in front of the cannula and two behind to secure the wound, with additional stitches as necessary. The tails of the first rat in each cage were marked with the experimental number for later identification and then rats were removed from the frame and placed in an incubation chamber at 37˚C for approximately 30 min. Animals remained in the chamber until they were fully recovered from the anaesthetic and able to stably support their own weight, at which time they were returned to their home cage. All rats were left for a minimum of 6 days before any further experimental procedures.

2.2.1 Infusions

Infusions were done using a Standard Infusion Only Harvard Pump 11 Plus Dual system (Harvard Apparatus, Cambridge, UK) and Hamilton 800 Series 5 µl glass syringes (Harvard Apparatus, Cambridge, UK). Infusion needles were attached to the syringes using fine bore polythene tubing (Portex, Kent, UK - inner diameter 0.38 mm, outer diameter 1.09 mm). The syringes and tubing were filled with sterile water and then a 2 µl air bubble was introduced to separate the infusion solution. Infusions were always conducted at a rate of 0.5 µl/min with a total of 1 µl delivered bilaterally.

It was ensured that any excess solution was removed from the tubing after the infusion and the injector and tube were rinsed in ethanol between animals to avoid any cross-contamination.

A minimum of six days following surgery, all animals received a practice infusion of sterile PBS to clear out the cannula and allow them to become familiar with the procedure. Animals were always infused one cage at a time and taken to the procedure room in the same transport boxes (odd numbered animals always in box A, even in box B). Rats were placed on the experimenter's lap and allowed to freely explore for the duration of the infusion. Dummy cannula were removed and the infusion needles inserted until fully flush with the guide cannula, resulting in a 1 mm protrusion into the hippocampus. The needles remained in place for 2 min after the end of the infusion to ensure maximal delivery of the solution. The injector was then removed and the dummy cannula replaced.

Animals received the experimental infusion 40 mins prior to the condition of interest and were returned to their home cages until required for testing. All infusions were counterbalanced so that home cage, conditioning chamber and any order effects were controlled. Post mortem histology was performed to ensure correct placement of the cannula.

2.2.2 Solutions

Diltiazem Hydrochloride (Sigma, Dorset, UK) was dissolved to stock concentration (100 nmol) in DEPC water. 0.1 M sterile PBS (pH 7.2) was filtered through a 0.22 µm Millipore syringe and used to dilute the stock solution of Diltiazem for a working solution (10 nmol) and as the control. Solutions were separated into 10µl aliquots and stored at -20˚c until use.

BDNF antisense was designed to target all BDNF transcripts, binding to exon IX. Synthetic phosphorothioate-modified antisense and missense control

oligonucleotides were created by Sigma (Dorset, UK), with the first 5' and last 3' bonds modified only. Sequences are listed in Table 2.2. Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to ensure missense did not bind to any other non-target mRNAs.

Table 2.2 BDNF antisense (aso) and missense sequences used

	Sequence 5' – 3'
$BDNF_{aso}$	tCTTCCCCTTTTAATGgT
Missense Control	aTACTTTCTGTTCTTGcC

2.3 Behaviour

2.3.1 Contextual Fear Conditioning

2.3.1.1 Apparatus

All sessions were undertaken in two standard modular test chambers for rats, interior measuring 30.5 cm (L) x 24.1 cm (W) x 21.0 cm (H), with aluminium side walls and clear polycarbonate rear and door (Med Associates Inc., Vermont, USA). Chambers had a grid floor consisting of 19 equally spaced bars positioned 1.6 cm above the floor, through which a 0.5 mA shock was delivered. This was controlled by a stand-alone aversive stimulator/scrambler (Med Associates Inc., Vermont, USA). Boxes were placed inside sound attenuating chambers 55.9 cm (L) x 55.9 cm (W) x 35.6 cm (H) (Med Associates Inc., Vermont, USA). The programmes for each session were controlled through Med-PC version IV research control and data acquisition system (Med Associates Inc., Vermont, USA). Behaviour was digitally recorded from cameras (JSP Electronics Ltd, China) positioned centrally above the chambers and viewed using Numeroscope software (Viewpoint, France) for offline analysis.

Animals were taken from home cages to the testing room in large transport boxes. The same box was used for each session.

2.3.1.2 Conditioning

Animals were placed in conditioning chambers for 2 mins before receiving a 0.5 mA scrambled foot-shock (US) for 2 secs. They remained in the chambers for 1 min after the shock before returning to home cages in the same transport boxes. Conditioning took place in the morning, a minimum of 30 mins after the start of the dark period in the holding room.

2.3.1.3 Recall

Three hrs, 24 hrs and/or 7 days later rats were returned to the same conditioning chamber (CS) for a 2 min recall session in the absence of the US. The recall session 3 hrs after CFC measured short-term memory and the 24 hr and 7 day sessions assessed long-term CFM.

2.3.1.4 Extinction

Twenty-four hrs following CFC rats were returned to the conditioning context for a 10 min session in the absence of the US to elicit extinction of the acquired memory. Consolidation of this extinction was then tested through subsequent recall sessions, as above.

2.3.1.5 Latent inhibition

Twenty-four hrs prior to conditioning rats were exposed to the conditioning context for 4 hrs. Animals were returned to their home cages overnight and received CFC the next day as described above (Conditioning) excepting time of day was aligned to offset of the 4 hr LI period. The 4 hr pre-exposure to the CS leads to reduced freezing following CFC (Lubow, 1973).

2.3.1.6 Control groups

Two control groups were used. Naïve animals were taken directly from home cages and a second Novelty group were exposed to the context in the absence of any US for 2 mins before being returned to home cages. Animals in the Novelty group were exposed to the context at the same time of day as conditioned animals and were sacrificed 2, 4 or 24 hrs following context exposure to correspond to the time point of interest. Naïve animals were sacrificed at the same time as cage-mates.

2.3.1.7 Analysis

Freezing behaviour, defined as complete immobility for 1 sec except for respiration, was scored every 10 secs blind to condition from offline digital recordings (as previously reported; Barnes and Thomas, 2008; Trent et al., 2015). The number of instances of freezing was recorded for each session that the animal undertook, as well as for separate pre and post US periods. The percentage freezing was calculated for each animal in each session and then averaged within experimental and control groups. Scores were checked for reliability by a minimum of two scorers.

Repeated measures ANOVAs were conducted for each cohort of animals, with all sessions included for analysis, with session as within-subjects factor. Mauchly's test of sphericity was used and if significant a Greenhouse-Geisser correction was applied. Post-hoc analyses between specific conditions of interest were conducted if repeated measures results were significant ($P < 0.05$).

2.3.2 Reversal Learning

2.3.2.1 Apparatus

Animals were run in sets of 4 using Bussey-Saksida rat touch screen chambers (Figure 2.5, A, Campden Instruments, 80604, Leics, UK) with accompanying Animal Behaviour Environment Test (ABET) II software (Whisker Client) (Campden Instruments, 89505). Chambers had a square environment area of 368 cm^2 with a

unique trapezoidal wall shape to focus the animal's attention on the screens. Walls were 345 mm high and chambers had a grid perforated floor (10 mm square with 14 mm pitch). The touchscreen was limited to two square openings (8 cm x 8 cm) by an acrylic mask secured in-front of the screen.

Liquid reward (10% sucrose solution) was delivered to the reward magazine via a peristalsis pump through clear tubing (0.8 mm x 1.6 mm).

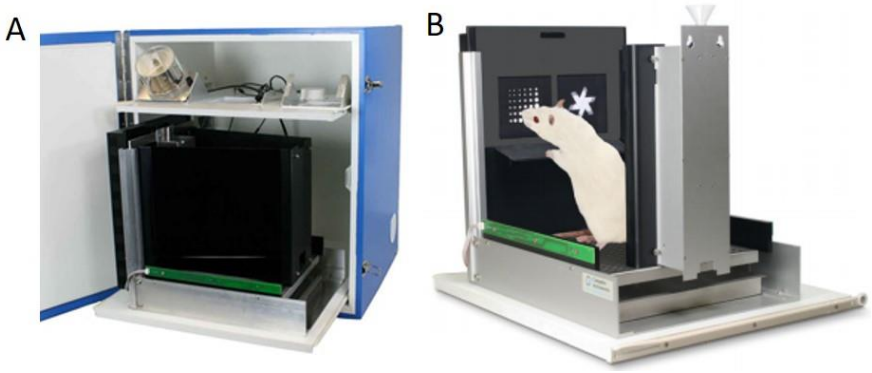

Figure 2.5 Bussey Saksida Touchscreen chamber set-up. A) the testing environment inside the outer chamber. B) The inside of the testing environment with the screen displaying two example stimuli. Images taken from http://www.limef.com/Downloads/bussey-chambers.pdf

2.3.2.2 Water restriction

Animals were placed on water restriction with a minimum of 2 hrs access to water per day (administered in experimental chambers and/or home cages). Water was removed for a maximum of 22 hrs in any 24 hr period and animals were weighed daily for the first week to ensure none dropped below 85% of natural body weight. Subsequently animals were weighed once a week during any water restricted periods. Animals underwent 5 days of water restriction before training to familiarise them with the procedure. During training, access to water was given immediately following each session. Water restriction was used in favour of food restriction following evidence to suggest that this produces a better response in rodents, specifically in terms of more stable body-weight fluctuations and exploratory behaviour (Tucci et al., 2006).

2.3.2.3 Habituation

Naïve animals were placed into Bussey chambers for a 30 min session. At the beginning of each session a brief (1000 ms) tone was played and the food tray light turned on. The magazine tray was primed with an initial liquid reward of 10% sucrose solution (6000 ms) made up fresh every 7 days. Rats were required to enter and leave the reward tray for the programme to continue and the light to turn off. There was a 5 sec delay before the start of each subsequent trial. The light was turned on coinciding with a tone and a further smaller reward of approximately 20-25 µl delivered. If the rat was still in the reward tray at the end of the 5 sec delay, then there was an additional 1 sec delay. This repeated until the end of the 30 min session or until 100 trials was reached. To move on to the next stage of training, animals must have completed 100 trials within the 30 min session for 2 consecutive days (Table 2.3).

Table 2.3 Criterion that were reached for each experimental condition before animals moved on to the next stage of training

Experimental Condition	Criterion
Habituation	100 trials completed in 30 mins for 2 consecutive days
Must-Touch training	100 trials with > 20 blank touches in 30 mins for 2 consecutive days
Visual Discrimination	> 80% correct for 2 consecutive days
Reversal	> 80% correct for 2 consecutive days
Extinction	5 sessions

2.3.2.4 Must-Touch training

Stimuli of various black and white shapes were displayed randomly one at a time on either the left or right side of the screen (Figure 2.5 B). The other side was left blank. The position of the stimuli was chosen pseudo-randomly in that an image would not

be displayed on the same side more than 3 times consecutively. The rat was required to nose poke the stimuli to receive the liquid reward. Touching the screen elicited a tone, accompanied by the food tray light and a 500 ms delivery of liquid reward. There was a 5 sec delay following the rat entering the food tray and a new stimuli being presented. Touching the blank screen gave no response. To progress to the next training phase, animals needed to complete 100 trials within 30 mins, with fewer blank screen touches on 2 consecutive days.

2.3.2.5 Visual discrimination

The food tray was primed with liquid reward and the light turned on. The programme was initialised by the rat exiting the food tray. Novel stimuli were presented simultaneously on both sides of the screen (Figure 2.6). As before, the position of the stimuli was pseudo randomised. The rat was required to touch the correct stimuli (S+) to elicit the tone/light and the reward. Collecting the reward led to the usual 5 sec delay before the next trial. If the incorrect stimuli (S-) was touched then the trial was terminated and the food tray light turned on for a 10 sec time-out period, with no reward. The animal had to then enter and exit the food tray to initiate the next trial. The correct stimuli type was counterbalanced between animals. Animals were required to make at least 80% correct responses on two consecutive days.

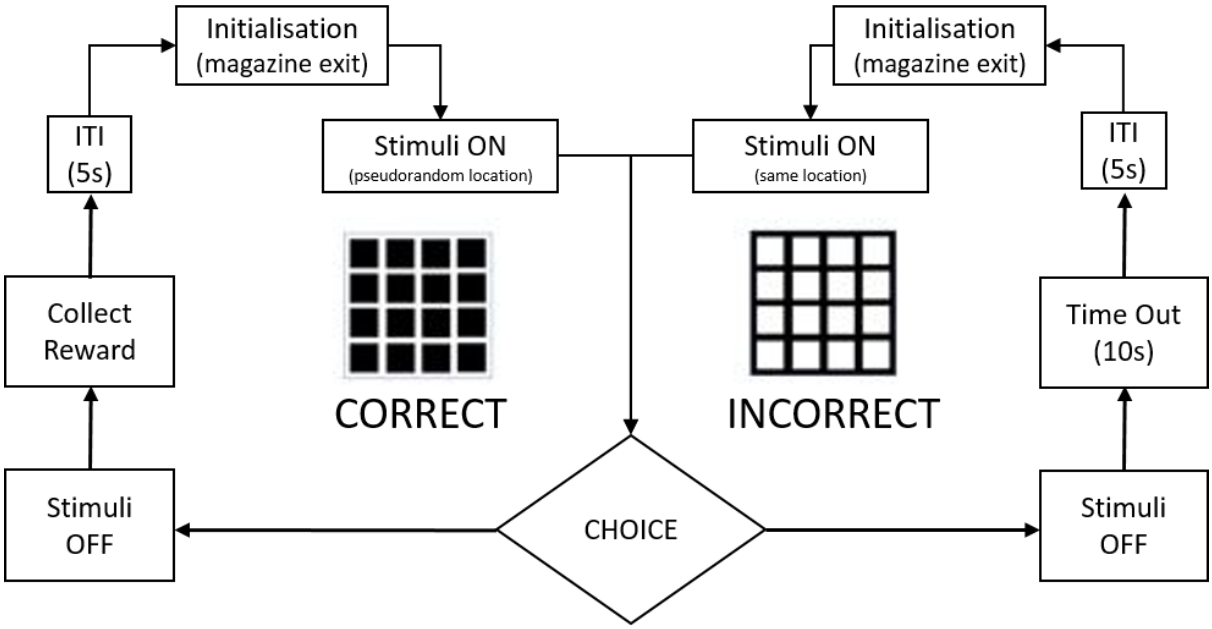

Figure 2.6 Flow diagram of experimental paradigm for visual discrimination and reversal conditions. Stimuli shown are those used in the experiment. Correct stimulus was counterbalanced across animals.

2.3.2.6 Reversal of visual discrimination

Once criterion was reached for visual discrimination the contingencies were reversed, with the previously incorrect stimuli now being rewarded. Training continued until the same criterion (80% correct for 2 consecutive days) was reached. Following this, animals underwent 5 sessions where no reward was given for correct responses (extinction trials).

2.3.2.7 Analysis

Trial numbers and number of correct/incorrect responses, as well as latencies, tray entries and beam breaks were recorded for each session.

The number of trials and sessions to reach criterion were totalled for each animal and averaged across WT and HET groups for all experimental conditions. One-way ANOVAs were conducted for each comparison when assumptions of normality and equal variance were met (Shapiro Wilks and Levene's tests respectively). In cases where variances were not equal, an independent student's t-test was conducted and the appropriate t-statistic used.

Below chance errors, defined as the number of errors made when an animal was performing significantly below chance (< 45%), were totalled for each animal and averaged across WT and HET groups for reversal sessions. This was used as a measure of perseverative behaviour following reversal. Above chance errors (errors made when animals were performing above chance, > 55%) were also calculated for each animal for both visual discrimination and reversal to assess ability to acquire initial learning as well as reversed contingencies. One-way ANOVAs were conducted between groups to assess for differences.

Similar comparisons were made between latencies for reward collection, correct and black touches; with the average time (ms) calculated for each stage of the experiment for both WT and HET animals.

2.3.3 Locomotor activity

Rats were assessed for basal levels of locomotor activity.

2.3.3.1 Apparatus and paradigm

Animals were placed into novel empty home-cage equivalent cages 36 cm x 47 cm, fitted with 2 continuous laser beams for 2 hrs on 3 consecutive days. A photobeam/rotometer activity system (Paul Fray Ltd, Cambridge) was used to record the number of beam breaks, as well as double beam breaks in quick succession to constitute a 'run' measure from one end of the box to the other.

2.3.3.2 Analysis

Total beam breaks and total runs were calculated for each animal for the 3 separate testing days. The average was calculated for all WT and all HET animals and repeated measures ANOVA was conducted across the 3 sessions. Post-hoc analyses were conducted where significant overall differences were observed, to determine which session/s may be disparate.

Beams breaks and runs were also totalled within 30 min bins for each of the 3 sessions, to assess habituation to the novel context within session (indicated by reducing activity (Xavier et al., 1991)). Repeated measures ANOVA was conducted across the four 30 min bins to determine the effect of time in the box and compared between genotype.

2.3.4 Startle Response

2.3.4.1 Apparatus and paradigm

Animals underwent a 30 min startle response session in a SR-Lab™ Startle Response System (San Diego Instruments, CA). They were placed in acrylic tubular chambers 20 cm long with an internal diameter of 8.9 cm. These are designed to reduce stress by restraint by allowing animals to turn while remaining over the sensor and ensuring reliable response readings. Chambers were positioned on top of the accelerometer sensor inside a Prime Isolation Cabinet, 40.6 cm (W) x 38.1 cm (D) x 58.4 cm (H).

Each session consisted of 91 trials and background noise was set at 70 dB. Test pulses were administered at 120 dB during the first half of the session and 105 dB in the second. Interleaved pre-pulse trials were administered of 4 dB, 8 dB and 16 dB to assess attenuation of the startle response for each sound level.

2.3.4.2 Analysis

Average response for each animal was calculated for the first 3 pulses and final 10 pulses of 120 dB and 105 dB alone to assess startle and any habituation in response. The mean differences in startle following each of 4 dB, 8 dB and 16 dB pre-pulses were calculated as a measure of pre-pulse inhibition for both 120 dB and 105 dB stimuli. One-Way ANOVAs were conducted to compare the mean values for each measure between WT and HET animals.

2.3.5 Open Field Exploration Test

An Open Field Exploration Test was used to assess basic activity levels and provide an initial screen for anxiety-related behaviours (Prut and Belzung, 2003).

2.3.5.1 Apparatus and paradigm

A black wooden arena was used, 1 m² with 50 cm high walls in a room with dimmed lighting (70 lux). A camera positioned centrally above the arena recorded a single track for each animal, processed using EthoVision XT software (Noldus, VA). A centre 'zone' was defined as the central 70 cm² of the arena to determine the amount of time spent at the perimeter and in the open area.

Animals were transported in their home cages to an adjacent holding room a minimum of 20 mins prior to testing. Each animal was taken to the testing room and placed into the arena in the corner closest to the door. Recording was started immediately and sessions lasted 10 mins. Animals were removed from the arena and returned to home cages. The arena was cleaned with 70% Sani-Cloth disposable disinfection wipes between each run (PDI, Flints, UK).

2.3.5.2 Analysis

Total distance, velocity (mean and max) and time spent in centre were extracted for each animal. Averages were calculated within WT and HET animals and one-way ANOVAs were conducted for each measure to compare genotypes.

2.3.6 Statistical approach

All statistical analyses were conducted using standard null hypothesis testing. Due to this choice of analysis, it is not possible to conclude absence of effects in some experiments which have small group sizes and may suffer from low power. For experiments where this may be the case and trends (or close to) were observed, caution is noted for concluding absence or specificity of effects.

2.4 Standard Lab Protocols

2.4.1 DEPC Treatment

For every litre of solution requiring treatment, 1 ml of diethylpyrocarbonate solution (DEPC, Sigma-Aldrich, Dorset, UK) was added. The solution was shaken vigorously and left for a minimum of 1 hr in a fume hood. Solutions were autoclaved and left to cool to RT before use.

2.4.2 Slide preparation

Poly-L-lysine (25 mg, Sigma-Aldrich, Dorset, UK) was dissolved in DEPC treated water and stored in 1 ml aliquots until use. Each 1 ml aliquot was diluted in 50 ml DEPC treated water for a working solution. Twin frost microscope slides (VWR International, Leics, UK) were dipped in Poly-L-lysine, air dried and stored at 4˚C until needed.

2.4.3 Sectioning and fixing of brain slices for histology and *in situ* hybridisation

Whole brains were taken from animals immediately post-mortem, fresh frozen on dry-ice and stored at -80˚C. Coronal brain sections of 14 µm were slices at -20˚C using a cryostat (Leica Microsystems CM1860UV). Slices were mounted on poly-L-lysine coated glass microscope slides and air-dried. Each slide had 1 slice from 5 or 6 brains, counterbalanced across experimental conditions. This allowed a series of slides to be produced that represented all animals in an experiment. Slides from each experiment were processed in parallel to ensure internal technical control.

Dried mounted slides were placed into glass slide racks and fixed using fresh 4% paraformaldehyde (PFA) solution. PFA (P6148, Sigma-Aldrich, Dorset, UK) was diluted in 1 x phosphate buffered saline (PBS, pH 7.2), stirred and heated at 60˚C until fully dissolved. The resulting solution was cooled to 4˚C and kept on ice while slides were fixed for 5 mins. Slides were then placed in 1 x PBS for 1 min and then

in 70% ethanol (EtOH) for 5 mins. All racks were stored in 95% EtOH at 4˚C until required.

2.4.4 Histology

A 1% thionin stock solution was made up using 1 g thionin acetate salt (Sigma, Dorset, UK). This was added to a buffer solution of 1 M sodium acetate solution and 1 M acetic acid solution (adjusted to pH 4.3) for a working solution of 0.1%. Slides were briefly rehydrated in double distilled water (ddH_20) before immersion in 0.1% thionin stain for 10 mins. A brief wash in ddH_20 and 70% EtOH were followed by differentiation in 70% EtOH + 2% 1 M acetic acid for as long as necessary (approximately 2 mins to remove excess thionin). Sections were serially dehydrated in 95% and 100% EtOH for 1 min and left overnight in Histo-Clear (Agar-Scientific, Essex, UK) to de-lipidate the tissue. Clear glass coverslips (Agar Scientific, Essex, UK) were secured onto the slides using DePeX mounting medium (VWR, Leics, UK). Excess mounting solution was removed with a razor blade and slides were cleaned with EtOH before viewing under a standard light microscope (Leica).

2.4.5 In situ hybridisation

The semi-quantitative *in situ* hybridisation (ISH) protocol using radiolabelled oligonucleotide (45mers) probes was similar to that previously described by Wisden & Morris (1994).

2.4.5.1 Oligonucleotide probe design

Oligonucleotide probes (45mers) were designed using the FASTA gene sequences from NCBI (http://www.ncbi.nlm.nih.gov/) for both human and rat genes of interest. Probes were designed to be 45 base-pairs long, have an AT:CG ratio close to 50% with a maximum of 3 bases difference and to have a maximum of 3 consecutive matching bases. Probes were identified that were homologous between rats and human and showed a maximum of 80% homology with other genes of interest. All

designed probes were tested for homology elsewhere in the genome by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and Blat (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start) (Table 2.4). The Blat function of the UCSC website was also used in conjunction with Ensembl (http://www.ensembl.org/index.html) to ensure probes designed to detect all variants of a gene (pan probes) were inclusive of all known transcripts.

Probes were synthesised by Sigma-Aldrich (Dorset, UK) and diluted to 1 µg/µl in phosphate buffer (PB, pH 7) on arrival. Aliquots of working solutions at 5 ng/µl diluted in sterile water were made up of each probe for ease of use and to minimise repetitive thawing or contamination.

Table 2.4 Oligonucleotide probe sequences for ISH including accession n˚ of targeted genes and the complementary nucleotide span to which the probes bind

Gene	Accession n˚	Nucleotide span	Probe sequence
Cacna1c	NM_012517.2	3940 - 3896	3’ - TCGAAGTAGGTGGAGTTGACCACGTACCACAC TTTGTACTGGTGC -5’
BDNFIX	All BDNF transcripts		3’ – CGAACCTTCTGGTCCTCATCCAGCAGCTCTTC GATCACGTGCTCA – 5’

2.4.5.2 Radiolabelling of probes

Probes were labelled using deoxyadenosine 5’- (α-thio) triphosphate [^{35}S] (dATP) (Perkin Elmer, MA, USA) which has a half-life of 87 days.

Each oligonucleotide probe (2 µl) was added to 4.5 µl deionized water, 2.5 µl terminal deoxynucleotidyl transferase buffer (Promega, WI, USA), 1.5 µl terminal

deoxynucleotidyl transferase (Promega, WI, USA) (kept at -20˚C) and 1.5 µl ^{35}S - dATP. The solution was incubated at 35˚C for 1 hr.

After incubation, 38 µl of deionised water was added to the above solution. Qiaquick Nucleotide Removal Kits (Qiagen, Manchester, UK) were used for purification of the probes following the enzymatic reaction. Protocol was conducted as manufacturer's recommendation. Ten volumes of buffer PNI were added to 1 volume of labelled probe and centrifuged for 1 min at 6000 rpm. The spin columns were washed twice with 500 µl of buffer PE for 1 min at 6000 rpm and flow-through discarded. Samples were spun for a further 1 min at 13,000 rpm to remove any residual EtOH. DNA was eluted by applying 50 µl buffer EB directly to the spin column membrane, incubating at RT for 1 min and centrifuging at 13,000 rpm for 1 min.

Once complete 2 µl of the solution was added to 2 ml of scintillation fluid (Perkin Elmer, MA, USA) and activity was measured using a Packard Tri-CARB 2100 TR liquid scintillation counter (Isocount laboratory services) before converting to activity per µl. Labelling was deemed successful if values were between 200,000 and 800,000 cpm.

2.4.5.3 In situ Hybridisation

Hybridisation buffer was made containing; 25 ml 100% deionised formamide, 10 ml 20 x SSC (Saline - Sodium Citrate buffer, containing 3 M NaCl and 0.3 M sodium citrate, PH 7.0), 2.5 ml 0.5 M sodium phosphate (pH 7), 0.5 ml 0.1 M sodium pyrophosphate, 5 ml 50 x Denhardts solution, 2.5 ml 4 mg/ml acid-alkali hydrolysed salmon sperm DNA, 1 ml 5 mg/ml polyadenylic acid, 50 µl 120 mg/ml heparin and 5 g Dextran sulphate (all purchased from Sigma-Aldrich, Dorset, UK). The solution was shaken vigorously and stored overnight at 4˚C until fully dissolved. It was made up to 50 ml with DEPC treated water and stored in foil at 4˚C until needed.

Three slides of brain sections per series per probe were selected for ISH, to provide 2 slides to define Total hybridisation levels and 1 to define the Non-Specific (NS) hybridisation signal. Radiolabelled probes were applied at a level of 200,000 cpm per slide. A master mix for each probe was made up to include; radiolabelled probe, 2 µl of Dithiothreitol (DTT, 1 M) and 100 µl HYB Buffer per slide. This solution was applied to 2 Total slides per series (100 µl per slide). Unlabelled probe was then added at a ratio of 8:1 labelled probe, and 100 µl applied to the remaining NS allocated slides. Parafilm (Sigma-Aldrich, Suffolk, UK) strips were used to spread and cover the solution across all brain sections on the slide and to form the necessary matrix for ISH to occur. All slides were sealed in humidified plastic chambers and incubated at 42˚C overnight.

Parafilm coverslips were removed in 1 x SSC at RT. Slides were then washed in 1 x SSC at 52˚c for 1 hr (2 x 30 min washes) before rinsing in 0.1 x SSC and dehydration in 70% and 95% ethanol (1 min each).

2.4.5.4 Film development

Slides were mounted in Amersham rapid development cassettes (Fisher Scientific, Loughborough, UK), with Carestream Biomax MR film (Anachem, Luton, UK) and a ^{14}C ladder (American Radiolabelled Chemicals, Saint Louis, USA) to allow quantification. The length of exposure depended on the level of expression of the GOI and ranged from 2 to 17 days. Autoradiograms were then developed in a dark room using an automatic developer (Photon Imaging Systems, Swindon, UK).

2.4.5.5 Analysis

Films were scanned using an Epson Perfection V330 flatbed Photo Scanner (Epson, Hertfordshire, UK) at high resolution to allow quantitative analysis of optical density. ImageJ 1.47v software (NIH, USA) was used to convert grey values of regions of interest (ROI) into pixel intensity values by creating a standard curve from the ^{14}C

ladder with known levels of radioactivity. A standard curve was created for each individual film to account for differences in development time.

Five samples were taken from each ROI, from each hemisphere and from each brain slice in the series. This resulted in 20 Total activity values and 10 NS values for each ROI per animal. The total of the NS values was subtracted from the Total activity to provide a Specific activity value. These were averaged across all animals in each condition. All values were normalised to the average of the control group in order to assess percentage change from baseline.

Normalised Specific Value = Control (Total activity – NS activity)/ Condition (Total activity – NS activity) * 100

Control values were also normalised to the average of the control group to determine within group variance.

2.4.5.6 Statistics

For learning regulated expression assays, one-way ANOVAs were conducted to determine the effect of condition on normalised density values. Analysis was separated by region. Where a significant effect was observed post-hoc Dunnett or Bonferroni analyses were conducted. For basal expression comparison for WT and HET animals, student's t-tests were used for each region.

2.4.6 ELISA immunoassays

2.4.6.1 Protein extraction

Approximately 100 µg of tissue was homogenised in 1 ml T-PER (Radio Immunoprecipitation Assay) buffer at RT using a hand borosilicate glass pestle and mortar (Fisher Scientific, Delaware, USA). RIPA buffer was made up of 150 mM sodium chloride with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 50 mM Tris (pH 8) and protease inhibitor treated. One cOmplete™ Mini EDTA – free Protease Inhibitor Cocktail tablet (Sigma-Aldrich,

Dorset, UK) was added per 10 ml of RIPA buffer. The amount of homogenising was kept as consistent as possible between samples. Samples were centrifuged, 12000 rpm for 20 mins at 4˚C. The supernatant was carefully aspirated and stored at -20˚C until required (-80˚C for long-term storage). The pellet was discarded.

2.4.6.2 Protein Assay

A BCA assay (Pierce BCA Protein Assay kit, Thermo Scientific, UK) was used to quantify the amount of protein because it was a method compatible with tissue prepared in T-PER buffer (Desjardins et al., 2009). Seven standards were created with concentrations between 2-0.5 µg/ml using T-PER as a diluent and an additional blank (containing only RIPA). Samples were diluted 10 x in T-PER buffer. Fifty µl was added to 1 ml reagent mix (1 part reagent B with 50 parts reagent A) and incubated at 37˚C for 30 mins. These were quickly cooled to RT and transferred to BRAND UV cuvettes (Sigma, Dorset, UK). Absorbance was measured at 562 nm using a WPA Biowave II UV Spectrophotometer (Biochrom, Cambridge, UK). A standard curve was created from the 7 standards and the equation of the line used to calculate the concentration of each sample. The absorbance of the blank was subtracted from each value to account for absorbance of the diluent.

2.4.6.3 Assay protocol

BlueGene competitive immunoassays for CACNA1C and CACNA1D were purchased from American Research Products Inc. (Massachusetts, USA). Rat BDNF sandwich ELISA Kit was purchased from Thermo Scientific (Delaware, USA). Protocols were conducted exactly to manufacturer's recommendations.

BDNF

All reagents and samples were equilibrated to RT before use. Seven standards were created for a dilution series ranging from 3000 pg/ml to 12.29 pg/ml and one blank to include only the assay diluents supplied. Additional controls were used for T-PER

only to determine any effects of the extraction buffer on the reaction. Samples were diluted 1:10 in extraction buffer to produce protein concentrations predicted to fall within the standard curve and kit detection range. 100 µl of standards, samples and T-PER control were added to wells; all reactions were run in duplicate. The plate was covered and incubated overnight at 4 ºC with gentle shaking. Wells were washed 4 times with 1 x Wash buffer supplied (300 µl per well), with all liquid removed between washes. The plate was inverted and blotted on clean paper towel to ensure complete removal following the fourth wash. 100 µl of biotinylated antibody was added to each well and incubated for 1 hour at RT. This was discarded and the wells washed as above, before adding 100 µl of Streptavidin – HRP solution. Plates were incubated for 45 mins followed by 4 more washes as previously. Finally, 100 µl of TMB Substrate was added to each well and incubated for 30 mins at RT in the dark with gentle shaking, followed by 50 µl of Stop Solution.

CACNA1C and CACNA1D

All kit components were brought to RT before use. Samples were diluted 1:2 in extraction buffer. 100 µl of standards, samples and T-PER control were added to wells with an additional 10 µl of supplied balance solution added to the samples and one set of control wells. 50 µl of HRP enzyme conjugate was added to each well, excluding the control wells, and the plate was covered and incubated at 37 ºC for 1 hour. The plate was washed manually 5 x with 1 x wash buffer, with all liquid aliquoted after each and blotted dry on clean paper towel following the final wash. The plate was covered and incubated for 15 mins at 37 ºC with a substrate for HRP enzyme, with 50 µl of supplied substrate A and substrate B added to each well. A stop solution was added (50 µl) and plates were evaluated within 5 mins.

2.4.6.4 Analysis

Plates were evaluated using a CLARIOstar microplate reader (BMG LABTECH Ltd, Bucks, UK) set at 450 nm and 550 nm. For analysis, 550 nm values were extracted

from 450 nm values to account for any optical imperfections in the plate. A standard curve was produced from the standards, with the equation of the line used to calculate the concentration for each sample. Values were multiplied by dilution ratio and normalised to sample protein concentration.

3 BASAL AND ACTIVITY-REGULATED EXPRESSION OF *CACNA1C*

3.1 Introduction

Changes in levels of gene expression in relation to certain behaviours or cognitive processes, strongly implicate those genes in the molecular processes underlying them. Long term potentiation and the synaptic plasticity underlying long-term memory formation require de novo gene transcription as well as protein synthesis (Impey et al., 1996; Hall et al., 2000; Nader et al., 2000). Previous research in contextual fear conditioning has shown changes in the expression of specific genes during different aspects of associative learning; identifying separable molecular signatures of distinct memory processes to include consolidation, recall and extinction (Hall et al., 2000; Lee et al., 2004; Kirtley and Thomas, 2010; Mizuno et al., 2012. For review see Quirk and Mueller, 2008).

Contextual fear conditioning (CFC) is a hippocampal dependent, single trial, associative learning task. It relies on the implicit formation of an association between an aversive stimulus (footshock) and a novel context. This results in a conditioned response (freezing behaviour) during subsequent exposures to the context in the absence of the footshock, indicative of contextual fear memory (CFM). Learning is rapid, requiring only a single association trial, and is robustly expressed when tested weeks later. This memory can be manipulated by prolonged re-exposure to the context which reduces conditioned freezing behaviour through the extinction of CFM. The implicit nature of this task further limits the confounds of motivation, as the recall of associations and resulting behaviour is seemingly automatic, based on a natural response to fear of discomfort. There is no need for pre-training or any additional manipulations to promote behaviour (e.g. hunger for food), which may differ dramatically between animal and across sessions. This allows the investigation of

potentially separable mechanisms underlying consolidation, recall and the extinction of a CFM in the same animals in single sessions.

Previous studies have focussed on regulated expression of immediate early genes Activity-Regulated Cytoskeletal-Associated Protein (*Arc*) and *Zif268* and neurotrophic factor *BDNF*. *Arc* has been found to be rapidly increased in the hippocampus following CFC, with expression changes evident from 15 mins (Czerniawski et al., 2011) and protein levels peaking at 6 hrs post training (Lee et al., 2004), though increases are also associated with exploration of a novel context (Guzowski et al., 1999). Re-exposure to a previously conditioned context for 3 mins has been found to lead to a similar increase in *Arc* expression in the CA1 and CA3 regions of the hippocampus, measured 90 mins after recall (Mamiya et al., 2009). In the same study mice that were re-exposed to the context for a 10 min extinction session did not exhibit increased *Arc* at the same time point, suggesting *Arc* is specifically required following recall of CFM.

The transcription of *Arc* is known to be regulated by *BDNF* (Ying et al., 2002). Similar profiles of *BDNF* expression are observed following CFC, with increases observed in the CA1 region of the hippocampus from 30 mins, returning to baseline at 24 hrs (Hall et al., 2000; Lubin et al., 2008). Furthermore, independent expression profiles have been found for different transcripts of *BDNF* suggesting more finely regulated transcription (Mizuno et al., 2012). Following re-exposure to the context for a short recall session, *BDNF* expression is significantly elevated at 30 mins, though this increase is not evident following a prolonged extinction session (Kirtley and Thomas, 2010). It has been proposed that this increased expression of *BDNF* (and the associated increase in *Arc*) is acting to constrain extinction learning (Kirtley and Thomas, 2010; Trent et al., 2015) and that the lower levels observed following prolonged recall indicate a removal of that constraint.

In contrast, *Zif268* has been found to be increased in the CA1 and CA3 regions of the hippocampus, 30 mins after exposure to a novel context alone; without an additional increase following experience of a footshock or the formation of an aversive association (Hall et al., 2000). This suggests this gene may play a more vital role in the formation of context representations rather than CFM, emphasising the need for novelty controls in these single trial experiments. Re-exposure to a trained context for 2 mins and 10 mins elicits a similar increase in expression 30 mins later in both conditions (Kirtley and Thomas, 2010). This suggests distinct roles for *Zif268* and *BDNF* in the processes of recall and extinction; with *Zif268* hypothesised to promote the persistence of CS-US associations, and *BDNF* more specifically providing a molecular switch between maintenance of CFM and its extinction (Kirtley and Thomas, 2010).

These different profiles of expression indicate separable molecular processes underlying different aspects of learning. Evidence supports a role for *BDNF* and *Arc* in the consolidation of CFM, with a potentially more complicated balance between *Arc, BDNF* and *Zif268* in its recall and extinction. It has also been observed that there may be a second phase of transcription (as well as protein synthesis) necessary for the consolidation of CFM (Igaz et al., 2002). There is evidence of increases of transcripts of *BDNF* 24 hrs after CFC (Mizuno et al., 2012) and there has been a second peak in ARC protein observed at 24 hrs, though no change in *Zif268* (Lonergan et al., 2010). This further highlights the importance of time-course expression profiles to distinguish the role of certain genes.

The transcription of these genes is mediated by the phosphorylation of CREB which is induced by calcium influx into the cell following synaptic activation (Sheng et al., 1991). There are multiple routes for calcium to enter the cell, including glutamate receptors, N-methyl-D-aspartate receptors (NMDA-R), AMPA-R and mGluR, as well as LVGCCs. Expression of subunits of NMDA-R and mGluR has been investigated

following LTP, though there is limited research into behaviourally induced expression changes of these channels in the hippocampus following CFC. Thomas et al., (1996), found increased NR2B and mGluR1c expression, evident from 24 hrs after the induction of LTP in dentate gyrus granule cells. These findings, in conjunction with evidence for two critical periods of mRNA transcription for consolidation, indicate the importance of investigating delayed expression to a minimum of 24 hrs in *Cacna1c*.

Cacna1c codes for the alpha-1 subunit of $Ca_v1.2$ calcium channels. These channels are known to be necessary for an NMDA-R independent memory formation (Moosmang et al., 2005) and calcium influx through these channels has been shown to mediate synaptic regulation of gene transcription (Murphy et al., 1991; Zheng et al., 2011). *BDNF has* additionally been found to be preferentially regulated by calcium influx through LVGCCs compared to NMDA-R (Ghosh et al., 1994; West et al., 2001). Although functionally implicated in certain types of learning and memory, there has been no investigation into potential effects of associative learning on expression levels of *Cacna1c*. Changes in expression may indicate a role for this gene in specific aspects of learning and may provide a link to already established molecular signatures of learning in rapidly transcribed genes, including *BDNF*.

The following experiments used *in situ* hybridisation techniques to quantify the baseline and relative expression of *Cacna1c* following specific aspects of associative learning in sub-regions of the hippocampus. The basal expression profile of *Cacna1c,* as well as CACNA1C protein is initially presented. MRNA was then assessed at different time points following CFC to establish an expression profile linked to consolidation of CFM; from 2 hrs to 24 hrs post conditioning. Expression was also measured following exposure to a novel context and a latent inhibition paradigm to separate out expression changes specifically related to consolidation, rather than novelty or experience of an aversive stimuli. Secondly, comparisons were conducted between expression following short and long recall sessions, the latter

designed to induce extinction of CFM. Experiments aimed to determine whether *Cacna1c* may have a specific role in distinct processes of associative learning.

3.2 Methods

3.2.1 Animals

Sixty-four male Lister Hooded rats (250 – 300 g) were housed as previously described on a reverse light-dark cycle with ad libitum access to food and water. Experiments were conducted in the dark period. Animals were sacrificed in home cages using a rising concentration of CO_z at specific time points following each behaviour of interest.

3.2.2 Behavioural procedure

3.2.2.1 Apparatus

For full description of the apparatus and paradigm used, see General Methods 2.3.1. Contextual fear conditioning took place in a rat conditioning box with a metal grid floor (Standard modular test chamber for rat, Med Associated Inc., Vermont, USA). Cages were cleaned with a 50% ethanol solution before and after each session, which also gave a mild background odour. Animals were taken from their home cages to the procedure room in large transport boxes. All animals in transport box A were placed in conditioning box A and animals in transport box B in conditioning box B. Digital recording commenced prior to the animal being placed in the box to ensure full recording of the trial.

3.2.2.2 Behaviour

For contextual fear conditioning, animals were placed into the novel conditioning chamber for 2 mins prior to receiving a 0.5 mA scrambled footshock for 2 secs. They remained in the chamber for an additional 1 min before being returned to their home cages. A 'Novelty' group was exposed to the context for 2 mins without receiving a footshock and were returned to home cages. Conditioned animals were sacrificed 2, 4, 8 and 24 hrs later. Naïve home cage litter mates were sacrificed at the same time. 'Novelty' animals were sacrificed 4 hrs after exposure.

A separate group of animals was pre-exposed to the context for 8 hrs before receiving the footshock to induce a latent inhibition effect (Holland and Bouton, 1999) and control for the experience of the footshock in the absence of an association formed with the context. A separate 'Nov/LI' group was used to control for the prolonged exposure to the context, spending 8 hrs in the box without a subsequent footshock before being returned to their home cage. Both groups were sacrificed 4 hrs after return to their home cages. N = 6 for all groups, except 2 and 8 hrs following CFC (n = 4).

For the separate investigation of recall and extinction. Eighteen animals underwent CFC as described. Forty-eight hrs later 12 of the animals were returned to the conditioning context; 6 for 2 mins and 6 for 10 mins. The 2 min exposure is designed to measure recall of the association without affecting CFM, whereas the prolonged re-exposure leads to extinction of CFM indicated by reduced freezing. The additional 6 animals were used as a 'No recall' control group and were sacrificed along with their cage mates 2 hrs after re-exposure to the context.

3.2.2.3 Analysis

Freezing behaviour, defined as complete immobility except for respiration for 1 sec, was manually scored blind to condition from digital recording. A visual decision on freezing was made every 10 seconds throughout each session, with number of instances divided by total number of bins to provide a % freezing for each animal (as done previously; Barnes and Thomas, 2008; Trent et al., 2015). Scores were checked for reliability by a minimum of 2 scorers. Scores were taken for each rat during the first 2 mins of exposure to the context (Pre-US) to check basal levels of freezing behaviour which may indicate anxiety. A second score was taken following the footshock (post-US) in animals that underwent conditioning, to measure acquisition of the association between shock and context and to ensure the latent inhibition manipulation produced the expected reduction in freezing behaviour. In animals that

underwent a recall session, scoring was conducted as above to establish successful recall of CFM and to confirm reduced freezing during extinction training. Scores were averaged within experimental group. Paired samples student's t-tests were conducted to determine whether freezing increased post-US compared to pre-US in groups designed to acquire CFM and also to determine decreased freezing in the prolonged extinction group (first 2 mins compared to the last 2 mins of the session). Repeated measures ANOVAs were used to compare freezing levels pre and post-US between LI and CFC groups as well as maintenance of freezing levels in recall and extinction groups. Levene's tests for equality of variances were conducted for all variables.

3.2.3 *In situ* hybridisation

3.2.3.1 Sectioning and hybridisation

For detailed methods see General Methods 2.4.6. Brains were removed immediately post-mortem, fresh frozen on dry-ice and stored at – 80°C. Coronal sections 14 µm thick were sliced from each brain through the hippocampus, mounted on poly-L-lysine coated microscope slides and air-dried at RT. Series were created to include one slice from each experimental condition. Slides were fixed in 4 % PFA solution followed by a 1 min wash in 1 x PBS and dehydration in 70% EtOH. All slides were stored at 4°C in 95% EtOH until required.

For hybridisation, 3 slides were selected from each series to produce 2 "total" slides and 1 "non-specific" control. A master mix was made to include hybridisation buffer, DTT and deoxyadenosine 5'- (α-thio) triphosphate [^{35}S] (dATP) labelled oligonucleotide probe specific to *Cacna1c*. 100 µl of master mix was added to each "total" slide and covered with Parafilm. Unlabelled probe was added to the master mix at a ratio of 8:1 before being added to the "non-specific" slides for each series. Slides were incubated overnight at 42°C. Parafilm covers were removed in 1 x SSC at RT, followed by 2 x 30 min washes in 1 x SSC at 52°C. Slides underwent a final

wash for 1 min in 0.1 x SSC at RT and were dehydrated in 70% and 95% EtOH and left to air dry. All slides were mounted together in a rapid development cassette for 7 days with Biomax MR film and a ^{14}C ladder for quantification. Autoradiograms were developed using an automatic developer.

3.2.3.2 Analysis

Films were analysed using ImageJ software (NIH). Values were converted using a standard curve calculated from the ^{14}C ladder. Measurements were taken from each region of interest and average across the 2 "total" slides. Values from each "non-specific" slide were subtracted before values were averaged within each experimental condition and normalised to Naïve. One-way ANOVAs were conducted to determine the effect of condition on normalised density values. Post-hoc Bonferroni tests were conducted where ANOVAs were significant. Tests of normality and equality of variances were conducted throughout and corrected where necessary.

3.2.4 ELISA immunoassays

3.2.4.1 Assay protocol

For detailed methods see General Methods 2.4.8. Protein was extracted from PFC, cerebellum and DG/CA3 region of the hippocampus in 1 ml T-PER extraction buffer. Protein concentration was measured using a BCA assay. A BlueGene competitive immunoassay for CACNA1C was used and protocols conducted exactly to manufacturer's guidelines. Wells were incubated with 100 µl of sample, a balance solution and HRP enzyme conjugate at 37°C for 1 hr. After washing, wells were incubated with a substrate for HRP enzyme for 15 mins before a stop solution was added. Plates were analysed immediately using a CLARIOstar microplate reader at 450 nm.

3.2.4.2 Analysis

A standard curve was produced from 6 standards and used to convert values to protein concentrations. For presentation, concentrations were normalised to PFC.

