The impact of genetic variation in *Cacna1c* and prepubertal stress on hippocampal function

A thesis presented for the degree of

Doctor of Philosophy

By

Anna Louise Moon

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Abstract

Schizophrenia and bipolar disorder are examples of disabling psychiatric conditions caused by both genetic and environmental factors. Genome-wide association studies have consistently demonstrated that variation in the gene *calcium voltage-gated channel subunit alpha1C* (*CACNA1C*) increases risk for psychiatric disorders. Early life stress has also been reliably and convincingly associated with increased risk for schizophrenia and bipolar disorder.

This thesis presents results concerning how genetic variation in *Cacna1c* and prepubertal stress can affect hippocampal function and structure, potentially through interacting together. PPS is shown to decrease *Cacna1c* in the CA1 and CA3 of the hippocampal formation, which is also seen in a small cohort of human ELS samples, suggesting a translational aspect of this work (Chapter 3). Delay, trace and unpaired auditory fear conditioning were conducted in *Cacna1c*$^{+/-}$ (Chapter 4) and PPS rats (Chapter 5) to examine how associative learning was processed within the hippocampus and amygdala in these animals. Both *Cacna1c*$^{+/-}$ rats and PPS rats demonstrate disrupted trace fear conditioning, a paradigm that explicitly requires the hippocampus. Trace conditioning has been associated with adult hippocampal neurogenesis, a process that is decreased at the cell proliferation level in *Cacna1c*$^{+/-}$ rats (Chapter 6), whereas PPS rats have increased immature neurons (Chapter 6). Environmental enrichment in *Cacna1c*$^{+/-}$ rats was not sufficient to correct these behavioural and molecular deficits (Chapter 8). Finally, *Cacna1c*$^{+/-}$ animals show PV and GAD67 expression changes in the dentate gyrus, suggesting a potential inhibitory deficit (Chapter 7). These results contribute to the literature implicating *Cacna1c*, stress and the hippocampus in the development of mental disorders.

Abbreviations

µl - Microlitres

ACC – Anterior Cingulate Cortex

ACTH – Adrenocorticotrophic Hormone

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

ANOVA – Analysis of variance

ARC – Activity-regulated cytoskeleton-associated protein

ASD – Autism Spectrum Disorder

BDNF – Brain Derived Neurotrophic Factor

BPD – Bipolar Disorder

BrdU – 5-Bromo-2'-deoxyuridine

BSA – Bovine serum albumin

CA – *Cornu Ammonis*

CACNA1C - Calcium Voltage-Gated Channel Subunit Alpha1C (gene)

CACNA1D - Calcium Voltage-Gated Channel Subunit Alpha1D (gene)

CACNA1I - Calcium Voltage-Gated Channel Subunit Alpha1I (gene)

CACNB2 – Calcium Voltage-Gated Channel Auxillary Subunit Beta 2 (gene)

CaM – Calmodulin

CaMKII – Calmodulin-dependent protein kinase II

Cav1.2 – Calcium Voltage-Gated Channel Subunit Alpha1C (L-type) (protein)

CO_2 – Carbon dioxide

CNV – Copy Number Variants

CREB – cAMP response element-binding protein

CRH – Corticotrophin-releasing Hormone

CS – Conditioned stimulus

DG – Dentate Gyrus

DNA – Deoxyribonucleic acid

ELS – Early life stress

ER – Entorhinal cortex

ERK – Extracellular signal-regulated Kinase

GABA – y-aminobutyric acid

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GR – Glucocorticoid Receptor

GWAS – Genome-wide Association Study

HPA – Hypothalamic-Pituitary-Adrenal axis

IP - Intraperitoneal

ISH – *in-situ* hybridisation

LTCC – L-Type Calcium Channel

LTP – Long-term potentiation

mA - Milliamps

MDD – Major Depressive Disorder

MR – Mineralcorticoid Receptor

mRNA – Messenger ribonucleic acid

NMDAR - N-methyl-D-asparate glutamate receptor

NR3C1 – Nuclear Receptor Subfamily 3 Group C

NSC – Neural stem cell

PBS – Phosphate Buffered Saline

PFA – Paraformaldehyde

PFC – Prefrontal cortex

PND – Postnatal day

PPI – Prepulse inhibition

PTSD – Post-traumatic Stress Disorder

qPCR – Quantitative Polymerase Chain Reaction

SEM – Standard error of the mean

SCZ – Schizophrenia

SGZ – Subgranular zone

SNP – Single Nucleotide Polymorphism

TBE – Tris/Borate/Ethylenediaminetetraacetic acid

TBS – Tris Buffered Saline

TS – Timothy Syndrome

US – Unconditional stimulus

VGCC – Voltage-Gated Calcium Channel

ZFN – Zinc Finger Nuclease

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Chapter 1: General Introduction

1.1 The association of genetic variation in *CACNA1C* with psychiatric disorders

1.1.1 Introduction

Risk for psychiatric disorders can be conferred by both genetic and environmental factors, and likely also through interactions between genes and the environment (Uher, 2014). Schizophrenia and bipolar disorder are two related psychiatric disorders that are associated with a severely impacted quality of life, inadequate clinical response and reduced life expectancy (Jones et al., 2006; Aykut et al., 2017). Therefore, there is a tremendous need for research into understanding the underlying mechanisms of the disorders in order to inform and improve therapeutics. Advances in genomic techniques in recent years has accelerated this research, however functional work is now essential to determine how risk genes work together and with environmental factors to confer risk for disorder.