3.3 Results

3.3.1 Basal expression of *Cacna1c*

ISH and ELISA immunoassays were conducted to determine the basal expression profile of *Cacna1c.* Basal levels of expression were compared between the PFC, cerebellum and hippocampus (Figure 3.1). Transcripts levels were highest in the dentate gyrus (DG) sub-region of the hippocampus, followed by the CA3 and the granule layer of the cerebellum. Expression in the PFC was highest in the medial PFC. Protein levels similarly were highest in the DG/CA3, with equal levels of CACNA1C observed in the PFC and cerebellum (Figure 3.2).

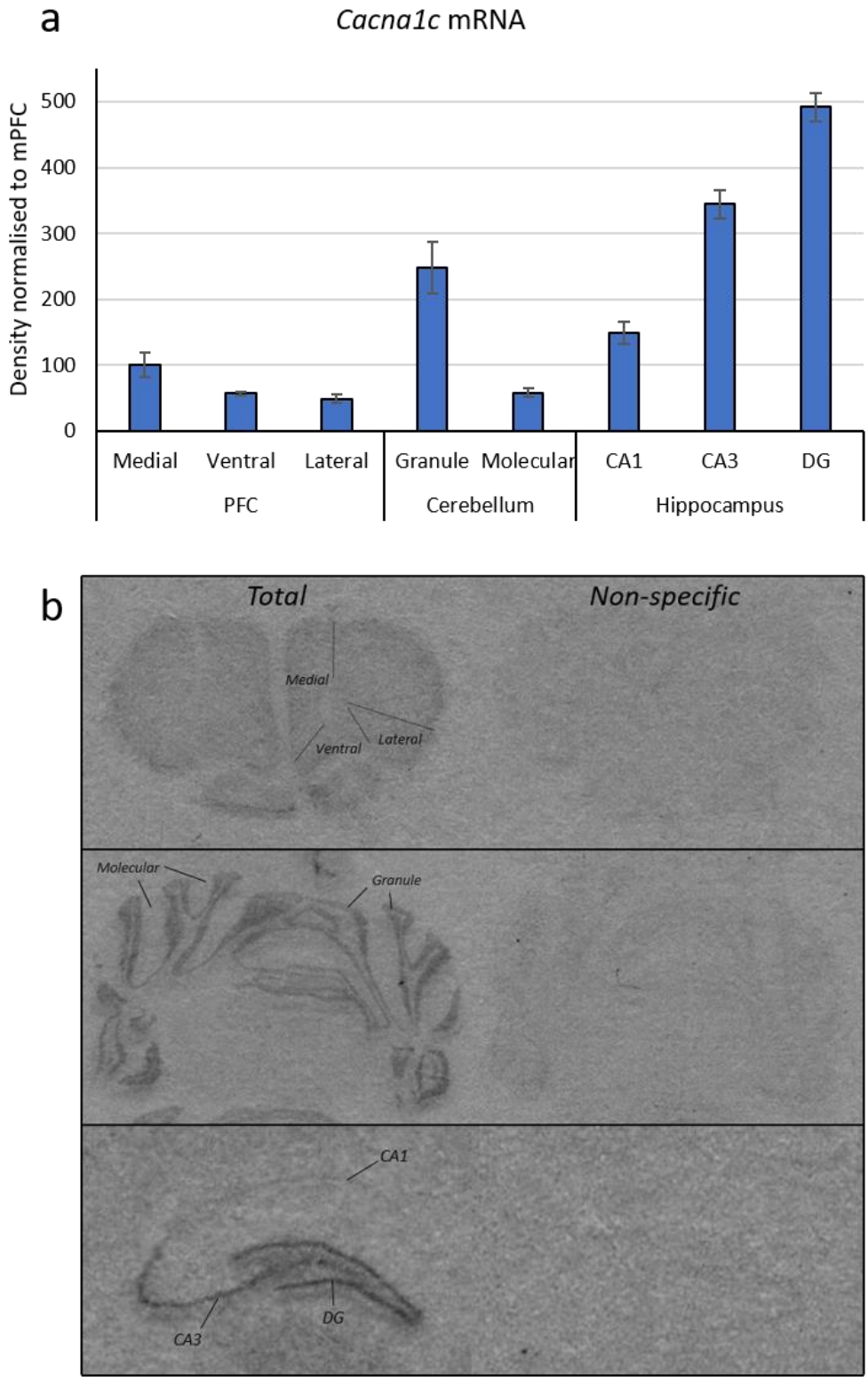

Figure 3.1 Basal expression of Cacna1c mRNA. a) Quantification of ISH density values, normalised to medial PFC. Greatest expression observed in the dentate gyrus. Bars represent mean density values. Error bars are +/- SEM. n = 4 b) Representative ISH autoradiogram images of Cacna1c expression in the PFC, cerebellum and hippocampus. Total binding of probe is shown on the left hand side and non-specific binding on the right. Specific binding values were calculated by subtracting 'non-specific' from 'total'.

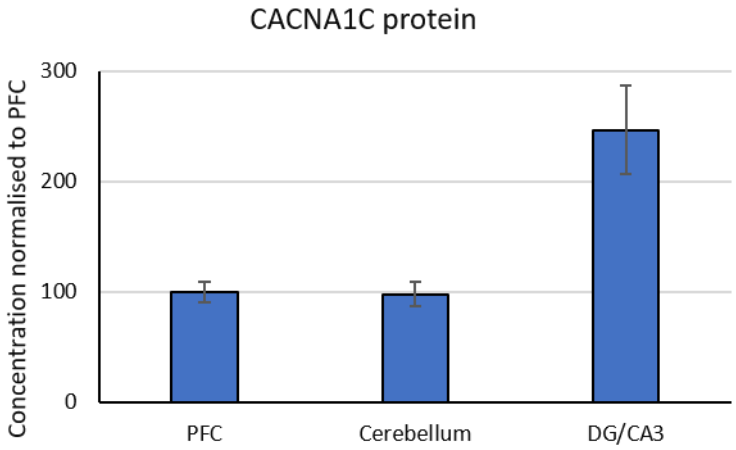

Figure 3.2 Basal expression of CACNA1C protein. Protein levels were highest in the DG/CA3 region of the hippocampus and similar in the PFC and cerebellum. Bars represent protein levels normalised to PFC. Error bars are +/- SEM. n = 6.

3.3.2 Expression of *Cacna1c* following consolidation of CFM

The expression of *Cacna1c* was compared in animals at 2, 4, 8 and 24 hrs after CFC and naïve home-cage controls, to determine whether expression was related to the consolidation of CFM. Expression was also compared in animals 2 hrs after exposure to the novel context for 2 mins (Novelty) or 8 hrs (Nov/LI), as well as a latent inhibition (LI) group that received CFC training following 8 hrs exposure to the context. These additional groups allowed comparisons which controlled for exposure to a novel context (brief and prolonged) and the experience of a footshock in the absence of association formation.

3.3.2.1 Conditioned animals exhibited the expected increase in freezing behaviour

Freezing behaviour was scored for all animals that were exposed to the training context (Figure 3.3). There were no differences in freezing behaviour across groups during the first 2 mins in the context ($F(3,20) = 1.990$, $P = 0.148$). In those animals that received a footshock (CFC and LI groups), freezing behaviour was scored 2 mins before the foot shock (Pre-US) and during the 1 min afterwards (Post-US). There was a significant interaction between test-phase and condition ($F(1,10) = 34.757$, $P < 0.001$, $\eta_p^2 = .777$). Animals in the CFC group exhibited significantly greater freezing following the footshock compared to those animals that experience prolonged pre-exposure to the context (LI) ($F(1,10) = 25.879$, $P < 0.001$, $\eta_p^2 = .721$), indicating successful acquisition of CFM.

3.3.2.2 Reduced expression of Cacna1c following exposure to a novel context

There was a significant effect of control condition on the expression of *Cacna1c* ($F(3,60) = 5.249$, $P = 0.003$), with reduced expression observed in the Novelty group compared to naïve animals ($P = 0.033$, Dunnett's 2-sided test) (Figure 3.4). There was no effect of region ($F(2,60) = 1.059$, $P = 0.353$) and no evidence of an interaction ($F(6,60) = 0.249$, $P = 0.958$). Significant changes in individual regions were observed in the CA1 ($t(10) = 2.933$, $P = 0.015$).

3.3.2.3 *Increased expression of Cacna1c 24 hrs after CFC*

Expression of *Cacna1c* differed at specific time points following CFC when compared with Novelty controls ($F(4,21) = 3.339$, $P = 0.029$). Post-hoc Bonferroni tests revealed significantly increased expression at 24 hrs post conditioning compared to Novelty controls ($P = 0.017$). Differences between Novelty and 24 hrs were found to be significant in the DG ($F(4,21) = 3.434$, $P = 0.026$) (Figure 3.4). There was no effect of CFC on expression in the CA1 or CA3 regions of the hippocampus independently ($F(4,21) = 0.149$, $P = 0.961$ and $F(4,21) = 0.666$, $P = 0.622$, respectively); though there was not a significant effect of region ($F(2,42) = 0.384$, $P = 0.684$) or a condition by region interaction ($F(8,42) = 0.768$, $P = 0.633$) to confirm region specific effects. There were no differences observed between expression 24 hrs following CFC and LI ($F(1,30) = 0.647$, $P = 0.427$).

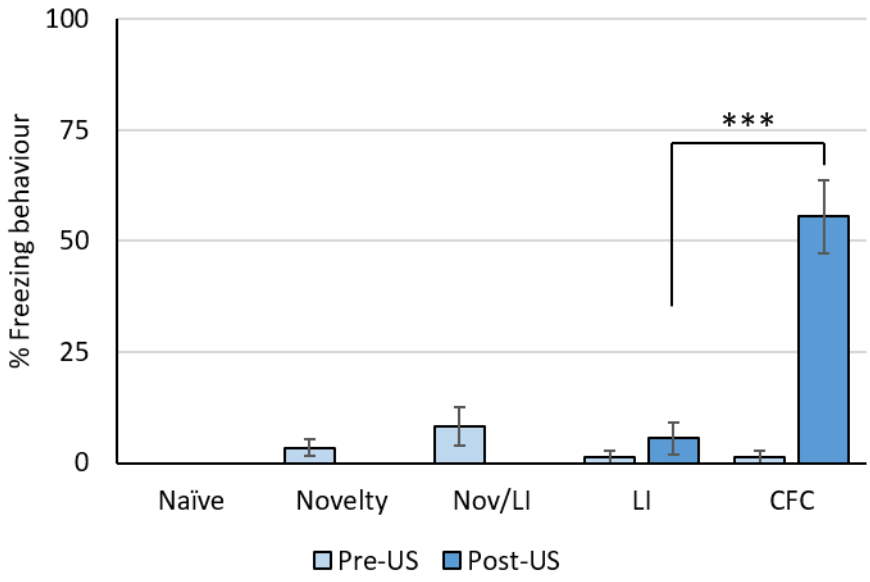

*Figure 3.3 Freezing behaviour by condition for Pre and Post US. % Freezing behaviour was scored for each group that was exposed to the context for the first 2 mins. Animals that experienced the footshock were scored for the 1 min following. Freezing behaviour in the CFC group was significantly greater than in the LI group that underwent prolonged pre-exposure to the context. Bars represent average freezing behaviour for each group at each phase. Error bars are SEM. *** P < 0.001.*

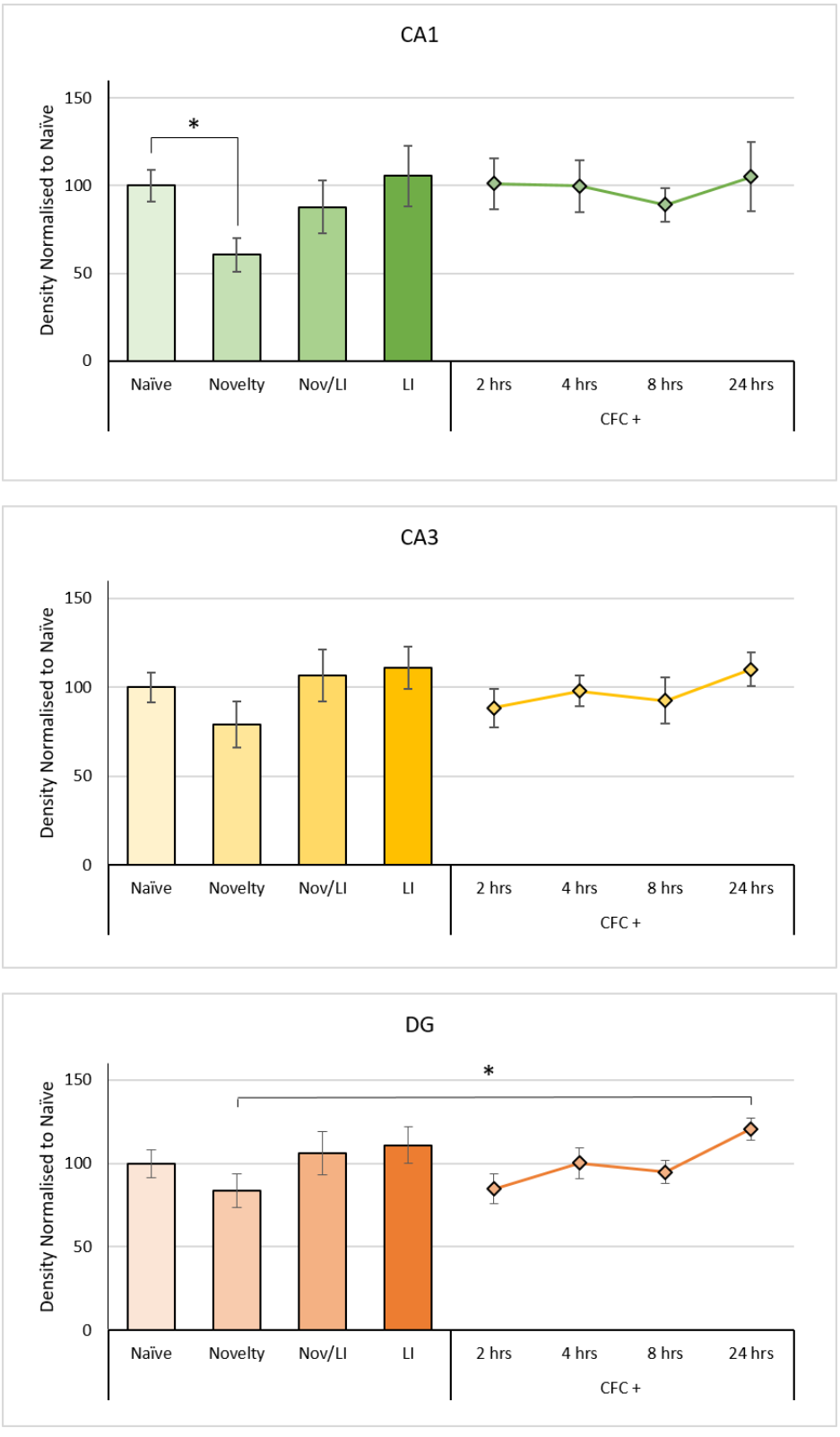

*Figure 3.4 Expression of Cacna1c in CA1, CA3 and DG sub-regions of the hippocampus following different learning experiences. There was a significant reduction in expression following exposure to novel context compared to naïve animals (P = 0.003). Expression following CFC showed an increase at 24 hrs compared to Novelty (P = 0.029). Bars represent mean optical density normalised to Naïve control. Error bars are +/- SEM. n = 6 for all groups except 2 and 8 hrs (n = 4). * P < 0.05*

3.3.3 Expression of *Cacna1c* following recall and extinction of CFM

A separate cohort of animals all underwent CFC. Comparisons were made between expression in animals that were re-exposed to the training context 48 hrs later for either a 2 min recall session or a prolonged 10 min extinction session and animals that were not re-exposed to the context.

3.3.3.1 Prolonged exposure to the conditioned context results in reduced freezing behaviour

All animals successfully acquired CFM, indicated by increased freezing following the footshock compared to the 2 mins before ($t(17) = -16.898$, $P < 0.001$, $\eta_p^2 = .944$).

Following CFC animals re-exposed to the trained context exhibited high levels of freezing indicative of successful recall of CFM (Figure 3.5) There were no differences observed between freezing behaviour during the 1 min following the footshock during CFC and the first 2 mins of recall ($F(1,10) = 0.059$, $P = 0.813$) with no differences between recall or extinction groups ($F(1,10) = 4.543$, $P = 0.059$) and no evidence of an interaction ($F(1,10) = 0.237$, $P = 0.637$).

The prolonged 10 min exposure to the context resulted in reduced levels of freezing indicative of the extinction of CFM. Freezing levels were significantly reduced in the last 2 mins of the session compared to the first ($t(5) = 9.690$, $P < 0.001$).

3.3.3.2 Reduced expression of Cacna1c following recall in the CA1

Cacna1c expression differed following recall and extinction in the CA1 region of the hippocampus (Figure 3.6: $F(2,15) = 4.628$, $P = 0.027$). Post-hoc Bonferroni tests revealed a significantly reduced expression in animals that underwent the short 2 min recall session compared to those that experienced the 10 min extinction session ($P = 0.029$). Neither group differed significantly from the 'No Recall' group.

There were no differences in expression in the CA3 or DG region of the hippocampus between any of the 3 groups ($F(2,15) = 0.358$, $P = 0.705$ and ($f(2,15) = 0.196$, $P = 0.824$, respectively).

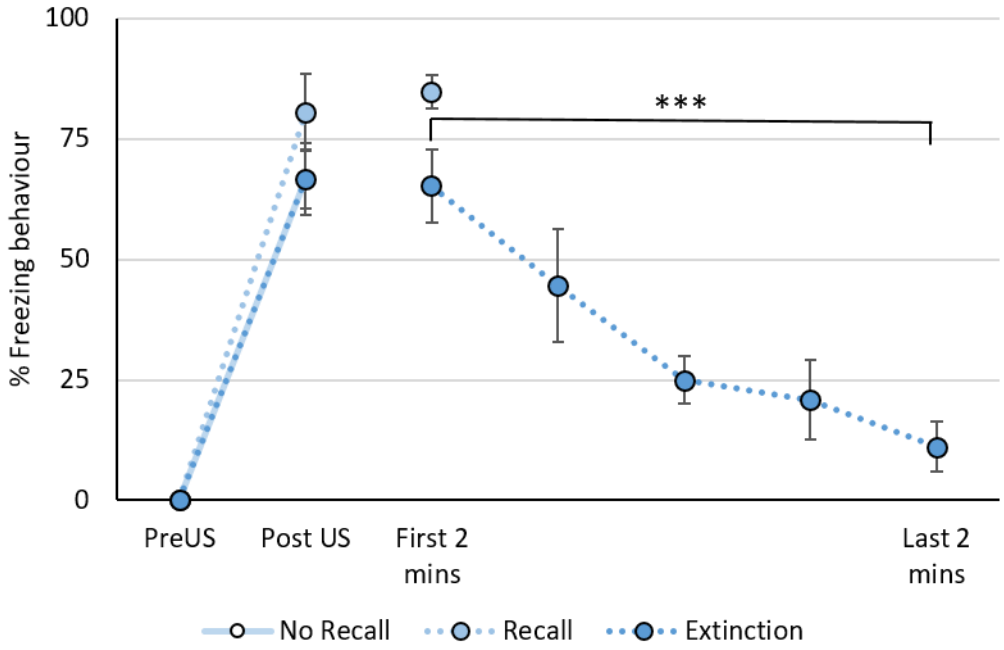

*Figure 3.5 Freezing behaviour in 'No recall', 'Recall' and 'Extinction' groups. PreUS: 2 mins prior to footshock, PostUS: 1 min following footshock. First 2 mins and Last 2 mins refer to recall session. Behaviour was scored in 2 min bins during the prolonged extinction phase to indicate within-session extinction. There was a significant increase in freezing following US presentation in all groups ($t(17) = -16.898$, $P =< 0.001$) indicating acquisition of CFM and significantly reduced freezing following prolonged re-exposure ($t(5) = 9.690$, $P < 0.001$), indicative of its extinction. Data points represent the average % freezing for each group during each testing phase. Error bars are SEM. *** $P < 0.001$*

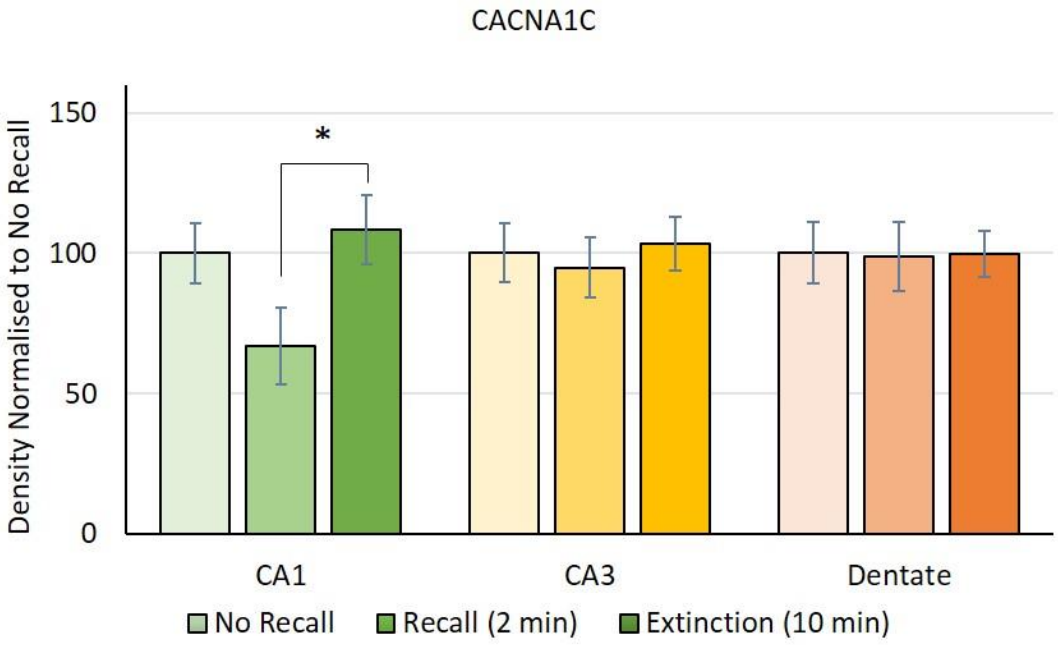

Figure 3.6 Expression of Cacna1c 2 hrs following a 2 min recall session or a 10 min recall session, normalised to animals that underwent no recall. There was a significant reduction in expression in CA1 region of the hippocampus following the 2 min recall session compared to the 10 min extinction session (P < 0.05 Bonferroni corrected).*

3.4 Discussion

The expression of *Cacna1c* was investigated following specific aspects of a hippocampal dependent contextual fear conditioning task. Of the regions that were investigated, basal expression of *Cacna1c* was found to be highest in the DG region of the hippocampus, followed by the CA3 and the granule layer of the cerebellum. The lowest levels were observed in the CA1, molecular layer of the cerebellum and the medial PFC. *In situ* hybridisation revealed decreased expression following exposure to a novel and a conditioned context, as well as increased expression 24 hrs following CFC.

3.4.1 Delayed regulated expression of *Cacna1c* in the dentate gyrus following CFC

There was an increase in expression of *Cacna1c* 24 hrs following CFC, compared to both novelty controls. Changes were significant in the dentate gyrus region of the hippocampus, with no effects observed at earlier time points investigated. This indicates that the expression of *Cacna1c* is related to the consolidation of CFM. The expression profiles of IEGs, with much earlier increases, indicate that the increase observed is unlikely to drive the initial changes in their expression, though it may be a consequence or compensatory response.

This increase in expression 24 hrs following CFC may indicate a role for $Ca_v1.2$ channels in a structural alteration in the synapse as a result of plasticity. Previous studies have found delayed upregulation of subunits of glutamate receptors in the dentate gyrus following the induction of LTP in hippocampus (Thomas et al., 1994, 1996). Thomas et al., (1996) found an increase in the NR2B subunit of NMDA receptors evident from 24 hrs, peaking at 48 hrs, along with increases in mGluR1c which only became evident at 96 hrs following induction of LTP. It was proposed that these late-phase profiles of expression may relate to cascades of events that are required for the maintenance of LTP and that increased glutamate receptors maybe reflect a switch from silent synapses to functional ones. Persistence of long-term

memory has also found to be related to delayed protein synthesis of BDNF at around 12 hrs following CFC (Bekinschtein et al., 2007). The increase of expression in *Cacna1c* observed here may indicate a delayed role in consolidation and maintenance of CFM. Alternatively, increased expression may be part of a structural consequence of synaptic plasticity induced by CFC as alluded to by Thomas et al, (1996). With the changes observed being greatest in the dentate gyrus, it is possible that this increase in *Cacna1c* in relation to learning could be associated with neurogenesis; a process known to be required for contextual fear conditioning (Saxe et al., 2006).

3.4.2 Selective reduced expression of *Cacna1c* following novelty exposure and recall

Results further indicated a reduction of *Cacna1c* following the exposure to both a novel context for 2 mins and a previously trained context during recall. Different expression levels of *Cacna1c* were observed following recall and extinction of CFM. Research has established that the process of extinction requires active learning of a CS – no US association rather than the degradation of the previous memory (Myers and Davis, 2002). In contrast, the process of recall involves short exposure to the previously trained context and has been postulated to involve molecular mechanisms which constrain extinction rather than an active re-consolidation of CFM (Trent et al., 2015). The reduced expression of *Cacna1c* following recall could be part of this active constrain on extinction, or could potentially reduce the activation of downstream mechanisms involved in extinction; further experiments would be necessary to investigate this fully.

These results indicate changes in the expression of *Cacna1c* that are related to the formation of CFM. Although relative levels of expression of *Cacna1c* do not appear to drive the increases in downstream signalling genes observed for specific aspects of learning in CFC, they may be relevant for persistence of CFM. Observing changes in expression of a gene is one way to establish its role in a particular

psychological process. However, the lack of early changes in expression does not confirm that this gene or related protein is not functionally related to the changes in IEG expression previously observed. CACNA1C forms the alpha-1 subunit of $Ca_v1.2$ channels and controls the voltage sensitivity of the channel pore. Calcium influx through these channels is known to regulate immediate early genes implicated in these processes and has been specifically found to regulate the transcription of *BDNF* (Ghosh et al., 1994; Zheng et al., 2012). Finely regulated calcium influx through these channels may therefore play a role in a subset of these processes, without an accompanying initial change in expression levels.

3.4.3 Limitations and future directions

It is worth noting that expression was only investigated in the hippocampus for this study and that changes may exist in different areas of the brain. The expression of *Arc* has previously been found to be upregulated in the amygdala and the hippocampus following short re-exposure to a trained context, whereas upregulation was observed in the amygdala and medial PFC following extinction (Mamiya et al., 2009). It is also possible that there may be transcript specific regulation, as has been observed in *BDNF* (Lubin et al., 2008). However, with multiple unconfirmed predicted transcripts coded by the *Cacan1c* gene (with over 40 coded by the human gene), this is difficult to discern at present.

This experiment was designed to establish whether changes in expression of *Cacna1c* were associated with consolidation, recall and extinction, potentially relating to those observed with IEGs. The time points selected therefore reflected the critical periods in which previous evidence has implicated expression changes in these processes. The observation of increased expression at 24 hrs following CFC in the DG would require additional timed comparison groups for novelty and latent inhibition to conclude that this is related specifically to a mechanism involved in the formation or persistence of CFM. It would also be pertinent to extend the time points out further

to establish when expression may return to baseline, as well as explore a complete time-course following recall.

Given the minimal changes in *Cacna1c* expression observed and previous findings regarding the role for calcium influx through LVGCCs in learning (Davis and Bauer, 2012), it would be logical to investigate whether there are separable roles for calcium influx through LVGCCs in the hippocampus in specific aspects of CFM processing.

3.5 Conclusion

Increases in the expression of *Cacna1c* following CFC suggest a role for the expression of this gene in the consolidation of CFM. Although expression changes do not seem to reflect changes observed in IEGs, the delayed increase may reflect a role for the expression of *Cacna1c* in the persistence of associative learning. These results further indicate a reduction in expression of *Cacna1c* following exposure to a context in the absence of new fear learning. It is likely that any distinction in the role of *Cacna1c* earlier in these aspects of learning will be revealed by investigating the functional regulation of calcium influx through LVGCCs.

4 INHIBITION OF L-TYPE VOLTAGE GATED CHANNELS WITH DILTIAZEM: EFFECTS ON ASSOCIATIVE LEARNING

4.1 Introduction

Calcium influx through L-type voltage gated calcium channels (LVGCCs) is known to be necessary for certain forms of NMDA receptor independent LTP and learning (Moosmang et al., 2005). Selective pharmacological inhibitors of these channels have been developed, which block or enhance this calcium flow into the cell, while leaving other routes unaffected. This provides the opportunity to investigate the effect of acute functional inhibition of LVGCCs on specific aspects of behaviour.

4.1.1 L-type calcium channel inhibitors

There are 3 main classes of LVGGC inhibitors currently available for clinical use; phenylalkylamines, dihydropyridines and benzothiazepines. These bind to three separate receptor sites on the alpha 1 subunit of the L-type calcium channels, which are allosterically linked (Hockerman et al., 1997). They are highly selective for L-type channels over other types of VGCC, however there is evidence of binding to other sodium and potassium channels at higher doses. Both $Ca_v1.2$ and $Ca_v1.3$ channels are affected by these inhibitors and due to their high homology there is currently no selective inhibitor for $Ca_v1.2$ channels.

Experimental investigation implicates the IIIS5, IIIS6 and IVS6 transmembrane segments of the α1 subunit in binding all three classes of these drugs. Individual amino acids have been identified which are necessary for high affinity binding of each type. Mutation of these specific amino acids leads to a reduction in inhibitor affinity to the level of that observed with other classes of calcium channels. The binding

regions for each drug class are in close proximity, with potential overlap in necessary residues. Because of this close proximity, it is suggested that phenylalkylamines and dihydropyridines bind to different faces of the IIIS6 and IVS6 transmembrane segments. L-type calcium channel affinity to all of the classes of drug are state and membrane potential dependent, with greater affinity to inactivated and open state channels.

Phenylalkylamines:

These inhibitors are thought to access calcium channels from the intracellular side, though evidence is mixed and may depend on cell-type. They inhibit L-type calcium currents by blocking channels directly through obstructing the transmembrane pore.

The binding site for phenylalkylamines is allosterically linked to that for benzothiazepines and dihydropyridines, in that it's binding will inhibit the binding of the other two. Although L-type calcium channels are most sensitive to phenylalkylamines, other calcium channels as well as some sodium and potassium channels have been found to be blocked by the drug verapamil at higher concentrations (Freeze et al., 2006) and affinity is influenced by channel state. It has a half-life of 2.8-7.4 hours and is soluble in water (70 mg/ml)

Dihydropyridines:

Dihydropyridines are calcium channel modulators that may act as channel agonists or antagonists depending on drug structure, membrane potential and the frequency of stimulation. Nifedipine and Nimodipine are dihydropyridine calcium channel blockers that target primarily L-type calcium channels. Unlike Verapamil they do not directly block the transmembrane pore, but bind to a site at which agonists increase calcium channel activity and antagonists decrease it. Nifedipine is highly insoluble in water and so for experimental purposes is usually dissolved in high concentrations of dimethyl sulfoxide (DMSO), which has been suggested to cause degeneration in the developing brain (Hanslick et al., 2009). It has a short half-life of 2 hours.

4.1.1.1 Benzothiazepines:

Research suggests that benzothiazepines are intermediate between dihydropyridines and phenylalkylamines in terms of blocking properties. Diltiazem is one of the main benzothiazepines used commercially. It is found to produce more tonic block than Verapamil but less than Nifedipine and more frequency dependent block than Nifedipine but less than Verapamil (Herzig et al., 1992). Although selective for L-type calcium channels, it will block other calcium channel types at much higher doses. Inhibition occurs via binding to an extracellular domain. It has a half-life of between 3 and 5 hours and is soluble in water (50mg/ml), making it more desirable for infusion into the brain compared to classes requiring alternative solvents.

4.1.2 Previous findings

All three classes of inhibitors have previously been used to study the role of LVGCCs in associative learning, primarily in mice. Both systemic administration and local infusion of the drugs have been investigated to determine effects on specific aspects of learning, however results are mixed and conclusions are incomplete.

4.1.2.1 Acquisition

In the context of fear conditioning, acquisition involves learning an association of a conditioned stimulus (CS) such as a tone or context with an aversive unconditioned stimulus (US) such as a mild foot shock. This aversive learned association is robust and rapidly acquired. Inhibition of L-type calcium channels has been found to not affect the acquisition or expression of fear conditioning. Cain et al., (2002), used systemic injection of two dihydropyridine LVGCC inhibitors (Nifedipine and Nimodipine) to test the role of LVGCCs in acquisition, expression and extinction of conditioned fear in mice. They found that acquisition was not affected by inhibition with 40mg/kg doses of Nifedipine at either 20 minutes or 50 minutes prior to conditioning. This suggests that neither the sensitivity to the foot shock or the processing of the US is impaired by inhibition of LVGCCs when inhibitors are

administered systemically. Local infusion of inhibitors into the amygdala similarly report no effects on the acquisition of CFM (e.g. Bauer et al., 2002).

4.1.2.2 Consolidation

Cain et al., (2002) further tested consolidation of the acquired memory with a two-minute drug-free recall test 24 hrs following conditioning. Systemic administration of Nifedipine 20 or 50 mins prior to training was found to have no effect on consolidation, however Nimodipine (15 mg/kg) was found to be state-dependent. Mice injected with a second dose of Nimodipine prior to the recall session showed comparable freezing levels to mice treated with vehicle prior to training, suggesting that the drug may provide an internal context or 'state' to which fear is conditioned. Suzuki et al., (2008) report similar results, finding that systemic injection of Nimodipine (8-32 mg/kg) immediately following, rather than prior to fear conditioning, also does not appear to affect recall in mice 24 hours later.

However, investigations into the consolidation of fear memory appear to show mixed results regarding the effects of LVGCC inhibition. Bauer et al., (2002) examined the effects of bi-lateral infusion of different doses of Verapamil into the lateral amygdala on auditory fear conditioning. In line with previous findings, there was no effect of infusion on post-shock freezing behaviour. However, 24 hrs later, rats infused with Verapamil showed a dose-dependent impairment in long-term memory for the tone CS. Only the two highest doses showed a significant effect on freezing behaviour. This impairment was not evident at 1, 3 or 6 hrs post conditioning, suggesting that inhibition of LVGCCs in the amygdala affects the consolidation of fear association but not its acquisition.

The contradictory results regarding long-term impairment of consolidation with pharmacological inhibitors may be due to the different drugs and doses used, administration method and time delay between infusion and conditioning. Localised injection in the lateral amygdala may have targeted more specific cell types to induce

the impairment observed at 24 hrs with Verapamil. However, the role of LVGCCs in consolidation of fear conditioning may also depend on the specific isoform of the L-type channel which may be expressed in different ratios in different tissue types.

4.1.2.3 Recall

The recall of conditioned fear memory has been found to involve dissociable molecular processes from consolidation (Lee et al., 2004). There does not appear to be any strong effects of LVGCC inhibition on the recall of conditioned fear memory. However, there are limited studies that have investigated in isolation the effects of inhibitor infusion directly before the primary recall session in the absence of any prior injections. Cain et al., (2002) conducted additional experiments to determine whether the effects of Nimodipine observed on consolidation were state-dependent. The systemic infusion prior to the recall session following drug free conditioning did not affect freezing behaviour, suggesting that the inhibition of LVGCCs does not affect the expression of conditioned fear memory during a subsequent recall session. Similar to consolidation, it is possible that more precise local infusions may produce different results.

4.1.2.4 Extinction

Extinction is a form of inhibitory learning (Bouton, 2004; Herry et al., 2010), in which conditioned responding to a stimulus is reduced in the absence of the reinforcer. Prolonged exposure to the CS in the absence of the US leads to new learning which inhibits the previously acquired association. The inhibition of previous learning rather than the degradation of memory is supported by findings that the original memory can be recovered spontaneously, through intended reminders or through pathology (Quirk and Mueller, 2008). There are seemingly considered to be two stages to extinction; training and recall.

Multiple studies have found an effect of LVGCC inhibition on extinction, though results are not entirely consistent. Cain et al., (2002) found that systemic infusion of

Nifedipine or Nimodipine prior to an extinction session led to a perseverance of freezing behaviour during training compared to vehicle infused mice, which showed a time dependent decline. They found the same results for both cued and context fear conditioning. In contrast to these results, Davis and Bauer, (2012) found no effect of local BLA infusions of Verapamil or Nifedipine on extinction training of cued fear conditioning. They found similar levels of freezing in rats infused with inhibitor and those infused with vehicle 15 minutes prior to the extinction training session. However, when tested 24 hours later it was found that animals infused with inhibitor prior to training showed an impaired retrieval of extinction learning, with greater levels of freezing observed. This suggests a distinct role for LVGCCs in extinction learning, however the precise role they play at different stages and in different regions of the brain is yet to be elucidated.

4.1.2.5 *Latent Inhibition*

Latent inhibition (LI) describes the conditions in which there is reduced association formed between the CS and US when there has been pre-exposure to the CS, compared to when the CS is novel. It takes longer for familiar stimuli to acquire meaning as a CS compared to novel stimuli, with the idea that a CS-No US association has been formed (Lubow, 1973). There are very few studies that have investigated the role of LVGCCs in LI, however impairments have been observed following systemic injection of two independent inhibitors (nifedipine and diltiazem).

Barad et al., (2004) injected mice before a CS pre-exposure session 1 day before fear conditioning and compared freezing behaviour recall 1 day later. Mice were injected systemically with either vehicle or Nifedipine (40mg/kg). Half the mice underwent the pre-exposure session one day before conditioning. Nifedipine was found to block LI completely, with no significant difference observed between mice pre-exposed to the context and those that were not. Further investigation determined that the effect was not due to state-dependent learning and that similar effects were

observed with diltiazem at the same concentration. The LI effect that was observed was not as extreme as previous observations, which may have been due to the one-day delay between pre-exposure and conditioning.

These previous findings suggest a specific role for LVGCCs in associative learning. However, there are conflicting and incomplete conclusions from the existing literature. Different studies have used very different experimental designs, different drugs and different methods of administration. Importantly the systemic administration of certain drugs has since been noted to cause an aversive response confounding the conclusions that can be made. For example, Mckinney et al., (2008) found that systemic injection of Nifedipine impairs locomotor activity as well as induces an aversive state which may itself act as a CS during fear conditioning.

To date, local infusion of LVGCC inhibitors has primarily focussed on the amygdala in relation to fear conditioning. It is well established that the hippocampus plays a vital role in contextual fear conditioning and associative learning (Eichenbaum, 2004; Matus-Amat et al., 2004). Furthermore, hippocampal function is robustly found to be altered in patients with schizophrenia and carriers of risk variants for the disorder as discussed previously (Heckers, 2001; Erk et al., 2014). Considering the identification of calcium channels and calcium signalling in risk for schizophrenia, it is pertinent to determine their role in regions relevant to the disorder.

4.2 Experimental aims

For the following experiments we use contextual fear conditioning as a model of hippocampal dependent associative learning. The experiments in this chapter aim to determine the effects of local inhibition of L-type voltage gated calcium channels in the hippocampus on specific aspects of associative learning, namely; acquisition, consolidation, recall, extinction and latent inhibition. Diltiazem was infused bilaterally

into the rat hippocampus prior to different context exposures and freezing behaviour was assessed as an index of association formation.

Diltiazem was chosen as the inhibitor for these experiments due to its intermediate properties of tonic block and frequency dependency. Although at the time of the experiments there was no available $Ca_v1.2$ selective drug, it is highly selective for LVGCCs over other types of voltage gated calcium channels expressed in the brain. It is highly soluble in water, allowing for PBS to be used as a diluent without the need for DMSO, making it more suitable for local infusion into the brain with lower toxicity. It has a biological half-life of 3.5-4 hours which is ideal for the intended acute inhibition, but still long enough to be appropriate for longer behavioural sessions.

4.3 Experiment 1 – Effect of intrahippocampal diltiazem on acquisition and short-term recall

The aim of this experiment was to assess the effect of local inhibition of calcium influx through LVGCCs on the acquisition and short term recall of contextual fear memory (CFM). Diltiazem or PBS was infused bilaterally into the hippocampus prior to conditioning and freezing behaviour was compared as a measure of learned association between the context and aversive stimuli. Behaviour was assessed in the context prior to any exposure to the footshock (US) to determine any baseline differences following the infusion. Scores were then taken immediately following the US in the same session and in the 2 min recall session 3 hrs later to assess CFM.

4.3.1 Method and Materials

4.3.1.1 Subjects

12 Adult male Lister Hooded rats (250-275 g, Charles Rivers, Margate, UK) were housed in pairs as previously described. All animals were given a minimum of five days from arrival to settle before being used in any experiments. At the end point of each experiment, animals were sacrificed using a rising concentration of CO_2 in a home cage culling chamber (Clinipath Equipment Limited, Hull, UK) and brains were removed and fresh frozen on dry-ice. One animal in the control group for this experiment showed no evidence of any conditioning following training and so was excluded from analysis, resulting in 6 animals in the diltiazem group and 5 in the PBS treated group.

4.3.1.2 Surgery

All surgery was conducted under anaesthetic (2.5-5% Isoflurane) on a stereotaxic frame (Bilaney, Kent UK) to ensure accurate targeting of the dorsal hippocampus (see General Methods). Double guide cannulae were inserted and secured to the skull targeted at + 0.19 cm from bregma in the coronal plane and – 0.35 cm in the

sagittal plane. Dummy cannulae were inserted to protect the guide from blockage or infection prior to and following infusions.

Rats were tail marked and left to recover in an incubation chamber at 37˚C for 30 mins. There was a minimum 6-day interval between surgery and behavioural procedures. Post-hoc histological analyses were conducted to establish correct placement of cannula (Figure 4.1; See General Methods 2.4.4 for full description).

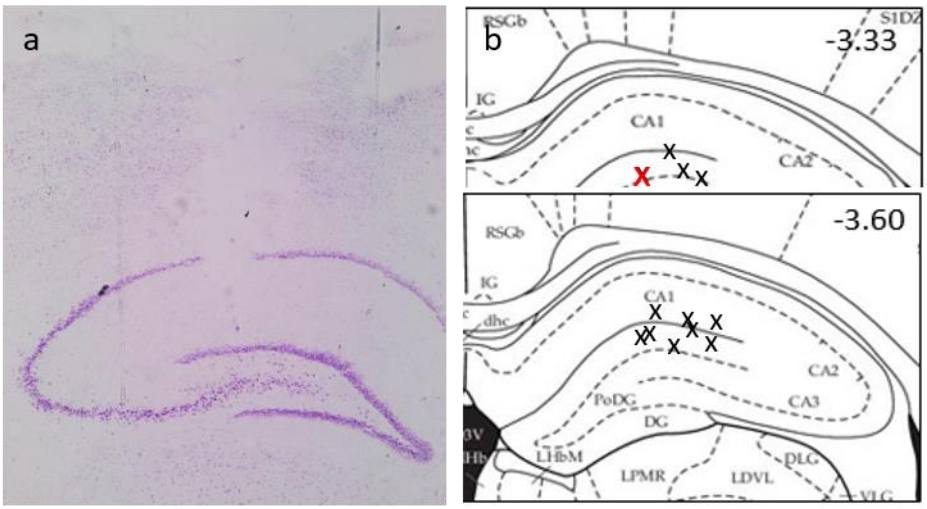

Figure 4.1 Histological analysis for cannula placement for infusions for Acquisition and STM experiment. a) Example thionin stained brain slice indicating guide cannula placement. b) Location of point of guide cannula for all animals. Crosses indicate focal point of infusion, red cross indicates animal excluded from analysis due to behaviour.

4.3.1.3 Infusions

All animals received a practice infusion of sterile PBS (pH 7.2) to clear out the cannula and allow them to become familiar with the procedure. Animals were always infused one cage at a time and taken to the procedure room in the same transport boxes (odd numbered animals always in box A, even in box B). Rats were placed on the experimenter's lap and allowed to freely explore for the duration of the infusion. Dummy cannulae were removed and the infusion needles inserted until fully flush with the guide cannula, resulting in a 1 mm protrusion into the hippocampus. The needles remained in place for 2 min after the end of the infusion to ensure maximal

delivery of the solution. The injector was then removed and the dummy cannula replaced.

Animals received the experimental infusion 40 mins prior to the condition of interest and were returned to their home cages until required for testing. Animals received infusions of either PBS (pH 7.2) or diltiazem (10 nmol) at a rate of 0.5 µl/min, with a total of 1 µl delivered bilaterally. All infusions were counterbalanced so that home cage, conditioning chamber and any order effects were controlled. Post mortem histology was performed (see General Methods) to ensure correct placement of the cannula.

4.3.1.4 Behavioural apparatus

For full description of the apparatus and paradigm used, see General Methods 2.3.1. Contextual fear conditioning was conducted as previously in a rat conditioning box with a metal grid floor. Animals were transported from home cages to the testing room in large transport boxes, with the same box used for each session. Digital recordings were taken for the full duration of each session.

Contextual Fear Conditioning

Animals were placed in conditioning chambers for 2 mins before receiving a 0.5 mA foot-shock for 2 secs. They remained in the chambers for 1 min after the shock before returning to home cages. To assess acquisition and short term recall of contextual fear memory, rats were returned to the conditioning chambers 3 hrs following training and then 24 hrs and 7 days later for a long term recall session. Rats were placed in the same context as training but in the absence of any US for 2 mins and then returned to home cages.

4.3.1.5 Analysis

Freezing behaviour was scored as previously described and checked for reliability by additional scorers. Scores were taken for each rat before the administration of the

footshock (Pre-US) to check basal levels of freezing behaviour which may indicate anxiety. A second score was taken following the footshock (post-US) to measure acquisition of the association between shock and context and scores were taken from each subsequent recall test to assess the consolidation of this association.

Freezing levels were compared between diltiazem and PBS treated animals using repeated measure ANOVA, unless otherwise stated. Greenhouse Geisser corrections were applied where Mauchly's test of sphericity was significant. Post-hoc one-way ANOVAs were conducted for individual test sessions based on a-priori hypotheses where appropriate.

4.3.2 Results

4.3.2.1 Intrahippocampal diltiazem has no effect on the acquisition or short-term recall of contextual fear memory

Diltiazem (10 nmol, 1 µl, 0.5 µl/min) or PBS was infused 40 mins prior to CFC (Figure 4.2). Repeated measures ANOVA showed a significant effect of session on freezing behaviour (session: $F(2,18) = 151.633$, $P<.001$, $\eta_p^2 = .94$). Increased freezing was evident post-US (M = 54.54%, SEM = 3.25%) and during the short-term recall session 3 hours later (M = 68.94%, SEM = 4.22%) compared to pre-US (0%, $t(10) = 16.785$, $P<.001$; $t(10) = 16.344$, $P<.001$ respectively), indicative of successful acquisition and recall of CFM.