1.1.2 Schizophrenia

Schizophrenia is a neurodevelopmental disorder with a prevalence of 1% within the general population (Sullivan et al., 2003). It is characterised by a broad spectrum of symptoms which are often reported within three clusters: positive (hallucinations and delusions), negative (anhedonia and social withdrawal) and cognitive (working memory and attentional deficits) (Andreasen and Olsen, 1982; Tamminga and Medoff, 2000). The onset of psychotic symptoms usually manifest between late adolescence and early adulthood, although cognitive impairments often occur long before this (Lewandowski et al., 2011). The neurodevelopmental hypothesis of schizophrenia proposes that development can, at least in part, be traced to genetic and/or environmental factors influencing brain development during the prenatal or

postnatal period (Murray and Lewis, 1987; Weinberger, 1987; Schmitt et al., 2014). Once the disease develops, sufferers can either recover completely, improve over time or, as about a third of patients experience, not show any improvement of symptoms despite medication, which can lead to severe psychosocial deterioration (Tamminga and Medoff, 2000).

Both dopaminergic and glutamatergic neurotransmission has been implicated in schizophrenia pathology. Current antipsychotics block dopamine D_2 receptors however, both genetic and pharmacological evidence also indicate a role for the N-methyl-D-asparate (NMDA) glutamate receptor complex and related proteins (Tamminga and Medoff, 2000; Hall et al., 2015). NMDAR antagonists produce schizophrenia-like symptoms in healthy humans and animal models (Coyle, 2012). Neuronal abnormalities described in schizophrenia also include altered pruning of glutamate synapses (Boksa, 2012) and changes in γ-aminobutyric acid (GABA) interneuron maturation (Akbarian et al., 1995; Beasley and Reynolds, 1997), although the complexities of post-mortem work mean that these abnormalities are far from confirmed.

1.1.3 Bipolar Disorder

Bipolar disorder (BPD) is characterised by recurrent episodes of depressive symptoms and at least one episode of sustained abnormally elevated mood, known as mania or hypomania depending on the severity, duration and whether or not psychosis is present (Merikangas et al., 2007). BPD is typically a chronic, relapsing and remitting illness that affects 1-2% of the population in the UK (Smith et al., 2013; Jann, 2014). Longitudinal studies have shown that depressive episodes are more likely than a manic or hypomanic cycle in general, but this varies between individuals (Baldessarini et al., 2010). It should be noted that whilst schizophrenia and BPD are technically different disorders, there is a large degree of symptom overlap; BPD

patients can present with psychotic symptoms whereas mood symptoms are also common in schizophrenia.

Lithium is the gold standard treatment for BPD, however only 30-50% of patients respond adequately and can become non-responsive during chronic use (Kujawa and Nemeroff, 2000). Another drug given to BPD is valproic acid/valproate which is prescribed to treat manic symptoms (Rosenberg, 2007). These two drugs both inhibit the collapse of sensory neuron growth cones and increase growth cone area via inositol signalling pathways and metabolism (Williams et al., 2002) although this may not be the only mechanism at work (Machado-Vieira et al., 2009). For example, valproic acid has also been found to increase GABA levels and affect signalling in Extracellular Signal-regulated Kinase (ERK) pathways (Rosenberg, 2007).

There is also evidence for the dysregulation of the glutamate/GABA system in BPD; glutamate levels are increased in BPD brains post-mortem and lithium treatment increases GABA levels (Sigitova et al., 2017).

1.1.4 Genetics of psychiatric disorders

Heritability estimates for both schizophrenia and BPD are around 80% (McGuffin et al., 2003; Sullivan et al., 2003; Barnett and Smoller, 2009; Gejman et al., 2010). The pattern of inheritance is not Mendelian and is derived from a large number of genetic variants within many genomic regions (Ripke et al., 2014) largely making small contributions to confer risk of disease. Despite this complexity, an increasing number of genetic variants have now been identified from large-scale collaborative genomic studies, leading to the identification of a number of risk loci for both schizophrenia and BPD, as well as other mood and psychiatric illnesses. Some of the genetic risk is thought to be derived from rare variants (<1% population frequency) in the form of copy number variants (CNVs), however a large body of research has examined the consequence of common single nucleotide polymorphisms (SNPs) (>1% population

frequency) on psychiatric disorder risk (Fromer et al., 2014; Purcell et al., 2014; Rees et al., 2014; Stefansson et al., 2014), which are further described below. This has resulted in the identification of several biological pathways potentially involved in increasing risk for psychotic disorders. Of these, some of the strongest evidence exists within specific subunits of voltage gated calcium channels, as well as calcium signalling pathways more generally.