There was no effect of diltiazem on freezing behaviour (group: $F(1,9) = 1.05$, $P = .332$, $\eta_p^2 = .10$) or an interaction between session and group (session x group: $F(2,18) = .801$, $P = .464$, $\eta_p^2 = .08$). When tested 3 hours following conditioning, diltiazem treated animals showed the same levels of freezing (M = 68.06%, SEM = 6.00%) compared to PBS treated animals (M = 70.00%, SEM = 6.58%). This suggests that there was no effect of infusion of diltiazem on initial context exploration, processing of the US, or acquisition and short-term recall of CFM.

4.3.2.2 Intrahippocampal diltiazem affects the recall of CFM at 24 hours and 7 days following conditioning

Animals were returned to the training context for further recall sessions at 24 hours and 7 days to assess the effects of diltiazem on consolidation. Repeated measures ANOVA across the two recall sessions showed no effect of session (session: $F(1,9) = 1.947$, $P = .196$, $\eta_p^2 = .18$) or an interaction between group and session (session x group: $F(1,9) = 3.017$, $P = .116$, $\eta_p^2 = .25$), suggesting freezing behaviour was maintained across the recall sessions.

There was a significant effect of diltiazem on freezing levels across recall sessions (group: $F(1,9) = 8.376$, $P = .018$, $\eta_p^2 = .48$), with reduced freezing observed in diltiazem treated animals. Follow up independent sample t-tests, with equal variances not assumed, revealed a significant difference between freezing in diltiazem (M = 9.72%, SEM = 2.56) and PBS treated animals (M = 46.67%, SEM = 19.81) at 7 days following conditioning ($t(4.717) = 4.126$, $P = .010$, $d = 2.59$). This suggests a specific effect of diltiazem infusion prior to training on the consolidation of CFM.

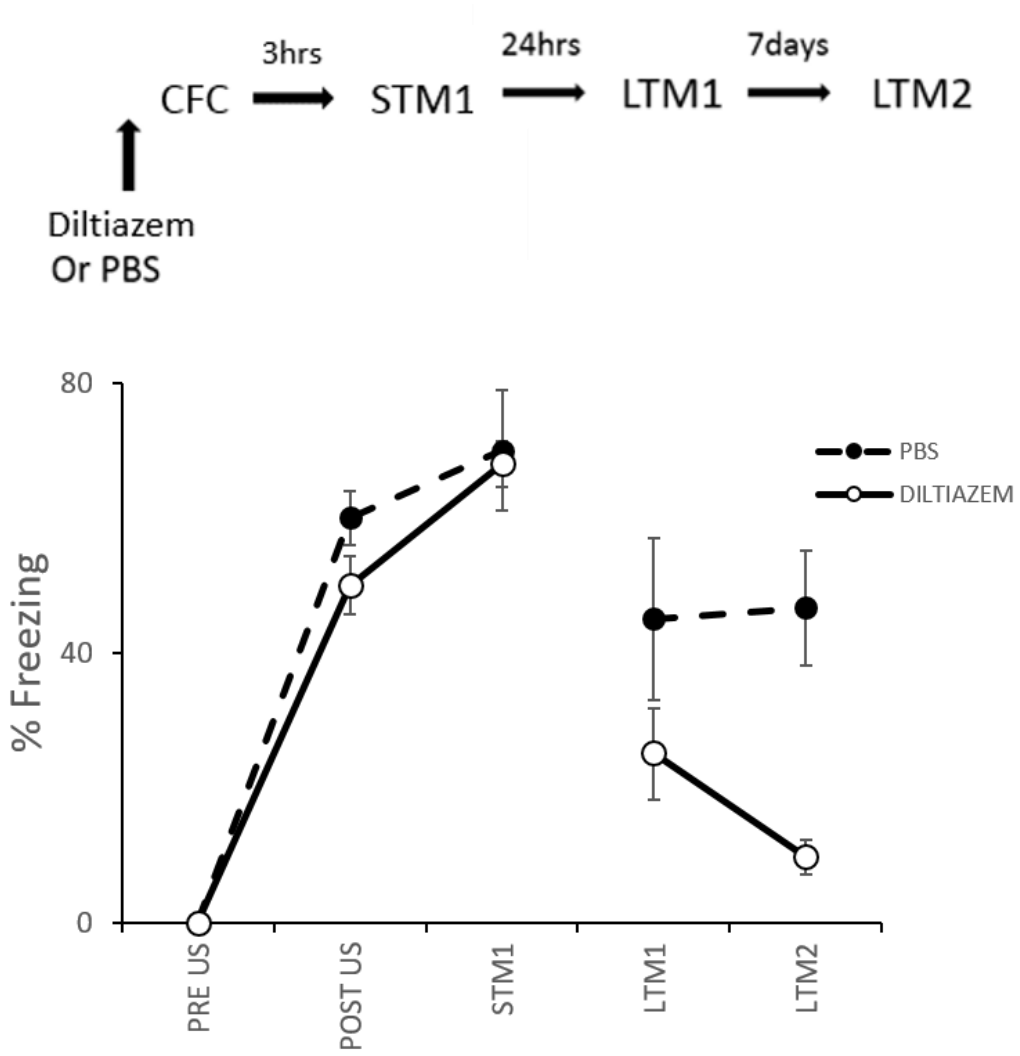

Figure 4.2 Intrahippocampal diltiazem has no effect on the acquisition of short term recall of CFM. Tests of consolidation at 24 hours and 7 days later indicate a significant effect of diltiazem on freezing behaviour during recall ($F(1,9) = 8.376$, $P = .018$) with no evidence of an interaction ($F(1,9) = 3.017$, $P = .116$). Data points represent average % freezing for each group. Error bars are SEM.

4.4 Experiment 2 – Effect of intrahippocampal diltiazem on consolidation

This experiment aimed to further assess the effect of diltiazem on consolidation of CFM without the confounding experience of a short term recall session. Re-exposure to the context 3 hrs following conditioning and recall of the association has been posited to lead to reactivation of the acquired CFM and induction of a labile state (Nader, 2003). This further experience of the context may therefore lead to new learning about its association with the US and influence future consolidation (Lee et al., 2004).

As experiment 1, diltiazem was infused bilaterally into the hippocampus prior to conditioning and freezing behaviour was assessed 24 hrs and 7 days later in the absence of any short-term recall test.

4.4.1 Methods and materials

4.4.1.1 Subjects

Twelve adult male Lister Hooded rats, housed as above. All animals underwent the full experiment and were included in analysis.

4.4.1.2 Surgery

As experiment 1. Histological analyses were conducted to confirm cannula placements (Figure 4.3)

4.4.1.3 Infusions

As experiment 1. Infusions of diltiazem or PBS were administered 40 mins prior to the conditioning session.

4.4.1.4 Contextual Fear Conditioning

Animals were conditioned as in experiment 1 and underwent 2 recall sessions at 24 hrs and 7 days following conditioning.

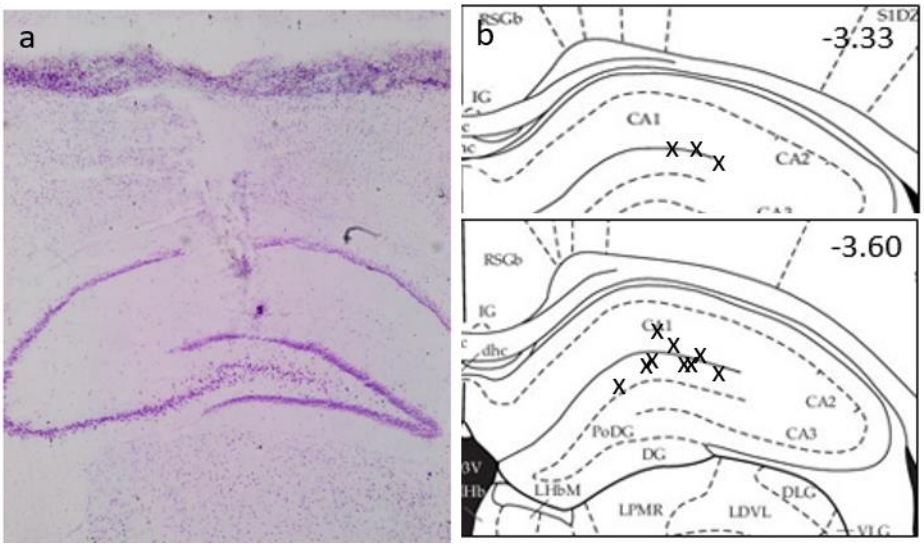

Figure 4.3 Histological analysis for cannula placement of infusions for Consolidation experiment. a) Example thionin stained brain slice indicating guide cannula placement. b) Location of point of guide cannula for all animals. Crosses indicate focal point of infusion.

4.4.2 Results

4.4.2.1 Hippocampal infusion of diltiazem impairs the consolidation of contextual fear memory

Animals were infused with diltiazem or PBS 40 mins prior to CFC. Repeated measures ANOVA across all sessions showed a significant effect of session (session: $F(3,30) = 28.262$, $P < 0.001$, $\eta_p^2 = .74$), in line with findings in experiment 1. There was a significant effect of diltiazem on freezing behaviour (Group: $F(1,10) = 10.465$, $P = .009$, $\eta_p^2 = .87$) and evidence of an interaction (session x group: $F(3, 30) = 12.112$, $P < .001$, $\eta_p^2 = .55$).

Post-hoc analyses across pre and post-US sessions showed that freezing behaviour was increased following CFC (session: $F(1,10) = 53.309$, $P < .001$, $\eta_p^2 = .84$), indicative of acquisition of CFM, with no effect of diltiazem (group: $F(1,10) = .013$, $P = .910$, $\eta_p^2 = .001$) or evidence of an interaction (group x session: $F(1,10) = .129$, $P = .727$, $\eta_p^2 = .013$), as shown in experiment 1.

However, repeated measures ANOVA across recall sessions showed significantly reduced freezing behaviour in diltiazem treated animals (M = 10.42%, SEM = 6.20%) compared to PBS treated animals (M = 51.39%, SEM = 6.20%) across both sessions ($F(1,10) = 21.838$, $P = .001$, $\eta_p^2 = .69$) with little or no evidence of a learned association (Figure 4.4). This concurs with findings in experiment 1, though further post-hoc analyses show reduced freezing evident at both 24 hours and 7 days later (One-way ANOVA: $F(1,10) = 25.415$, $P = .001$, $\eta_p^2 = .72$; $F(1,10) = 14.257$, $P = .004$, $\eta_p^2 = .59$ respectively). In line with experiment 1, this suggests inhibition of LVGCCs during training selectively impairs the consolidation of CFM.

In addition there was a significant effect of recall session on freezing behaviour observed (session: $F(1,10) = 9.494$, $P = .012$, $\eta_p^2 = .49$) with reduced freezing at 7 days compared to 24 hours following CFC. This was in conjunction with an interaction

between group and session ($F(1,10) = 6.798$, $P = .026$, $\eta_p^2 = .41$), with reduced freezing only evident in PBS treated animals. This suggests there may be between session extinction of CFM in PBS treated animals, as previously reported.

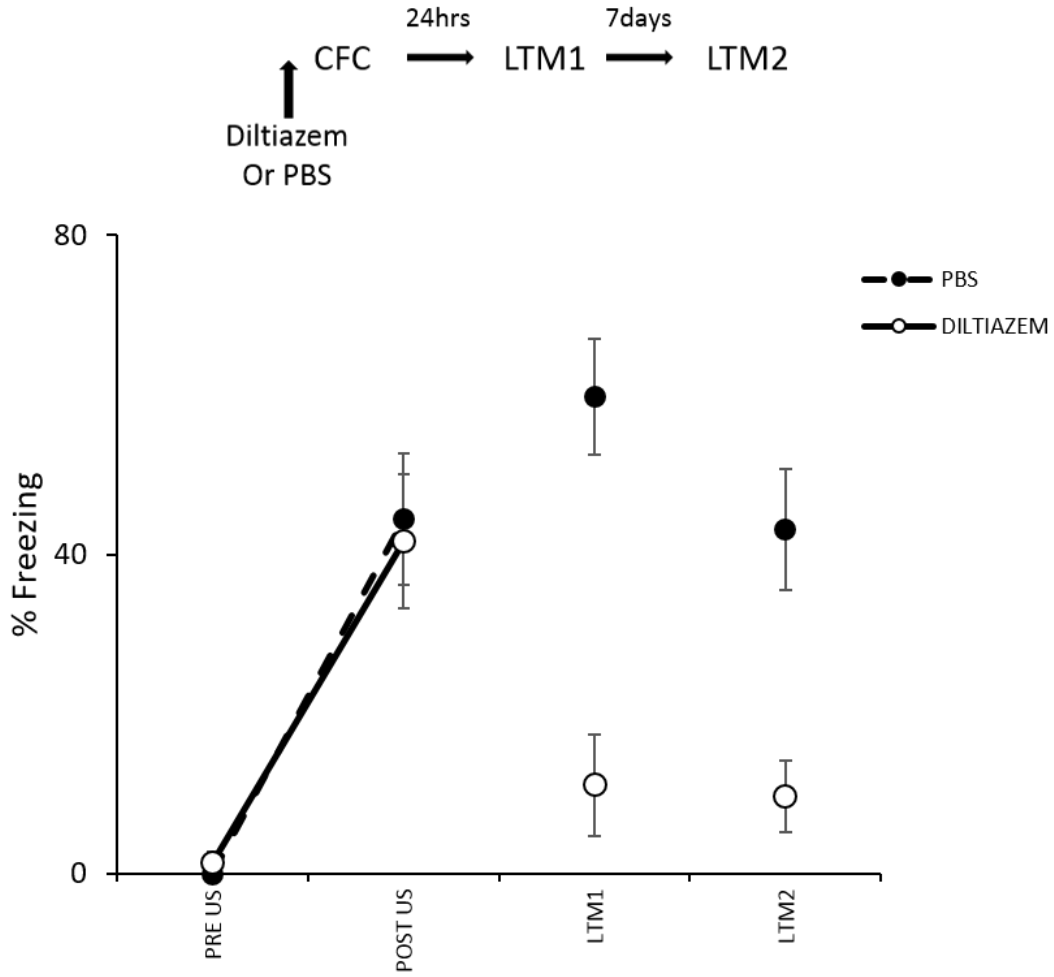

Figure 4.4 Intrahippocampal infusion of L-type VGCC inhibitor diltiazem impairs consolidation of contextual fear memory. There was reduced freezing in diltiazem treated animals at recall 24 hours and 7 days later (Session x Group, F(1,10)=6.798, P = 0.026). Data points represent average % freezing for each group at each testing phase. Error bars are SEM.

4.5 Experiment 3 - Effect of intrahippocampal diltiazem on recall

Previous studies have shown that the recall of associative memory involves separable molecular processes from those activated by consolidation (Lee et al., 2004). With such strong effects of diltiazem observed on consolidation, this experiment aimed to investigate the effects of inhibiting LVGCCs on the recall of CFM.

Animals underwent CFC drug free. The following day they received infusions of diltiazem or PBS 40 mins prior to a 2 min recall session. Freezing was assessed during this session and in 2 subsequent recall sessions 24 hrs and 7 days later to determine the immediate effect of inhibition and any future recall attempts.

4.5.1 Methods and Materials

4.5.1.1 Subjects

14 animals were housed in pairs as previously described.

4.5.1.2 Surgery

As experiment 1. Histological analyses were conducted to confirm cannula placement (Figure 4.5).

4.5.1.3 Infusions

Infusions occurred 40 mins prior to the first recall session 24 hrs following drug free training. Solutions were administered as experiment 1.

4.5.1.4 Behaviour

Animals were conditioned drug free with a 3 min CFC session as experiment 1. A 2 min recall session occurred 24 hrs later, with diltiazem or PBS infused 40 mins before exposure to the context. There were two subsequent drug free recall sessions 24 hrs and 7 days later.

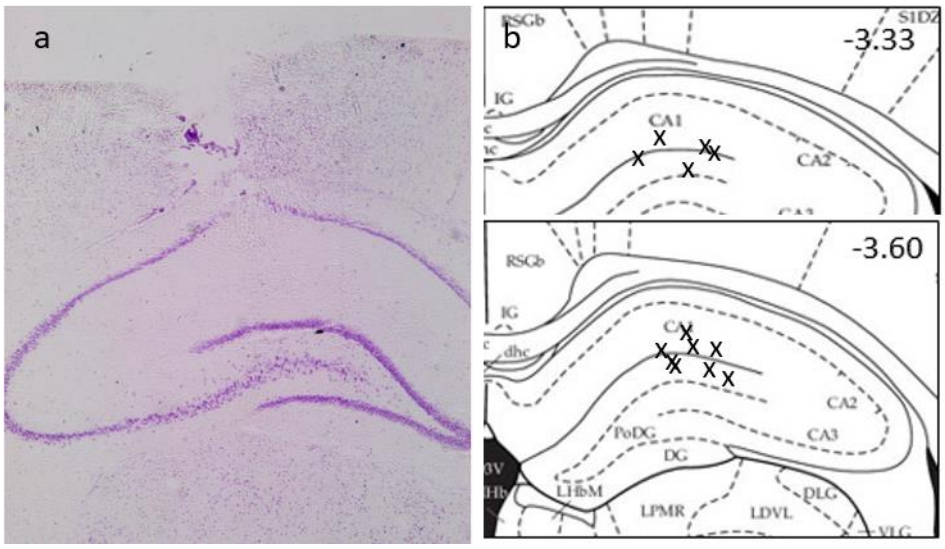

Figure 4.5 Histological analysis for cannula placement for Recall experiments a) Example thionin stained brain slice indicating guide cannula placement. b) Location of point of guide cannula for all animals. Crosses indicate focal point of infusion.

4.5.2 Results

4.5.2.1 Intrahippocampal infusions of diltiazem prior to retrieval has no effect on the recall of CFM

Diltiazem or PBS was infused 40 mins prior to a 2 min recall session 24 hrs following drug free CFC. Animals showed the expected conditioned freezing behaviour prior to infusions (pre-US vs post-US, Figure 4.6), with increased freezing observed post-US (session: $F(1,12) = 180.390$, $P < .001$, $\eta_p^2 = .94$). There was no difference between animals that subsequently received infusions of diltiazem or PBS (group: $F(1,12) = 2.362$, $P = .15$, $\eta_p^2 = .16$) and no evidence of an interaction ($F(1,12) = .878$, $P = .367$, $\eta_p^2 = .07$).

There was no effect of diltiazem on freezing behaviour across recall sessions; 40 mins following the infusion, nor at 24 hours and 7 days later when tested drug free (group: $F(1,12) = .320$, $P = .582$, $\eta_p^2 = .03$). As experiments 1 and 2, there was evidence of between session extinction of CFM, with reduced freezing observed across subsequent recall sessions (session: $F(2,24) = 5.032$, $P = .015$, $\eta_p^2 = .30$), though there was no indication of an interaction between group and session (session x group: $F(2,24) = 1.305$, $P = .290$, $\eta_p^2 = .10$). This suggests there is no effect of inhibition of LVGCCs prior to retrieval on the recall of CFM.

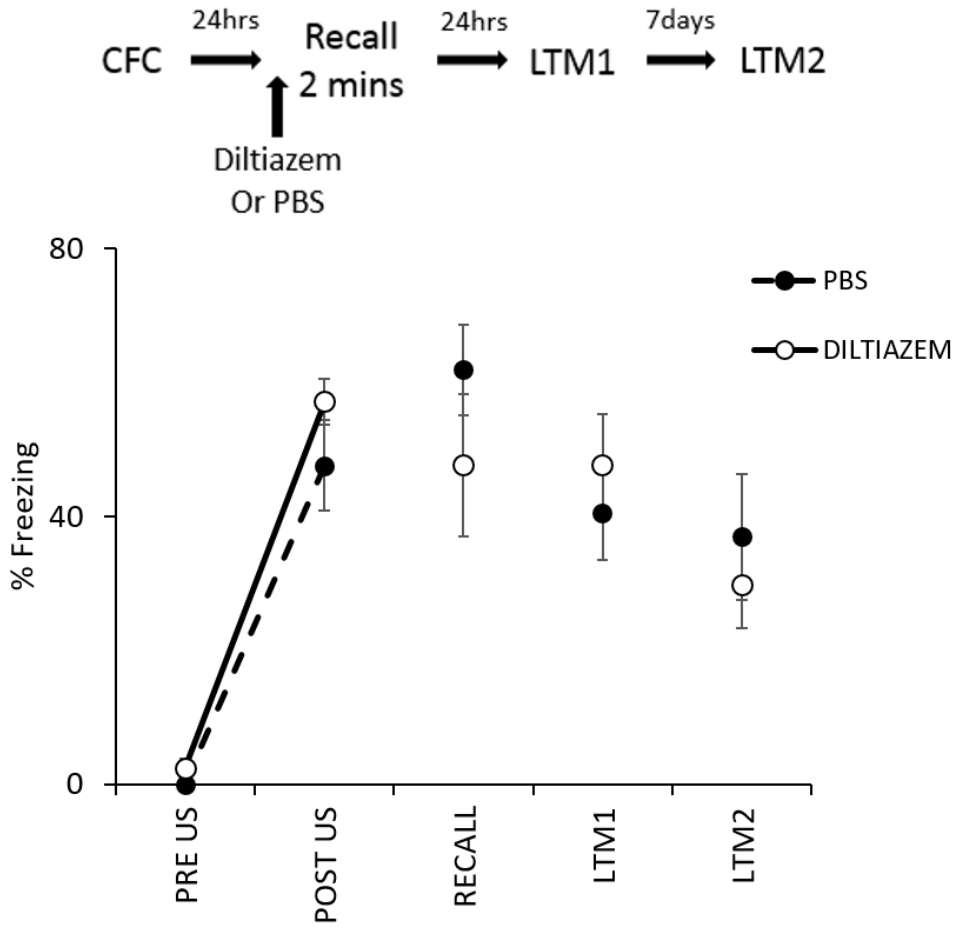

Figure 4.6 Intrahippocampal infusion of diltiazem prior to recall has no effect on freezing behaviour or on recall 24hours and 7 days later ($F(1,12) = 0.32$, $P = 0.582$). Data points represent average % freezing. Error bars are SEM.

4.6 Experiment 4 – Effect of intrahippocampal diltiazem on extinction learning

Prolonged exposure to a previously conditioned context in the absence of the US results in a reduction in learned freezing behaviour, termed extinction (Bouton, 1993). Evidence suggests that this extinction occurs due to new learning of a CS-No US association which competes with and inhibits previous conditioning (Myers and Davis, 2002).

This experiment aimed to investigate whether the infusion of diltiazem into the hippocampus prior to an extinction session interfered with extinction training (i.e. the reduction of freezing seen within session) and/or the consolidation of extinction when tested 24 hrs and 7 days later.

4.6.1 Methods and Materials

4.6.1.1 Subjects

16 animals were housed as previously described. One animal was excluded from analysis following post-hoc histological analysis of cannulae placement, suggesting incorrect infusion location (Figure 4.7). This resulted in 8 animals in the diltiazem group and 7 in the PBS control group for final analysis.

4.6.1.2 Surgery

As experiment 1. Histological analyses were conducted to confirm cannula placement (Figure 4.7)

4.6.1.3 Infusions

Diltiazem or PBS was infused prior to a 10 min recall session 24 hrs following drug free CFC. Solutions were prepared and administered as experiment 1.

4.6.1.4 Behaviour

Animals underwent 3 min CFC as experiments 1-3. 24 hrs later animals were re-exposed to the training context for 10 mins to induce extinction of the learned association. Freezing behaviour was scored every 10 secs throughout the entire extinction session. Behaviour was then assessed 24 hrs and 7 days later during 2 min US free recall sessions, as experiments 1-3.

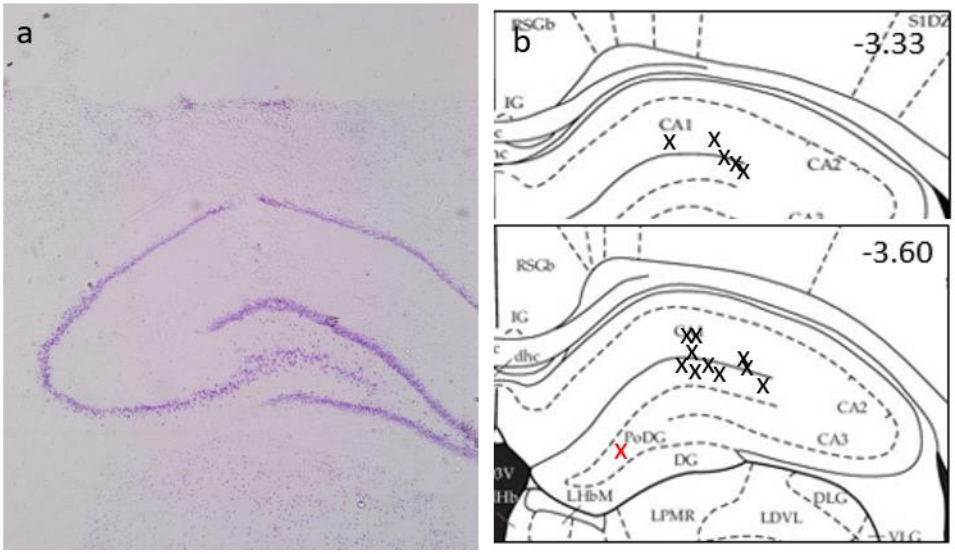

Figure 4.7 Histological analysis for cannula placement for Extinction experiments a) Example thionin stained brain slice indicating guide cannula placement. b) Location of point of guide cannula for all animals. Crosses indicate focal point of infusion. The red cross indicates the site of infusion for the 1 animal excluded from analyses.

4.6.2 Results

All animals acquired the learned association drug free on testing day one (repeated measures ANOVA: $F(1,13) = 109.975$, $P < .001$, $\eta_p^2 = .89$), with no difference between animals which subsequently received infusions of diltiazem or PBS ($F(1,13) = .095$, $P = .763$, $\eta_p^2 = .01$), or any evidence of an interaction ($F(1,13) = .085$, $P = .775$, $\eta_p^2 = .01$).

4.6.2.1 Intrahippocampal diltiazem has no effect on the acquisition of extinction learning

All animals showed the expected reduction in freezing behaviour within the 10 min extinction session (Figure 4.8: $F(1,13) = 18.635$, $P = .001$, $\eta_p^2 = .59$), indicating successful extinction acquisition. There was no effect of diltiazem on freezing behaviour during the extinction session ($F(1,13) = 2.415$, $P = .144$, $\eta_p^2 = .16$) and no evidence of an interaction ($F(1,13) = 2.099$, $P = .171$, $\eta_p^2 = .14$).

4.6.2.2 Intrahippocampal diltiazem specifically affects the consolidation of extinction learning

To assess the consolidation of extinction animals were returned to the context 24 hrs and 7 days following training. There was a significant effect of diltiazem on freezing behaviour, with treated animals showing greater freezing 24 hrs and 7 days later (Figure 4.8: $F(1,13) = 8.92$, $P = 0.011$, $\eta_p^2 = .41$). There was no evidence of changes in freezing behaviour between the two recall sessions ($F(1,13) = .012$, $P = .916$, $\eta_p^2 = .001$) nor an interaction with group ($F(1,13) = 2.630$, $P = .129$, $\eta_p^2 = .17$). This implicates a specific effect of diltiazem on the consolidation of extinction learning.

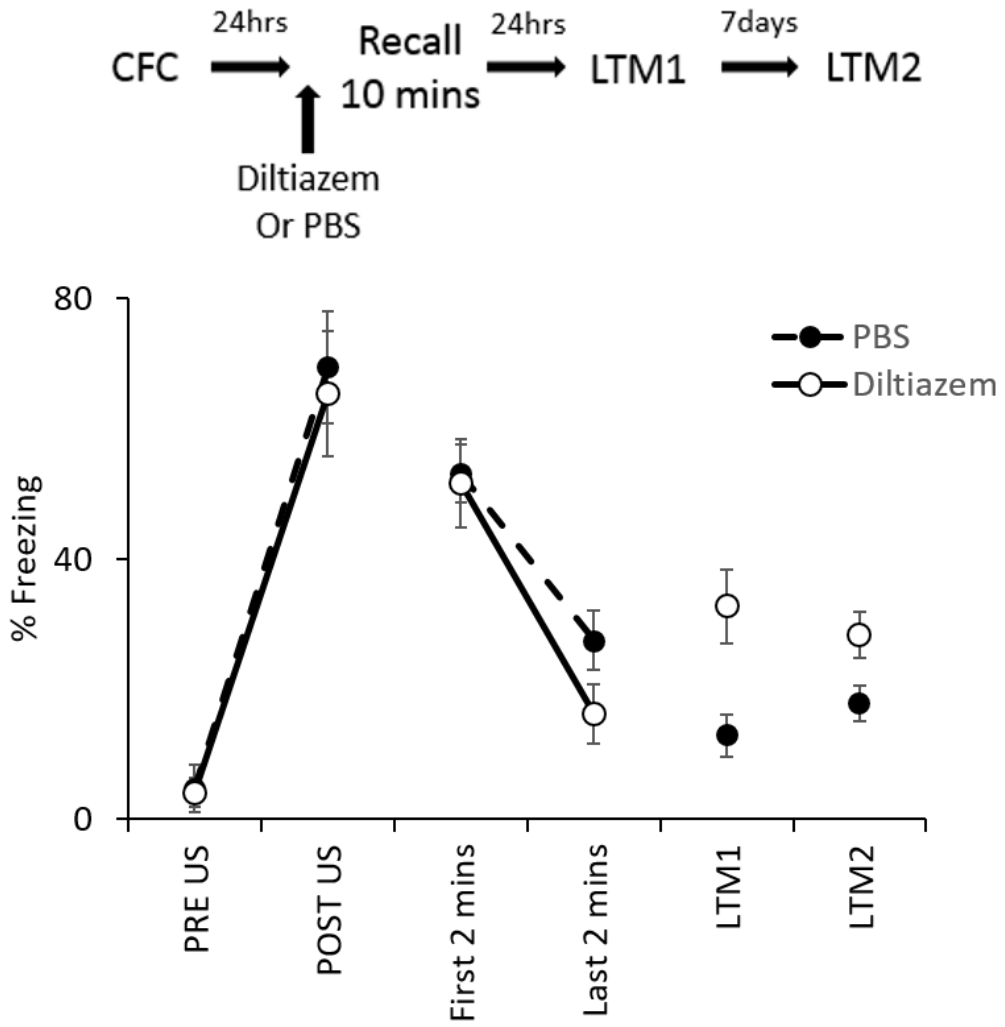

Figure 4.8 Intrahippocampal infusion of L-type VGCC inhibitor diltiazem impairs extinction consolidation but not training. There was no effect of diltiazem on within session extinction ($F(1,13) = 2.415$, $P = 0.144$), however freezing was greater in diltiazem treated animals when tested 24 hours and 7 days post extinction training ($F(1,13) = 8.92$, $P = 0.011$). Data point represent average % freezing. Error bars are SEM.

4.7 Experiment 5 – Effect of intrahippocampal diltiazem on latent inhibition

Similar to extinction learning, pre-exposure to a context prior to conditioning can interfere with the formation of association with an US. This process is called latent inhibition (LI) and refers to the fact that it is harder for familiar stimuli to gain predictive status as a CS than novel stimuli (Kiernan and Westbrook, 1993).

Following the observed deficits in extinction consolidation, this experiment aimed to investigate the effects of infusion of diltiazem prior to a 4 hr LI session, 24 hrs before CFC. Animals experiencing prolonged exposure to the context prior to training, would be expected to show reduced conditioned freezing behaviour compared to those conditioned to a novel context, as in experiments 1-4.

4.7.1 Methods and Materials

4.7.1.1 Subjects

12 animals were housed as experiments 1-4.

4.7.1.2 Surgery

As experiment 1. Histological analyses were conducted to confirm cannula placements (Figure 4.9).

4.7.1.3 Infusions

Diltiazem or PBS was administered 40 mins prior to a 4 hr LI session. Solutions and procedure were as experiments 1-4.

4.7.1.4 Behaviour

Animals underwent a 4 hr pre-exposure LI session in the training context 24 hrs before drug free CFC. There were two subsequent 2 min recall sessions 24 hrs and 7 days later, as experiments 1-4. Freezing was scored fully for the first 2 mins and

last 2 mins of the LI session and behaviour was observed during the session to assure no confounding experiences.

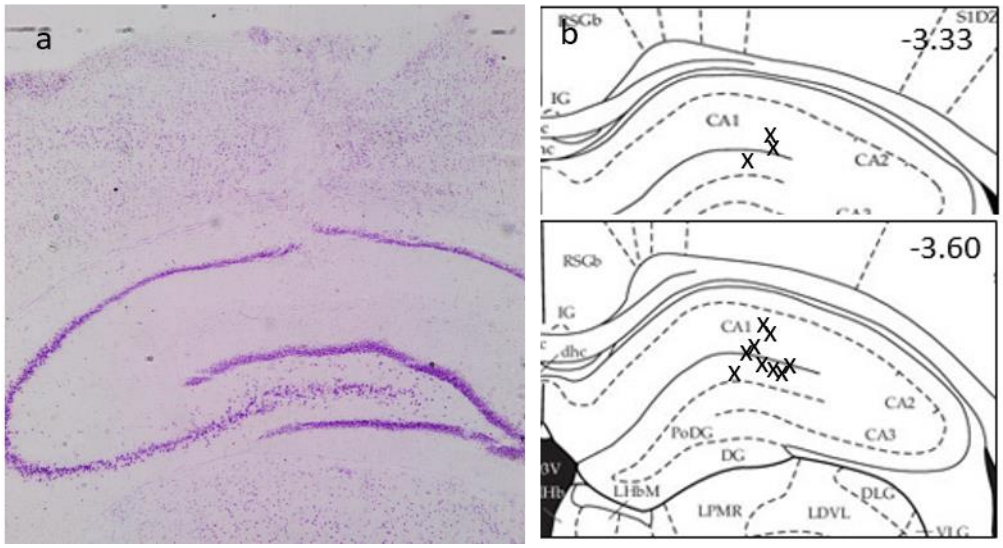

Figure 4.9 Histological analysis for cannula placement for Latent Inhibition experiments a) Example thionin stained brain slice indicating guide cannula placement. b) Location of point of guide cannula for all animals. Crosses indicate focal point of infusion.

4.7.2 Results

4.7.2.1 Intrahippocampal diltiazem impairs latent inhibition of CFM

Across the full experiment, repeated measures ANOVA with Greenhouse-Geisser correction, revealed a significant effect of session on freezing behaviour ($F(2.313, 23.127) = 22.732$, $P < .001$, $\eta_p^2 = .69$), an effect of diltiazem ($F(1,10) = 11.113$, $P = .008$, $\eta_p^2 = .53$) and evidence of an interaction ($F(2.213, 23.127) = 5.381$, $P = .009$, $\eta_p^2 = .35$). This indicates an effect of diltiazem infusion on behaviours related to latent inhibition of CFM. As experiments 1 - 4, there was no effect of infusion on initial exploratory behaviour of the context (Independent samples t-test, equal variances not assumed: $t(5) = -1.000$, $P = .363$), or on behaviour during the LI session, suggesting no adverse behavioural side-effects of diltiazem infusion.

During CFC 24 hrs following the LI session, there was a significant effect of conditioning on freezing behaviour (Figure 4.10: $F(1,10) = 7.692$, $P = .020$, $\eta_p^2 = .44$),

however there was also a trend towards an interaction between group and session ($F(1,10) = 4.923$, $P = .051$, $\eta_p^2 = .33$), suggesting that the conditioning may have had different effects on diltiazem and PBS treated animals. There was no main effect of diltiazem on freezing behaviour ($F(1,10) = 2.609$, $P = .137$, $\eta_p^2 = .21$).

Animals were returned to the context 24 hrs and 7 days later to assess whether CFM had been consolidated. There was significantly greater freezing in the diltiazem infused (M = 55.56%, SEM = 7.35%) compared to PBS infused (M = 19.44%, SEM = 7.35%) animals (Figure 4.10: $F(1,10) = 12.071$, $P = 006$, $\eta_p^2 = .55$). Diltiazem treated animals formed a CFM despite prolonged pre-exposure to the context, whereas PBS treated animals showed the expected effects of latent inhibition, with reduced levels of freezing. This suggests that inhibition of LVGCCs impairs latent inhibition of CFM.

As previous experiments there was evidence of between session extinction with reduced freezing observed during the second recall ($F(1,10) = 9.412$, $P = .012$, $\eta_p^2 = .49$), though no evidence of an interaction ($F(1,10) = 1.324$, $P = .277$, $\eta_p^2 = .117$).

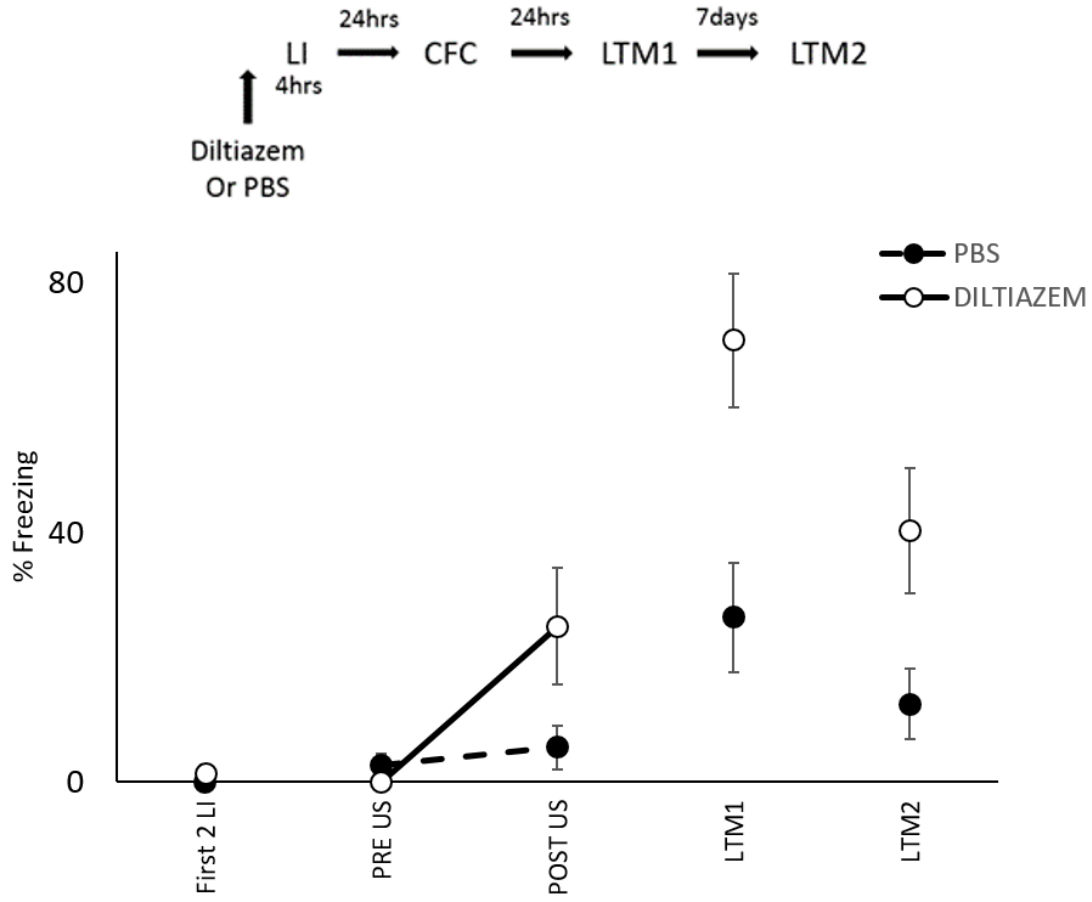

Figure 4.10 Infusion of diltiazem impairs the latent inhibition of CFM. Diltiazem treated animals showed increased freezing following LI compared to PBS treated animals ($F(1,10) = 11.113$, $P = 0.008$). Data points represent average % freezing for each group. Error bars are SEM

4.8 Experiment 6 – Effect of intrahippocampal *BDNF* antisense on latent inhibition

As previously discussed, calcium influx through LVGCCs has been found to regulate transcription of *BDNF.* Studies have robustly identified a role for *BDNF* activity in the consolidation and extinction of contextual fear memory. Infusion of *BDNF* antisense into the hippocampus led to an impairment in CFM formation (Lee et al., 2004) and heterozygous knockout mice have shown similarly impaired consolidation, rescued by recombinant BDNF infused into the hippocampus (Liu et al., 2004). Elevated BDNF levels have further been found to constrain extinction learning (Kirtley and Thomas, 2010). Given the implication of this downstream mechanism from LVGCC in consolidation and extinction, it is pertinent to investigate whether this extends to latent inhibition.

The following experiment aimed to determine whether knockdown of *BDNF,* using antisense infused into the hippocampus, produced a similar deficit in latent inhibition as observed with diltiazem. Results would contribute to our understanding of potential downstream or convergent mechanisms which may be affected by acute block of LVGCCs.

4.8.1 Methods and materials

4.8.1.1 Subjects

12 adult male Lister Hooded rats (250 – 275 g) were housed as experiment 1. One animal was excluded from analysis due to post-hoc histology, leaving 6 animals in the control group and 5 in the experimental group (Figure 4.11).

4.8.1.2 Surgery

As experiment 1. Histological analyses were conducted to confirm cannula placements location (Figure 4.11).

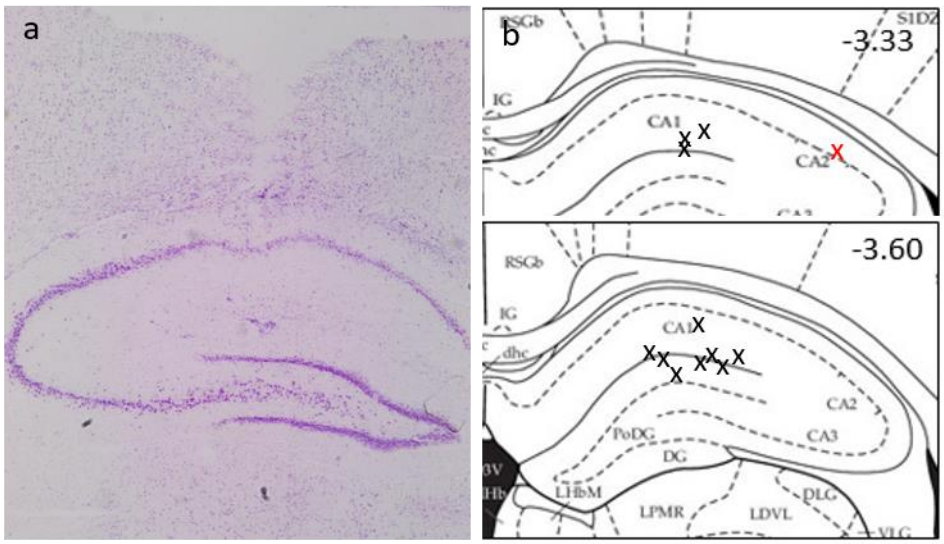

Figure 4.11 Histological analysis for cannula placement for Recall experiments a) Example thionin stained brain slice indicating guide cannula placement. b) Location of point of guide cannula for all animals. Crosses indicate focal point of infusion. The red cross indicates the infusion location of the animal that was subsequently excluded from analyses.

4.8.1.3 Infusions

BDNF antisense and missense were synthesised from Sigma, as PAGE-purified phosphorothioate end-capped 18-mer sequences. They were re-suspended in sterile PBS (PH 7.4) to a concentration of 1 nmol/µl. Sequences (See Table 4.1) were BLAST searched in NCBI database to ensure no off-target effects were predicted. Control missense sequences were made up of the same 18 nucleotides as the antisense and were similarly subjected to BLAST searches to ensure no match to any nucleotide in the rat.

Table 4.1 Sequences for BDNF antisense and missense

$BDNF_{aso}$	5' - TCT TCC CCT TTT AAT GGT - 3'
$BDNF_{mso}$	5' - ATA CTT TCT GTT CTT GCC – 3'

BDNF antisense or missense was infused locally into the hippocampus as experiments 1-5, albeit with longer infusion time (1 µl per hemisphere at a rate of 0.125 µl/min) and at 90 mins prior to experiment. As before, cannula remained in

place for an additional 2 minutes to ensure full delivery of the solutions. Animals were returned to home cages until required for testing.

4.8.1.4 Behaviour

All animals underwent the same LI behavioural paradigm as experiment 5, with a 4 hr pre-exposure session on day one in the absence of any US and CFC 24 hrs later. Recall was assessed at 24 hrs and 7 days as previously. Analysis was conducted as experiments 1 – 5.

4.8.2 Results

$BDNF_{aso}$ infused locally into the hippocampus significantly impaired the latent inhibition of CFM (Figure 4.12). Repeated measured ANOVA with a Greenhouse-Geiser correction showed an effect of session on freezing behaviour ($F(1.763, 15.866) = 37.816$, $P < .0001$, , $\eta_p^2 = .808$), an effect of genotype ($F(1,9) = 13.600$, $P = .005$, , $\eta_p^2 = .602$) and a significant interaction ($F(1.763, 15.866) = 4.941$, $P = 0.025$, , $\eta_p^2 = .354$).

Post-hoc ANOVAs reveal a significant greater levels of freezing in animals infused with $BDNF_{aso}$ compared to missense in both long term recall sessions (LTM1: $F(1,9) = 32.479$, $P < .0001$; LTM2: $F(1,9) = 28.512$, $P = .039$). There was no difference in freezing behaviour during the first 2 mins of LI, pre-US or post-US. This suggests there was no confounding effects of infusion on freezing behaviour prior to CFC.

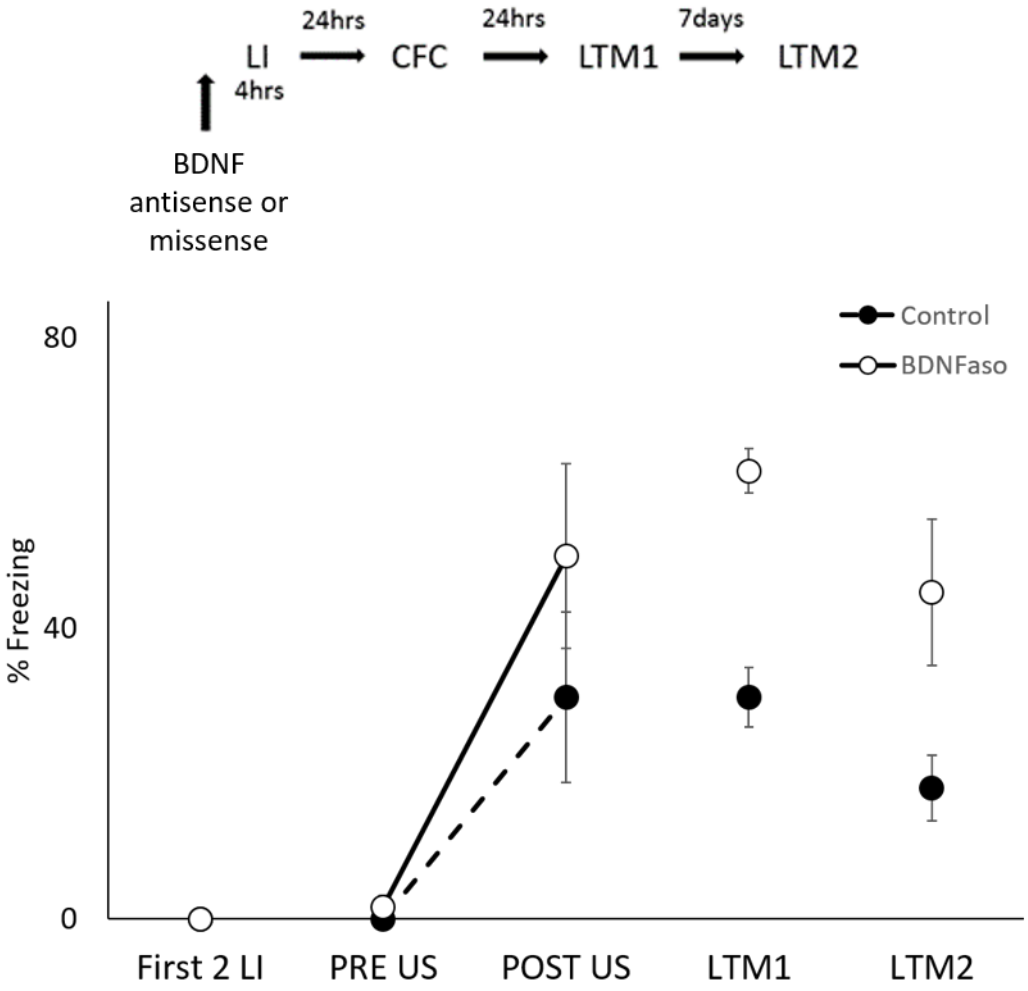

Figure 4.12 Infusion of BDNFaso impairs the latent inhibition of CFM compared to infusion of a missense control F(1.763,15.866) = 37.816, P < 0.0001. Data points represent average % freezing for each group. Error bars are SEM.

4.9 Summary

Local infusion of diltiazem into the hippocampus impairs the consolidation of CFM. It further impairs the consolidation of extinction learning and latent inhibition, while having no effect on the acquisition or recall of CFM. Inhibition of *BDNF* with local infusion of antisense into the hippocampus, results in a similar impairment in latent inhibition as that observed with diltiazem.

4.10 Discussion

Diltiazem is an L-type specific calcium channel inhibitor that acutely blocks the influx of calcium into the cell through these channels. By timing infusion of this drug prior to specific behavioural testing sessions it is possible to discern the role of these channels in different aspects of associative learning. Diltiazem was infused bilaterally into the hippocampus to assess regional specific effects on behaviour. Results suggest a specific role for hippocampal LVGCCs in the consolidation of CFM as well as inhibitory learning processes; the consolidation of extinction and latent inhibition. Additional investigation into the effect of inhibition of *BDNF* suggests a putative convergent downstream mechanism which may link calcium influx through LVGCCs with behaviour.

4.10.1 LVGCCs in the hippocampus are necessary for the consolidation of CFM

Inhibition of LVGCCs prior to CFC selectively impairs the consolidation of CFM while leaving acquisition and short term recall intact. One can thus infer that calcium influx through these channels in the hippocampus is necessary for long-term consolidation. Importantly there was no aversive response to the drug that may have affected behaviour, with no evidence of anxiety related behaviour on exposure to the context. The necessity of calcium influx through these channels for consolidation concurs with previous findings with infusion into the basolateral amygdala (Bauer et al., 2002), suggesting a conserved role for LVGCCs across different brain regions. It also further confirms that the hippocampus, in addition to the amygdala, is an essential part of the network involved in successful consolidation of contextual associative learning (Phillips and LeDoux, 1992; Kim et al., 1993; Frohardt et al., 2000).

Previous studies have focussed primarily on cued fear conditioning rather than contextual conditioning in relation to LVGCCs. As the hippocampus is required for the processing of contextual information (Holland and Bouton, 1999), it may be that the localised role of LVGCCs observed in these experiments is specific to the

consolidation of more complex contextual associations. Evidence supports the requirement of the hippocampus for contextual fear conditioning, whereas the amygdala is found to be necessary for both contextual and cued fear associations (Phillips and LeDoux, 1992). Our results demonstrate that it is LVGCCs within the hippocampus that are a necessary part of its function in consolidation.

The results observed are not in line with systemic infusion of inhibitors which have found no effect on consolidation for either cued or contextual fear conditioning (e.g. Cain et al., 2005). It is possible that systemic infusion may have had peripheral effects due to the expression of these channels in the heart (Treinys and Jurevicius, 2008), or that this method of drug delivery led to inadequate/inaccurate dosing in the brain regions that are specifically involved in fear memory. Furthermore systemic infusion has been found to produce aversive effects, resulting in conditioning occurring to the drug itself in addition to the intended CS (Moosmang et al., 2005), which may in part explain the preserved freezing observed.

It is not possible to conclude whether calcium influx through LVGCCs is sufficient for the consolidation of CFM, but these experiments do imply that it is necessary. Previous studies have shown that NMDA receptor activity is also necessary for the acquisition and consolidation of CFM (Fanselow and Kim, 1994; Dalton et al., 2012; Davis and Bauer, 2012), suggesting that it might be the combined signalling through these channels that creates the appropriate message for successful consolidation, whether of initial learning or extinction.

4.10.2 Inhibition of LVGCCs impairs inhibitory learning processes of extinction and latent inhibition

The current results suggest calcium influx through LVGCCs is necessary for the consolidation of extinction, as well as the latent inhibition of CFM. Similar to acquisition of CFM there was no effect of inhibition on extinction training, however

recovery of conditioned freezing was evident 24 hours and 7 days later. This extends the role of LVGCCs beyond the consolidation of an aversive CS-US association, to include CS-no US associations.

The results support the notion that extinction is an active process that requires additional learning regarding a previously conditioned stimuli, rather than a degradation of the original memory (Quirk, 2002; Bouton, 2004). Inhibition did not affect the recall of CFM, but showed a selective impairment on the processing of a prolonged exposure to the context. This could indicate the activation of separate processes following continued exposure, or as has been suggested previously, a threshold effect of a single process that leads to competing inhibitory associations of CS-No US that becomes stronger with longer exposure (Trent et al., 2015).

Importantly, the process of extinction is not simply the formation of a separate new association, with the effect of inhibition simply being generalised to consolidation. Wang et al., (2012), found that infusion of LVGCC inhibitors into the dorsal hippocampus impaired initial learning of CFC, but had no effect on the consolidation of a second CFC trial. The effect observed on extinction consolidation therefore suggests a separate process that is relevant for the processing of relevant competing information regarding previously salient stimuli.

These findings are in agreement with those observed previously in the amygdala (Davis and Bauer, 2012). Similar to consolidation, this suggests a conserved role for LVGCCs in extinction learning across brain regions. In contrast, systemic infusions of inhibitors prior to training were found to result in perseverance of freezing within the extinction session. As previously discussed, this may be due to peripheral effects of the drug or to a global inhibition masking relevant network effects. To address the concern of drug effects during training, Suzuki et al., (2008) administered the inhibitor immediately after extinction. They found that systemic infusion resulted in recovery of freezing behaviour when tested 24 hrs later in agreement with the current findings.

They further investigated the combined effects of LVGCC inhibition and anisomycin on recall and subsequent expression of CFM. They found that blockage of protein synthesis with anisomycin immediately before or after a short recall session resulted in a deficit in conditioned freezing 24 hrs later. However, this deficit was completely rescued by inhibition of LVGCCs. They conclude that this implicates LVGCCs in the destabilisation of reactivated memory, however this can also be interpreted in-line with present findings as the facilitation of inhibitory extinction learning.

The effect of inhibition observed on LI effects is in agreement with that found previously. Barad et al., (2004) found the LI effect was completely abolished with systemic infusion of LVGCC inhibitor, with freezing behaviour being indistinguishable from animals conditioned to a novel context. This is the first experiment to investigate the local infusion of inhibitor in the hippocampus on the latent inhibition of CFM, as well as to determine its lasting effects on subsequent behaviour. Findings suggest that the processes involved may be similar to that of consolidation and extinction, extending the role of LVGCCs to the formation of potentially relevant contextual representations of novel stimuli.

4.10.3 Downstream signalling effects and gene transcription

An important distinction to make is that there were no effects of diltiazem on behaviour when the drug was present; rather differences were observed during subsequent drug free sessions. This highlights the notion that calcium influx through LVGCCs in this instance is required for longer term processing rather than immediate response, likely requiring de novo gene transcription. It is well documented that calcium influx through these channels activates specific downstream signalling processes and facilitates gene transcription (see Barbado et al., 2009). For example, calcineurin regulates gene transcription through nuclear translocation of the NF-ATc family of transcription factors; a process dependent upon calcium influx through LVGCCs (Graef et al., 1999). Furthermore, there is growing evidence of selective

regulation of isoforms of Brain Derived Neurotrophic Factor (*BDNF)* by LVGCC specific calcium influx (Tabuchi et al., 2000; Zheng et al., 2011), which is known to play a significant role in synaptic plasticity and learning (Cunha et al., 2010). The delayed effect on behaviour we observe is therefore likely due to the disruption of these downstream mechanisms at the time of learning and subsequent absence of necessary gene transcription, the consequence of which only becomes apparent hours or days later.

This effect on downstream mechanisms is supported by the current results showing deficits of latent inhibition following inhibition of *BDNF*. Similar impairments following inhibition of LVGCCs and *BDNF* suggest that they are part of the same molecular mechanism involved in this process. Given the evidence supporting the regulation of *BDNF* by calcium influx through LVGCCS and previous findings with consolidation (Lee et al., 2004; Liu et al., 2004), it is viable that *BDNF* is a key component which is disrupted by inhibition of LVGCCs.

4.10.4 Distinguishing between L-type channel sub-types

Although diltiazem is specific to L-type channels at the dose used, inhibition does affect both types of L-type channel expressed in the brain; $Ca_v1.2$ and $Ca_v1.3$. The more robustly identified risk gene *CACNA1C* codes for the alpha-1 sub-unit of $Ca_v1.2$ channels, however at present it is impossible to discern the role of these specific channels with pharmacological methods. Previous studies combining genetic and pharmacological approaches have suggested that $Ca_v1.3$ channels may be involved specifically in the consolidation of CFM whereas $Ca_v1.2$ may be necessary for extinction (Moosmang et al., 2005). Separable roles of NMDA receptors (GluN2A and GluN2B) have been found in relation to consolidation and extinction of a conditioned fear response (Dalton et al., 2012). Inhibition of GluN2A was found to selectively affect consolidation, whereas inhibition of GluN2B was found to affect extinction.

As with NMDA receptors, it could be the case that different sub-types are relevant for the initial formation of CS-US conditioning and others for subsequent relevant CS-No US associations. In the absence of sub-unit specific inhibitors, genetic manipulations may be able to more successfully separate their function in relation to specific behaviours.

4.11 Conclusions

These experiments demonstrate a selective role for calcium influx through LVGCCs in specific aspects of associative learning; consolidation, extinction and latent inhibition. Inhibition of LVGCCs resulted in both deficits in conditioned behaviour as well as its recovery or erroneous perseverance. This makes it highly unlikely that diltiazem may be having an effect due to simple damage of the system. It instead indicates a role for LVGCCs in the formation of both new and competing associative memories, as well as potentially relevant contextual representations, which are then used to inform subsequent behaviour.

5 BASIC CHARACTERISATION OF *CACNA1C* HETEROZYGOUS KNOCK-OUT RATS

5.1 Introduction

Previous experiments using diltiazem have revealed a role for LVGCCs in specific aspects of associative learning. However, as discussed, these experiments are unable to distinguish between the sub-types of LVGCC, $Ca_v1.2$ and $Ca_v1.3$. To address the specific role of *Cacna1c* and $Ca_v1.2$ channels in these processes we utilised a global heterozygous *Cacna1c* knock-out rat model (Sage Research Labs – See Methods 2.1.2).

The investigation of effects of reduced expression of a gene complements pharmacological studies in allowing assumptions to be made about the necessity of the gene product in specific processes. Above and beyond this, it also provides a method for understanding how this gene may be relevant in psychiatric illness and how alterations in its expression may manifest functionally in specific symptoms. Common variation in the *CACNA1C* gene associated with risk for schizophrenia and bipolar disorder, has been linked to decreased expression in certain regions of the brain, including the cerebellum (Roussos et al., 2014; Eckart et al., 2016)(see Chapter 8 for more detailed discussion in humans). Furthermore, exome sequencing has reported an enrichment in rare deleterious mutations in calcium channel subunits, to include *CACNA1C*, in people with schizophrenia (Purcell et al., 2014b), supporting the investigation of a reduced expression model.

Conditional *Cacna1c* knock-outs and region specific knock-outs have been extremely valuable for understanding the roles of $Ca_v1.2$. However, these models do not necessarily reflect relevant disease state. Genetic risk is present throughout the life-course and will likely affect central and peripheral systems across development.

Chronic haploinsufficiency may therefore be a better model when considering relevance to psychiatric illness. There are no instances of people with complete deletion of the *CACNA1C* gene and homozygous knockout in rodents is embryonically lethal (due to its vital role in cardiac function, Goonasekera et al., 2012); therefore, investigating the subtle effects of expression manipulation, rather than gene absence is more relevant when considering symptoms of disease. It is worth noting that overexpression of $Ca_v1.2$ in mice has been found to cause a slowly progressing hypertrophy and eventual heart failure (Muth et al., 2001). This complicates the investigation of a global over expression model of *Cacna1c* for investigation of more complex long term behaviour.

A newly characterised haplo-insufficient rat model was chosen for study. Rats allow the modelling of more complex behaviours and cognitive processes that better reflect neuropsychological testing in humans with relevance to disease (Iannaccone and Jacob, 2009). Their physiology is also closer to humans than that of mice and their systems should respond in a more similar way to any pharmacological manipulations that may be used at a later date. Larger brain sizes also make certain techniques more practical in rats. It further provides greater continuity with regard to previous experiments with LVGCC inhibition and expression profiling.

It was important to establish the validity of this novel rat model in relation to *Cacna1c* expression and to conduct some basic characterisation of behaviour; including locomotor activity, anxiety behaviour and startle response, which potentially could impact on later learning paradigms. This is particularly pertinent to fear conditioning, as freezing behaviour is used as the measure of associative memory formation and expression. Changes in activity levels could confound both conditioned and unconditioned fear associated behaviours. In addition, it has been found that anxiety can affect both the acquisition and extinction of fear memories in people (Lissek et al., 2005).

Genetic knockout models of *Cacna1c* have, to date, all been in mice. Global heterozygous models have shown minimal differences in baseline behaviour measures. Dao et al., (2010) found no differences between WT and HET mice on motor coordination using an accelerating rotarod task, as well as no differences in pain sensitivity or muscle strength, measured by a hot plate and hanging wire test respectively. Similarly, there have been no differences reported in activity during an open field or home-cage locomotor paradigms in male mice with either global haploinsufficiency or forebrain specific knockout of *Cacna1c* (Dao et al., 2010; Lee et al., 2012). However, there has been subtle indication of reduced locomotor activity in female HET mice compared to WT (Dao et al., 2010).

Conflicting results regarding anxiety have been reported. Lee et al., (2012) found that both global haploinsufficiency of *Cacna1c* and forebrain specific knockdown led to decreased time spent in the open arm of an elevated plus maze (EPM), an indication of increased anxiety. However, they found no differences in behaviour in an open field exploration task (OF) or during a light-dark conflict test (LDC) in mice with a global knock-down of *Cacna1c*. Conversely, Dao et al., (2010) found no effects of globally reduced *Cacna1c* on any of the above tasks in male mice and additionally no effects on physiological response to stress/anxiety. However, female HET mice showed mildly increased anxiety. Overall, studies suggest that forebrain specific knockout of *Cacna1c* rather than global haploinsufficiency may lead to anxiety-like behaviour.

There has been minimal investigation of the effects of reduced *Cacna1c* on startle response. Dao et al., (2010) reported no effects of genotype on acoustic startle response in male mice at pulse intensities from 80 – 120 dB, though they did observe an attenuated response at 120 dB in female mice. The acoustic startle task is also utilised to assess pre-pulse inhibition (PPI), though this does not appear to have been investigated in *Cacna1c* knockout models at present. Deficits in pre-pulse inhibition

are well-established in schizophrenia and this measure is a reliable quantitative phenotype for potential sensorimotor gating deficits in rodents (Swerdlow et al., 2000, 2008).

Compensation from increases in *Cacna1d* expression have been investigated across previous models. There does not appear to be evidence to support increased *Cacna1d* expression in the brain of HET mice, either with global haploinsufficiency (Dao et al., 2010) or with region specific knockout (Langwieser et al., 2010). Increases of *Cacna1d* expression have been found in the heart, though this does not appear to be sufficient to fully compensate *Cacna1c* gene function (Rosati et al., 2011). One study did identify a compensation effect, observing increases of calcium permeable AMPA receptors (cp-AMPAR) in the lateral amygdala of conditional CNS knockout mice and LVGCC independent LTP (Langwieser et al., 2010). This has been suggested to be part of a mechanistic adaptation to LVGCC inactivity, through the alternative use of cp-AMPAR, as has been observed in hippocampal neurons in vitro (Thiagarajan et al., 2005). However, this effect is unlikely to be a significant mechanism in heterozygous knockout models due to the continued presence of functioning LVGCCs. The upregulation of other calcium channels in haploinsufficiency models has not been reported.

These experiments aimed to establish the validity of the *Cacna1c* heterozygous rat model and characterise basal expression levels. The expression of *Cacna1c* and *Cacna1d* was explored to determine successful reduction of the target gene and assess potential compensation. Basal expression of *BDNF* was also measured to investigate potential effects on known downstream mechanisms. Basic characterisation of activity and anxiety levels were conducted to ensure no confounding effects on later learning paradigms. Animals were tested for activity levels and habituation during repeated exposure to a novel home cage environment. An additional measure of activity was obtained from an open field exploration task

and anxiety was assessed based on time spent in the centre of the open field arena. Animals underwent an acoustic startle response test to determine startle reactivity and to measure pre-pulse inhibition.

5.2 Experiment 1 - Gene expression in *Cacna1c* HET animals

For the full description of the design and production of the genetic mutation in *Cacna1c* and development of the model see General Methods, 2.1.2. Before any behavioural experiments were conducted on these animals it was vital to establish the validity of the model. Protein and mRNA levels were measured to determine successful reduction of expression of *Cacna1c* in different regions of the brain. *Cacna1d* expression was measured to determine whether there were any compensatory increases in the other LVGCC expressed in the brain. Levels of LVGCC regulated and learning associated gene *BDNF* were also measured as a potential indicator of a downstream effect of altered LVGCC function (as previously discussed, General Introduction).

5.2.1 Methods and materials

5.2.1.1 Subjects

Thirty-eight adult male Sprague Dawley animals were obtained from Charles River and housed in groups of 1 – 4 on a reverse light-dark cycle (lights on at 10am) as previously described. Animals were given 7 days to settle before sacrifice in home cages with a rising concentration of CO_2 (Clinipath Equipment Limited, Hull, UK). Whole brains were taken from 10 of the animals (5 WT and 5 HET) and fresh frozen immediately post-mortem for *in situ* hybridisation analysis. Microdissections were manually taken from the remaining animals and fresh frozen on dry-ice, to include PFC, cerebellum, CA1 and DG/CA3. These were used for extractions of mRNA and protein for RT-qPCR and ELISA assays.

5.2.1.2 Transcript quantification by in situ hybridisation (ISH)

For detailed methods see General Methods 2.4.6. 14µm coronal slices of the PFC, cerebellum and hippocampus were taken from the ten whole brain samples and mounted on poly-L-lysine coated slides (VWR International, Leics, UK). Two series

of five brains per slide were created with WT and HET brains represented in both series. Slides were fixed with 4% PFA, dehyrdrated and stored in 95% ethanol until use. The 45 bp oligonucleotide probes for *Cacna1c* and *BDNFIX* (see Chapter 3) were radiolabelled using deoxyadenosine 5'- (α-thio) triphosphate [^{35}S] (dATP) (Perkin Elmer, MA, USA). Three slides from each series per brain region were hybridised separately to each labelled probe, two "Total" slides and one "Non-specific". Hybridisation was performed overnight at 42 °C, followed by two 30 min washes in 1 x SSC at 52 °C. Slides underwent additional washes in 0.1 x SSC, 70% and 95% EtOH for 1 min each and left to air dry before apposing to Carestream Biomax MR film (Anachem, Luton, UK). Sections were exposed for 1 week before being developed using an automatic developer (Photon Imaging Systems, Swindon, UK).

Analysis was conducted using imageJ software as described previously, with optical density values quantified using a ^{14}C ladder. Ten measurements (five bilateral) were made for each region of interest from each brain slice. Specifically, CA1, CA3 and DG regions of the hippocampus, the granule layer of the cerebellum and the medial PFC. Regional values were averaged within animal for the two "Total" slides as a technical repeat and the "Non-specific" average was subtracted to give a "Specific" value for each animal. Values were normalised to mean WT by region and averaged within genotype. Student's t-tests were conducted for each region to compare expression between WT and HET. Bonferroni corrections were applied where there were no prior hypotheses. Dunnet's tests were used when comparing to control group.

5.2.1.3 Protein quantification by ELISA assay

5.2.1.3.1 Protein extraction

For detailed protocol, see General Methods, 2.4.7. Approximately 100 µg of tissue was homogenised in 1 ml T-PER (Tissue Protein Extraction Reagent, Thermo

Scientific, UK) with protease inhibitors. Samples were centrifuged at 10,000 x g for 5 mins and the supernatant taken for analysis. Protein concentration was measured using a BCA assay (Pierce BCA Protein Assay Kit, Thermo Scientific, UK).

5.2.1.3.2 Immunoassays

BlueGene ELISA kits (American Research Products Ltd, MA, USA) were used to quantify protein of the two calcium channels genes using the competitive enzyme technique. Experiments were conducted as per manufacturer's protocols. Samples were diluted 1:2 in extraction buffer and added to wells, along with a balance solution and enzyme conjugate and incubated for 1 hour at 37°C. Six standards were run alongside to provide a standard curve for quantification as well as a blank control.

A sandwich ELISA (Thermo Scientific, UK) was used to quantify BDNF protein levels. Samples were diluted 1:10 in extraction buffer before adding to wells along with 7 standards and a blank control. The plate was incubated overnight at 4°C, before washing 4 x and adding biotinylated antibody.

Wells for all plates were incubated with a substrate for HRP enzyme, followed by a stop solution. Plates were measured within 5 mins at 450 nm in a microplate reader. A standard curve was plotted from the standards to calculate protein concentration of each sample from intensity readings. Student's t-tests were conducted to compare expression between WT and HET animals for each region. Values were normalised to WT for graphical representation.

5.2.2 Results

5.2.2.1 Reduction of CACNA1C expression in HET animals compared to WT across brain regions

The expression of *Cacna1c* was assessed by ISH. HET animals showed an average 48% reduction of *Cacna1c* mRNA in the hippocampus, 37% reduction in the cerebellum and 17% in the PFC (Figure 5.1). Reduced *Cacna1c* mRNA levels were observed in HET animals in the CA1 and CA3 sub-regions of the hippocampus and the cerebellum ($t(8) = 2.306$, $P = 0.05$; $t(8) = 4.945$, $P = 0.001$; and $t(6.003) = 3.117$, $P = 0.021$ respectively). mRNA expression levels in the DG and PFC of HET animals were found not to be normally distributed, therefore log10 transformations were conducted. There was significantly reduced expression of *Cacna1c* in HET animals in the DG sub-region of the hippocampus and a strong trend towards a reduction in the PFC ($t(8) = 4.101$, $P = 0.003$ and $t(8) = 2.290$, $P = 0.051$ respectively).

Protein levels were measured using an ELISA assay for PFC, cerebellum and DG/CA3 regions of the hippocampus (Figure 5.2). There was a significant reduction of CACNA1C expression in the cerebellum ($t(10) = 2.751$, $P = 0.020$). Expression in the PFC was not normally distributed and a log10 transformation did not result in normality, therefore a non-parametric Mann-Whitney-U test was conducted. Significantly reduced expression was observed in HET animals compared to WT ($U = 5.00$, $P = 0.041$). Expression in the DG/CA3 did not significantly differ between WT and HET animals ($t(10) = 1.136$, $P = 0.283$).

There were no differences observed between WT and HET animals in the level of CACNA1D protein measured from the same protein extractions as above (Figure 5.3) (*PFC*: $t(10) = -1.674$, $P = 0.125$; *cerebellum*: $t(10) = 1.064$, $P = 0.312$; *DG/CA3:* $t(10) = 0.226$, $P = 0.826$).

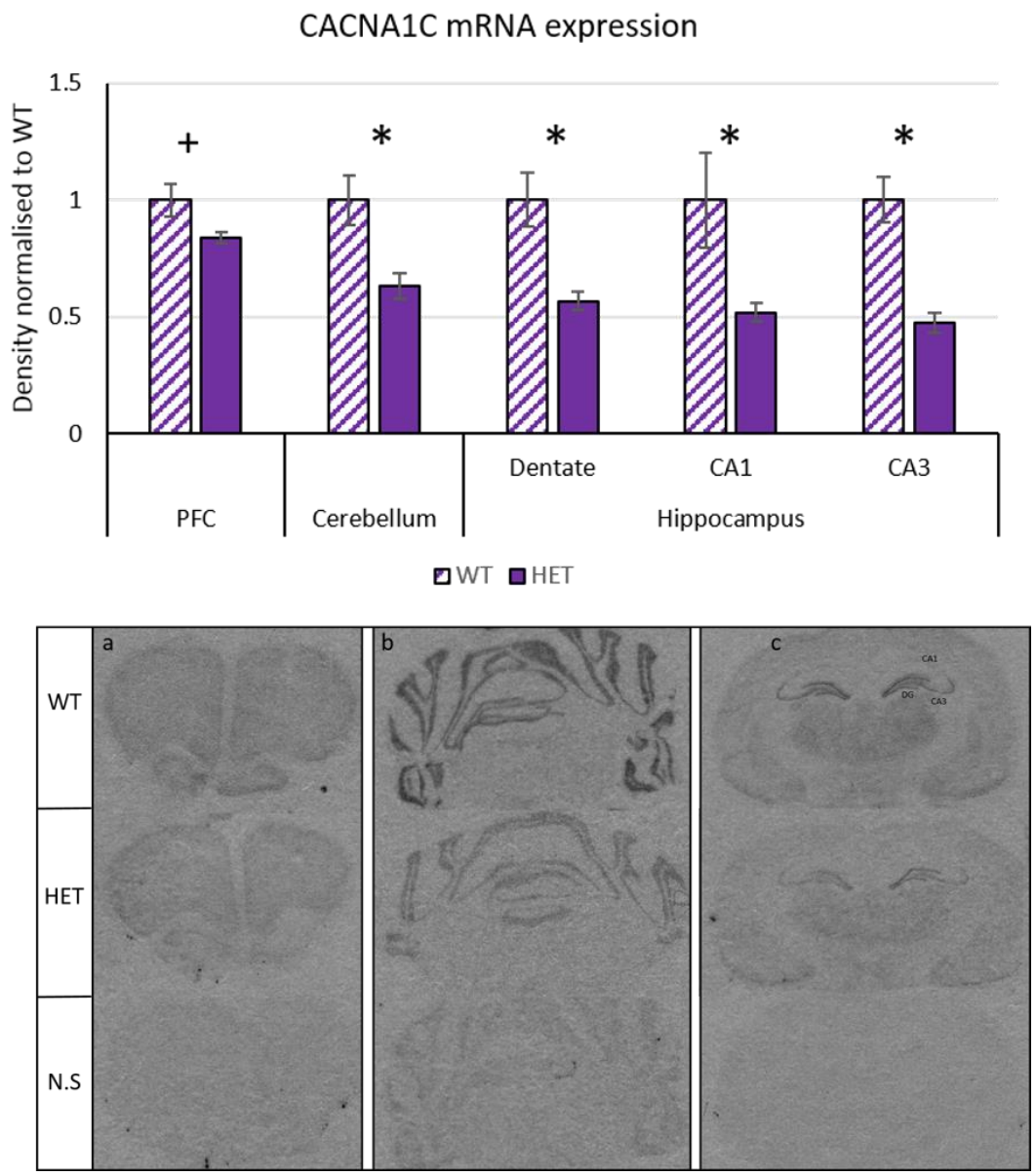

*Figure 5.1 CACNA1C mRNA expression in PFC, cerebellum and hippocampus. HET animals show reduced expression across brain regions (17 – 48%). Bars represent mean value normalised to WT for each genotype, error bars are SEM. * P < 0.05, + P <0.06. Representative ISH for WT, HET and non-specific binding (N.S.) for; a) PFC; b) Cerebellum; c) Hippocampus CA1, CA3 and DG regions labelled.*

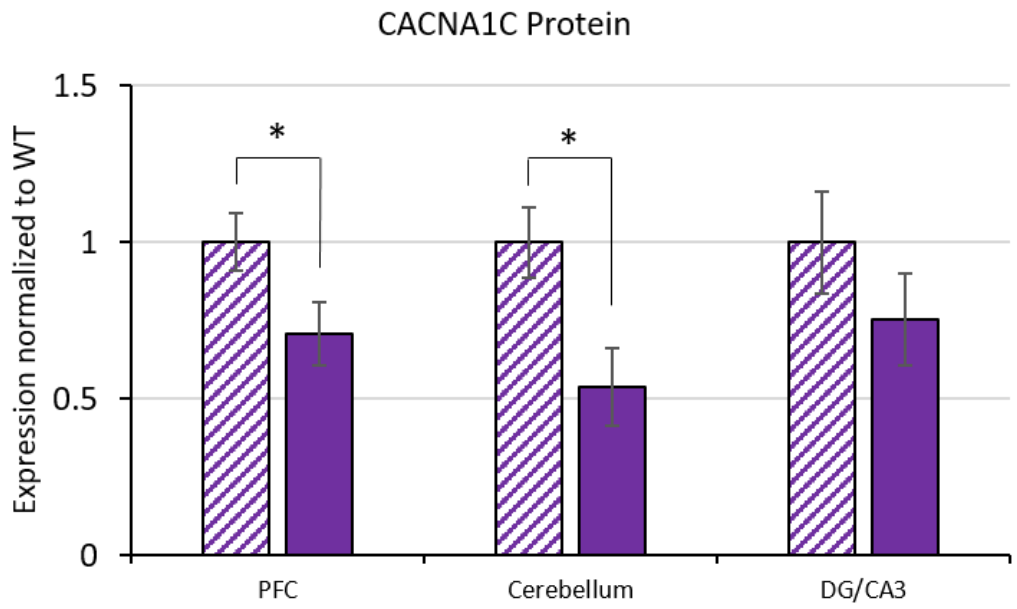

*Figure 5.2 CACNA1C protein expression in PFC, cerebellum and DG/CA3 region of the hippocampus. Significantly reduced expression in the PFC and cerebellum. Bars represent mean fold change normalised to WT, error bars are SEM. * P < 0.05.*

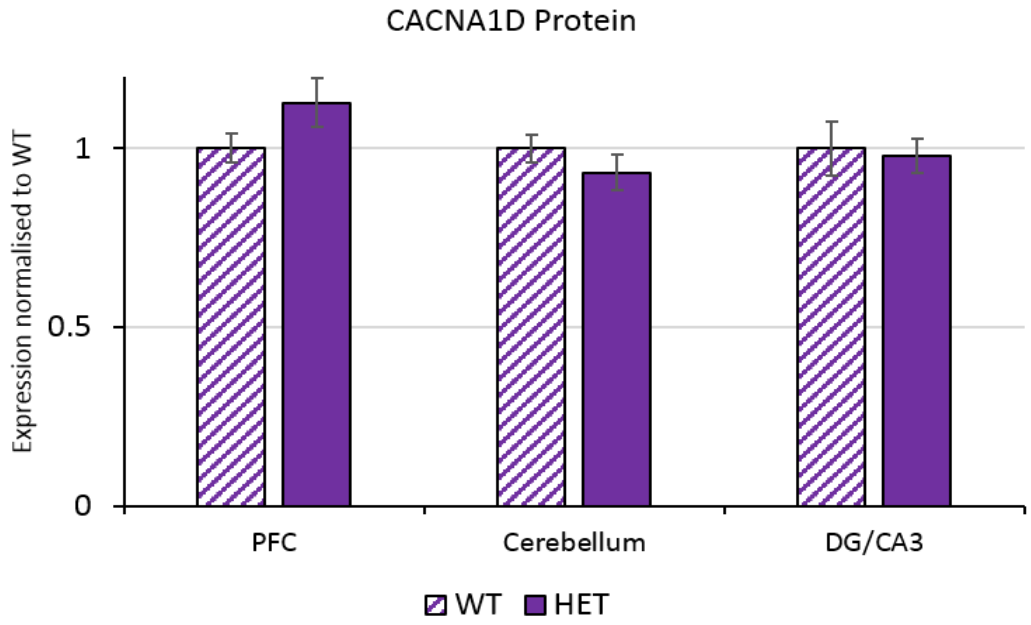

Figure 5.3 CACNA1D protein expression in PFC, cerebellum and DG.CA3 region of the hippocampus. No significant differences between WT and HET animals in any region. Bars represent mean fold change normalised to WT, error bars are SEM.

5.2.2.2 Reduced BDNFIX expression in HET animals

Repeated measures ANOVA found a region by genotype interaction effect on the expression of total *BDNF (BDNFIX)* ($F(4,32) = 2.672$, $P = 0.050$). HET animals were found to have a 22% reduction in total *BDNF* (*BDNFIX)* mRNA levels in the PFC ($t(8) = 2.316$, $P = 0.049$), though this did not survive correction for multiple comparisons (Figure 5.4). There was a trend towards increased *BDNF* expression in HET animals in the DG region of the hippocampus ($t(8) = -2.177$, $P = 0.061$), but no evidence to support differences in expression in the CA1 or CA3 sub-regions ($t(8) = -1.418$, $P = 0.194$; $t(8) = -0.558$, $P = 0.592$). No differences in expression were observed in the cerebellum ($t(8) = -0.885$, $P = 0.402$).

There were no changes in BDNF protein levels between WT and HET animals (Figure 5.5). The ELISA assay showed similar levels of BDNF expression in the DG/CA3 region of the hippocampus, cerebellum and PFC ($t(10) = 0.011$, $P = 0.991$; $t(10) = 0.104$, $P = 0.919$; $t(10) = 0.521$, $P = 0.614$ respectively).

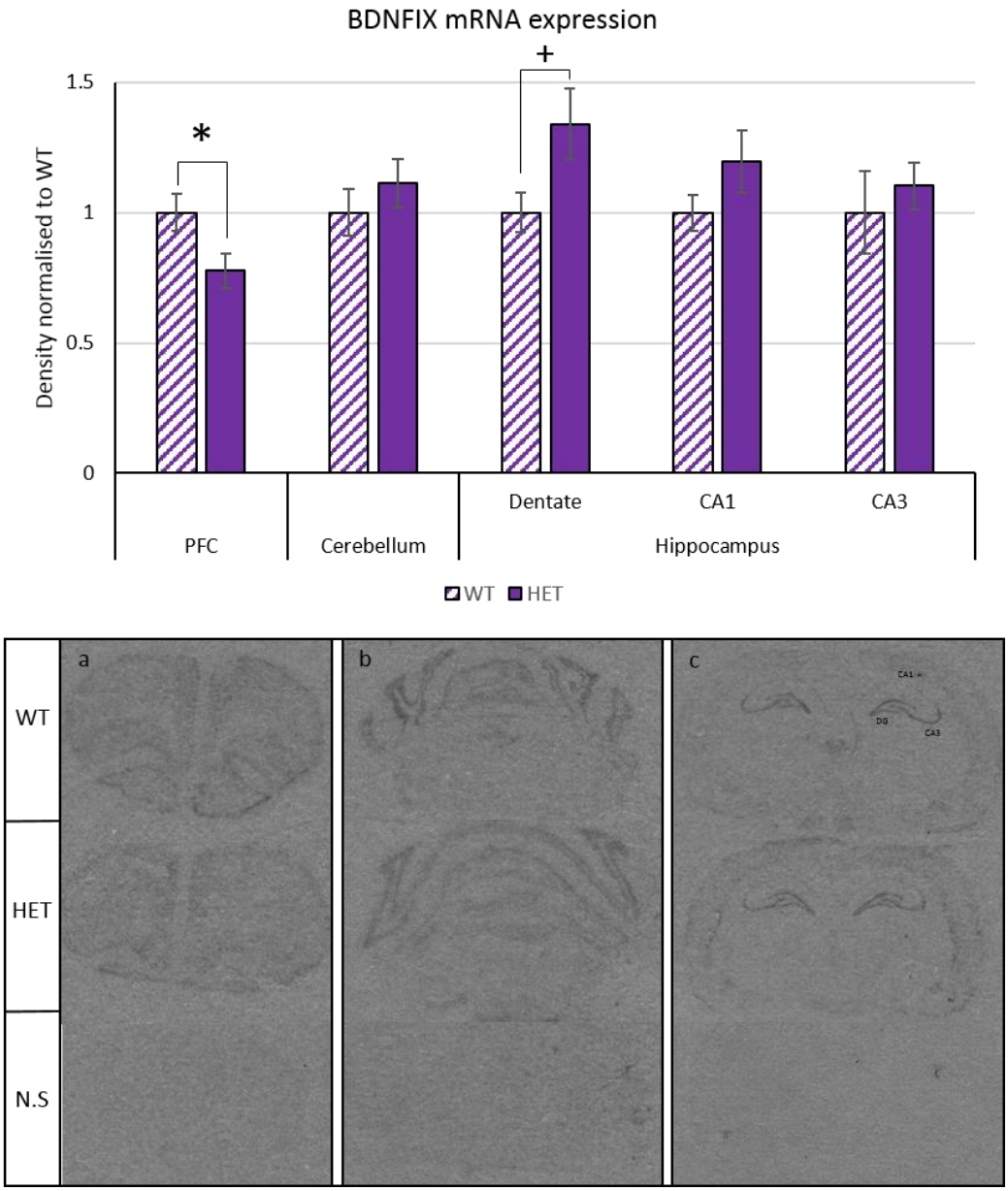

*Figure 5.4 BDNFIX mRNA expression levels compared between WT and HET animals in PFC, cerebellum and hippocampus. There was a region by genotype interaction effect with results showing a 22% reduction in PFC in HET animals and a trend towards increased expression in the hippocampus. There was no evidence of changes in cerebellum, CA1 or CA3 regions. Bars represent mean expression normalised to WT and error bars are SEM. * P < 0.05. + P < 0.1 a) Representative ISH of PFC, b) cerebellum and c) hippocampus, for WT and HET animals and a non-specific (N.S) control.*

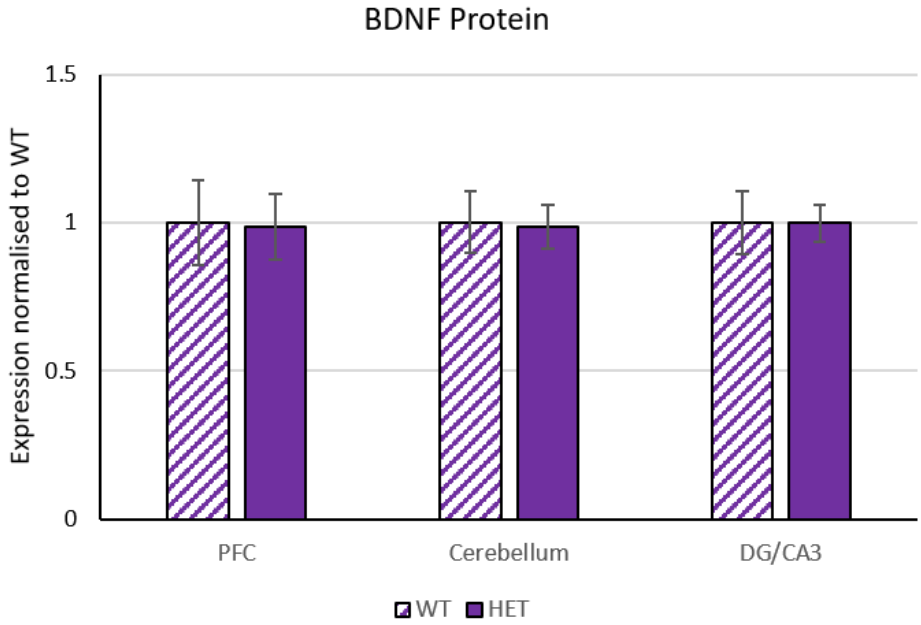

Figure 5.5 BDNF protein expression in WT and HET animals in PFC, cerebellum and DG/CA3 region of the hippocampus. No differences observed between WT and HET. Bars represent mean concentration of BDNF for each genotype in each region of interest (pg/ml), error bars are SEM.

5.3 Experiment 2 - Basic behavioural characterisation of HET and WT animals

The following experiments assessed the basal activity and anxiety levels in WT and HET animals. Exposure to a novel home-cage environment was used to assess locomotor activity and an open field exploration was conducted as a further measure of activity in a mildly aversive context and an indication of any anxiety-related behaviour. Startle response was further compared between WT and HET animals using an auditory startle response paradigm. The same paradigm was used to conduct analyses of pre-pulse inhibition, as a measure of sensorimotor gating, a common phenotype assessed in relation to schizophrenia.

5.3.1 Methods

5.3.1.1 Animals

Thirty-two Sprague Dawley rats, 16 WT and 16 HET litter mates, were housed in groups of 2-4 with ad libitum access to food and water. All animals were weighed before any experiments occurred and handled for 5 days to habituate. Weights ranged from 267 g to 486 g. There was no difference in weight between genotype ($t(30) = 1.086$, $P = 0.286$).

5.3.1.2 Locomotor activity

Behaviour

Activity levels were tested in empty home-cage equivalent contexts for 2 hrs on 3 consecutive days (See General Methods). Animals were taken in groups of 8 to the testing room in home-cages and transferred to individual testing boxes. The number of beam breaks and the number of runs (2 consecutive independent beam breaks, indicative of rapid movement from one end of the cage to the other) were counted for each animal, split into four 30 min bins.

Analysis

Shapiro-Wilks tests revealed that data were not normally distributed. All variables underwent a square root transformation before analysis to attain a normal distribution; a log transformation was unsuitable with the high incidence of '0' values later in the sessions. Total beam breaks and runs were compared between WT and HET animals using independent samples student's t-tests, as measures of locomotor activity. Levene's tests for equality of variances were conducted and corrections used where stated. Differences within the sessions and across the 3 testing days were compared within animals and between genotype using repeated measures ANOVA, as a measure of habituation to a novel context. As behaviour was consistent across the 3 testing days, data was averaged across the 3 days for each animal for

within session analyses. Between session analyses were conducted on total beam breaks and runs. Greenhouse-Geisser corrections were applied where unequal variances were observed. One WT animal was excluded due to concerns that the apparatus recording may have temporarily malfunctioned with data being more than 3 standard deviations away from the mean.

5.3.1.3 Open Field Exploration

Behaviour

Animals were placed into an arena (1 m^2) for 10 mins (See General Methods). A central zone was defined as the centre 70 cm x 70 cm square. Distance and speed travelled were recorded for each animal as an additional measure of activity. Duration of time spent in the central zone and in the perimeter of the arena were recorded, with greater time spent in the central zone indicating reduced anxiety.

Analysis

Student's t-tests were used to compare distance, speed and time in the central zone between WT and HET animals. Shapiro Wilk's test found a non-normal distribution for time spent in the central zone, therefore a log10 transformation was conducted prior to a student's t-test. Levene's test for equality of variances found no significant results for any variable.

5.3.1.4 Startle response and pre-pulse inhibition

Behaviour

Animals underwent a 30 min startle response session in a SR-Lab™ Startle Response System, consisting of 91 trials. Background noise level was set to 70 dB and test pulses were administered at 120 dB and 105 dB. Pre-pulse trials were interleaved throughout the session at 4 dB, 8 dB and 16 dB to determine levels of pre-pulse inhibition. At the end of the session there were 6 additional pulses of increasing intensity; 70 dB, 80 dB, 90 dB, 100 dB, 110 dB and 120 dB to measure

increasing startle response. An accelerometer sensor measured the amount of movement of each animal in response to each test pulse.

Analysis

Repeated measures ANOVA was used to analyse the effect of increased pulse intensity and genotype on startle response; as well as to determine the effect of increased pre-pulse intensity. Student's t-tests were used to compare overall startle response for 120 dB pulses alone and 105 dB pulses between WT and HET animals. One HET animal was excluded from analyses due to values being greater than 2 standard deviations away from the mean. Levene's tests for equality of variance were conducted and Greenhouse-Geisser corrections were applied where necessary. Shapiro-Wilk's tests for normality found no significant effects.

5.4 Results

5.4.1 Reduced expression of *Cacna1c* does not affect activity levels or habituation to a novel environment

There was no difference in the total number of beam breaks or runs made between WT and HET animals across the full 3 days of testing (t(29) = -1.666, P = 0.107 and t(29) = -1.526, P = 0.138 respectively) (Figure 5.6).

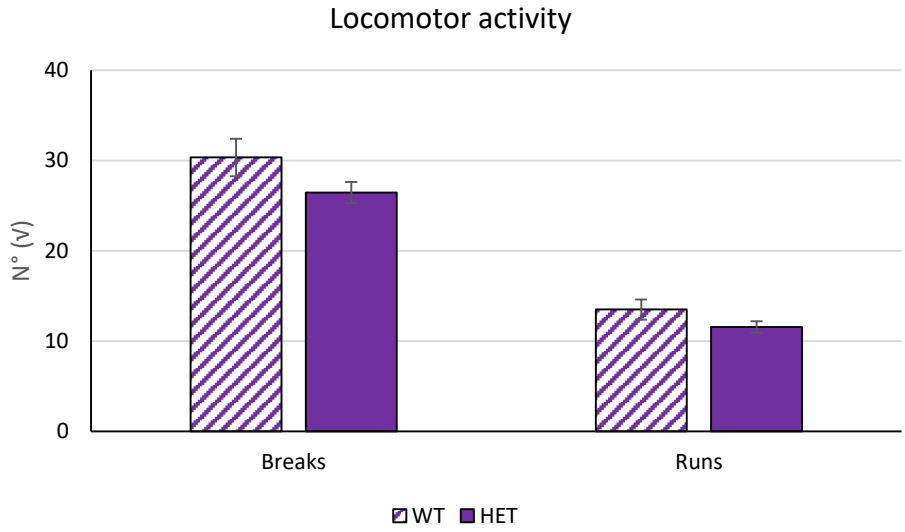

Figure 5.6 Total number of "Breaks" and "Runs" for WT and HET animals across the 3 consecutive test days. No differences were observed between WT and HET animals, P > 0.05. Bars represent mean value for each genotype, transformed by square-root, error bars are SEM. Y-axis is the square root of counts.

All animals showed normal habituation to context exposure within session, with fewer beam breaks and runs being made in the last half hour of the sessions compared to the first. The number of beam breaks and runs were compared between WT and HET split into four 30 min bins across the 2-hour sessions (Figure 5.7). Repeated measures ANOVA showed a significant effect of time within session on number of beam breaks ($F(2.069, 59.990)_{\varepsilon = 0.690} = 184.494$, $P < 0.001$, $\eta_p^2 = 0.864$). There was no evidence of an interaction, though a trend towards an effect of genotype ($F(2.069, 59.990)_{\varepsilon = 0.690} = 0.173$, $P = 0.849$ and $F(1,29) = 3.407$, $P = 0.075$ respectively).

Similarly, there was a significant effect of time within session on number of runs ($F(1.942, 56.332)_{\varepsilon = 0.647} = 187.085$, $P < 0.001$, $\eta_p^2 = 0.866$). There was no evidence of an interaction but a trend towards an effect of genotype ($F(1.942, 56.332)_{\varepsilon = 0.647} = 0.427$, $P = 0.649$ and $F(1,29) = 3.620$, $P = 0.067$ respectively). Post hoc analyses revealed that both trends towards genotype effects were driven by differences in the third 30-minute bin (Breaks: $P = 0.047$, Runs: $P = 0.026$), with no effects observed in the first hour or last 30 minutes of the task.

All animals showed between session habituation with fewer breaks and runs observed on testing day 3 compared to day 1. Repeated measures ANOVA reported a significant effect of day on beam breaks and runs ($F(2,58) = 5.465$, $P = 0.007$, $\eta_p^2 = 0.159$ and $F(2,58) = 5.695$, $P = 0.006$, $\eta_p^2 = 0.164$ respectively). There was no evidence of an interaction between day and genotype for either beam breaks or runs ($F(2,58) = 0.169$, $P = 0.845$ and $F(2,58) = 0.543$, $P = 0.584$ respectively). Beam breaks across the 3 days did not differ by genotype, though a trend was reported for runs ($F(1,29) = 2.893$, $P = 0.100$ and $F(1,29) = 3.354$, $P = 0.077$ respectively). Post-hoc analysis of separate days indicated that this was driven by a trend on day 2 towards reduced runs in HET animals ($F(1,29) = 3.013$, $P = 0.093$), with no effects on day 1 or day 3 ($P = 0.212$ and $P = 0.510$ respectively).

Power analysis of these comparisons indicate that sample sizes were adequate (at a level of 0.8) to detect large effect sizes (< 0.6). Previous studies have reported large effects of genotype on similar tasks (e.g. Miyakawa et al., 2003), however given the trends observed, it is possible that small effects of genotype on locomotor activity may have been neglected. Results should therefore be interpreted with caution in relation to the absence of effects and will be taken into account for future experiments where this may influence interpretations.

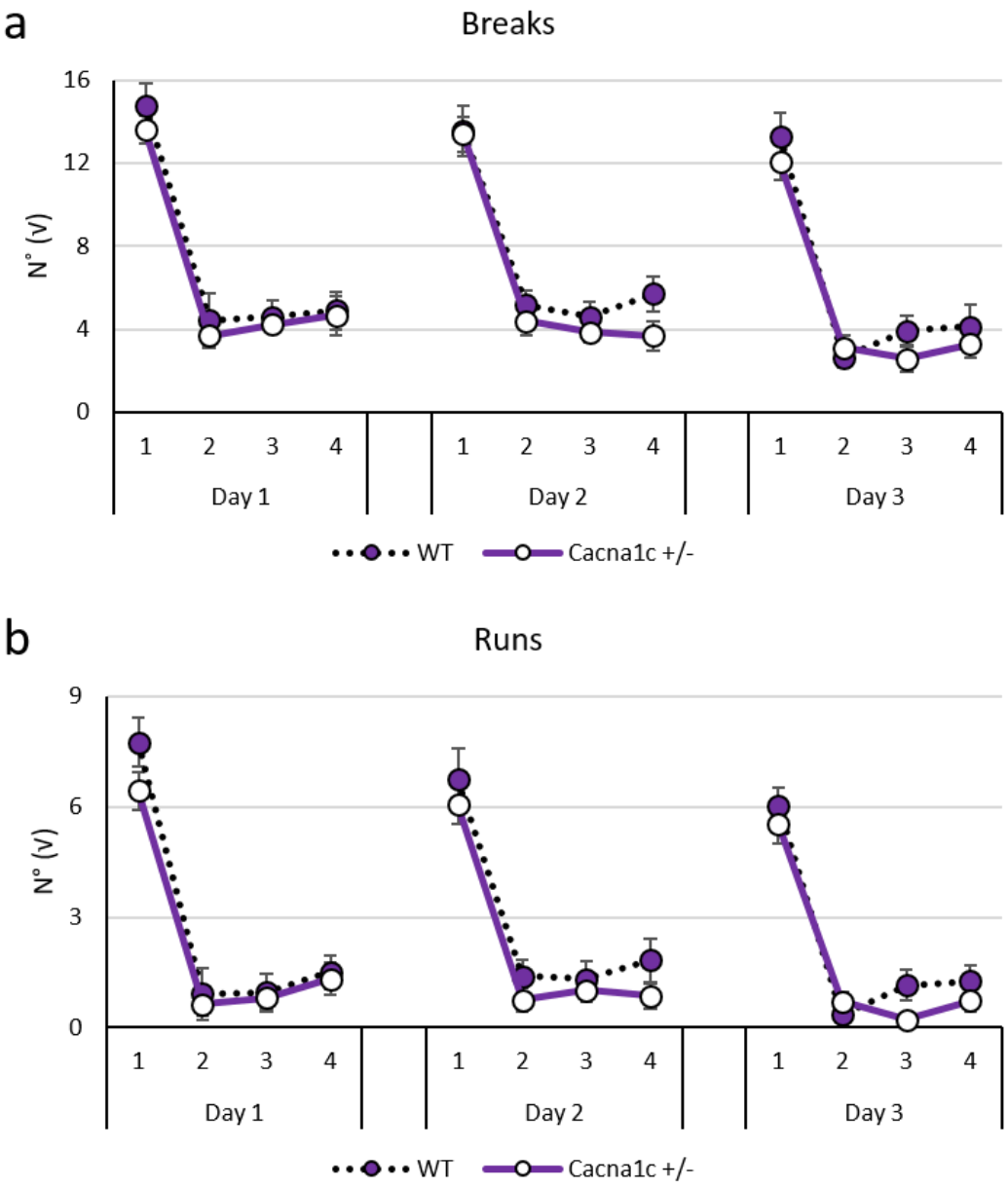

Figure 5.7 Locomotor activity split by test day. a) Number of beam breaks for WT and HET animals during three consecutive days of 2-hour exposure to a novel home-cage style environment. Numbers 1-4 represent individual 30 min bins within the 2-hour recording period. No differences observed between genotype, P > 0.05. b) As graph a. for "runs", recorded as two different consecutive beam breaks indicating movement from one end of the cage to another. Data points represent mean number of breaks or runs for each genotype (transformed by square-root), error bars are SEM.

5.4.2 Reduced expression of *Cacna1c* does not affect activity or anxiety levels in an Open Field Exploration task.

There was no difference in activity levels between WT and HET animals, with similar distance travelled and similar mean and max velocities during the task ($t(30) = -0.294$, $P = 0.771$; $t(30) = -0.293$, $P = 0.771$; and $t(30) = -0.064$, $P = 0.950$ respectively) Figure 5.8).

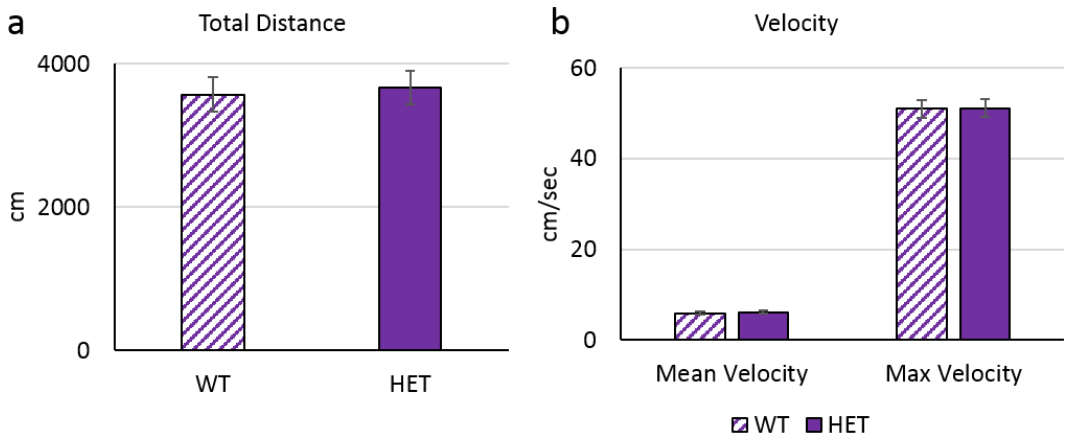

Figure 5.8 Open Field Activity Levels. a) No difference between WT and HET animals in the distance travelled during the task, P = 0.771. b) No difference in the mean or max velocity travelled between WT and HET animals, P = 0.771 and P = 0.950 respectively. Bars represent mean values for each genotype, errors bars are SEM.

There was no difference in anxiety levels between WT and HET animals (Figure 5.9), indicated by a similar amount of time spent in the central zone during the 10 min exploration ($t(30) = 1.567$, $P = 0.128$).

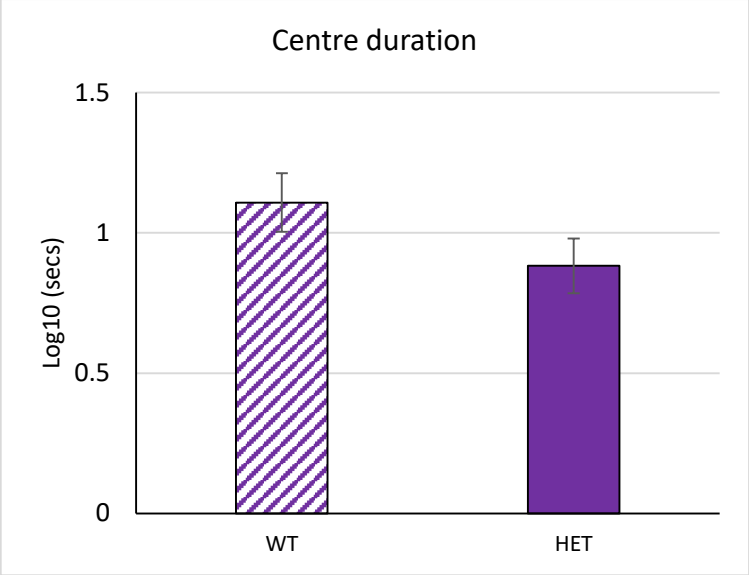

Figure 5.9 Duration spent in central zone of arena for WT and HET animals. No differences observed between genotype. Data presented has undergone a log10 transformation. Bars represent means for each genotype and error bars are SEM.

5.4.3 Reduced expression of Cacna1c does not affect startle response or pre-pulse inhibition

Animals were compared on auditory startle response and effects of pre-pulse inhibition (Figure 5.10). There was no effect of genotype on startle to 120 dB or 105 dB test pulses compared to 70 dB background noise ($t(29) = 1.070$, $P = 0.293$ and $t(29) = -0.240$, $P = 0.813$ respectively). Increasing test pulses in increments of 10 dB, from 70 dB to 120 dB, resulted in the expected increase in startle response ($F(1.444, 41.872)_{\varepsilon = 0.298} = 145.237$, $P < 0.001$, $\eta_p^2 = 0.834$), with no effect of genotype observed or an interaction ($F(1,29) = 0.987$, $P = 0.329$ and $F(1.444, 41.872)_{\varepsilon = 0.298} = 0.696$, $P = 0.460$).

Pre pulses of 4 dB, 8 dB and 16 dB were interleaved between trials to measure pre-pulse inhibition of startle response to test pulses. There was a significant effect of pre-pulse intensity on percentage inhibition of startle to 120 dB pulses ($F(1.292, 37.469)_{\varepsilon = 0.646} = 50.581$, $P < 0.001$, $\eta_p^2 = 0.636$), though no differences observed between genotypes or evidence of an interaction ($F(1,29) = 1.462$, $P = 0.236$ and $F(1.292, 37.469)_{\varepsilon = 0.646} = 0.041$, $P = 0.895$ respectively).

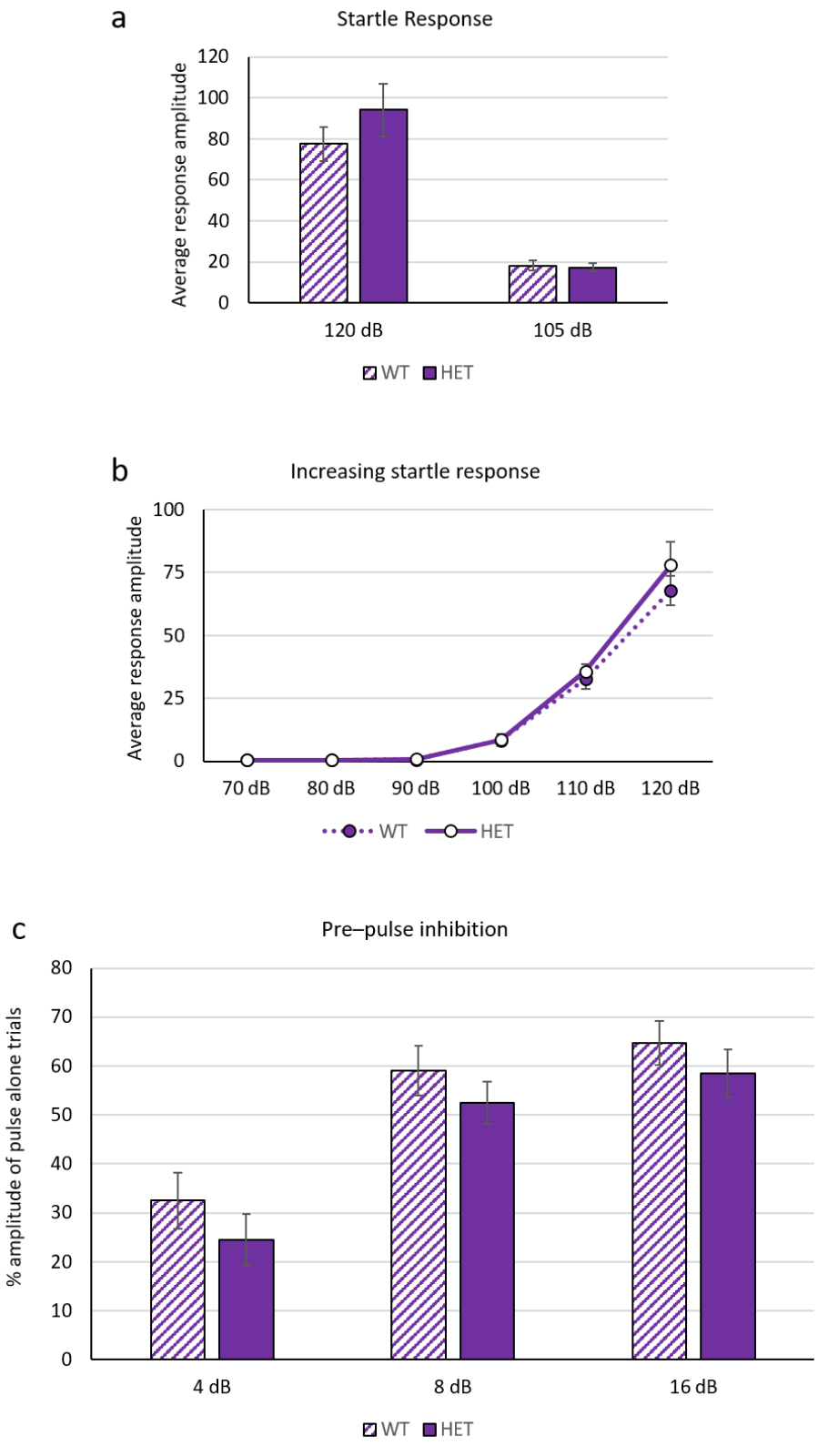

Figure 5.10 Startle response compared between WT and HET animals. a) Average response amplitude to 120 dB and 105 dB pulse alone trials. b) Effect of increasing startle response between 70 dB and 120 dB. c) Percentage inhibition of response to 120 dB test pulses for 4 dB, 8 dB and 16 dB pre-pulses. No differences were observed between WT and HET animals (P> 0.05). Bars and data points correspond to average amplitude of response for each genotype. Error bars are +/- SEM.

5.5 Discussion

5.5.1 Reduced expression of *Cacna1c* in heterozygous rats with no evidence of compensation by *Cacna1d*

The model used for all subsequent experiments shows a significant reduction of *Cacna1c* transcript in the cerebellum and the hippocampus and a trend towards reduced expression in the PFC. Protein levels of CACNA1C were significantly reduced in the cerebellum and PFC, though reductions did not reach significance in the DG/CA3 region of the hippocampus. There is no evidence of compensation in the expression of the highly homologous gene *Cacna1d,* which codes for the other LVGCC that is expressed in the CNS. This suggests that differences in behaviour observed between WT and HET animals can be attributed to the selective reduction of *Cacna1c*.

5.5.2 Reduction in *Cacna1c* is associated with alterations of *BDNF* mRNA expression with no effects on protein

HET animals showed reduced expression of total *BDNFIX* transcript in the mPFC and a trend towards an increase in expression in the DG region of the hippocampus. Whether the effects are truly specific to sub-regions of the hippocampus requires further investigation. Reduced BDNF has been observed in post-mortem PFC tissue of patients with schizophrenia (Weickert et al., 2003) and dysregulated expression of BDNF and its associated TrkB receptor has been reported in post-mortem patient hippocampus (Takahashi et al., 2000). Furthermore, processes known to require BDNF, including hippocampal neurogenesis (Lee et al., 2002), are found to be disrupted in mental illness (Schoenfeld and Cameron, 2015).

Previous findings have indicated a reduction in *BDNFIX* expression in the forebrain of *Cacna1c* knockout mice (Lee et al., 2016), though region specificity was not investigated. It was found that reduced *Cacna1c* in these mice was associated with

increased cell death of young hippocampal neurons, with no effects on proliferation. In contrast to these findings in mice, we observe only a small decrease in *BDNF* mRNA in the PFC, with no effects on protein. This may be due to the difference in model used, with a forebrain specific knockout in *Cacna1c* expression having larger downstream consequences. Although basal differences in expression of *BDNF* in the hippocampus were not significant, it would be pertinent to investigate expression in this region following tasks which are known to involve BDNF dependent mechanisms including learning and neurogenesis.

As discussed (in the introduction) calcium influx through LVGCCs preferentially regulates CREB-dependent transcription of *BDNF* through the rapid activation of signalling cascades in response to stimulation (West et al., 2001). The reduced expression of *Cacna1c,* in the absence of changes in *Cacna1d,* could therefore lead to decreased calcium influx through these channels, affecting the transcription of *BDNF* as observed in the PFC. Calcium influx through LVGCCs is thought to preferentially activate transcription of *BDNFIV* which is an activity-dependent transcript. Mutant mice with a selective disruption of this transcript have been found to show only a modest decrease in basal levels of *BDNFIX*, whereas significantly reduced activity-induced *BDNF* was seen compared to WT (Sakata et al., 2009). The indications of subtle differences in basal expression observed here may therefore become more pronounced following procedures known to induce synaptic plasticity and transcription of activity-dependent *BDNF*.

Future experiments should investigate activity dependent expression of BDNF protein and *BDNFIV* transcript in these animals, as well as potentially determining sub-cellular localisation. This will be discussed in more detail in the General Discussion.

5.5.3 No effects of reduced expression of CACNA1C on activity levels, anxiety or startle response

The current results primarily concur with previous studies that have found no effect of reduced CACNA1C on locomotor activity (Dao et al., 2010; Lee et al., 2012). There were no effects of genotype in a mildly aversive open field, though there were trends towards effects following prolonged exposure to a home-cage like environment. This suggests that activity levels are not likely to confound later experiments involving short exposure to a novel or aversive context. However, it is important to take into account that there may be small effects on locomotion that may not have been detected and which may have a greater influence on tasks involving prolonged exposure. There was also no effect of genotype on habituation following repeated exposures to a novel environment, indicated by reduced activity. The expected decrease in ambulation within and across sessions, indicates that animals are able to form and recall a representation of the context (Terry, 1979).

There were no differences observed between WT and HET animals in measures of anxiety related behaviour. Global heterozygous knockout mice models have similarly been found to display comparable levels of anxiety with WTs across multiple tasks, including Open Field, and Light-Dark avoidance, though evidence is conflicted regarding the Elevated Plus Maze task (Dao et al., 2010). Forebrain specific knockout of *Cacna1c* has been associated with increased anxiety related behaviours across all three of these anxiety tasks. This is thought to be due to the more extreme reduction in regional expression induced by a conditional knockout (Lee et al., 2012). It is worth noting that the open field task is only one measure of anxiety-related behaviour and is thought to potentially only represent one dimension of anxiety (Ramos, 2008). It is therefore possible that HET animals may differ on other anxiety-related tasks. For a more detailed assessment of anxiety in these animals it would

be pertinent to conduct multiple tests of anxiety related behaviour to include the Elevated Plus Maze.

WT and HET animals did not differ in reactivity during the acoustic startle response test and there was no evidence of any deficits in pre-pulse inhibition in HET animals. This is in line with the previous study in *Cacna1c* knockout mice, which found no effects of genotype in startle response in males (Dao et al., 2010). There does not appear to be any previously published investigation of the effects of reduced *Cacna1c* on PPI in rodents. Studies of PPI in humans have reported no deficits associated with the risk allele for schizophrenia in *CACNA1C* (Roussos et al., 2011). Reduced expression of *Cacna1c* may not have effects on sensorimotor gating or this task may not be sensitive enough to reveal differences which may exist in these animals.

5.5.4 Limitations of a genetic knockdown model

A chronic global knockdown model does have limitations including potential compensation effects and rearing environment. In accord with previous studies, there was no evidence of compensation by the other closely related *Cacna1d* gene. One previous study did observe compensation through an increase in calcium permeable AMPA receptors, though this has only been reported in forebrain specific knockout mice (Langwieser et al., 2010). Regardless of compensation effects, any behavioural differences observed in these animals would indicate a necessary function for *Cacna1c*, or at least one that cannot be fully compensated for by other mechanisms.

5.6 Conclusions

The novel heterozygous rat model displays a reduction in *Cacna1c* expression across different regions of the brain, including the mPFC, cerebellum and hippocampus, with no evidence of compensation in the expression of other brain specific LVGCC varaints. Reduced *Cacna1c* was associated with reduced expression of basal *BDNF* mRNA specifically in the PFC, indicative of effects on relevant downstream mechanisms. There is no evidence of abnormalities in locomotor activity or anxiety levels in the model, reducing potential confounds on subsequent investigations of associative learning and memory.

6 THE EFFECT OF GENETIC KNOCK-DOWN OF CACNA1C ON AVERSIVE CONDITIONING

6.1 Introduction

Pharmacological inhibition has identified a role for LVGCCs in specific aspects of contextual fear conditioning, conserved across different brain regions. However, at present, pharmacological methods cannot distinguish between $Ca_v1.2$ and $Ca_v1.3$ channels (coded for by *CACNA1C* and *CACNA1D* genes respectively). Genetic knock-down of the individual genes can be utilised to understand the relative contribution and potentially separable roles these channels may play in aversive conditioning.

As discussed previously, intronic variation in *CACNA1C* has been associated with risk for psychiatric illness and dysregulated expression of the gene (Quinn et al., 2010; Gershon et al., 2014; Yoshimizu et al., 2014). Investigating the effect of reduced expression of *Cacna1c* on specific aspects of learning known to be disrupted in schizophrenia, may help to understand the molecular mechanisms which link genetic risk with relevant symptoms.

To date, the majority of studies have utilised mice for investigation of the effects of haploinsufficiency of *Cacna1c.* Evidence supports the role of $Ca_v1.2$ in NMDAR-independent L-LTP and spatial learning (Moosmang et al., 2005), however there are a limited number of studies that have investigated the effect of reduced expression on associative learning. Previous findings suggest there may be a limited role for $Ca_v1.2$ channels in the consolidation and extinction of CFM, however results are inconclusive and based on region specific knockouts (White et al., 2008; Langwieser et al., 2010). At present, there do not appear to be any studies that have investigated effects on latent inhibition.

Moosmang et al., (2005) found that inactivation of the *Cacna1c gene,* primarily in the hippocampus and neocortex of mice, led to deficits in protein synthesis dependent, NMDAR-independent LTP in the CA1 region of the hippocampus. In conjunction with this, they found deficits in spatial learning and an impairment in CREB activation. They conclude that this demonstrates a necessary role for $Ca_v1.2$ channels in certain types of hippocampal-dependent synaptic plasticity and spatial learning. This is supported by findings in mice with a forebrain specific conditional deletion of *Cacna1c* in excitatory neurons (White et al., 2008). There was no difference between knockout and wild-type animals during acquisition of spatial learning, however when tested 30 days later knockout mice demonstrated a deficit in recall, indicative of an effect on the consolidation of remote spatial learning.

In these same mice Mckinney et al., (2008) investigated the effects on CFC. Mice were conditioned over two days in the same context to an un-cued footshock. There were no differences observed between genotypes for the acquisition or consolidation of CFM, with both groups showing the expected increase in freezing following training. This corresponds to additional studies, suggesting that the consolidation of CFM may instead rely on $Ca_v1.3$ channels (McKinney and Murphy, 2006; Berger and Bartsch, 2014).

There was also no effect of *Cacna1c* knockdown on the extinction of CFM in these mice. Both WT and knockout animals showed the expected reduction in freezing during prolonged re-exposure to the context and maintained reduced levels when assessed during a recall session 24 hrs later. It was therefore concluded that $Ca_v1.2$ channels do not have a necessary role in extinction, or account for the effect observed with inhibition of LVGCCs more generally. In contradiction to this, Busquet et al., (2008) found that in mice genetically modified to possess dihydropyridine insensitive $Ca_v1.2$ channels, the effect of systemic infusion of Nifedipine (an LVGCC inhibitor) on extinction was completely abolished. However, further investigation

suggested that the role of $Ca_v1.2$ channels was mediated by a peripheral mechanism rather than a central one, with no effects observed with intracerebroventricular administration of Nifedipine. This may be explained by the observation that Nifedipine has been found to produce aversive effects when injected peripherally (as previously discussed) which may lead to conditioning to the drug alone. The effects may therefore be abolished in animals with dihydropyridine insensitive $Ca_v1.2$ channels because these aversive effects are not experienced. $Ca_v1.3$ knockout mice have also been found to show no deficits in the acquisition or consolidation of extinction learning (McKinney and Murphy, 2006; Busquet et al., 2008). Together, these results are unable to explain the contribution of specific L-type channels to the effect observed with LVGCC inhibitors locally infused into the brain.

One factor that may influence the differences observed is the effect of chronic haploinsufficiency compared to acute inhibition. Langwieser et al., (2010) also found that chronic CNS specific deletion of *Cacna1c* had no effect on the consolidation of CFM compared to acute inhibition of LVGCC, even though they confirmed that the effect of inhibition was specifically driven by $Ca_v1.2$ channels. On further investigation, they found that chronic knockout of *Cacna1c* led to compensatory changes in the amygdala. They observed a homeostatic switch in plasticity from an LVGCC-dependent mechanism to one which utilises post-synaptic calcium-permeable AMPA receptors (cp-AMPAR). There was a significant increase in the expression of GluA1 in knockout animals, suggesting a compensatory mechanism in response to a lack of $Ca_v1.2$ channels. This could explain some of the discrepancy in previous results, both between genetic models and also in relation to acute pharmacological inhibition. However, they only investigated the effects in the amygdala and in relation to the consolidation of CFM. Whether this mechanism is able to compensate fully for the potential function of $Ca_v1.2$ channels in extinction and other inhibitory learning processes is yet to be elucidated.

Other fear learning related behaviours have been found to be affected by localised deletion of *Cacna1c*. Jeon et al., (2010) found that deletion of *Cacna1c* in the anterior cingulate cortex (ACC) of mice led to an impairment in observational fear learning. Mice that observed other mice receiving repetitive foot-shocks developed freezing behaviour even though they did not experience the aversive stimuli themselves. This effect was impaired in knockout mice, with reduced freezing behaviour observed, suggesting that $Ca_v1.2$ channels in the ACC are necessary for observational social fear. Interestingly these mice did not show any deficits in classical fear conditioning, further highlighting the potential significance of regional specificity in knockdown models.

Although forebrain specific and conditional knockout models are incredibly useful for understanding region specific roles of these genes and to establish direct causality, they are not necessarily representative of the disease state to which CACNA1C has been associated. As previously discussed, the risk SNP identified as being associated with schizophrenia and bipolar, is associated with significantly reduced expression of CACNA1C in the cerebellum (Gershon et al., 2014). Furthermore, rare deleterious variants in calcium channel subunits have been found to be associated with risk for schizophrenia (Purcell et al., 2014b). It is therefore pertinent to use models which reflect this when investigating disease relevant behaviour, which not all previous models have. The effect of a chronic global haploinsufficiency of *Cacna1c* on contextual fear conditioning has yet to be investigated, and as of yet, no studies have investigated any of these behaviours in rats. Considering the association of CACNA1C with schizophrenia, it would also be highly relevant to investigate the effect of reduced expression on latent inhibition due to deficits observed in patients, in addition to those aspects of associative learning addressed previously.

Previous results aiming to determine the separable role of $Ca_v1.2$ and $Ca_v1.3$ channels in CFC are conflicting and rely on the comparison of multiple models across multiple methodologies. To help elucidate the relative contribution of $Ca_v1.2$ channels to the effect observed with acute LVGCC inhibitors and to understand the role of *Cacna1c* more fully, it is necessary to investigate these behaviours across one disease relevant model.

6.2 Experimental aims

The aim of the following experiments was to determine the effect of chronic global haploinsufficiency of *Cacna1c* in Sprague Dawley rats on specific aspects of associative learning: acquisition, consolidation, extinction and latent inhibition. As previously, CFC was used as a model of hippocampal dependent associative learning. Heterozygous knock-out rats were compared to wild-type litter mates for each behavioural paradigm. This is the first study to assess the effect of genetic knockdown of *Cacna1c* on associative learning in rats and specifically the first study to assess the effect of any form of reduced expression on latent inhibition.

Experiment 1 – Effect of *Cacna1c* knockdown on the acquisition and consolidation of CFM

Experiment 2 – Effect of knockdown on the extinction of CFM and the consolidation of extinction

Experiment 3 – Effect of knockdown on the latent inhibition of CFC

6.3 Experiment 1 – Effects of reduced expression on the acquisition and consolidation of CFM

This experiment aimed to determine whether reduced expression of *Cacna1c* affected the acquisition, short term recall and/or the consolidation of CFM.

6.3.1 Methods and materials

6.3.1.1 Animals

14 animals (8 WT and 6 HET) were housed as previously described. All animals were given a minimum of five days from arrival to settle before being used in any experiments. Animals were sacrificed at the end of the experiment using a rising concentration of CO_2. Brains were removed and fresh frozen on dry-ice and tail biopsies were taken for post-mortem confirmation of genotypes.

6.3.1.2 Behaviour

Animals underwent the standard CFC paradigm as described previously. They received conditioning in the morning, followed by recall sessions at 3 hrs, 24 hrs and 7 days. Shock intensity was maintained from previous experiments at 0.5 mA following confirmation of conditioning in Sprague Dawley control animals at this level.

6.3.1.3 Analysis

Freezing was scored blind to genotype for each session from digital recordings. Genotypes were confirmed post-mortem and freezing behaviour was compared between WT and HET animals using repeated measures ANOVA, unless otherwise stated. Greenhouse Geisser corrections were applied where Mauchly's test of sphericity was significant. Post-hoc one-way ANOVAs were conducted for individual test sessions based on a-priori hypotheses where appropriate.

6.3.2 Results

6.3.2.1 Reduced expression of Cacna1c does not affect the acquisition or consolidation of CFM

All animals showed the expected increase in freezing following exposure to the US, with increased freezing observed immediately following shock and at 3 hrs, 24 hrs and 7 days later (Test: $F(4,48) = 29.624$, $P < 0.001$, $\eta_p^2 = .71$). There was no difference in behaviour between genotypes (Genotype: $F(1,12) = .385$, $P = .546$, $\eta_p^2 = .03$) and no evidence of an interaction between genotype and test session (Test x Genotype: $F(4, 48) = .533$, $P = .712$, $\eta_p^2 = .04$). This indicates that a reduction in *Cacna1c* does not affect the acquisition or consolidation of CFM (Figure 6.1).

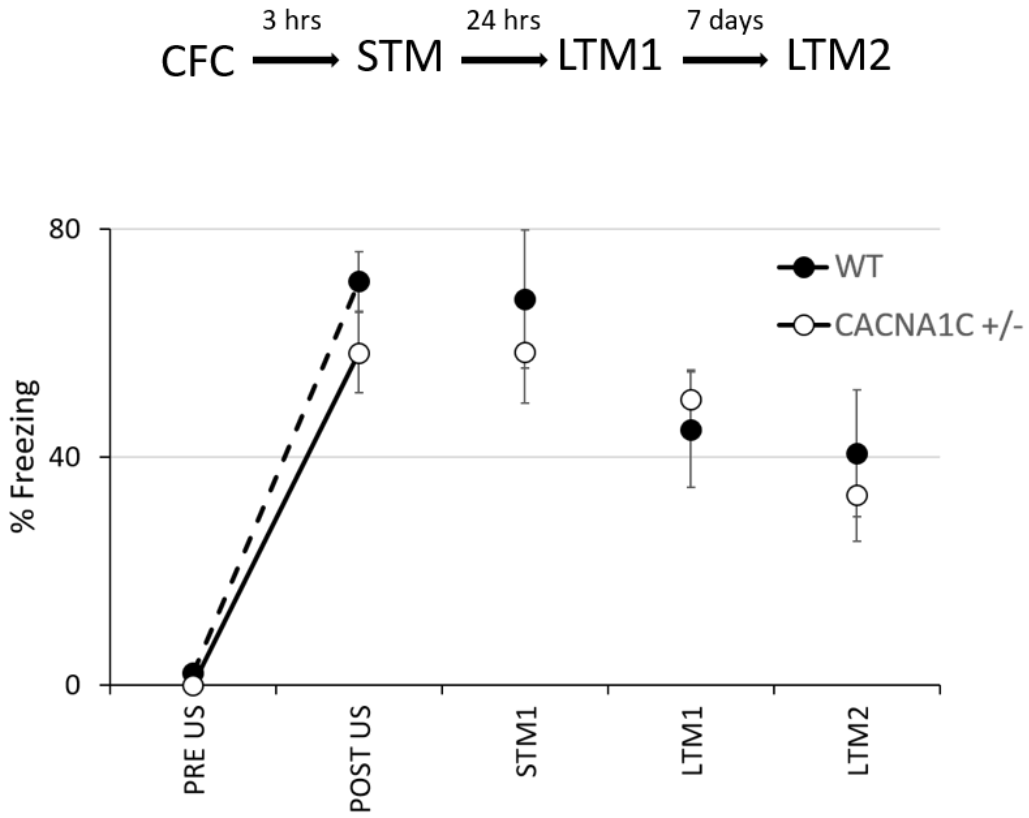

Figure 6.1 Cacna1c haploinsufficiency does not affect the acquisition, short term recall or consolidation of CFM ($F(1,12) = 0.385$, $P = 0.546$) (STM1: 3 hour recall; LTM1: 24 hour recall; LTM2: 7 day recall). Data points represent average % freezing for each genotype. Error bars are SEM.

6.4 Experiment 2 – Effects of reduced expression on the extinction of CFM

As *Cacna1c* HET knockout animals were able to successfully consolidate contextual fear associations, it allowed further investigation of post retrieval processes and other aspects of inhibitory learning. This experiment aimed to establish whether haploinsufficiency of *Cacna1c* affects the acquisition and/or consolidation of extinction. Animals underwent CFC as in experiment 1 and were then re-exposed to the training context for a prolonged recall session, designed to induce extinction of CFM. Acquisition of extinction was assessed by freezing levels within this session and the consolidation of extinction was determined by the freezing during recall sessions 24 hrs and 7 days later. Successful extinction was determined by reduced freezing within the 10 min session and maintained low levels of freezing during recall.

6.4.1 Methods and materials

6.4.1.1 Animals

32 male Sprague Dawley rats (16 HET and 16 WT) were housed as previously described with littermates in cages of 2 - 4.

6.4.1.2 Behaviour

All animals underwent CFC as previously described. 24 hrs later they were returned to the conditioning chambers for a 10 min extinction training session in the absence of the US. They were re-exposed to the same context for two 2 min recall sessions 24 hrs and 7 days following extinction to assess the consolidation of extinction.

6.4.1.3 Analysis

Analysis was conducted as experiment 1. During the 10 min extinction session, freezing behaviour was scored as previously, averaged across 2 min bins. Freezing behaviour is presented for the first 2 mins and last 2 only, though profiles throughout the 10 mins were checked for consistency.

6.4.2 Results

6.4.2.1 Reduced expression of Cacna1c does not affect the acquisition or the consolidation of extinction learning

There was no difference in freezing behaviour between *Cacna1c* +/- knockout and WT animals (Figure 6.2). Repeated measures ANOVA with a Greenhouse-Geisser correction showed a significant effect of test on freezing behaviour ($F(3.113, 93.386) = 14.809$, $P < .0001$) with no indication of an effect of genotype ($F(1,30) = .850$, $P = .364$, η_p^2 .028) or an interaction ($F(3.113, 93.386) = .604$, $P = .620$, η_p^2 .02).

As previously, all animals acquired the initial CFM ($F(1,30) = 107.143$, $P < .001$, $\eta_p^2 = .781$) with no evidence of a difference between genotype ($F(1,30) = .042$, $P = .839$, $\eta_p^2 = .001$) or an interaction ($F(1,30) = .00$, $P = 1.00$). The 10 min extinction trial successfully reduced freezing within session for both groups ($F(1,30) = 7.479$, $P = .010$, η_p^2 .20).

Results suggest that there is no effect of reduced expression of *Cacna1c* on freezing behaviour during an extinction training session or during recall of extinction when tested 24 hrs and 7 days later.

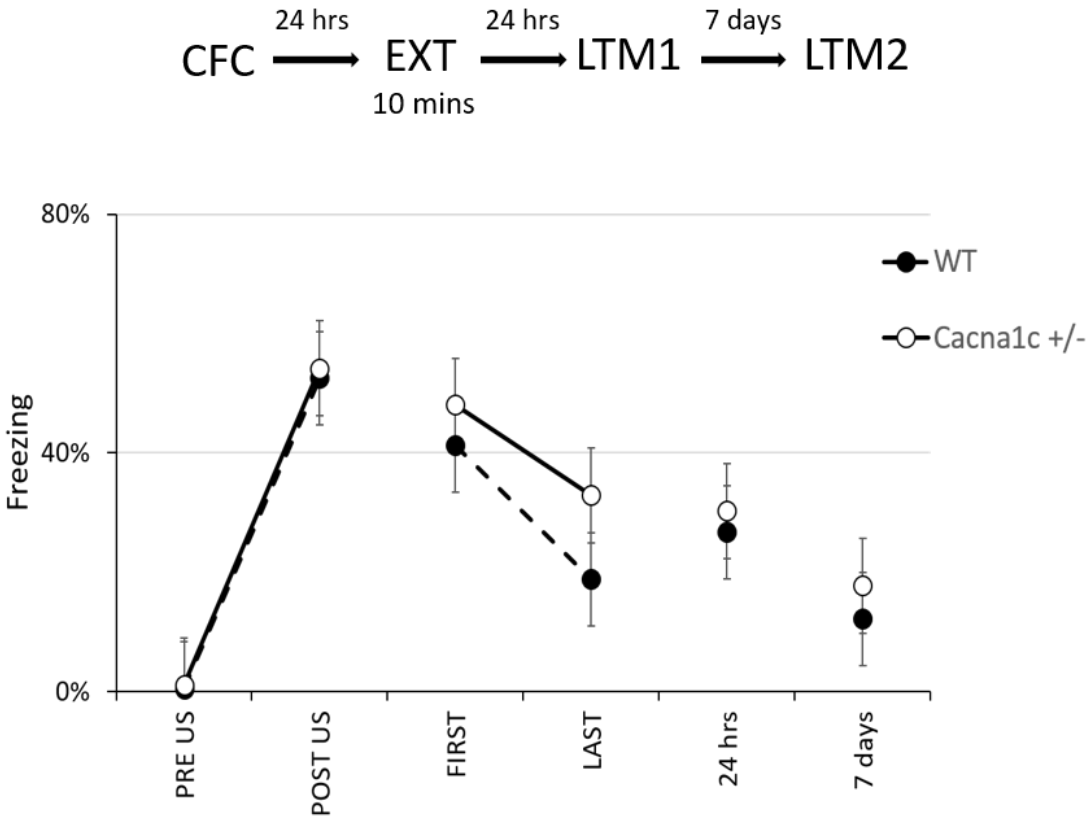

Figure 6.2 Reduced Cacna1c expression does not affect the acquisition of consolidation of extinction learning (F(1,30) = .850, P = .364). FIRST: Freezing during first 2 mins of the 10 min extinction session; LAST: Last 2 minutes of the 10 min extinction session. Data points represent average % freezing for each genotype. Error bars are SEM.

6.5 Experiment 3 – Effects of reduced expression on latent inhibition

This experiment aimed to investigate the effects of reduced *Cacna1c* on the latent inhibition of CFM. This involved prolonged exposure to the context for 4 hrs prior to CFC, so that the context was no longer novel at training. Familiarity with the CS reduces the association formed with the US, as discussed previously (See Chapter 4.), indicated by a blunted freezing response following training. Animals underwent a 4 hr LI session 24 hrs before CFC, then underwent training and recall sessions as experiment 1.

6.5.1 Methods and materials

6.5.1.1 Animals

17 animals (7 WT and 10 HET) were housed as previously described with littermates in cages of 3 or 4. One WT and one HET animal were excluded from analysis due to an incorrect shock intensity being used for CFC, leaving 6 WT and 9 HET animals for comparison.

6.5.1.2 Behaviour

Animals were exposed to the training context for 4 hrs on the morning of day one. CFC was conducted 24 hrs later to coincide with the offset time of LI. Recall sessions at 24 hrs and 7 days later were conducted as experiment 1.

6.5.1.3 Analysis

Behaviour was scored for the first 2 mins of the LI session to establish baseline freezing. The full LI digital recording was inspected to ensure no abnormal behaviour or faults which may confound the experience. Statistical comparisons were as experiment 1.

6.5.2 Results

6.5.2.1 Genetic knockdown of Cacna1c affects the latent inhibition of CFM

Cacna1c HET animals showed increased freezing compared to WT animals following CFC, indicating a reduced latent inhibition effect (Figure 6.3).

HET and WT animal freezing behaviour was compared across the whole of the task. Repeated measures ANOVA with a Greenhouse-Geisser correction showed a significant effect of test session on freezing behaviour ($F(2.496, 32.453) = 58.781$, $P < 0.001$, $\eta_p^2 = .819$), an effect of genotype ($F(1,13) = 35.080$, $P < .001$, $\eta_p^2 = .730$) and an interaction ($F(2.496, 32.453) = 14.927$, $P < .001$, $\eta_p^2 = .534$).

Cacna1c knockout animals showed significantly greater freezing levels post US (two tailed, independent samples t-test: $t(13) = -2.289$, $P = .039$). They also showed higher freezing levels during recall sessions at 24 hrs (M = 87.96%, SEM = 5.22%) and 7 days (M = 76.85%, SEM = 8.54%) following CFC compared to WT animals (M = 34.72%, SEM = 9.48%; M = 19.44%, SEM = 7.66% respectively; $t(13) = -5.337$, $P < .001$ and $t(13) = -4.692$, $P < .001$). There was no freezing behaviour observed in either group during the first 2 minutes of LI and no effect of genotype on freezing behaviour pre-US ($t(13) = -1.183$, $P = .258$), confirming these animals did not show abnormal behaviour prior to CFC, with differences isolated to sessions following training.

Results demonstrate a deficit in the latent inhibition of CFM in *Cacna1c* HET animals, with increased freezing behaviour indicating the formation of an association despite pre-exposure to the CS.

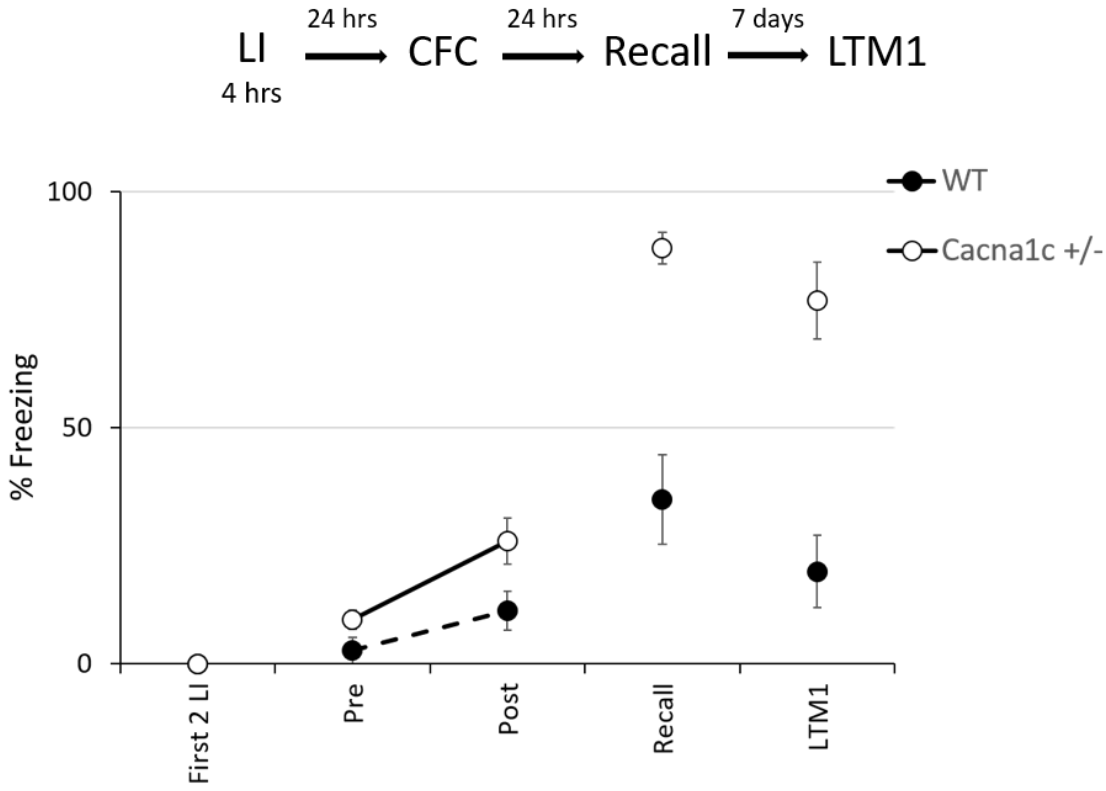

Figure 6.3 Cacna1c HET knockout animals show a deficit in the latent inhibition of CFM ($F(2.496, 32.453) = 58.781$, $P < 0.001$). Pre: 2 mins prior to footshock; Post: 1 min following footshock; Recall: 24 hrs following CFC; LTM1: 7 days later. Data points represent average % freezing. Error bars are SEM.

6.6 Summary

Global chronic knockdown of *Cacna1c* in Sprague Dawley rats has no effect on the acquisition, consolidation or extinction of CFM. However, *Cacna1c* knockdown animals show a significant deficit in the latent inhibition of CFM; demonstrating the inappropriate formation of an association between an US and a familiar CS, which is maintained at 7 days.

6.7 Discussion

This is the first study investigating the effect of chronic global haploinsufficiency of *Cacna1c* in rats on specific aspects of contextual fear conditioning. While there was no evidence of a general learning impairment, there was a specific deficit observed in the latent inhibition of CFM, a behaviour found to be altered in people with schizophrenia (Escobar et al., 2002).

6.7.1 Reduced expression of Cav1.2 channels does not affect the formation of hippocampal dependent associative memory

In agreement with previous studies, there was no evidence of a deficit in the acquisition or consolidation of CFM in *Cacna1c* knockout rats. Similar to findings across knockout mouse models, changes in expression of *Cacna1c* do not lead to a general learning impairment of associative memory. Although in these experiments animals only had approximately a 50% reduction of CACNA1C, other previous studies have shown similar lack of effects with complete brain specific knockout (Mckinney et al., 2008; Langwieser et al., 2010). It is therefore viable that CACNA1C expression may not be necessary for the consolidation of hippocampal dependent CFM.

Previous studies have identified a more critical role for $Ca_v1.3$ channels in consolidation. McKinney and Murphy, (2006) investigated the effect of a knockout of *Cacna1d* on CFC. They found that knockout mice were specifically impaired on the consolidation of CFM, which they concluded was due to a deficit in the formation of associations rather than an inability to encode the context. These findings in conjunction with those presented above, suggest that the impairment in consolidation of CFM observed with acute LVGCC inhibition is primarily driven by the block of calcium influx through $Ca_v1.3$ channels. Furthermore, $Ca_v1.2$ channels are neither necessary nor sufficient for the acquisition and consolidation of CFM.

Although cognitive impairments are a key symptom in people with schizophrenia, a general impairment in learning is not commonly observed in patients (Rushe et al., 1999). It is therefore more likely that risk genes that have been identified, to include *Cacna1c,* will have subtler effects on cognition.

6.7.2 $Ca_v1.2$ channels are not necessary for the acquisition or consolidation of extinction of CFM

There was no evidence of a difference between *Cacna1c* knockout and wild-type rats in extinction learning following CFC. This concurs with previous findings with conditional knockouts in mice that found no effect of brain specific reduced expression on extinction learning or consolidation (Mckinney et al., 2008). However, separable effects on extinction and latent inhibition disagree with previous findings that suggest they may involve the same molecular mechanisms (Barad et al., 2004). Although both may involve calcium influx through LVGCCs, there are clearly mechanisms yet to be discerned that distinguish between inhibition following pre-exposure and re-exposure following training.

Previous evidence also suggests that the knockdown of $Ca_v1.3$ channels does not affect extinction (McKinney and Murphy, 2006). This is at odds with the pharmacological findings which demonstrate an effect on the consolidation of extinction learning. Mckinney et al., (2008) investigated the effects of Nifedipine to see if they could replicate the findings of Cain et al., (2005). They administered the LVGCC inhibitor systemically and found similar effects on extinction, however they attributed this to an effect of the drug on locomotor activity. This conclusion does not explain the consistent findings of an effect of LVGCC inhibitors infused locally into the brain, or the findings observed with different classes of inhibitor. It is possible that the consolidation of extinction relies on calcium influx through both $Ca_v1.2$ and $Ca_v1.3$ channels, the complete inhibition of which is only achieved by a pharmacological blockade and not through partial genetic knockdown of either

channel. Alternatively, as suggested by Busquet et al., (2008) there may be a peripherally mediated mechanism, or potentially off-target central effects of the inhibitors which are not yet fully understood.

The current findings in conjunction with previous studies cannot yet fully explain the effect of LVGCC inhibitors on extinction learning. It is possible that calcium influx through $Ca_v1.2$ channels play a role in extinction, however there may be compensation mechanisms that are not yet fully characterised.

6.7.3 Reduced expression of *Cacna1c* leads to the formation of inappropriate aversive associations with familiar stimuli

Cacna1c knockout rats had a profound impairment in the latent inhibition of CFM. This implies a vital role for $Ca_v1.2$ channels for inhibiting the formation of associations between a CS and an aversive US, based on prior information. As there was no observed deficit in the acquisition of CFM or the subsequent acquisition of extinction, it can be assumed that *Cacna1c* knock out rats are capable of acquiring both CS-US and CS-no US associations under some circumstances. There may be a requirement of prior salience to form new CS-no US associations (as in the case of extinction), or it may be an inability to transfer prior learned irrelevance contingencies to current experience. Inappropriate associations are then formed based on a response to the contingencies of their current environment rather than previous information.

The process of latent inhibition has been suggested to be impaired in schizophrenia, though results are conflicting. Escobar et al., (2002) proposed that people with schizophrenia have an inability to compare and express stored representations. This is in line with Cohen and Serven-Schreiber's cognitive model which suggests that patients can successfully process contextual information but have difficulties in maintaining and using it to inhibit subsequent inappropriate responses (Cohen et al., 1992). The data presented here fits with these models, as well as with the observed

impairment in people with schizophrenia in the processing of neural signals of prediction error (Gradin et al., 2011). Deficits in LI have been observed in patients (Gray et al., 1995a; Escobar et al., 2002) as well as a more general formation of abnormal associations (Jensen et al., 2008). However, there has been found to be a difference in impairment between acute and chronic episodes, with impairment being seen only in those patients suffering acute psychotic episodes (Gray et al., 1995b). Different observations have been theorised to possibly relate to medication status of participants (Lubow et al., 1987) indicating a likely interaction of these processes with the dopamine system.

Previous studies have found that the administration of amphetamine, an indirect dopamine agonist, leads to impairments in latent inhibition in both rats (Weiner et al., 1984, 1988; Young et al., 1993) and humans (Gray et al., 1992; Salgado et al., 2000). This has led to latent inhibition being seen as a viable behaviour to model positive symptoms of psychosis, due to the disruption of the dopamine system in patients and the effective use of dopamine antagonists in treatment. Interestingly amphetamine administration has been found to have an effect on the function and expression of $Ca_v1.2$ channels (Andres et al., 2015a; Cameron et al., 2015). Acute administration of methamphetamine in human cells in vitro has been found to block calcium influx through LVGCCs, whereas longer exposure leads to a specific upregulation of *CACNA1C* (Andres et al., 2015b). A more direct link between dopamine and LVGCCs has been postulated in primary mid-brain mouse neurons, with endogenous dopamine through D2R receptors found to suppress the activation of LVGCCs and the generation of synchronous oscillations (Yasumoto et al., 2004). Whether there is a direct mechanism which links the similar behavioural effects on latent inhibition of reduced expression of *Cacna1c* and dopamine agonists is yet to be fully elucidated; though it is possible that converging effects are mediated through BDNF.

Previous results (Chapter 3) suggest a similar effect of reduced *BDNF* on latent inhibition and studies have found that BDNF controls and maintains the expression of the dopamine D_3 receptors (Guillin et al., 2001; Sokoloff et al., 2002). Furthermore, BDNF has been implicated in methamphetamine-induced release of dopamine and its link to psychosis (Narita et al., 2003; Manning et al., 2015). Future studies should explicitly investigate a link between LVGCC, BDNF and dopamine in latent inhibition, as well as the possibility to rescue behavioural deficits with the manipulation of dopamine and/or BDNF in *Cacna1c* knockout animals.

6.7.4 Limitations and future directions

It is possible that compensatory mechanisms may have developed in these animals that are able to fulfil some of the functionality of *Cacna1c* and the role that Cav1.2 channels may play in a 'normal' brain. It has previously been shown that conditional CNS knockout of *Cacna1c* can lead to an increase in calcium-permeable AMPAR, though this was only investigated in the amygdala (Langwieser et al., 2010). Although it is possible that compensation mechanisms have evolved in response to reduced *Cacna1c* that may be able to fulfil a role in extinction, it is evident that normal expression levels of *Cacna1c* are necessary for the latent inhibition of CFM. Future studies would need to investigate this more fully; to determine if there is a compensation by cp-AMPAR and whether this is sufficient for the acquisition and consolidation of extinction, as well as initial learning as previously observed (Langwieser et al., 2010). It is also possible, that any compensation that may have occurred may not be as stable as the natural mechanisms involving $Ca_v1.2$ channels. Further investigation into any potential environmental interactions, including stress, could help interrogate their ability to fully compensate for the function of *CACNA1C.*

It is not yet known whether the deficits observed here are specific to aversive associations or whether they may additionally affect reward based association formation. The same brain regions (primarily the ventral striatum) have been found

to be activated in response to prediction error regardless of valence in healthy controls (Jensen et al., 2007). With the added accordant impairments in LI seen with the manipulation of the dopamine system, it would be pertinent to translate this work into reward learning. Future studies will aim to investigate whether deficits in *Cacna1c* knockout animals generalise to inhibition of reward learning or if they are specific to aversive associations.

6.8 Conclusions

Chronic global haploinsufficiency of *Cacna1c* in rats causes a profound specific deficit in latent inhibition of CFM. This is the first study to investigate this aspect of associative learning in a reduced expression model of *Cacna1c* and suggests a necessary role for $Ca_v1.2$ channels. Latent inhibition has been found to be impaired in people with schizophrenia and has been further linked with dysregulation of the dopamine system. It is possible that dysfunction of signalling through $Ca_v1.2$ channels may play a role in the etiology of these related symptoms, with a potential convergence and/or interaction with BDNF and dopamine, requiring further investigation.

7 EFFECT OF GENETIC KNOCKDOWN OF CACNA1C ON REVERSAL LEARNING

7.1 Introduction

Reversal learning involves the inhibition of a previous learnt association between a stimuli and reward and the acquisition of a new opposite contingency or rule. It is a necessary form of behavioural flexibility which allows new experience to influence subsequent behaviour (Floresco et al., 2009). Deficits in this form of learning can lead to maladaptive, perseverative behaviour that is not supported by, or beneficial in, the current environment. Impairments in this task have been reliably observed in patients with schizophrenia and bipolar disorder (Elliott et al., 1995; Waltz and Gold, 2007a; McKirdy et al., 2009; Adleman et al., 2011). Experimental studies have shown that reversal learning can be dissociated, to an extent, into specific phases which require separable functional mechanisms and neural circuitry. These include initial acquisition of contingencies, reversal and inhibition of prior contingencies and the increased control of behaviour to respond to previously unrewarded stimuli. These highly dissociable components of behaviour and neurobiology can be useful in relating a particular pattern of effects in a single cohort of animals, to discrete psychologies and brain functions. Importantly homologous cortical-striatal networks have been found to be involved in these processes in both rats and humans (Balleine and O'Doherty, 2010) and the development of a touchscreen based paradigm for rodents makes reversal learning a highly translatable function (Bussey et al., 2012). The molecular mechanisms underlying the separate phases of reversal, and the potential role of risk genes for schizophrenia in these processes, is yet to be fully understood.

Neural circuitry to include the frontal cortex (specifically ventral medial prefrontal cortex (vmPFC) and orbitofrontal cortex (OFC)), medial temporal lobe and dorsal

medial striatum (DLS), have been reliably found to play an important role in reversal learning tasks, through lesion studies (Clarke et al., 2008; Castañé et al., 2010; Graybeal et al., 2011) and human imaging (Cools et al., 2002; Ghahremani et al., 2010). The DLS and dopamine signalling more broadly is also known to be necessary for reversal learning through inhibitor studies (e.g. Lee et al., 2007). Evidence has further identified a role of the amygdala in the updating and change of reward associations, however results are conflicting (e.g. Izquierdo and Murray, 2007; Clarke et al., 2008; Rudebeck and Murray, 2008). It is postulated that it may play a specific role in the use of positive reinforcement for the adaptation of subsequent behaviour (Rudebeck and Murray, 2008).

Dysfunction in these key brain regions and dopamine signalling are known to be prevalent in neuropsychiatric disorders, including schizophrenia, bipolar and autism (e.g. Drevets et al., 1997; Goldberg et al., 1999; Culbreth et al., 2015). It has been suggested that reversal learning assays can be exploited to provide greater specificity in identifying PFC dysfunction (Clark et al., 2004) in patients with schizophrenia, with behavioural deficits only evident during specific phases of the task.

Acquisition of visual discrimination

In a typical reward based reversal task using visual cues, performance is initially at chance level with approximately equal responses to each of two visual stimuli. As the association with reward is learned, the rewarded stimulus is selected more often and performance increases (Figure 7.1). As discrimination is incrementally acquired, responses become quicker and eventually behaviour becomes over-trained to the point of being habitual (Reading et al., 1991).

Genetic knockout studies have identified a role for N-methyl-D-aspartate receptor (NMDAR) NR2A subunit in the acquisition and maintenance of stable associations between stimuli and reward (Brigman et al., 2008). Despite the implication of the

dopamine system and NMDAR function, patients with schizophrenia have been found to successfully acquire discrimination at this phase (Elliott et al., 1995; Waltz and Gold, 2007a; Leeson et al., 2009). However, some studies have observed subtle deficits in patients in first-episode psychosis (Murray et al., 2008a) as well as stable outpatients (Reddy et al., 2016).

Inhibition of previous contingencies (Early reversal)

Once a pre-determined criterion is reached for discrimination, contingencies are reversed and the previously unrewarded stimulus now predicts reward. Performance is reduced to below chance level as responses are initially still made to the previously rewarded stimuli (Figure 7.1). Previous studies have referred to this as "Early" reversal (Graybeal et al., 2011; Bussey et al., 2012). Errors made at this stage are termed perseverative errors, referring to the inappropriate continuation of behaviour driven by prior associations (Bussey et al., 2012). Correct response to the newly reversed association requires the inhibition of the prior contingency and the comparison of new information with stored representations (Floresco et al., 2009). Animals must be able to employ cognitive control networks to inhibit the habitual responding acquired during the "Visual discrimination" phase of the task. This is typically manifested by increased response latencies and uncertainty in reward collection, as behaviour shifts from habitual to goal-directed (Balleine and O'Doherty, 2010). Goal directed behaviour is postulated to be more flexible, relying on the associations formed between action and outcome, compared to the habitual response system which relies on stimuli and response associations (Keramati et al., 2011).

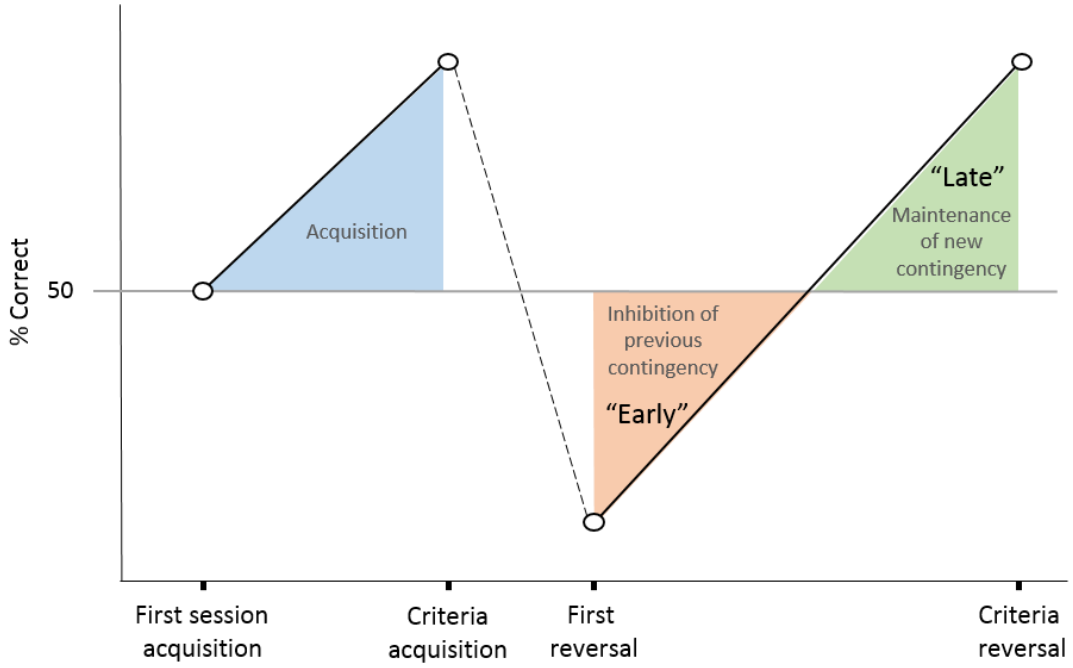

Figure 7.1 Progression of performance throughout the Reversal Learning task identifying relatively distinct phases of behaviour. Evidence suggests separable psychological and neural mechanisms are involved in the acquisition, inhibition and maintenance of competing contingencies.

The dopamine system has been more specifically implicated in this early stage of reversal with inhibitor studies, with dopamine neuron signalling from the midbrain to the striatum known to involved in the balance between habitual and goal-directed decision making (Keramati et al., 2011). It has been reported that the D2/D3 receptor antagonist raclopride specifically affected reversal learning in monkeys, with a greater number of errors made in treated animals compared to controls; with no effects on subsequent learning or the retention of the initial discrimination (Lee et al., 2007). Similar effects were observed with the D2 agonist bromocriptine in healthy humans (Mehta et al., 2001). D1-receptor agonism has been found to impair early reversal in mice (Izquierdo et al., 2006). Intraperitoneal injection of SKF81297 prior to each reversal session resulted in fewer correct responses in the first sessions of reversal but differences were absent by the third session with no effect on the time taken to reach reversal criteria. These findings support the role of the dopaminergic midbrain in the reversal but not acquisition of contingencies.

This early phase of reversal has been found to be significantly impaired in people with schizophrenia and bipolar disorder (Elliott et al., 1995; Crider, 1997; Waltz and Gold, 2007a; McKirdy et al., 2009; Adleman et al., 2011; Reddy et al., 2016). Elliott et al., (1995) found that patients were unimpaired on the initial acquisition of associations between stimuli and reward. However, when contingencies were reversed, patients took much longer to adjust their behaviour, demonstrating a perseverance of prior associations. There is no evidence that these impairments are related to medication status (Schlagenhauf et al., 2014), appearing relatively stable over time and there has been no association found with intelligence (Reddy et al., 2016). Imaging studies have further found that performance on this task in patients is correlated with activation of regions of the cognitive control network known to be necessary for this phase of the task (Schlagenhauf et al., 2014; Culbreth et al., 2015).

Animal models of relevance to schizophrenia have also been found to have specific deficits in "Early" reversal. Impairments have been observed in Phencyclidine (an NMDA-receptor antagonist) treated animals, with reduced performance levels following reversal (McLean et al., 2009). These were found to be significantly improved with IP injection of D1-like receptor antagonist SKF- 38393. An investigation of behavioural effects of homozygous knockout of schizophrenia risk gene *Dlg2* in mice has similarly reported increased perseveration in the early stages of reversal, extending to later reversal when a more demanding task was employed (Nithianantharajah et al., 2013).

Acquisition of new contingencies (Late reversal)

In comparison to the earlier stages of reversal in which inhibition of prior behaviour is required, later sessions necessitate the learning and maintenance of a new response contingency (Bussey et al., 2012). Performance increases incrementally, as in the "Visual discrimination" phase, until criteria is reached.

Studies have found that abnormalities at this stage can be dissociated from those in "Early" reversal, with more errors being made during late sessions, without increased perseverative errors (Chudasama and Robbins, 2003; Brigman et al., 2008). Inactivation of the dorsal medial striatum has been found to specifically impair rats' ability to maintain a new choice response pattern (Ragozzino, 2007). Lesions of the infralimbic PFC were also reported to result in impaired reversal learning, but in the absence of increased perseverative errors (Chudasama and Robbins, 2003). Animals were observed to make more learning related errors, indicative of problems in acquiring the new contingency rather than inhibiting the old.

Conversely, facilitations of "Late" reversal have been reported in relation to abnormalities of vmPFC function (Graybeal et al., 2011; Bryce and Howland, 2015). Graybeal et al. (2011) found that excitotoxic vmPFC lesions following acquisition resulted in fewer errors specifically during late reversal, with fewer sessions required to reach criteria. They suggest that this facilitation may be due to the vmPFC providing a brake on learning and that dysfunction of this region leads to deficits in this inhibition; promoting learning in subcortical regions. Interestingly they were able to rescue this facilitation with local infusions of BDNF into the vmPFC, suggesting this effect is mediated by stress effects on the regulation of BDNF in this region (Duman, 2004; Gourley et al., 2009). Conflicting evidence is likely due to the different timings of vmPFC lesion.

Human imaging studies during this late phase have identified altered activation in brain regions of patients required for cognitive control; finding a correlation with activation and behaviour consistent with deficits in forming adequate correct response representations (Culbreth et al., 2015). It is evident that normal reversal learning requires the coherent communication between multiple brain regions, with separable mechanisms more/less vital at different stages. Abnormalities during specific phases may indicate impairments in distinct underlying circuitry and

mechanisms. Determining whether individual risk genes for schizophrenia have a role in specific stages of reversal learning would help to elucidate the functional link between risk and the symptoms observed in patients.

CACNA1C and $Ca_v1.2$ channels more broadly have been found to be important for behaviours that are mediated by the mesolimbic dopamine system, specifically in relation to depression related behaviours and stimulant sensitisation (Bhat et al., 2012; Berger and Bartsch, 2014; Kabir et al., 2016). These channels have also been implicated in the processing of reward (Wessa et al., 2010; Bhat et al., 2012; Lancaster et al., 2014) . People with the risk associated variant in CACNA1C were found to have blunted reward responsiveness, without an observed effect on discriminability (Lancaster et al., 2014). Evidence has also suggested a direct role for $Ca_v1.2$ channels in the presynaptic regulation of mesolimbic dopamine signalling (Terrillion, 2015), as well as a role in mediating the inhibitory or excitatory consequences of D1 receptor activation (Hernandez-Lopez et al., 1997). Despite the extensive evidence of the relevance of CACNA1C for regions implicated in reversal learning and the impairments observed in patients with schizophrenia, to date no study has investigated the specific role of CACNA1C in this task.

Using a translational touchscreen reversal learning paradigm based on the one developed by Bussey et al, the current study aimed to establish the effects of reduced expression of *Cacna1c* on behaviour. Given the impairments in patients and previously observed effects on inhibitory aversive learning, it is pertinent to determine whether effects translate to the inhibition of reward associations within a dopamine dependent paradigm. It is hypothesised that a reduced expression of *Cacna1c* would result in impairments in “Early” reversal; when there is the requirement to elicit an inhibition of behavioural response to prior contingencies.

7.2 Methods and materials

7.2.1 Animals

26 male Sprague Dawley *Cacna1c* heterozygous knockout rats were used, aged between 89 and 111 days. All animals were given at least 2 weeks from arrival before any training procedures started. Animals were housed with littermates in groups of 1-4 depending on their size before transport from Charles River. Animals were placed on water restriction on testing days to maximise motivation for reward. Water restriction was conducted in accordance with Home Office regulations. One rat was euthanised due to the development of an aggressive tumour during the visual discrimination phase, therefore analyses were conducted on the 25 remaining animals.

7.2.2 Equipment

Animals were run in sets of 4 using Bussey-Saksida rat touch screen chambers (Campden Instruments, 80604, Leics) with accompanying Animal Behaviour Environment Test (ABET) II software (Campden Instruments, 89505). Touch screen chambers were used over operant boxes to allow a more direct translation to human paradigms (as tested in CANTAB). All sessions were completed in the afternoon.

7.2.3 Reversal learning paradigm

Animals were habituated to the apparatus and the delivery of reward prior to stimuli presentation. Liquid reward (10% sucrose solution) was used throughout the experiment. The order of sessions and criteria for each session are listed in Table 7.1. For a full description of conditions and trials see General Methods 2.3.2. Habituation and Must-Touch training were categorised as pre-training conditions, to familiarise rats with the apparatus and the required nose-poke of the touch screen to receive reward in the magazine. In brief, during habituation a light was turned on coinciding with a tone and delivery of reward. Animals were required to enter the

food tray to collect the reward and then exit to elicit the start of the next trial, with a 5s inter-trial interval (ITI). Once criteria were reached (Table 7.1) animals moved on to Must-Touch training, where they were required to nose-poke a stimulus (consisting of randomised white shapes on a black background) in favour of a blank screen to receive reward. Animals progressed to Visual Discrimination once criteria performance was reached. Visual Discrimination and Reversal involved the distinction between two stimuli to receive reward (Figure 7.2).

Table 7.1 Order of experimental conditions for Reversal Learning paradigm and criteria for each session.

Experimental Condition	Criterion
Habituation	100 trials completed in 30 mins for 2 consecutive days
Must-Touch training	100 trials with < 20 blank touches in 30 mins for 2 consecutive days
Visual Discrimination	> 80% correct for 2 consecutive days
Reversal	> 80% correct for 2 consecutive days

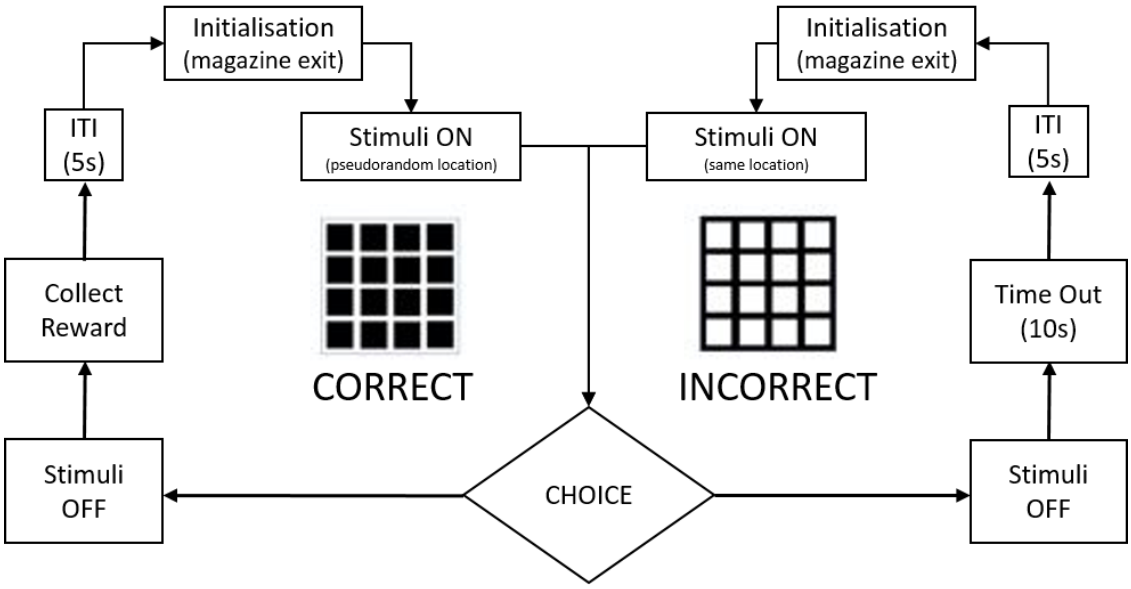

Figure 7.2 Schematic of trials used for Visual Discrimination and Reversal sessions for correct and incorrect responses.

7.2.4 Analysis

Chi-squared analysis of differences was used to establish whether there was a significant difference in completion rate between WT and HET animals across the whole task (defined as ability to successfully reach criteria in the Reversal stage) and for specific training phases.

Number of sessions to reach criterion were recorded for each condition, to determine how quickly animals acquired the contingencies. Animals were only included if they had reached criterion for that training phase. Correct responses and number of errors for each session were recorded to establish performance across phases and perseveration. Number of tray entries and beam breaks were used as a general measure of activity levels and interest in reward. Student's t-tests were used to compare mean values between WT and HET for each training phase. One-tailed tests were applied where prior hypotheses indicated direction of effect.

Latencies to respond to stimuli and latencies to collect reward were recorded to indicate speed of decision and association with reward. Repeated measures ANOVA were conducted for latencies within each task phase to compare averages for the first and last session of each phase. Due to low trial numbers in the first session following reversal, latencies were averaged across the first 3 sessions. For consistency, this was also done for both "Visual discrimination" and "Late" reversal measurements.

Where unequal variances were observed using Levene's test, Greenhouse Geisser corrections were applied. Normality of variables was assessed using Shapiro-Wilk's tests. Where significant statistics were observed a log10 transformation was conducted to produce a normal distribution. Non-normal distributions were only observed for response latencies (where stated). One outlier (defined as ± 2 standard deviations from the mean) was excluded from error rate analysis.

7.3 Results

The final experiment consisted of 120 sessions with 12 animals completing the full reversal paradigm. All animals completed the Habituation phase of the paradigm, though 2 animals were unable to reach criteria for Must Touch phase. 21 animals reached criteria for Visual Discrimination though only 19 were able to return to chance level performance following reversal of contingencies.

7.3.1 Performance trajectory concurs with previous studies in Sprague Dawley rats

Performance followed the expected trajectory as seen in previous reversal learning studies (Figure 7.3). Performance on first "Visual discrimination" session was approximately 50%, increasing to 80% criteria level through the training phase. Following reversal of contingencies, performance dropped to significantly below chance (16.85%, $t(20) = -10.835$, $P < 0.001$) with no differences between genotypes ($t(19) = 1.471$, $P = 0.158$).

This cohort of Sprague Dawley rats took more sessions to reach criteria than previous experiments have described in the same strain (Bussey et al., 2008), with an average of 25 sessions required to reach initial discrimination criteria and 41 sessions to reach reversal, compared to < 15 reported by Bussey et al. They had comparable trial numbers to reach criterion, just performing fewer trials during each session. Importantly for subsequent analyses there were no differences in beam breaks or tray entries between WT and HET rats during pre-training ($t(44) = -.783$, $P = 0.446$ and $t(37.943) = 0.338$, $P = 0.810$ respectively), nor in the overall number of trials completed between WT and HET animals during "Visual Discrimination" ($t(21) = -1.162$, $P = 0.258$) or "Reversal" ($t(19) = -1.007$, $P = 0.327$); suggesting a sub-strain effect rather than a phenotype relevant to reduced expression of *Cacna1c.*

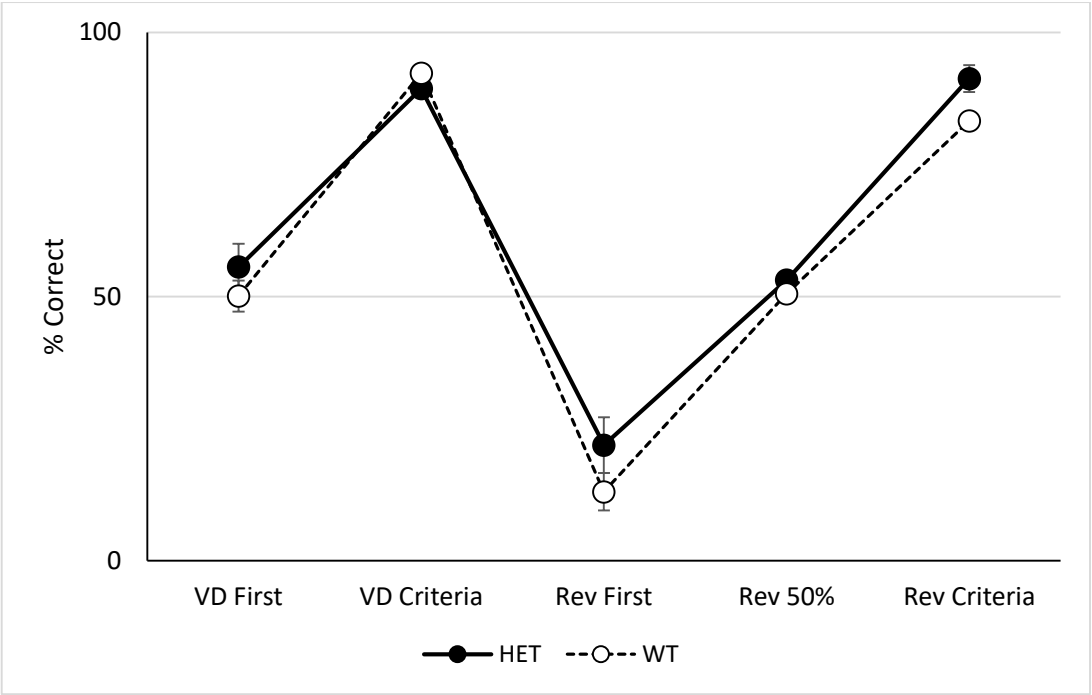

Figure 7.3 Performance by genotype across reversal learning. VD: Visual Discrimination; Rev: Reversal; First: First session of training phase; 50%: Session in which performance returns to approximately 50% following reversal. Criteria: Last session of training phase when determined criteria are reached. Expected reduced performance following reversal, with no differences between genotype.

7.3.2 No effects of genotype on Habituation or Must Touch training stages

7.3.2.1 Habituation

Both genotypes successfully habituated to the testing chamber and the reward protocol, with no differences in number of sessions or trials to reach criteria ($t(21) = -0.249$, $P = .806$ and $t(21) = -7.86$, $P = 0.441$ respectively: Figure 7.4 a and b). Repeated measures ANOVA comparing first and last session reward collection latency by genotype revealed a significant effect of session ($F(1,21) = 15.837$, $P = 0.001$, $\eta_p^2 = 0.430$) indicating a decrease in latency (Figure 7.4 c), with no effect of genotype ($F(1,21) = 1.350$, $P = 0.258$) or evidence of an interaction ($F(1,21) = 1.275$, $P = 0.272$); demonstrating both groups habituated to reward delivery and collection. All animals reached criterion and progressed to “Must Touch” phase of the task.

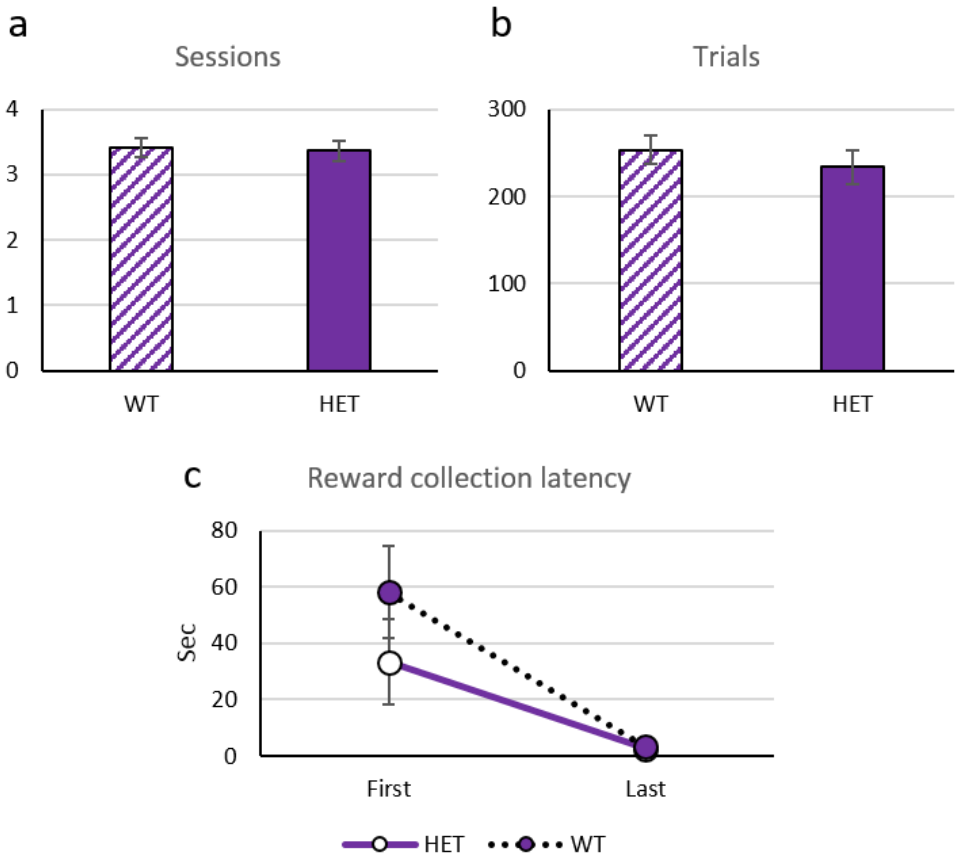

Figure 7.4 a) Sessions and b) Trials to criteria for WT and HET animals for "Habituation". There was no difference between genotype for either measure; P > 0.05. c) Average reward collection latency for first and last sessions with habituation. Significant reduction in latency with no differences observed between genotype or an interaction; P > 0.05. (WT: n = 13, HET: n = 12)

7.3.2.2 Must Touch

Both groups were able to learn to nose-poke a stimulus in favour of a blank screen to receive reward. There was no difference between genotypes in number of sessions or trials to reach criteria ((t(18.206) = 1.323, P = 0.202 and t(21) = -0.092, P = 0.928 respectively)(Figure 7.5 a and b). The expected reduction in correct touch latency and reward collection latency between first and last sessions was observed (F(1,21) = 26.265, P < 0.001, η_p^2 = 0.556, and F(1,21) = 28.648, P < 0.001, η_p^2 = 0.577 respectively), with no difference by genotype (F(1,21) = 0.006, P = 0.939) and no evidence of an interaction (F(1,21) = 0.042, P = 0.839; F(1,21) = 0.015, P = 0.905)(Figure 7.5 c and d). Two animals did not reach criteria (1 WT and 1 HET) and so did not progress to “Visual discrimination”.

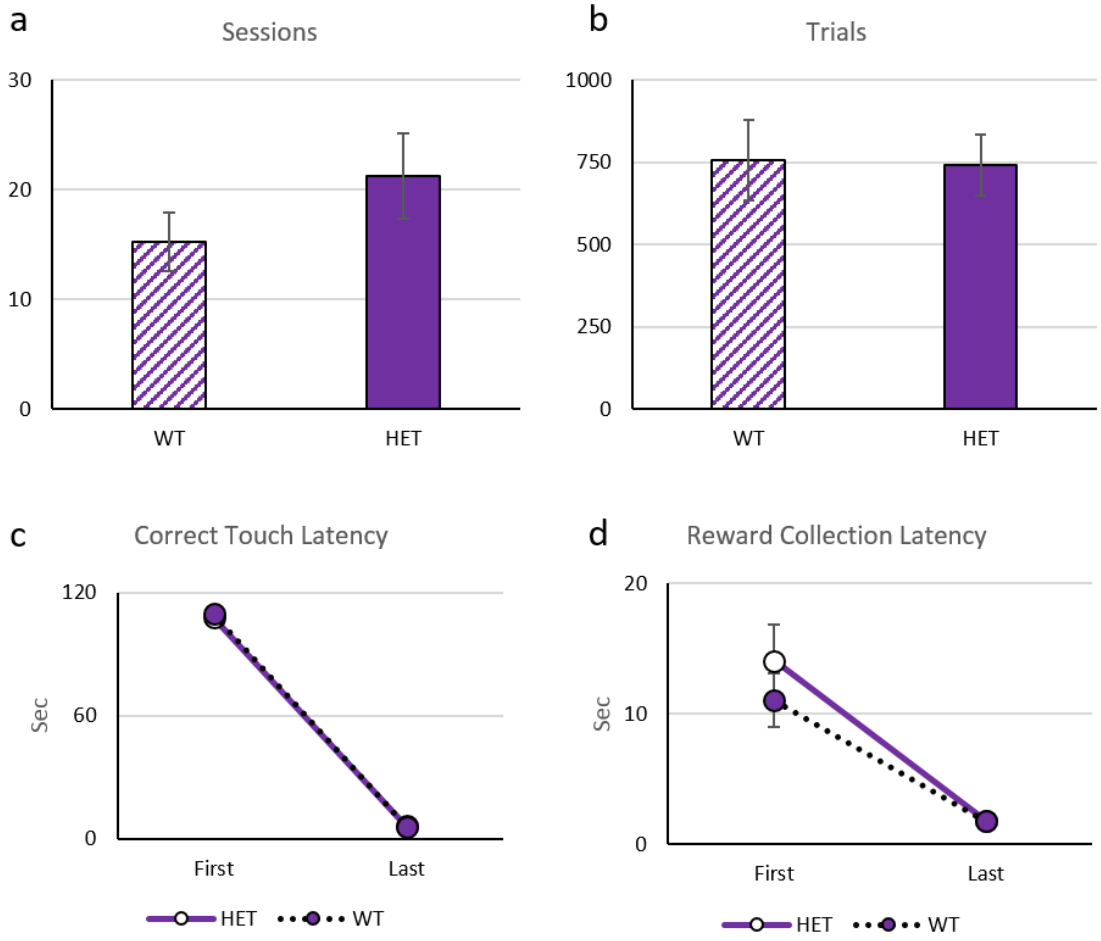

Figure 7.5 a) Session and b) Trial numbers to criteria for Must Touch condition by genotype. There was no difference between WT and HET animals, P > 0.05. b) Latency for animals to make a correct choice by nose-poking the touch-screen from the start of the trial and c) latency to collect reward for the first and last sessions of Must Touch by genotype. Significant reduction in latencies between sessions; P < 0.001 with no effect of genotype, P > 0.05. (WT: n = 12, HET: n = 11)

7.3.3 Fewer HET animals return to chance level performance following reversal

Of the 13 WT animals, 12 reached criteria for “Visual discrimination” (92%) and 9 of 11 HETs (82%). There was no significant difference in the proportion of completion between genotypes ($\chi(1) = 2.390$, $P = 0.122$). A total of 12 out of 23 rats completed the full reversal task. Eight of those that completed were WT and 4 were HET. Test for equality of proportions across the whole task did not show a difference between genotypes ($\chi(1) = 2.112$, $P = 0.146$). Further analyses were conducted to compare the proportion of WT and HET animals that were able to complete the “Early” reversal stage, indicating a successful inhibition of response to prior contingency. There was a trend towards fewer HETs completing this stage, with all 12 WT animals successfully completing, compared to 7 out of the 9 HET animals (78%) ($\chi(1) = 2.947$, $P = 0.086$, Cramer’s $V = 0.375$) (Figure 7.6). These results should be interpreted with caution due to the low number of animals in each group and the low minimum expected count for a chi-squared test (<5). Fisher’s exact test reported a p value of 0.17.

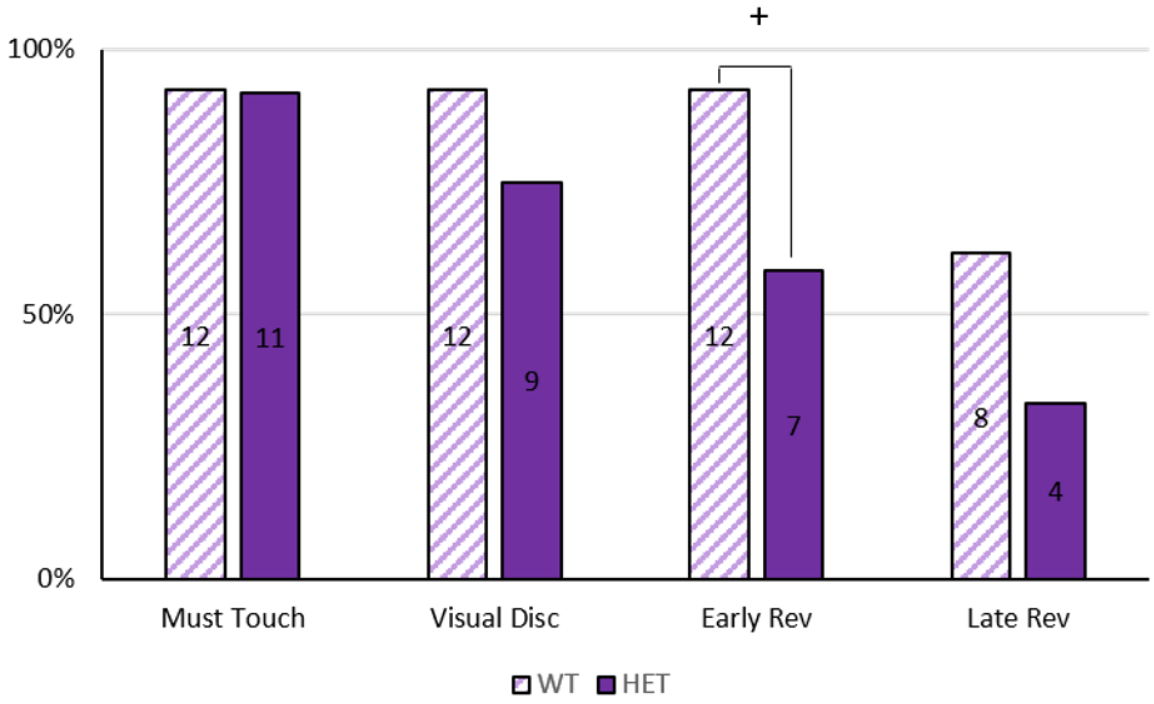

Figure 7.6 Completion rates (% of animals of each genotype) for each experimental condition from Must Touch to Reversal Criterion. Trend towards fewer HET animals compared to WT completing Early rev stage of 50% performance levels following reversal. + P < 0.10.

7.3.4 Indication of fewer sessions required for HET animals to reach criteria in "Late" reversal

Sessions to reach criteria were compared between genotypes for "Visual discrimination", "Reversal" and "Early" and "Late" phase reversal separately (Figure 7.7). There was no difference between genotype in the number of sessions to reach criteria for "Visual Discrimination" ($t(19) = -0.833$, $P = 0.415$), though a trend towards fewer sessions to criteria in "Reversal" in HET animals ($t(10) = -1.977$, $P = 0.076$). Analysis of the two stages of reversal (Early and Late) indicate that this trend is likely driven by fewer sessions in those HET animals that complete the task, specifically in the "Late" reversal once performance has returned to chance level ($t(10) = -2.202$, $P = 0.052$).

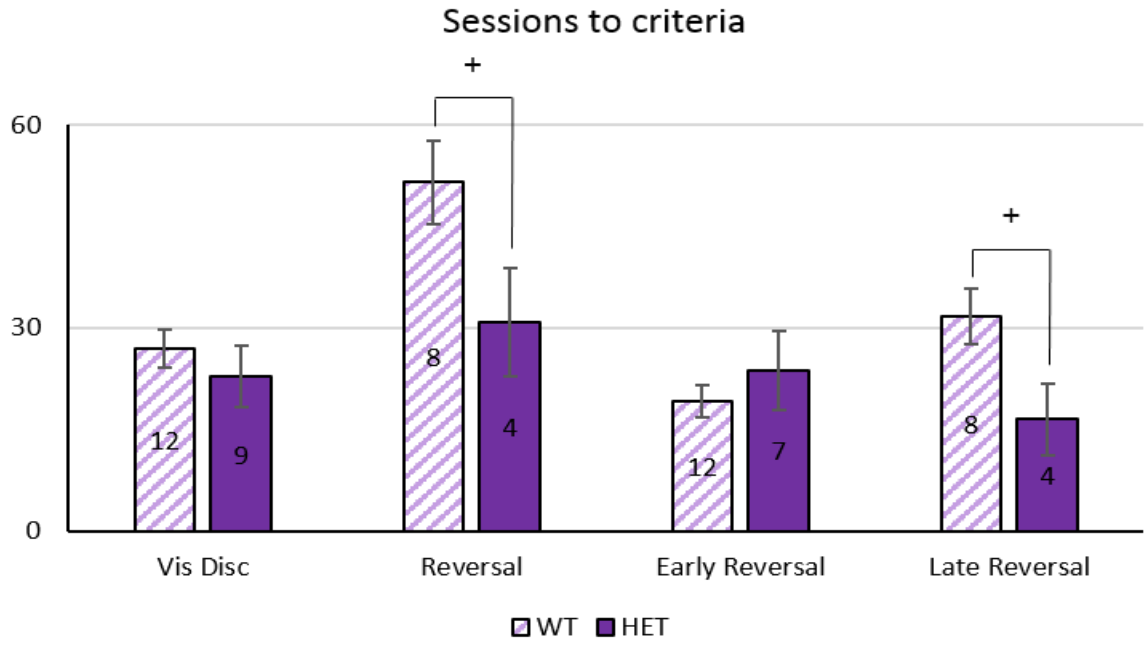

Figure 7.7 Number of sessions to reach criteria for "Visual Discrimination" (Vis Disc) and "Reversal" for WT and HET animals. Early reversal refers to the number of sessions completed before performance returned to chance level following reversal. Late reversal refers to the number of sessions to reach criteria once at chance level. Error bars are ± SEM. Labels inside bars are the number of animals included in each analysis. No significant differences between genotypes, though trends observed in reversal driven by fewer sessions to criteria in HETs during Late reversal. + P < 0.08.

Due to the trends observed, further analyses were conducted to compare "Early" reversal when separated by animals that completed or failed to complete the task. A two x two ANOVA with genotype and completion as the fixed variables found a significant effect of completion ($F(1,15) = 4.561$, $P = 0.050$, $\eta_p^2 = 0.233$), as well as a significant interaction between completion and genotype ($F(1,15) = 6.266$, $P = 0.024$ $\eta_p^2 = 0.295$). Post-hoc student's t-tests show greater sessions to chance required in HET animals that do not complete the task ($t(5) = 2.104$, $P = 0.045$, one-tailed), whereas there is no significant difference between those that complete (Figure 7.8). Greater numbers are required for this comparison to be sufficiently powered.

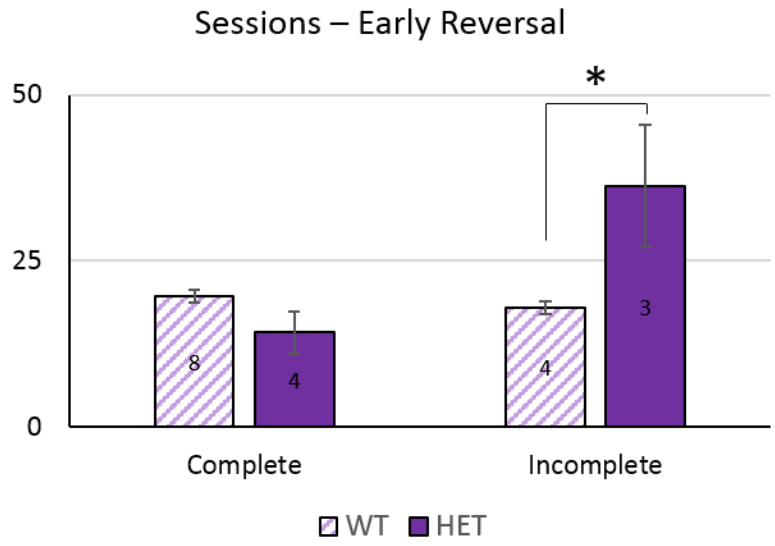

*Figure 7.8 Sessions in Early reversal split by genotype and by reversal completion status. Animals that completed the full reversal task took fewer sessions to reach 50% performance than those that failed ($F(1,15) = 4.561$, $P = 0.05$). Significant interaction between genotype and completion ($F(1,15) = 6.266$, $P = 0.024$). Post-hoc student's t-test show a significant effect of genotype between animals that do not complete the task. * $P < 0.05$*

7.3.5 HET animals make an increased number of errors during early reversal

The number of errors were compared between WT and HET animals during “Visual discrimination”, “Early” and “Late” reversal phases. A repeated measures ANOVA was conducted across phases for those animals that completed the full task. There was no effect of genotype ($F(1,9) = 0.554$, $P = 0.476$) or phase ($F(2,18) = 1.348$, $P = 0.285$) but there was a significant interaction ($F(2,18) = 4.946$, $P = 0.019$, $\eta_p^2 = 0.355$). Further post-hoc analyses to include all animals which completed each stage found there was no difference between the numbers of errors made during visual discrimination ($t(21) = -0.001$, $P = 1$). HET animals made significantly more errors compared to WT during “Early” reversal ($t(17) = 2.176$, $P = 0.044$, $d' = 1.03$), however there was a trend towards fewer errors made in “Late” reversal ($t(9) = -2.005$, $P = 0.076$) (Figure 7.9).

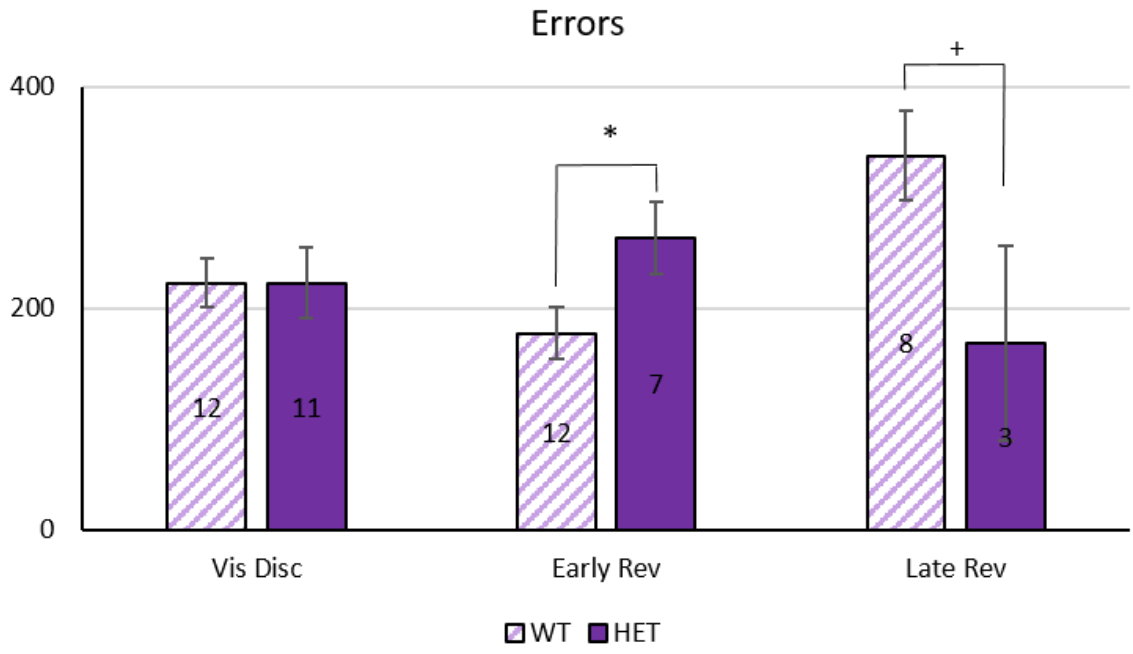

*Figure 7.9 Number or errors made by WT and HET animals during "Visual Discrimination", "Early" and "Late" reversal. Error bars represent SEM, labels within bars refer to the number of animals included in each analysis. Significantly more errors were made by HET animals during “Early” reversal with a trend towards fewer in “Late” reversal. One animal was excluded from “Late” reversal analysis due to value being 2 sd above the mean. * P < 0.05, + P < 0.10*

7.3.6 HET animals respond faster to stimuli following reversal with no differences in reward collection

There are no differences between WT and HET animals in response latency during "Visual discrimination", though animals responded faster to stimuli during the last sessions of the phase (Figure 7.10). A significant effect of session was observed ($F(1,19) = 7.322$, $P = 0.014$, $\eta_p^2 = 0.278$, *log10 transformed to normal distribution*), with no effect of genotype ($F(1,19) = 1.134$, $P = 0.300$) or an interaction ($F(1,19) = 2.487$, $P = 0.131$).

During "Early" reversal HET animals respond significantly faster to stimuli presentation compared to WT. Repeated measures ANOVA showed no effect of session ($F(1,16) = 2.240$, $P = 0.154$), but a significant effect of genotype ($F(1,16) = 10.921$, $P = 0.004$, $\eta_p^2 = 0.406$, *log10 transformed to normal distribution*). There was no evidence of an interaction ($F(1,16) = 0.273$, $P = 0.608$).

During the "Late" reversal stage a significant effect of session was observed ($F(1,10) = 9.421$, $P = 0.012$ $\eta_p^2 = 0.485$, *log10 transformed to normal distribution*), with faster responding seen at the end of the reversal phase. HET animals appeared to respond faster to stimuli, however this was not supported statistically. There was no difference between genotype ($F(1,10) = 2.770$, $P = 0.127$) or evidence or an interaction ($F(1,10) = 0.375$, $P = 0.554$).

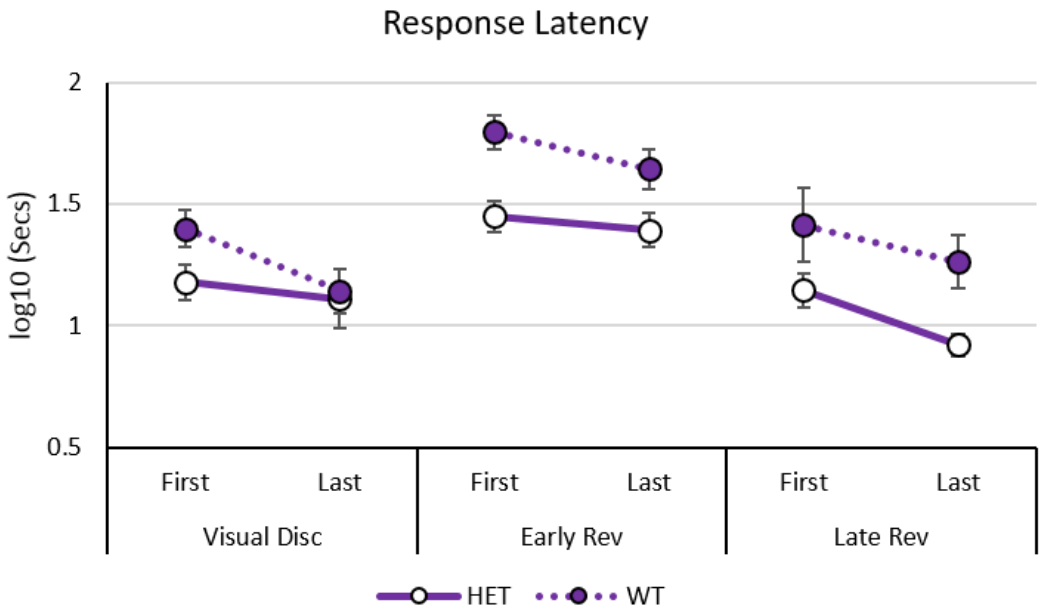

Figure 7.10 Response latencies for WT and HET animals compared at start and end of "Visual discrimination", "Early" and "Late" reversal. Latencies are averaged across the first 3 and last 3 sessions of each phase respectively. Error bars are SEM. Data presented has undergone a log10 transformation due to non-normal distribution. Significant effect of session in "Visual discrimination" and "Late" reversal and significantly faster responding in HET animals during "Early" reversal, $P < 0.05$.

There were no differences between genotype for reward collection latency during "Visual discrimination", "Early" or "Late" reversal. Repeated measures ANOVA showed an effect of session in "Visual discrimination ($F(1,19) = 4.458$, $P = 0.048$, $\eta_p^2 = 0.19$, *log10 transformed to normal distribution*) indicating faster reward collection at the end of the phase, though no effect of genotype ($F(1,19) = 0.044$, $P = 0.836$) or an interaction ($F(1,19) = 0.000$, $P = 0.991$). No effects were observed during "Early" reversal for session ($F(1,16) = 0.171$, $P = 0.684$) or genotype ($F(1,16) = 0.492$, $P = 0.493$) and there was no significant interaction ($F(1,16) = 1.225$, $P = 0.285$). Similarly no effects were observed during "Late" reversal for session ($F(1,10) = 0.621$, $P = 0.449$), or genotype ($F(1,10) = 0.384$, $P = 0.550$) and no evidence of an interaction ($F(1,10) = 1.767$, $P = 0.213$) (Figure 7.11).

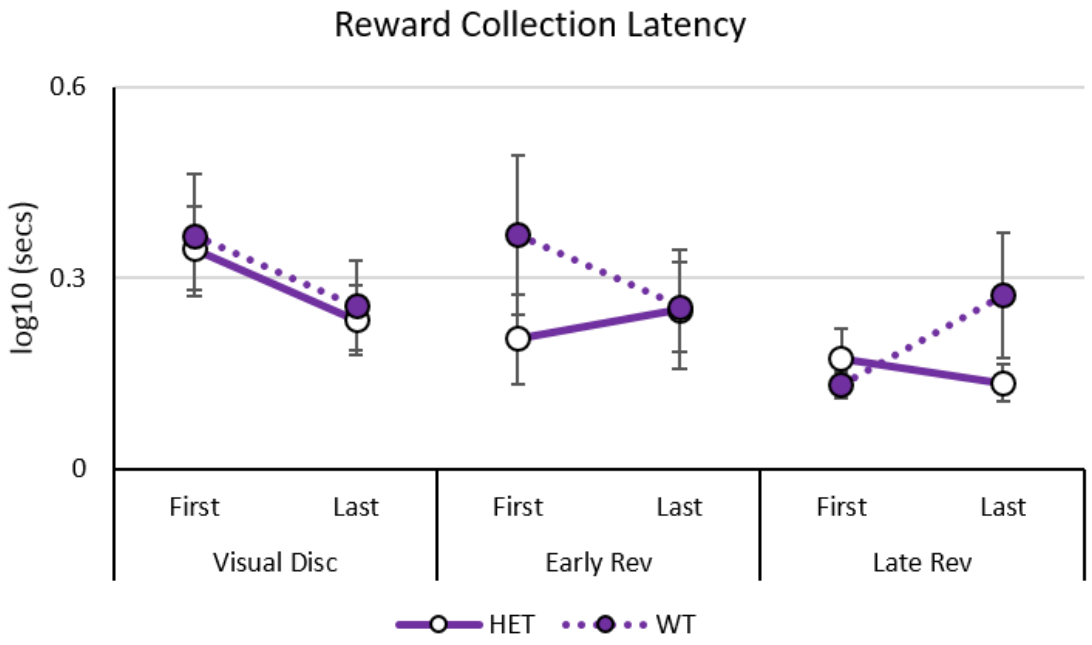

Figure 7.11 Reward Collection Latencies compared between WT and HET for "Visual Discrimination", "Early" and "Late" reversal. Reduced latency to collect reward was observed during "Visual Discrimination" only ($P < 0.05$). Latencies are averaged across the first 3 and last 3 sessions of each phase respectively. Error bars are SEM. Data presented has undergone a log10 transformation due to non-normal distribution. There was no significant difference between genotypes. $P > 0.05$.

7.4 Discussion

Cacna1c heterozygous knockout rats were compared to wildtypes during a touchscreen reward based reversal learning task. Results indicate that reduced expression of *Cacna1c* has no effect on the acquisition of visual discrimination, implying no effects on basic learning of reward associations, motivation or motor function (Mar et al., 2014). Altered behaviour was observed in HET animals specifically during the reversal learning stage indicative of behavioural inflexibility.

Reduced expression of Cacna1c affects inhibition of responding to prior contingencies

There was a trend towards fewer HET than WT rats completing the early stage of reversal to return to chance level performance. Further investigation of this trend, with greater numbers of animals is needed to determine its significance. HET animals that did return to chance levels made significantly more perseverative errors during this phase of the task and were faster to respond to stimuli than WT. This indicates that the normal expression of *Cacna1c* is not required for the acquisition of initial novel stimulus-reward associations. Behaviour associated with reduced expression instead indicates a specific impairment of the comparison and update of new information with existing representations, with a deficit in the appropriate inhibition of response to prior contingencies.

These current findings provide further support for separable mechanisms underlying the acquisition of reward associations and their adaptation or reversal (Cools et al., 2002; Chudasama and Robbins, 2003; Ghahremani et al., 2010). Specific impairment observed in “Early” reversal concurs with findings in genetic knockout of other disease relevant genes having a role in behavioural flexibility. Homozygous knockout mice for *Dlg2* show similar increases in perseverative errors during early stages of reversal (Nithianantharajah et al., 2013). Importantly, similar impairments

are consistently evident in patients with schizophrenia (Waltz and Gold, 2007a; Floresco et al., 2009; McKirdy et al., 2009; Culbreth et al., 2015). Patients are found to make increased perseverative errors and complete fewer reversal sessions than healthy controls in probabilistic reversal learning paradigms. The coherence of findings may suggest a convergence of risk gene related dysfunction on mechanisms of cognitive flexibility.

Decreased reaction times in HET animals during the reversal phase suggest lack of inhibition may be due to a propensity to continue to favour habitual responding instead of goal-directed decision processing, though it should be noted that current work did not carry out specific tests for goal-directed/habitual responding, such as differential sensitivity to reinforce devaluation. Dual-process theories of decision making indicate a competition that occurs between habitual and goal-directed responses, to maximise reward and minimise threat (Rangel et al., 2008); with goal-directed seemingly flexible but slow and habitual rapid but inflexible (Keramati et al., 2011). For decision making to be optimal, the appropriate inhibitory control must be exerted over the rapid habitual responses when outcomes change (Eagle et al., 2008; Izquierdo and Jentsch, 2012). Habitual responding is known to, in part, rely on glutamatergic projections from the infralimbic cortex to the dorsolateral striatum, with phasic dopamine signalling from the midbrain to areas of the striatum being necessary for the formation of stimulus-response associations (Redgrave et al., 2010). In comparison, action-outcome associations underlying goal directed behaviour rely heavily on the associative loop, comprising of the dorsomedial striatum and areas of the paralimbic cortex (Lanciego et al., 2012). Separate manipulation of tonic and phasic dopamine signalling to the nucleus accumbens has been found to selectively impair initial discrimination and response switching, with a disconnection of the mPFC leading to perseverative behaviour (Grace et al., 2007). Furthermore, tonic activity of dopamine neurons has been found to directly influence the

competition that occurs between response strategies by modulating the perceived cost of goal-directed behaviour (Keramati et al., 2011).

Both genotypes are able to acquire the initial discrimination, indicated by successfully reaching criteria and a significant reduction in response latencies. However, HET animals continue to respond faster than WT in early reversal with increased perseverative errors. This pattern of behaviour is consistent with the suggestion that habitual decision processes are functional in the mutants; whereas there is an impairment in switching from habitual to goal-directed responding to match the requirements of the changing contingencies. This could be a result of a deficit in the cost/benefit comparison required to facilitate an appropriate inhibitory control of habitual responding and allow a switch to goal-directed behaviour.

People who carry the variant in *Cacna1c* associated with increased risk of schizophrenia have been found to show blunted reward responsiveness (Lancaster et al., 2014) and patients with schizophrenia are known to have increased synthesis of dopamine (Breier et al., 1997). It is possible that reduced expression of *Cacna1c* leads to impairments in reward value representation through effects on tonic dopamine, modulating the perceived benefit of goal-directed behaviour and a perseveration of habitual responding.

The neural circuitry implicated in this task is also known to have a role in emotion regulation (Bechara et al., 2000; Davidson et al., 2000). Emotion regulation is a prominent symptom across psychiatric illness and is specifically evident in schizophrenia and bipolar disorder. It is conceivable that dysfunction in HETs in regions involved in this reversal task, could also manifest in dysregulation of emotion regulation and may partly underlie the shared genetic risk that CACNA1C has with these disorders.

Reduced Cacna1c may facilitate learning of new contingencies in a subgroup of animals

It appears that there was a subgroup of behaviourally distinct HET animals that were able to complete the reversal learning task. These animals demonstrated faster acquisition of the new contingency and reduced response latencies compared to WT.

Previous studies have observed a similar pattern of behaviour with lesions of the vmPFC and also acute moderate stress following successful visual discrimination (Graybeal et al., 2011). It was found that lesioned animals were faster to acquire the reversed contingency and made fewer errors in late reversal than sham controls. Similar effects were observed following a forced swim test, however response latencies were increased. The facilitation produced in late reversal with acute stress was prevented with local infusion of BDNF into the vmPFC. Although the pattern of responding in stressed animals may differ in some respect from that seen in lesioned or HET animals completing the task, this study highlights the potential role dysregulated *BDNF* may have in the facilitation of reversal observed. Given previous findings with these animals and evidence of reduced BDNF expression in conditional *Cacna1c* knockout mice (Lee et al., 2016), it would be pertinent to investigate these effects more fully, as well as determine the effects of manipulation of BDNF at different stages of reversal.

Studies into the effects of stress on learning have shown that under stress people and animals utilise a simpler habit based approach rather than goal directed behaviour (Schwabe and Wolf, 2009; Braun and Hauber, 2013). Although there appears to be a facilitation in later stages of reversal in those HET animals that are able to complete the task, response latencies still indicate a propensity for habit based responding rather than goal directed decision making. This highlights the potential value of future studies which aim to establish the effect of added stress on

specific learning behaviours in these animals, as well as manipulations of task difficulty (as in *Dlg2* mice, Nithianantharajah et al., 2013).

It could be the case that certain animals have developed a compensatory mechanism that does not rely on the presence of *Cacna1c* at normal expression levels and are therefore able to perform the task. It is only when comparing the subtle aspects of latency that the sub-optimal nature of this strategy may be revealed. The current task was not designed, nor is it sufficiently powered, to be able to investigate differences within HET animals, however future studies would look to determine potential environmental interactors that may influence these behaviours.

Limitations and future directions

The low completion rates in HETs, although a phenotype in itself, makes it difficult to compare the subtler effects which may exist on inhibitory learning. Statistical analyses of these low numbers have questionable validity, with the results from chi squared tests with minimum expected values of less than 5 requiring further confirmation. As the experiment was terminated before all animals reached criteria, it is not possible to conclude whether animals are unable to reverse responses or if they are simply slower to acquire. It may be the case that the task procedure was not optimised for Sprague Dawley rats. Previous studies have found reduced activity in Sprague Dawley animals, though comparison between Sprague Dawley and Long Evans strains in the touchscreen tasks have previously found no difference (Bussey et al., 2008). Sprague Dawley animals are reported to have reduced visual acuity which may have also made the discrimination between squares and grid more difficult (Prusky, Harker, Douglas and Whishaw 2002). Animals in this task performed fewer trials in individual sessions than previously reported (Bussey et al., 2008). This may explain the greater number of sessions required to reach the criteria, which were originally set at 100 trials. Stimulus choice and criteria may therefore need adjusting in future experiments to achieve greater completion rates. A larger cohort of animals

is necessary, with optimised conditions, to ensure sufficient numbers reach criteria for more powerful analyses across the full course of the task. A greater completion rate would also allow a more formal interrogation of habitual responding in mutant animals, through an additional phase of reward devaluation, though this may require investigation in a separate cohort.

The seeming existence of sub-groups of HET animals with opposing behavioural phenotypes indicates additional variables interacting with genotype. The method of breeding these animals meant that pups may have been reared by a *Cacna1c* heterozygous mother, which may have had an additional effect on the pre and/or postnatal environment. This will be discussed in more detail in the General Discussion, however it is worth noting that the HET animals that showed facilitation in late reversal were all born to a WT mother. Potential parenting effects and the influence of stress already discussed, highlight the need to consider environmental interactors when making conclusions about the effect of chronic genetic knockdown.

Future studies will need to discern the effects of reduced *Cacna1c* on specific aspects of dopamine signalling directly, to include tonic and phasic signalling and region specific analyses. Dopamine levels could be inferred by indirect measurement of tyrosine hydroxylase or quantified directly by dialysis tubes implanted stereotaxically into regions of interest. Determining the regulation of *BDNF* within more specific regions of the brain, particularly the PFC, would also be valuable to inform potential normalisation of behaviour.

7.5 Conclusions

The normal expression of *Cacna1c* is required for the appropriate behavioural adaptation to changing reward contingencies in a translational touchscreen reversal learning task. Behaviour is consistent with an inability to appropriately inhibit rapid habitual responding to optimise decision making processes. This supports the role

for *Cacna1c* in cognitive processes known to be impaired in patients with schizophrenia, as well as those relying on dopamine signalling in cortical-striatal neural circuits. Species homology of the neural circuitry involved in this task and the utilisation of the touchscreen paradigm enhances translation to humans. These findings contribute to our understanding of how the CACNA1C gene may confer risk for psychiatric illness, by identifying effects on specific learning related mechanisms and cognitive flexibility in a reduced expression model.

8 RISK ASSOCIATED VARIATION IN CACNA1C IN HUMANS: EFFECTS ON GENE EXPRESSION AND REVERSAL LEARNING BEHAVIOUR

8.1 Introduction

As previously discussed, common variation in CACNA1C is robustly associated with risk for multiple psychiatric disorders (e.g. Ripke et al., 2014). Like many other SNPs found to be associated with schizophrenia, the risk variants rs1006737 and rs2007044 are not located in an exonic region (Ripke et al., 2014) but instead reside in the large intron 3 of the gene. There is no evidence that these common variants lead to any changes in the sequence of the protein and at present their exact functional effects are still under debate. It is important to discern the effect of these variants on biological mechanisms, in order to better understand their contribution to the pathology of schizophrenia and other psychiatric illnesses and to help guide the development of novel treatment targets.

Two key approaches have been utilised to further understand the functional relevance of risk variants and how they may confer susceptibility to disease. First, the potential for SNPs to act as expression quantitative trait loci (eQTL) has been explored. EQTLs are genomic loci at which variation is found to affect transcript expression. Those loci found to affect expression of genes in close proximity (+/- 1 MB) are termed cis-eQTLs and effects on gene expression outside this region are termed trans-eQTLs. Typically, cis-eQTLs have a more pronounced effect on expression and are thought to more likely indicate a direct functional effect of variation on that specific gene (McKenzie et al., 2014). Trans-eQTLs may reflect an indirect relationship between variant and expression, though may provide an insight into overlapping biological mechanisms where multiple genes are affected.

Secondly, associations between risk variants and specific behaviours have been investigated to identify endophenotypes which may better link risk to known biological mechanisms (Flint and Munafò, 2007). Studies have explored multiple levels of endophenotype, to include cognitive function across multiple domains, brain imaging of structure and connectivity, and functional imaging to assess effects on neural activation (e.g. Zhang et al., 2012; Paulus et al., 2013; Lancaster et al., 2015).

It has been repeatedly found that GWAS risk loci are enriched for eQTLs (Nicolae et al., 2010; Richards et al., 2012) as well as cis-regulatory elements (CREs). Roussos et al., (2014) found that schizophrenia related SNPs were enriched for certain functional annotation categories, to include enhancers and promoter regions. A specific investigation of CACNA1C found that the risk allele at rs1006737 is associated with decreased gene expression ($P = 1.88 \times 10^{-5}$) in post-mortem brain tissue and hiPSC-derived neurons. Using chromosome confirmation capture Roussos et al., further identified a 1.4 kb enhancer region within the risk loci that physically interacts with a proximal gene promoter. This is supported by recent findings which examined the expression of CACNA1C in the superior temporal gyrus (STG) of post-mortem human brain tissue; with decreased expression observed in risk allele carriers (Eckart et al., 2016). They further investigated the potential of transcript specific changes in expression, finding similar effects in all three different types of transcscript studied. Reduced expression has also been observed in the cerebellum of people carrying the risk allele at rs1006737 (Gershon et al., 2014) across multiple transcripts, though no effect was observed in the parietal cortex.

In contrast, Bigos et al., (2010), found that the risk allele predicted increased CACNA1C expression in the dorsolateral prefrontal cortex (dPFC) of post-mortem brain samples. However, the samples ranged from gestational week 14 through to old age, as well as including samples of Caucasian and African American origin, which may have confounded results. Yoshimizu et al., (2014), similarly found an

increased expression of CACNA1C associated with the risk allele in functional induced neurons (iNs), in conjunction with an enhanced L-type calcium current density. Cell lines were derived from both healthy controls and a mixed population of patients (including schizophrenia, bipolar and major depressive disorder), which could contribute to differences observed.

Conflicting evidence of effects of rs1006737 on expression of CACNA1C suggests there may be brain region specific and potentially transcript specific regulation. The online database BRAINEAC (www.braineac.org) provides expression data from the UK Brain Expression Consortium (UKBEC), across different regions of the brain and with multiple probe sets detecting different transcripts. Gene expression can be stratified by risk SNP, both those located within or near the gene and those located elsewhere in the genome. This database provides a useful tool for investigating changes in expression associated with risk variants across multiple brain regions in the same individuals, which previous studies did not fully achieve. Effects on relevant downstream gene expression can further be interrogated to identify potential downstream biological mechanisms which are altered by risk variation.

With the existing evidence of altered *CACNA1C* expression in risk allele carriers, it becomes more informative to investigate the associated effects on other endophenotypes including measures of behaviour in an attempt to determine the functional effects of such genetic variation on brain systems. Associations with rs1006737 have been quite extensively studied across domains of cognition, to include verbal fluency, attention and memory, though evidence is conflicting on the effect of variation.

Previous studies suggest an effect of the risk allele on working memory in healthy controls and patients (Zhang et al., 2012). Using tasks aimed to measure interference control and goal maintenance (a spatial N-back and a dot pattern expectancy task), it was found that healthy risk allele carriers made more errors

compared to non-risk allele carriers. This was consistent with findings in people with schizophrenia; interestingly, in people with bipolar disorder, those who carried the risk allele were found to make fewer errors on the tasks. Further deficits have been observed in logical memory in patients (Hori et al., 2012) and verbal working memory in healthy controls (Dietsche et al., 2014). In contrast, Rolstad et al., (2016) found no effects of the risk allele in CACNA1C across multiple domains of cognition. The neuropsychological test battery was administered to assess performance on: memory/learning, speed/attention, executive function, verbal functioning and visuospatial function (see Sparding et al., 2015 for full description of battery). Rolstad et al., found no association of genotype in CACNA1C with performance in any cognitive domain.

Aside from type I and type II errors, these conflicting results could be due to common variation having effects on more specific aspects of cognition rather than resulting in a broad deficit. Cognitive test batteries are more often designed to assess these broader deficits. In addition, it is likely to be combined genetic risk load, rather than an individual risk SNP, that results in the more profound deficits observed in people with psychiatric illness. This would also explain differences observed between patient subgroups. Identifying the subtle deficits associated with common variation and looking for a potential convergence, would help to identify molecular mechanisms and pathways which may be affected.

To date, no study has looked at whether genotype at the relevant CACNA1C risk SNPs is associated with behaviour on a reversal learning task. This task allows the investigation of not only associative learning and reward response, but allows the interrogation of behaviour flexibility, a cognitive domain known to be affected in individuals with schizophrenia (as discussed previously, Chapter 7, e.g. Floresco et al., 2009). Previous studies have found an effect of rs1006737 on brain activation during reversal learning with enhanced amygdala activity seen in response to reward

in risk allele carriers (Wessa et al., 2010). However, there has been no investigation into the potential effects on specific aspects of reversal learning.

8.2 Aims

Interrogation of the online human tissue expression database BRAINEAC (www.braineac.org), aimed to identify whether risk variants in *CACNA1C* (rs1006737 and rs2007044), known to be associated with psychiatric illness, are associated with changes in expression of *CACNA1C*. In light of previous findings with *Cacna1c* heterozygous knockout models, analyses additionally aimed to establish whether there are any effects of the risk alleles on the expression of specific *BDNF* transcripts.

Further analyses aimed to determine whether these same risk variants in *CACNA1C* are able to predict performance on a probabilistic reversal learning task. Behavioural and genotype data at specific loci was extracted from existing data in a cohort of healthy controls (Lancaster et al., 2015). The number of risk alleles carried at rs1006737 and rs200747 were investigated for association with total performance on the task and performance following reversal.

8.3 Methods and materials

8.3.1 Analysing gene expression associated with risk SNPs using BRAINEAC

The BRAINEAC online database (www.braineac.org) is the product of the UK Brain Expression Consortium (UKBEC) which provides data on the regulation and alternative splicing of genes expressed in the human brain. The current database includes samples from 134 brains from individuals with no evidence of neurodegenerative disorders. Up to 10 regions of interest were collected from control individuals by both the Medical Research Council Sudden Death Brain and Tissue Bank, and the Sun Health Research Institute, to include: cerebellum (CRBL), hippocampus (HIPP), substantia nigra (SNIG), frontal cortex (FCTX), temporal cortex (TCTX), occipital cortex (OCTX), medulla (MEDU), putamen (PUTM), thalamus (THAL) and intralobular white matter (WHMT). Of the 134 samples 102 were male, with a mean age of 59 (16 – 102). For full RNA extraction and genotyping methods used in producing this data, see www.braineac.org and for quality control parameters see Trabzuni et al., (2011).

Using the online general user interface, the expression of CACNA1C and BDNF was stratified by rs1006737 and rs2007044 for each individual probe set available, across the 10 brain regions. Where significant ($P < 0.05$) changes were observed, the probe set was cross-linked to the specific transcript/s which it identifies using Affymetrix – NetAffx™ Analysis Centre (www.affymetrix.com/analysis/index.affyx). For BDNF this resulted in transcripts being separable by probes, allowing specific comparisons between them and for CACNA1C expression changes could be compared between sets of transcripts.

8.3.2 Acquisition of behavioural and genotype data for reversal learning analyses

Novel analyses were conducted on behavioural and genetic data collected by Lancaster et al., (2015).

8.3.2.1 Probabilistic reversal learning behavioural task

One hundred participants were recruited (aged 19 – 47) from staff and students at Cardiff University. Ethical approval was given by the ethics committee of the School of Psychology and informed consent was obtained from each participant prior to the study. Participants had no history of psychiatric illness (themselves or first degree relative) and did not report taking any psychotropic medication or illegal substances. A total of 84 participants (49 female) were used for analysis, after exclusion for quality control of genetic data and/or missing information.

Participants were asked to make a choice between a blue and a green stimulus presented simultaneously (Figure 8.1). A correct response was followed by a white 'smiley' face and rewarded with a +1 p reward and an incorrect response was followed by a red 'frowny' face and a -1 p punishment. Participants were told to maximise their 'earnings' throughout the task. After 7 – 11 trials the contingencies were reversed and the previously rewarded stimuli was now punished. There were 12 reversal sessions in total (108 choice trials). Each session consisted of 1 or 2 'probabilistic errors' in which the correct response was punished to ensure sufficient difficulty in the task, as well as provide additional measures of behavioural flexibility.

"Total Earnings" were calculated for each participant for the full task (in pence) based on the amount of rewards and punishments they received throughout. Performance following the first contingency reversal was calculated as percentage correct for that session block – "Reversal Accuracy".

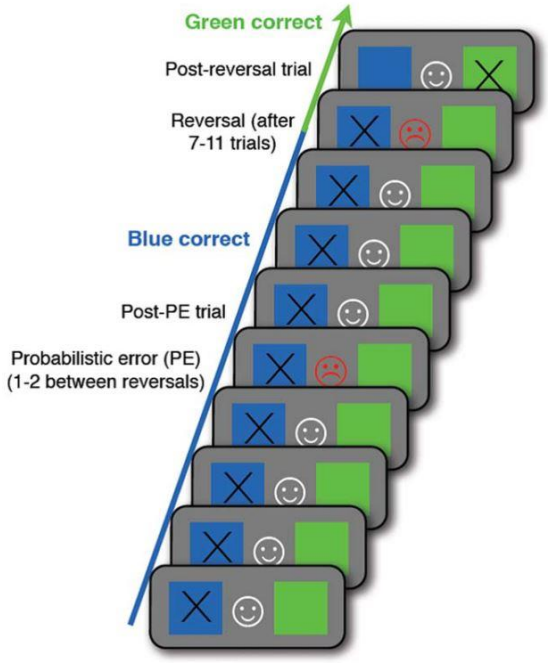

Figure 8.1 Reversal learning paradigm. Participants were presented with a choice between blue or green squares. Correct choices were rewarded with monetary winning and a white 'smiley' face and incorrect responses with a red 'frowny' face and a monetary fine. One or two probabilistic error trials were included within each session block in which the 'correct' response was punished. There were 7 – 11 trials in each block before contingency reversal (Figure from Lancaster et al., 2016).

8.3.2.2 DNA extraction and genotyping

Samples of saliva were collected by Lancaster et al., from participants for genotyping using Oragene DNA (OG-500) kits (DNA Genotek Inc., Ottawa, Canada). Genotyping was conducted using a custom HumanCoreExome BeadChip Kit (Illumina, San Diego, USA) which included 570,038 variants. Data was quality controlled in PLINK (whole genome data analysis toolset, (Purcell et al., 2007)). Participants were removed if data suggested non-European ancestry, ambiguity of sex, relation to other participants (through identity of descent) or incomplete genotyping <97%. Genotypes were then imputed by estimating haplotypes using SHAPEIT and imputing genotypes from the reference set 1000 Genomes (December 2013, release 1000 genomes haplotypes Phase 1 integrated variant set) using IMPUTE2 (Default parameters were used as detailed in Howie et al., 2009 & Delaneau et al., 2012).

For the purpose of the current analysis genotypes were extracted from the full SNP dataset for rs1006737 and rs200747 only.

8.3.3 Behavioural Analyses

Performance was compared between risk allele and non-risk allele for rs1006737 and rs2007044 separately. Total earnings (pence) and accuracy following reversal (%) were used as the dependent variables for analysis to provide measures of general performance on the task as well as behavioural flexibility.

Student's t-tests were conducted in IBM SPSS Statistics 20 to determine if there was an allelic association with performance; with the hypothesis that the risk allele would be associated with decreased performance. Shapiro-Wilk and Levene's statistics were used to test for normality and homogeneity of variance.

8.4 Results

8.4.1 Risk associated variation in CACNA1C selectively reduces expression in the cerebellum

The BRAINEAC human expression online database was interrogated to determine if schizophrenia risk variants in *CACNA1C* are associated with changes in expression. CACNA1C and BDNF were analysed from up to 10 brain regions across multiple transcripts. Allele frequency at rs1006737 was A: 29.1% G: 70.9% and at rs2007044 was G: 36.9% A: 63.1%, full genotype splits can be seen in Table 8.1.

Table 8.1 Sample number for each genotype of rs1006737 and rs2007044. Risk allele (A) frequency: 29.1% and (G) 36.9% respectively

Rs1006737		Rs2007044	
Genotype	Nº	Genotype	Nº
AA	11	GG	17
AG	56	GA	65
GG	67	AA	52

At a whole transcript level (using the winsorised mean across all probe-sets) there was a significant effect of genotype at both rs1006737 and rs2007044 on the expression of CACNA1C in the cerebellum (Figure 8.2: $P = 8.5 \times 10^{-7}$ and $P = 6.1 \times 10^{-9}$ respectively). There were 130 cerebellum samples included in the analysis. There was no effect of genotype on expression in any other brain region at this level ($P = 0.16 – 0.87$).

Observations at individual probe-set level showed effects on expression in the cerebellum in 46 / 57 probe-sets (81%), though magnitude and significance levels varied. Following previous work which showed different functionality of splice variants around exon 32 (Tang et al., 2004b), additional comparisons were made between probe sets that could distinguish between subsets of transcripts. Probe set

3400895 and 3400898 distinguish between transcripts that contain or skip exon 32 respectively. Both sets showed significantly reduced expression of CACNA1C in the cerebellum in risk allele carriers, suggesting that effects are broad across different types of transcripts.

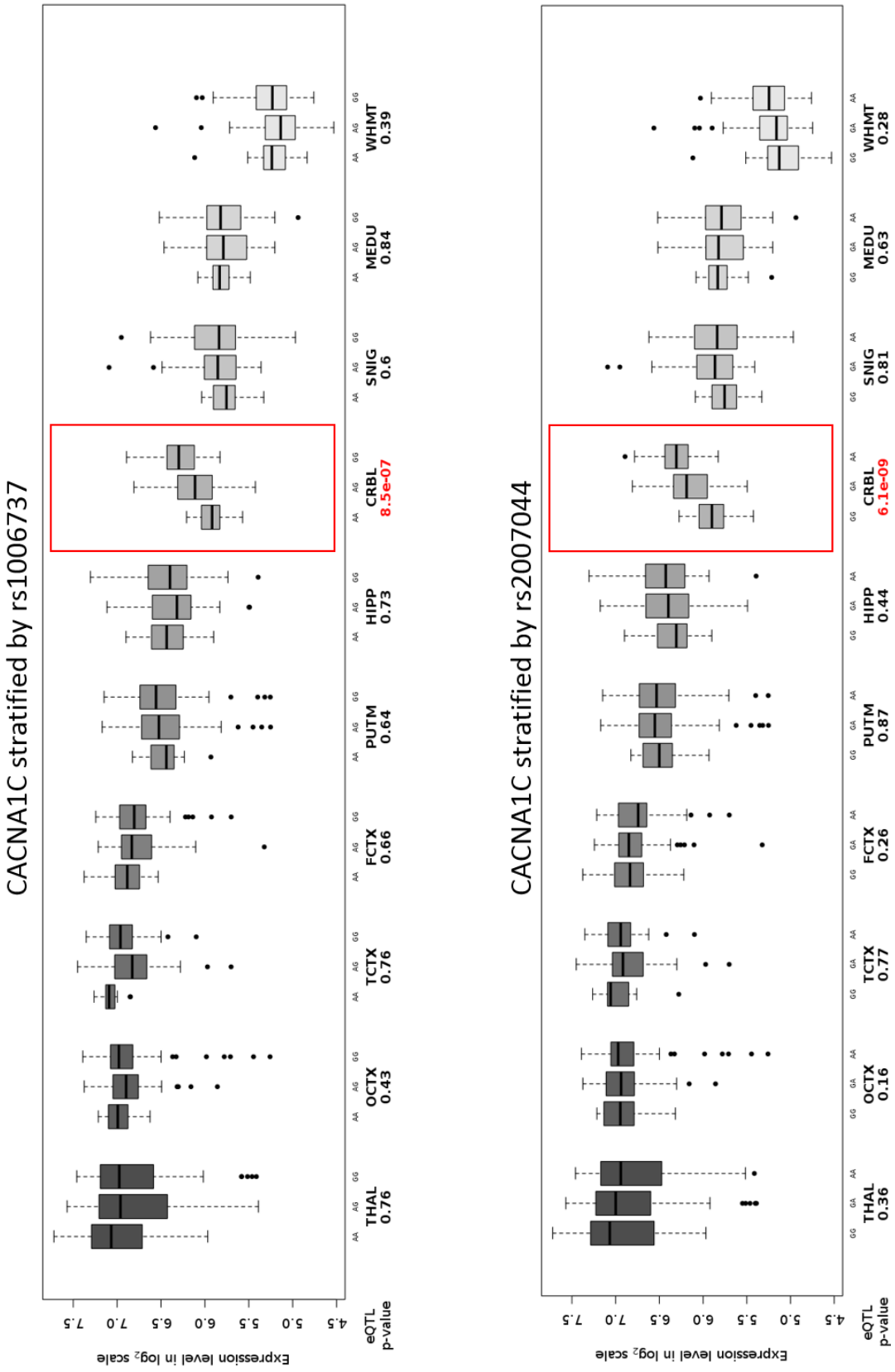

Figure 8.2 Box plots of expression changes in CACNA1C stratified by rs1006737 and rs2007044 across multiple brain regions. Both risk SNPs are associated with reduced expression specifically in the cerebellum (outlined in red). Figures adapted from www.braineac.org.

8.4.2 Risk variants in CACNA1C are associated with region specific changes in BDNF

The same risk variants were applied to stratify the expression levels of BDNF across different regions of the brain on the basis of *CACNA1C* genotype. At the average transcript level (winsorised mean across probe sets) there is no effect of genotype at rs1006737 or rs2007044 on the expression of total BDNF in any brain region.

The transcripts of BDNF are formed from the alternative splicing of unique 5' exons (I – VIII) with a common protein coding 3' exon IX. Due to these structural features, the 17 known human transcripts can be separately targeted by different probe-sets to the individual 5' exons, in addition to a global measure of BDNF mRNA with probes targeting the protein coding exon IX (Pruunsild et al., 2007).

Probe sets that identified exon IX (affymetrix exprID: 336247 & 336244; detecting all transcripts of BDNF) show a significant association of BDNF expression in the substantia nigra dopaminergic regions, with genotype at rs1006737 and rs2007044 (Figure 8.3). Both CACNA1C risk alleles are associated with decreased expression of global levels of BDNF in this specific brain region (P = 0.011 and P = 0.002 respectively), with no effects observed elsewhere.

Further analyses were focussed on exons 1, 2, 4 and 6 as BDNFI, II, IV and VI transcripts are most highly expressed in neurons (Rousseaud et al., 2015). Probe sets specific to exon 1 (affymetrix exprID: 336274) revealed an association between BDNF expression and genotype at rs1006737 (P = 0.013). The risk allele is associated with increased expression of BDNFI specifically in the hippocampus (Figure 8.4a). Exon two specific probe-sets show a similar association between risk allele and BDNF expression in the hippocampus (affymetrix exprID: 336273; HIPP: P = 0.011), with an additional effect in the frontal cortex (FCTX: P = 0.007) with the risk allele at rs1006737 associated with decreased BDNFIIa, BDNFIIb and BDNFIIc

(Figure 8.4). There was no indication of effects of genotype on BDNFIV or BDNFVI expression levels.

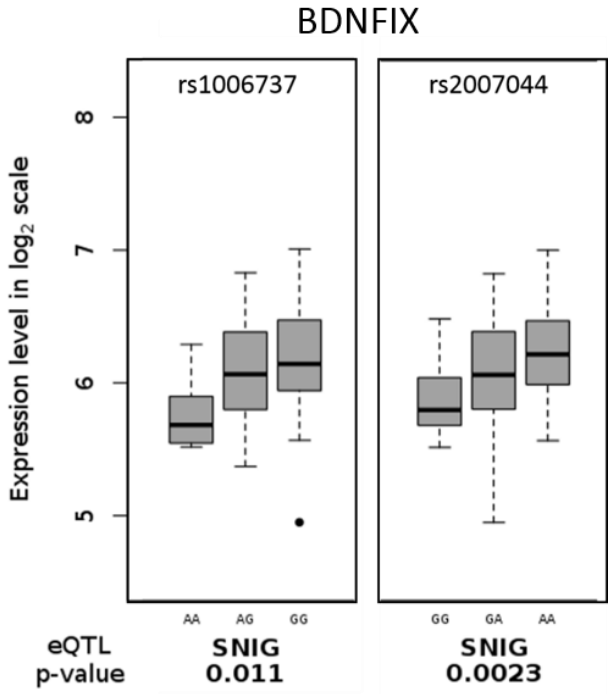

Figure 8.3 Expression levels of BDNF exon IX (affymetrix exprID: 336247; present in all transcripts) in substantia nigra (SNIG) stratified by genotype at rs1006737 and rs2007044. Risk allele is associated with decreased expression of BDNF in the substantia nigra. Figure adapted from www.braineac.org.

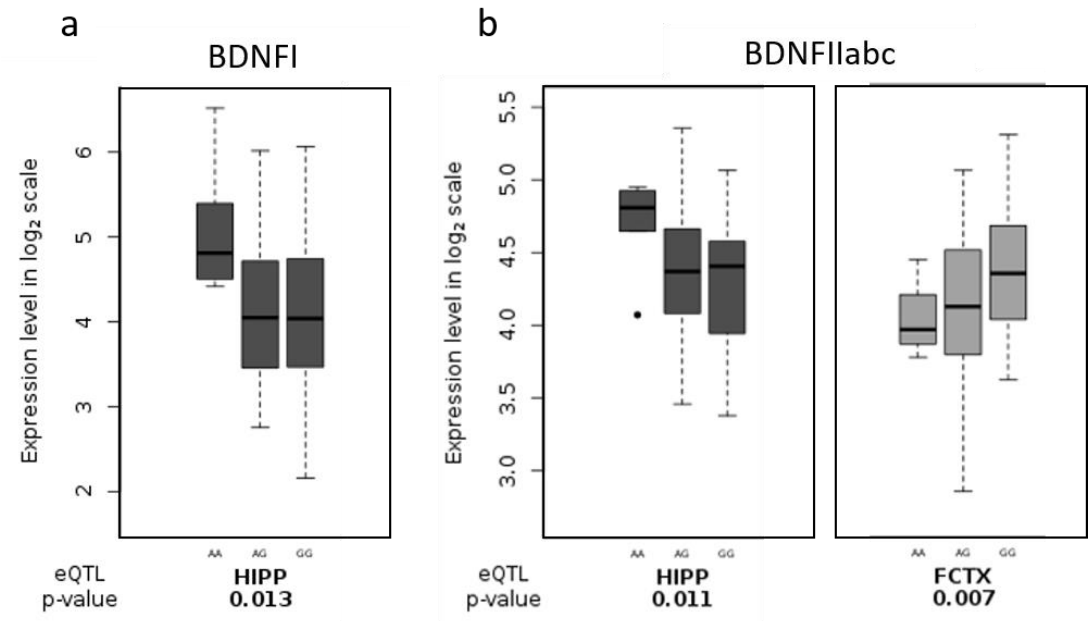

Figure 8.4 Expression levels of BDNF exon I and II stratified by rs1006737. a) BDNFI - affymetrix exprID 336274: Increased expression in the hippocampus in risk allele carriers, P = 0.013. b) BDNFIIabc – affymetrix exprID 336273: Increased expression in the hippocampus (P = 0.011) and decreased expression in the frontal cortex (P = 0.007) in risk allele carriers. Figure adapted from www.braineac.org.

8.4.3 Association of individual risk SNPs with reversal learning performance

Data was analysed from healthy participants who completed a probabilistic reversal learning task and provided saliva samples for genotyping. The effect of genotype at the two identified risk variants in *CACNA1C* (rs1006737 and rs2007044) on "Total Earnings" and "Reversal Accuracy" was explored. Reversal accuracy was specifically investigated to correspond to the stage of this task most commonly found to be impaired in patients with schizophrenia (Waltz and Gold, 2007a; McKirdy et al., 2009), as well as to most closely correspond to the stage at which deficits were observed in *Cacna1c* heterozygous knockout rats.

A total of 84 participants were used for analysis of rs1006737. There were 37 people who were non-risk homozygous (GG), 32 heterozygous (GA) and 15 risk homozygous (AA), giving a minor allele frequency of 37%. The variant is within Hardy Weinberg Equilibrium ($\chi(1) = 1.664$, $P = 0.20$) Genotypes were missing for two of the participants for rs2007044, therefore a total of 82 were used for analysis. For rs2007044 there were 27 people who were non-risk homozygous (AA), 39 heterozygous (AG) and 16 risk homozygous (GG) carriers, resulting in a minor allele frequency of 43%. Analysis was conducted to determine whether there was an allelic association with performance during the first reversal and across the whole of the task.

Total earnings on the task ranged from -12p to 50p and performance on first reversal ranged from 9.09% to 100%.

8.4.4 Schizophrenia risk variants are associated with accuracy of reversal learning

The risk allele in both rs1006737 and rs2007044 was associated with fewer correct responses during the first reversal session (Figure 8.5). Average performance associated with the risk allele (A) at rs1006737 was 64.08% (SEM = 2.66) compared to 71.27% (SEM = 1.90) associated with the non-risk allele (G); and at rs2007044

average performance associated with the risk allele (G) was 64.66% (SEM = 2.41) compared to 71.69% (SEM = 2.04). Student's t-tests between revealed a significant effect of risk allele at rs1006737 and rs2007044 with "Reversal Accuracy" ($t(166) = -2.242$, $P = 0.026$ and $t(165) = -2.230$, $P = 0.027$ respectively).

There were no significant effects of risk allele at rs1006737 ($t(166) = -1.562$, $P = 0.120$) or rs2007044 on "Total Earnings" ($t(165) = -1.122$, $P = 0.264$)(Figure 8.6).

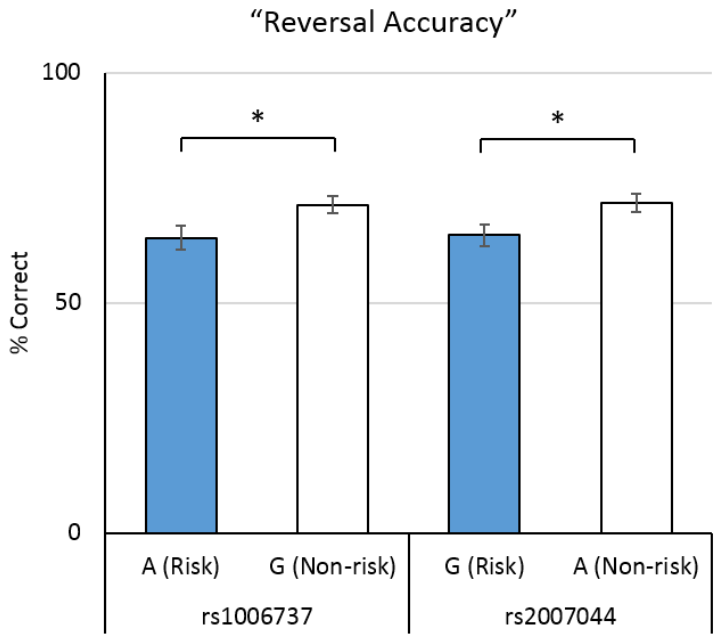

*Figure 8.5 Comparison of reversal accuracy following first reversal for risk and non-risk alleles at rs1006737 and rs2007044. Risk allele was associated with significantly reduced performance compared to non-risk allele carriers. Bars represent average % correct. Error bars are SEM. * $P < 0.05$.*

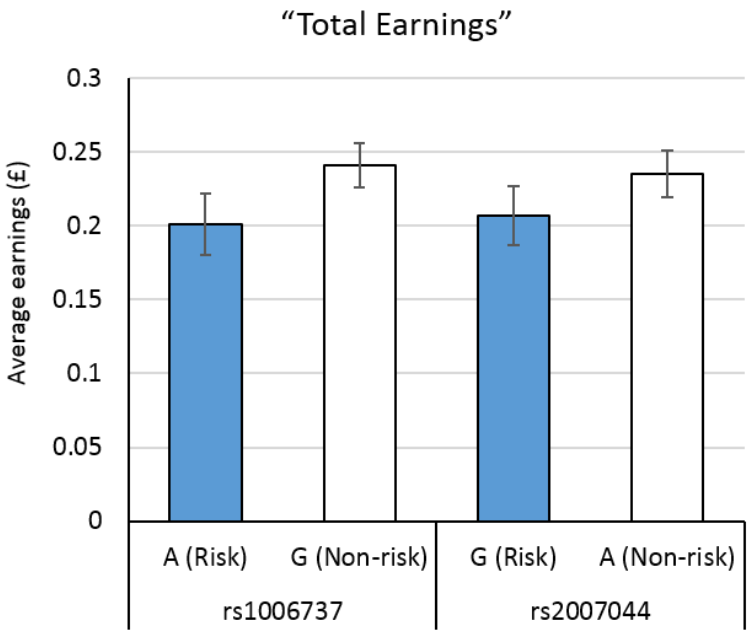

Figure 8.6 Comparison of "Total Earnings" across the whole task in risk allele and non-risk allele carriers in rs1006737 and rs2007044. There were no significant differences between risk and non-risk groups, P > 0.05. Bars represent average total earnings for each allele. Error bars are SEM.

8.5 Discussion

The results presented in this chapter demonstrate an effect of genotype at disease associated risk SNPs in CACNA1C on the expression of CACNA1C and transcripts of BDNF in healthy controls. They further suggest an effect of risk allele on specific aspects of reversal learning that are associated with behavioural flexibility.

8.5.1 Risk variants are associated with decreased expression of *CACNA1C* and region specific regulation of *BDNF*

Schizophrenia related risk alleles are associated with decreased expression of *CACNA1C* in the cerebellum of healthy controls and regulation of *BDNF* in the substantia nigra, hippocampus and frontal cortex. Decreased expression of *CACNA1C* is in line with some previous findings (Gershon et al., 2013; Eckart et al., 2016), though effects here were only observed in the cerebellum. Functional implications of the risk associated SNP on the expression of *CACNA1C* highlight the importance of understanding the role of this gene in disease relevant behaviours. Previous studies have suggested a key role for the cerebellum in reversal learning. Patients with focal vascular lesions of the cerebellum were found to have specific deficits in reward based reversal learning (Thoma et al., 2008) and imaging studies have indicated activation of the cerebellum in response to unpredicted rewards and an association with prediction error (Ramnania et al., 2004). These suggest that the mechanism associating reduced expression in the cerebellum with deficits in reversal learning is worth further investigation. Region specificity of decreases and evidence of increases elsewhere (Bigos et al., 2010; Yoshimizu et al., 2014) suggests that it may be the dysregulation of this gene rather than a loss or gain of function that leads to impairments. Therefore, it is important to understand effects on convergent downstream mechanisms and where/when they may be relevant. The data presented only relates to basal resting conditions, therefore it is possible that the full

consequences of expression dysregulation may only be observed during evoked behaviour.

These are the first analyses to identify expression changes in a relevant downstream gene associated with the risk SNP in *CACNA1C*. Changes in expression of *BDNF* provide an indication of the pathways which may be affected by risk variation at this locus. The most consistent finding across risk SNPs was the reduction of BDNFIX in the substantia nigra in risk allele carriers. Alterations of activation in people with schizophrenia in the substantia nigra and ventral tegmental area have been observed in relation to reward processing (Murray et al., 2008b). Additionally, structural changes in the substantia nigra have been identified in patients with schizophrenia, with reduced synaptic terminal size reported (Kolomeets and Uranova, 1999). The substantia nigra is known to play a role in movement and reward processing, particularly through the dopaminergic input to the striatum (Schultz, 2000; Howe and Dombeck, 2016). BDNF has been reported to be important for promoting the survival of dopaminergic neurons in this region (Hyman et al., 1991) and for regulating the normal expression of the D_3 receptor (Guillin et al., 2001). Reduced BDNF in the substantia nigra and behaviour known to recruit the same region, appear to be associated with an increased risk of schizophrenia. This could provide a mechanistic link between common genetic risk and observed endophenotypes. It further provides a viable link between common risk SNPs in CACNA1C and the well-supported "dopamine hypothesis" of schizophrenia.

Transcript specific regulation of BDNF in distinct brain regions, implicates a more finely tuned effect of risk variation. Different transcripts have different temporal and spatial properties in basal and activated conditions, with transcripts I and IV reported to be more activity dependent (Zheng et al., 2012). BDNFIX and BDNFIV specifically have been found to be increased in the hippocampus following associative learning paradigms (Hall et al., 2000; Rattiner et al., 2004; Zheng et al., 2012). Furthermore,

selective inhibition of activity dependent BDNF in the CA1 region of the hippocampus has been previously found to impair performance on tasks requiring behavioural flexibility, while keeping other forms of learning intact (Sakata et al., 2013). It is therefore plausible that changes in expression level of activity regulated transcripts in the hippocampus of risk allele carriers could have an effect on similar learning mechanisms in reversal learning. Further investigation of activity regulated effects on expression and behaviour in reduced expression models of CACNA1C would help to understand these effects.

These analyses do not provide a causative link between risk in *CACNA1C* and specific changes in *BDNF* expression associated with behavioural deficits. They do, however, provide a putative mechanism through which a single risk SNP acting as a cis-eQTL, affects downstream gene expression of another gene, having multiple distinct effects on different regions of the brain. This, in combination with other risk variations, may be part of a core set of neurobiological features which affect distinct behaviours associated with schizophrenia.

8.5.2 Risk allele carriers show impaired reversal learning performance

It was found that the risk allele at both variants was associated with fewer correct responses following the first reversal of contingency in a probabilistic reversal learning task. This contrast measures participants' behavioural flexibility. Results suggest that the risk variant may be affecting molecular mechanisms that are important for the comparison and update of existing information to inform subsequent behaviour. Impairment in different forms of behavioural flexibility is reliably observed in patients with schizophrenia (Murray et al., 2008a; Floresco et al., 2009; McKirdy et al., 2009). An fMRI study of patients found alterations in brain activation in regions linked to cognitive control in schizophrenia compared to controls (Culbreth et al., 2015). People with schizophrenia were found to achieve fewer reversals during a similar probabilistic reversal learning task, showing evidence of reduced behavioural

adaptation to incorrect responses. The current results suggest cognitive control may be one feature of cognitive impairment to which risk variation in CACNA1C is contributing and may inform future investigation into molecular mechanisms underlying abnormalities observed in patients.

A previous study found an association between rs1006737 and reward responsiveness, with risk allele carriers not acquiring a bias to an implicitly more commonly rewarded stimuli (Lancaster et al., 2014). These effects suggest that differences in reward responsiveness could partially explain the results observed with the current analysis, rather than differences in reversal learning or behavioural flexibility per se. However, as no effects were observed on performance across the full task, it can be assumed that participants in this study were able to acquire contingencies in this explicitly rewarded paradigm. Rs1006737 has also been found to be associated with altered emotion responsiveness and recognition, processes known to require overlapping neural circuits with reversal learning (Bechara et al., 2000). It was reported that greater time was needed to recognise emotion in faces in healthy risk allele carriers (Nieratschker et al., 2015) and reduced activation has been observed in the ventrolateral PFC in bipolar risk allele carriers compared to relatives and controls (Jogia et al., 2011).

The current results are in line with previous evidence suggesting genotype at rs1006737 is associated with deficits in learning and memory (Hori et al., 2012; Zhang et al., 2012; Dietsche et al., 2014); extending findings with verbal, logical and spatial learning to include reward based reversal. In contrast, Rolstad et al., (2016) reported no effects of risk variants in *CACNA1C* or *BDNF* with cognition. The current results concur with Rolstad et al., regarding performance across the whole task, with significant effects only seen when probing specific behaviours indicative of behaviour flexibility. This highlights the need to explore specific aspects of behaviour in relation to common variation as well as broader deficits, to aid identification of

endophenotypes. The pleiomorphic nature of schizophrenia, bipolar and other related psychiatric illnesses would support a subtler effect of common variation, potentially leading to a susceptibility rather than a clear deficit in cognition.

Further analysis of the same participants in this study using fMRI imaging data, has revealed a significant negative association between schizophrenia risk profile scores and activation of fronto-striatal brain regions with choice behaviour on this reversal learning task (Lancaster et al., 2015). Thus these common genetic variations may be associated with alterations in frontal-striatal networks that play a role in processing of reward contingencies and choice decision, but not to reward outcome. The convergence of current findings with single risk SNPs and those seen with broader risk profile scores and patients, would support further investigation of the role of CACNA1C and associated downstream pathways.

8.5.3 Limitations and future directions

Expression data are generated from post-mortem brain tissue and therefore cannot not convey test condition expression levels or changes which may be relevant for specific behaviours. Considering the fact that BDNF has been identified as an activity regulated gene important for synaptic function (Lanahan and Worley, 1998), it could be the case that these differences become more profound following learning or that they extend to different transcripts. Further investigation in animal models would help to determine activity dependent regulation, which would be fundamental to understanding effects relevant for neuroplasticity and learning.

It is worth noting that rs1006737 and rs2007044 are in high linkage disequilibrium (LD) with an r^2 of 0.794. Although not in complete LD, it is possible that they may be representing the same signal. It is also not known whether either of the risk SNPs investigated are the functional variant or whether another risk SNP in LD may be causative of changes in expression and associated behavioural deficits. Brief

analysis across other identified SNPs in CACNA1C related with psychiatric illness (Smoller et al., 2013) showed consistent effects on BDNF expression. Future analyses may look to establish any convergent effects of other risk variation on this pathway.

8.6 Conclusions

Risk variation in *CACNA1C* has shared effects on the expression of *CACNA1C* and *BDNF* and performance in a reversal learning task. The regulation of *BDNF* expression in specific brain regions, including the substantia nigra, may provide a molecular link between genetic risk for schizophrenia and phenotypes of behavioural flexibility that are known to rely on dopamine; as well as putatively link risk with well-established dopamine dysregulation observed in patients. Future studies need to establish whether there is a causal relationship, the effects of activity dependent regulation and investigate the potential for rescue of behavioural deficits in relevant animal models.

9 DISCUSSION

L-type calcium channels and *CACNA1C* more specifically, have been reliably associated with risk for psychiatric illness. Common variation in *CACNA1C* and other calcium channel subunits has been associated with increased risk for schizophrenia and bipolar disorder and there is an enrichment of rare deleterious mutations in L-type calcium channel subunits (Purcell et al., 2014a; Ripke et al., 2014). Calcium signalling is known to play a vital role in synaptic plasticity and learning and memory, and there appears to be a convergence of identified risk genes of schizophrenia on the synapse and associative learning (Harrison and Weinberger, 2005; Hall et al., 2009, 2015; Pocklington et al., 2015). The specific role that LVGCCs and *CACNA1C* have in aspects of learning that are relevant for symptoms of psychiatric illness is yet to be fully elucidated.

This thesis thus aimed to identify the role of LVGCCs and *CACNA1C* in specific aspects of associative learning. In understanding the molecular mechanisms which may link genetic risk for schizophrenia and specific cognitive symptoms of illness, it is hoped that potential novel treatment targets may be identified.

9.1 Summary of findings

9.1.1 Selective role for LVGCCs and *Cacna1c* in specific aspects of associative and inhibitory learning

Calcium influx through LVGCCS was found to have a specific role in the consolidation, extinction and latent inhibition of CFM. Reduced *Cacna1c* expression using a newly characterised HET model impaired the latent inhibition of CFM and the inhibition of previously acquired reward associations in a reversal learning task. This indicates a select role for LVGCCs in the appropriate formation and maintenance of CFM, with a distinct role for *Cacna1c,* and associated $Ca_v1.2$ channels, in inhibitory learning processes.

The investigation of expression of *Cacna1c* in a hippocampal-dependent CFC paradigm revealed activity-regulated gene expression related to specific experiences. Following conditions in which associative learning is expected to occur, latent inhibition, consolidation and extinction, normal basal expression of *Cacna1c* was maintained in those regions associated with CFC. In contrast, brief exposure to a novel or conditioned context, which does not support associative learning, was found to be associated with decreased expression of *Cacna1c* in the CA1 region of the hippocampus. Activity-regulated expression of *Cacna1c* following specific associative learning events, implicates a role for this gene in the formation and maintenance of appropriate Pavlovian fear associations, though not in their recall/expression.

The functional investigation of the role for calcium influx through LVGCCs was conducted using the same CFC paradigm. Local inhibition of LVGCCs in the hippocampus via infusion of diltiazem was found to have a specific effect on the consolidation of CFM and the consolidation of extinction. These results concur with previous findings with local infusion in the basolateral amygdala (Davis and Bauer, 2012), suggesting a conserved role for LVGCCs in the network of brain regions involved in CFC. This was the first study to investigate the effect of local inhibition of LVGCCs on the latent inhibition of CFM. Latent inhibition was found to be impaired following infusions of diltiazem, with strong associations formed to the context regardless of context pre-exposure. There were no effects of inhibiting calcium influx on the acquisition of CFM or on its recall. The lack of effects at recall corresponds to the decreased expression of *Cacna1c* observed in expression studies, supporting a limited role for calcium influx through these channels in related processes. Results suggest a specific role for LVGCCs in the hippocampus, in processes which utilise prediction error to signal learning and/or the appropriate update of existing representations.

Constitutively reduced expression of *Cacna1c* was found to have no effect on the acquisition or consolidation of reward or aversive associations. Wild-type and knockout animals were able to successfully form associations between a novel context and a footshock during CFC and could learn to associate select visual stimuli with reward in a reversal learning task. In contrast to hippocampal specific inhibition of LVGCCs, select deficits in these animals were only observed in relation to the latent inhibition of CFM. This implies that the normal expression of *Cacna1c* is necessary for the inhibition of inappropriate aversive contextual association formation. Whether this is driven by disrupted mechanisms in the hippocampus only, cannot be concluded from the current data. However, the overlap with deficits observed with local inhibition of LVGCCs, would indicate at least a contributory role for $Ca_v1.2$ channels in the hippocampus.

The different profile of deficits observed between local inhibition of LVGCCs and global genetic knockdown of *Cacna1c* during CFC, supports previous findings for distinct roles of $Ca_v1.2$ and $Ca_v1.3$ channels. The different expression profiles (Hell et al., 1993; Leitch et al., 2009) and biophysical properties of $Ca_v1.2$ and $Ca_v1.3$ channels (Catterall et al., 2005), suggest that subtypes have different functional roles. Previous investigations of knock-out mice have specified a role for $Ca_v1.3$ channels in the consolidation of CFM, with no effects on extinction (McKinney and Murphy, 2006) and have suggested that $Ca_v1.2$ channels are not necessary for either consolidation or extinction (Mckinney et al., 2008). The current results support this dissociation in the hippocampus, with consolidation more like to rely on calcium influx through $Ca_v1.3$ channels and a newly identified role for $Ca_v1.2$ in latent inhibition. The exact contribution of LVGCC subtypes to extinction is still yet to be fully determined.

Effects of reduced expression on inhibitory learning in latent inhibition were found to translate to reward based reversal learning in the same rat model. Reduced

expression of *Cacna1c* was found to lead to deficits in the inhibition of prior reward contingencies and behavioural flexibility. Mutant animals showed difficulties in reversing responses to new contingencies, with greater perseverative errors observed in the early stage of reversal. This suggests that normal expression of *Cacna1c* may be necessary for the comparison and update of new information with existing contingencies in an appetitive task, as was seen in aversive conditioning. Furthermore, results showed faster reaction times following reversal compared to wild-types, indicative of continued 'over-trained' responding, rather than appropriate activation of behavioural inhibition.

Although results suggest effects of reduced expression on inhibitory learning across aversive and reward learning, there were no deficits observed in PPI of acoustic startle response. This reflects the potential for distinct psychological functions and neural processes underlying latent inhibition and PPI. Even though these two measures have been used previously as attentional models in relation to deficits in schizophrenia, previous studies have found separable effects of environmental manipulations. Wilkinson et al. (1994), found that social isolation from rearing age in rats selectively disrupted PPI of the acoustic startle response, with no evidence of effects on latent inhibition. It has been suggested that latent inhibition recruits associative processes, rather than the 'sensory gating' processes attributed to PPI. This further supports a selective role for *Cacna1c* in aspects of associative learning, rather than a broader role in attention-related processing.

9.1.2 Implicated downstream effects on *BDNF* and relation to the dopamine system

Basal levels of *BDNF* were found to be decreased in the PFC in *Cacna1c* mutant rats. Analysis of gene expression in humans, revealed similar dysregulation of *BDNF* associated with risk alleles in *CACNA1C.* Additional experiments indicated an effect of intrahippocampal *BDNF* antisense on latent inhibition, as was observed with both LVGCC inhibition and with reduced expression of *Cacna1c* within this region.

Local infusion of *BDNF* antisense into the hippocampus, prior to extended pre-exposure, resulted in abnormal CFC 24 hrs later, with an inappropriate association formed between the CS and the US. This implicates *BDNF* expression in the same process which requires calcium influx through LVGCCs and the normal expression of *Cacna1c*; though this is not indicative of a direct functional relationship with the effects observed with diltiazem. There is reliable evidence of activity-regulated expression of *BDNF* by calcium influx through LVGCCs (Tabuchi et al., 2000; Tao et al., 2002; Zheng et al., 2011, 2012), though this is the first evidence of their convergence on latent inhibition.

Reduced expression of *Cacna1c* in rats was found to be associated with reduced expression of basal *BDNF* in the PFC and a trend towards an increase in expression in the dentate gyrus region of the hippocampus. In humans, risk alleles in *CACNA1C* were associated with decreased expression in *CACNA1C* and a similar profile of expression changes in *BDNF* as observed in the rat, though with additional decreases observed in the substantia nigra. In humans, there were changes in the expression of specific transcripts of *BDNF. BDNFI* was found to be increased in the hippocampus, whereas *BDNFII* was increased in the hippocampus and decreased in the PFC. Total *BDNF* (*BDNFIX)* expression was found to be reduced in the substantia nigra in association with *CACNA1C* risk alleles. Although there was no change in basal protein expression of BDNF in rats, the change in *BDNF* mRNA observed suggests there may be transcript specific dysregulation of *BDNF,* related to a decrease in *Cacna1c* expression. Further investigation is warranted to establish the effects of reduced *Cacna1c* expression on activity-regulated *BDNF* transcripts and protein expression.

BDNF is highly expressed by dopaminergic neurons (Seroogy et al., 1994; Baquet et al., 2005). It has been found to be a key regulator of the mesolimbic dopamine pathway, with activation of TrkB receptors promoting dopamine release in the nucleus

accumbens (Goggi et al., 2003). Evidence has found that *BDNF* released from dopamine neurons is responsible for normal expression of the dopamine D_3 receptor in the nucleus accumbens (Guillin et al., 2001; Sokoloff et al., 2002). Interestingly, dopamine D_2/D_3 receptors have been found to have a specific role in the reversal of reward associations in monkeys, with no effects on initial learning (Lee et al., 2007), as was observed in the *Cacna1c* knockout rats.

The distinct deficits that were observed in *Cacna1c* knockout animals are in behaviours that are known to be dependent on dopamine signalling, specifically latent inhibition and reward learning (Young et al., 2005). The mesolimbic dopamine pathway has a vital role in approach and avoidance behaviour. Dopamine neurons in the ventral tegmental area (VTA) and their projections to the nucleus accumbens are necessary for animals to process emotionally salient stimuli and form associations to inform future behaviour (Wise, 2004; Schultz, 2006). Although the current work did not directly measure the levels of dopamine in the brain of *Cacna1c* animals, behavioural deficits in these processes could indicate a downstream effect of reduced *Cacna1c* on dopamine signalling.

The present data suggests that calcium channel manipulation may be having a downstream effect on dopamine pathways, with effects on similar behaviours. Furthermore, the effects on *BDNF* provide a direct molecular link between LVGCC and dopamine regulation, placing it within currently supported hypotheses of the etiology of schizophrenia.

9.1.3 Translation of findings to humans

In addition to the effects on *BDNF* expression already discussed, similar impairments in reversal learning were observed in humans associated with risk alleles in *CACNA1C.* Risk alleles were found to be associated with decreased performance following the reversal of contingencies. The same risk alleles were found to be

associated with decreased expression of *CACNA1C* in the cerebellum. This demonstrates a translation of findings across species, identifying a disease relevant phenotype and further supports the use of rats with reduced expression of *Cacna1c* to investigate underlying molecular mechanisms.

The use of the touchscreen reversal learning task in the rat studies allows greater translation between species, as similar tasks can be conducted in rodents and humans. Importantly, touchscreen reversal learning is known to engage similar underlying neural networks in both rats and humans (Bussey et al., 2012). Observing similar impairments associated with changes in *CACNA1C* expression, suggests that its role is conserved across species. Importantly, the results in humans specifically provide an association with disease relevant variation. The overlap of findings would indicate that it is likely an effect of the risk allele on *CACNA1C* expression that may be affecting the observed behaviours.

Being able to translate animal findings to humans and specifically to disease relevant variations, is incredibly important for the ultimate goal of improving treatments. Understanding the role of Cacna1c in the rat in specific aspects of learning and memory, allows those behaviours to be specifically tested in humans. The additional identification of molecular markers that are associated with dysfunction in both species, suggests that the mechanisms underlying impairments are conserved. The dysregulation of *BDNF* associated with reduced *CACNA1C* expression in both rats and humans supports this conserved mechanism in relation to learning impairments. Further investigation in the rat model is necessary to fully understand these mechanisms, with the continued translation between rodent and human. The identification of potential treatment targets in the rat would therefore more likely be relevant to the human disease state.

9.2 Relation to symptoms of psychiatric illness

Cognitive deficits are a key feature of multiple psychiatric illnesses (Millan et al., 2012). The deficits that these studies found to be associated with reduced *Cacna1c* expression are observed in patients with schizophrenia. Deficits have been specifically reported in the reversal of reward discriminations (Crider, 1997; Waltz and Gold, 2007b; Reddy et al., 2016) and aspects of inhibitory associative learning (Swerdlow et al., 1996; Holt et al., 2009). The inability to ignore irrelevant stimuli, inhibit inappropriate behaviour or correctly compare and update representations, can be seen to manifest in other core symptoms of illness.

Deficits in associative inhibitory learning can clearly be linked to the development of positive symptoms in schizophrenia and related psychiatric illnesses. False beliefs and delusions are a key feature of psychosis and rely on the formation and persistence of beliefs, despite contrary evidence and experience (Coltheart, 2010). It has been theorised that these abnormal beliefs can arise from impairments in a prediction error signal (Fletcher and Frith, 2009), with evidence of disrupted processing seen in patients using fMRI (Corlett et al., 2007). The deficits observed in latent inhibition indicate errors in the use of previous experience to predict current outcomes and the formation of inappropriate associations to irrelevant stimuli. Predictive value given to irrelevant stimuli can lead to multiple conflicting associations, which require an increasingly convoluted model and set of beliefs to make sense of. This can lead to the resistant delusions that are evident in psychosis. Furthermore, beliefs and predictions about experiences influence how they are perceived. Abnormalities in perception could further manifest in hallucinations and have reciprocal effects on beliefs.

The appropriate adaptation and update of associations is vital for the regulation of emotional response and related approach or avoidance behaviour (Laviolette and Grace, 2006). Emotion dysregulation is a key symptom observed in patients with

bipolar disorder, with both automatic and voluntary emotion regulation seemingly impaired (Phillips et al., 2008). Deficits in fear learning and the perseveration of reward associations, can lead to emotional valence being assigned and maintained to irrelevant stimuli. This leads to emotion dysregulation and can lead to inappropriate reinforcement of behaviours. Similar deficits in emotional associative learning can be seen to underlie addictive behaviour. Appetitive associations can be formed with neutral/irrelevant environmental stimuli, which can trigger drug seeking behaviour. Reduced behavioural flexibility can lead to perseverance of drug seeking, even in the absence of expected reward, leading to addiction (Laviolette and Grace, 2006).

The appropriate formation and regulation of fear associations is clearly also relevant for anxiety disorders, including post-traumatic stress disorder (PTSD). The development of PTSD is not due to the experience of a traumatic event in itself, but due to a pathological memory of that event and associations which inappropriately trigger those memories (Rubin et al., 2008). Inappropriate formation of threat associations can also be seen to underlie symptoms prevalent in generalised anxiety disorder. Patients are found to have biased memory for aversive stimuli (Coles et al., 2007). Increased negative associations with irrelevant stimuli can easily lead to the characteristic feelings of being 'on edge' and a sense of dread, especially when experiences are not predictable. Salience given to stimuli that would normally be perceived as irrelevant, could also lead to difficulties in concentration and attention.

Given the overlap of genetic risk of *CACNA1C* with multiple disorders, it is unsurprising that the deficits observed in these studies could potentially manifest in a broad spectrum of symptoms which cross diagnostic boundaries. *CACNA1C* is just one of many genes, across over 100 genetic loci, that has been associated with increased risk for schizophrenia (Ripke et al., 2014). The role of this gene in associative learning indicates how changes in expression may contribute to

symptoms of disease. The convergence of genetic risk on synaptic plasticity and calcium signalling more specifically, suggests that the combined polygenic burden could have a detrimental effect on these processes, contributing to symptom development.

9.3 Limitations

9.3.1 Behavioural paradigms

The behavioural paradigms used in these studies are designed to assess different aspects of learning, though each method has limitations. CFC is an inherently implicit task, which although great for discerning basic functional roles, may not be directly representative of the more cognitive decisions on behavioural control that may manifest in everyday life. Single trial learning allows very precise manipulation and separation of underlying biological processes, which was necessary to discern the separable roles of LVGCCs. However, it cannot be assumed these processes would completely translate to more complex situations, with multiple changing contingencies, more representative of learning in natural settings.

The CFC experiments all compared behavioural effects between animals, rather than using a within-subjects design. It is difficult to re-test associations in the same animal with different manipulations, without using multiple stimuli which may add increased variation and confound results. It is therefore not possible to determine the strength of a latent inhibition effect in individual animals, rather only compare group effects using this method. It has also been found that different strains of rat can respond differently during CFC (Graham et al., 2009), with Sprague Dawley rats found to freeze more in certain instances. The current experiments are all comparing effects within strain and therefore this does not negate the differences observed, however it is worth considering for future experimental design.

Compared to CFC, behavioural measures during reversal learning primarily only become apparent when averaged across a large number of sessions. This paradigm is very useful for determining effects on different psychological processes known to be engaged during certain stages of the paradigm, though it is less useful in separating out specific biological mechanisms. It requires extended training periods and performance may have been influenced by additional variables, including hedonic/motivational factors (Mar et al., 2014). There was no evidence of an overt difference in motivation, however, it is postulated that reward related behaviour can be separated into three psychological components; liking (hedonic), wanting (incentive salience) and learning (acquired predictive association to cues)(Berridge et al., 2009), which this paradigm is not specifically designed to delineate. The combined use of aversive, implicit and reward based instrumental learning go part way to addressing some of these limitations, with a convergence on inhibitory learning processes suggesting a shared phenotype.

Although the touchscreen operant platform used in reversal allows greater translation between species, there are still necessary differences between tasks in rodents and humans; most evident in difficulty, instruction and motivation. The human reversal paradigm had to employ probabilistic contingencies in order for the task to be difficult enough to produce any divergence in behaviour, whereas the paradigm used in the rats had a simple correct/incorrect reward dichotomy. Human participants were also working for a monetary reward, whereas the rats were responding to a desire for liquid reward following water deprivation, likely resulting in different motivational drives. The convergence of findings across species supports shared mechanisms, however differences in behaviour should be interpreted with caution.

9.3.2 Pharmacological and genetic manipulations

Both pharmacological and genetic approaches to investigating gene function have limitations, however, by combining the two approaches they can complement one

another to address some of these issues. Pharmacological inhibitors have different affinity to different channel subtypes and different activation states (Hockerman et al., 1997). It is therefore not possible to conclude the exact proportion of channels that are inhibited in vivo, during specific behavioural paradigms. It is also possible that inhibitors can have off-target effects that may not be fully understood, with some LVGCC inhibitors being found to affect sodium, potassium and nicotinic acetylcholine receptors at high doses (Wheeler et al., 2006). Acute, local pharmacological inhibition does allow the precise timing of inhibition that permits greater distinction between psychological processes. Specific genetic knockdown of genes of interest is able to provide greater specificity over the cause of effect, however does have limitations relating to the separation of functional roles in related processes. It is not always possible to determine if impairments in two processes indicate a role for that gene in both, or that one may be a behavioural consequence of the other. The combination of pharmacology and genetic approaches complement each other to partially address these limitations and makes convergent findings more reliable.

The use of a chronic genetic knockdown model has additional limitations regarding a potential interaction with parent genotype in early life. Some animals were bred to a wild-type mother with a mutant father and some to a mutant mother with a wild-type father. It is possible that the pre-natal or post-natal environment provided by a mutant mother, could affect the development of the offspring, as well as interact with their genetic status. It is well established that rearing behaviour and experience during development affects behaviour in later life (Würbel, 2001; Pryce and Feldon, 2003; Fone and Porkess, 2008; Lupien et al., 2009). The psychiatric illnesses to which *CACAN1C* has been associated are primarily developmental and therefore an effect on early life likely plays a vital role. The investigation of the effects of pre and post-natal environment on adult behaviour was beyond the scope of this thesis, however future studies should look to investigate the potential interaction between genotype

of the pup, the mother and early environment. Differences in maternal care have been found to affect the development of psychopathology (Champagne and Curley, 2009). Preliminary analyses comparing adult mutant and wild-type rats from different mothers has indicated a potential interaction affecting extinction behaviour and pre-pulse inhibition, as well as a potential involvement in the differences observed in mutant animals in reversal learning. There is no evidence of any imprinting of the *Cacna1c* gene in mice or humans that could have subsequently affected gene dosage in the mutant offspring (Falls et al., 1999) and results presented here regarding the expression of *Cacna1c* further negate a gene dosage effect. Further investigation would be relevant to discern potential environmental and interaction effects that may be contributing to behavioural phenotypes.

9.3.3 "Modelling" schizophrenia

It is worth emphasising that any animal model investigating the function of psychiatric risk associated genes is not a "model of psychiatric illness". Animal models rely on indexed behavioural measures to make inferences about symptoms which may be 'uniquely' human. Single gene models cannot take into account the complex polygenic background in which specific risk variants occur, or the environmental factors which may contribute to symptom development. They do allow the interrogation of the functional role of the gene that has been associated with risk for illness, contributing to our understanding of how alterations in function may manifest in specific symptoms. Importantly, they also allow the investigation of related molecular processes that may be important for identifying novel treatment targets. Utilising translational approaches and aligning animal model findings with those observed with risk variation in humans, can help bridge the gap between gene function and disease mechanism.

9.4 Future directions

Given the convergence of findings on *BDNF* and previous implications of its regulation by calcium influx through LVGCCs (Tabuchi et al., 2000; West et al., 2001; Zheng et al., 2011), it would be pertinent to investigate activity-regulated expression of different *BDNF* transcripts in *Cacna1c* animals as well as protein. The preferential regulation of *BDNFI* and *BDNFIV* by calcium influx through LVGCCs (Tabuchi et al., 2000; Zheng et al., 2012) would suggest that these transcripts may be specifically reduced in the *Cacna1c* knockout model. As these transcripts are known to be activity dependent (Aid et al., 2007), differences may only become apparent following activation. Considering the specific role of *Cacna1c* observed in inhibitory learning processes, it would also be worth investigating the cleavage of BDNF and the relative ratio between proBDNF and its mature form in *Cacna1c* knockout animals. The different forms of BDNF have been found to have opposing effects on synaptic plasticity in the hippocampus (Pang et al., 2004; Park and Poo, 2013) with mature BDNF activating the TrkB receptor and proBDNF activating p75 receptors and their different downstream effector mechanisms. Activation of these receptors is thought to differentially promote LTP and LTD in the hippocampus (Woo et al., 2005). Effects on the ratio of pro BDNF : mature BDNF in *Cacna1c* animals could provide a synaptic plasticity mechanism through which these channels are affecting inhibitory learning processes. Previous studies have further observed differential methylation of the *BDNF* gene following different aspects of contextual fear conditioning (Lubin et al., 2008; Mizuno et al., 2012). It would therefore also be beneficial to determine whether there may be differential methylation of *BDNF* promoters in the *Cacna1c* knockout animals.

Given the implication of a specific role for LVGCCs in the hippocampus in relation to learning, as well as activity regulated expression of *Cacna1c* in the dentate gyrus, it would be relevant to explore potential differences in neurogenesis in association with

reduced *Cacna1c.* A recent study reported enhanced cell death in young hippocampal neurons in forebrain specific $Ca_v1.2$ knockout mice (Lee et al., 2016). Effects on neurogenesis may provide a further mechanistic link between *Cacna1c* and deficits in hippocampal dependent inhibitory learning (Kempermann et al., 2015).

As discussed, findings further indicate deficits in processes that are known to rely on dopamine signalling (Young et al., 2005), including specifically latent inhibition and reward reversal. The role of dopamine in latent inhibition has been extensively studied in relation to schizophrenia (Gray et al., 1995a). The administration of D-amphetamine, an indirect dopamine agonist, abolishes the latent inhibition effect in rats (Weiner et al., 1984, 1988) and humans (Gray et al., 1992; Salgado et al., 2000). This affect has been found to be reversed by co-administration of antipsychotics, known to function as dopamine antagonists (Solomon et al., 1981; Warburton et al., 1994). Furthermore, association between a CS and US has been found to be associated with increased dopamine release in the nucleus accumbens, which is absent following pre-exposure and latent inhibition (Young et al., 1993, 2005). It was therefore suggested that dopamine is important for controlling the salience of pre-exposed stimuli and subsequent association formation. The deficits observed with manipulation of LVGCCs are consistent with effects on this system in relation to latent inhibition.

There is an established role for dopamine in reward learning, with dopamine neurons responding to reward presentation and being activated by cues predictive of reward (Mirenowicz and Schultz, 1996; Stuber et al., 2008). Furthermore, it has been shown that dopamine responses correspond to prediction error, with increased levels to unexpected reward, rather than a general reward response (Waelti et al., 2001; Howe and Dombeck, 2016). Alterations of dopamine have also been found to affect reversal learning behaviour (Izquierdo et al., 2006; Clarke et al., 2011; den Ouden et al., 2013; Costa et al., 2015), with specific effects observed during the reversal stage,

with increased perseverative behaviour, reflective of the current results in the *Cacna1c* knockout rats.

It would be essential to measure dopamine levels in *Cacna1c* knockout rats, to determine whether reduced expression of *Cacna1c* has specific effects on dopamine levels and activity-dependent regulation. This could initially be conducted by measuring the expression of tyrosine hydroxylase as a proxy measure, though quantification of dopamine using microdialysis would be optimal. It would also be relevant to measure the functional link between calcium influx through LVGCCs, BDNF and dopamine in relation to latent inhibition.

Observed reduced expression of *BDNF* in *Cacna1c* knockout animals suggests that it might be a promising downstream target for rescue of the specific deficits seen in learning. A sphingosine-1 phosphate receptor modulator, Fingolimod, has been found to increase the levels of *BDNF* in the mouse brain (Deogracias et al., 2012). Preliminary experiments conducted have reproduced these findings in the Sprague Dawley rat brain, with acute intra-peritoneal injection of Fingolimod found to increase *BDNF* in both the hippocampus and PFC. Future experiments aim to investigate the effect of administration of this drug on the deficits in latent inhibition, as well as other interventions known to modulate *BDNF* levels in the brain, such as environment enrichment and exercise (Sleiman et al., 2016).

LVGCCs have been identified as promising therapeutic targets themselves, due to their selective pharmacological properties and are currently targeted with inhibitors for the treatment of angina and hypertension (Triggle, 2007). There has been substantial investigation into the efficacy of LVGCC inhibitors for the treatment of symptoms of bipolar disorder (See Cipriani et al., 2016 for review). The results do not support a conclusive benefit for inhibitors in the treatment of mania and there appears to be significant side effects observed with manipulation of LVGCC function more broadly, including headaches, blood pressure and nausea (Caillard, 1985;

Silverstone and Birkett, 2000). The upregulation of LVGCC expression has also been found to cause hypertrophy and eventual heart failure (Muth et al., 2001). LVGCCs have high levels of peripheral expression which are likely driving a large proportion of side-effects observed and there are currently no brain isoform specific modulators of channel (Cipriani et al., 2016). Targeting brain specific mechanisms which may be affected by abnormal LVGCC signalling, rather than LVGCCs themselves, could help bypass some of the issues of peripherally mediated side-effects.

Preliminary data further suggests an interaction of parent genotype with subsequent adult genotype. Therefore, future experiments should aim to investigate the effect of genetic knock-down of *Cacna1c* on rearing behaviour and potentially the prenatal environment of mothers. This provides a direct investigation of the link between genetic and environmental effects of one gene associated with increased risk for schizophrenia. It may also reveal further deficits in behaviours which may be predisposed by dysregulated *Cacna1c* expression, if not directly influenced, for example in PPI and extinction of CFM. Cross-fostering experiments would also help to determine whether effects on the mother's behaviour interact with genotype, as well as potentially identify an early intervention period.

9.5 Conclusions

L-type voltage gated calcium channels have a vital role in the consolidation and appropriate manipulation of contextual fear memory. *Cacna1c* appears to have a selective role in aspects of inhibitory learning of both aversive and reward based associations, suggesting the inappropriate use of information to update representations and adapt behaviour. Related behavioural deficits are found to be associated with disease relevant variation in human healthy controls. Reduced expression of *Cacna1c*, in both rodents and humans, is associated with region specific changes in *BDNF* expression.

Deficits observed are reflective of impaired psychologies which may underlie symptoms in patients with schizophrenia and other psychiatric illnesses. The translation of research across species when considering inherently human disease, is vital in determining symptom relevant etiology. The current findings contribute to our understanding of how genetic risk in *CACNA1C* may result in specific cognitive learning deficits. Future studies should further explore transcript specific and learning regulated changes in *BDNF* expression in these models and investigate a causal link between *CACNA1C*, *BDNF* and dopamine, with the potential to rescue behavioural deficits by selective *BDNF* manipulation.

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