1.1.5 Genome-wide association studies

Genome-wide association studies (GWAS) utilise a large number of subjects with a particular disorder (along with matched controls) to examine the frequencies of common genetic variants represented by single nucleotide polymorphisms (SNPs). SNPs are point mutations that are found within the genome and occur in the population at a frequency of >1 in 100, therefore they identify common variation (Collins and Sullivan, 2013). It is important however to mention that when implicating SNPs in a particular disorder, it is difficult to determine whether the implicated SNP directly causes the biological effect, or it is in linkage disequilibrium with the causative variant. The effect size of individual SNPs associated with psychiatric disorder is normally very small (odds ratios of <1.2) (Cariaga-Martinez et al., 2016). However, recent GWAS studies have shown that collectively this common variation can account for a large amount of the heritability of psychiatric disorders (Cichon et al., 2009; Sullivan, 2015; Sullivan et al., 2018).

GWAS studies have identified SNPs in calcium channel genes as risk alleles in a whole range of psychiatric disorders. One of the first and now well replicated GWAS findings in psychiatric illness was the association of SNP rs1006737 within the *CACNA1C* gene with BPD (Sklar et al., 2008), implicating the A allele with increased risk of disorder. *CACNA1C (calcium voltage-gated channel subunit alpha1c)* encodes a specific subunit of L-type voltage gated calcium channels (LTCCs) called $Ca_V1.2$ (Catterall, 2011). This association with BPD was confirmed in a larger data set

(Ferreira *et al.*, 2008) and subsequent studies showed a further association of this SNP with schizophrenia (Table 1.1). There is also some evidence that rs1006737 may also be implicated in autism and major depressive disorder, although these studies are less replicated than studies examining BPD and schizophrenia (Table 1.1).

Table 1.1: Summary of published association studies of SNPs within CACNA1C with psychiatric/neurodevelopmental disorders (BPD = bipolar disorder, SCZ = schizophrenia, MDD = major depressive disorder, ADHD = attention deficit hyperactivity disorder)

SNP	Disorder	Risk allele	Main references
rs1006737	BPD	A	(Ferreira et al., 2008) (Sklar et al., 2008) (Green et al., 2013) (Gonzalez et al., 2013) (Liu et al., 2011) (Ruderfer et al., 2014) (Lett et al., 2011)
	SCZ	A	(Green et al., 2009) (Nyegaard et al., 2010) (He et al., 2014) (Ivorra et al., 2014) (Guan et al., 2014) (Zheng et al., 2014) (Hori et al., 2012) (Ruderfer et al., 2014)
	Autism	G	(Li et al., 2015)
	MDD	A	(Liu et al., 2011) (Green et al., 2009) (Wray et al., 2012) (Casamassima et al., 2010)
rs4765905	SCZ	A	(Hamshere et al., 2013) (Takahashi et al., 2015)
	Autism	G	(Li et al., 2015)
rs4765913	BPD	A	(Ripke et al., 2014) (Mühleisen et al., 2014)
	SCZ	A	(Ripke et al., 2014)
	MDD	A	(Ripke et al., 2014)
rs1024582	BPD, SCZ, ADHD, MDD, Autism	A	(Smoller et al., 2013)
rs2007044	SCZ	A	(Ripke et al., 2014) (Pardiñas et al., 2018)
rs7297582	BPD	T	(Liu et al., 2011)
	MDD	T	(Liu et al., 2011)
rs12898315	SCZ	A	(Pardiñas et al., 2018)
rs10744560	BPD	T	(Stahl et al., 2018)

The Psychiatric Genetics Consortium GWAS identified 108 significant loci associated with schizophrenia; including three calcium channel SNPs. *CACNA1C* (SNP rs2007044) showed a highly significant p value with an odds ratio of 1.088 for the rare allele. *CACNB2* (SNP rs7893279) also was implicated (OR: 1.125) and another SNP in *CACNA1I* (Chr22_39987017_D) reached genome-wide significance with an odds ratio of 1.07 (Ripke et al., 2014).

Interestingly, in a five disorder meta-analysis (Smoller et al., 2013), *CACNA1C* SNPs (along with *CACNB2*) were found to have shared effects across attention deficit hyperactivity disorder (ADHD), autism spectrum disorder, BPD, SCZ and major depressive disorder (Smoller et al., 2013). This suggests that common variation in *CACNA1C* may be associated with particular symptom clusters across disorders instead of one particular disorder. Having said this, the association with BPD and SCZ was strongest (Table 1.1).

The majority of risk-associated SNPs are in known linkage disequilibrium with each other, except rs7297582 and rs12898315. All SNPs listed in Table 1.1 are located in intron 3 within non-coding regions, where other SNPs associated with schizophrenia have been located (Roussos et al., 2014). Intron 3 of *CACNA1C* is a region with high levels of linkage disequilibrium and multiple sites identified as potential gene expression regulators by the ENCyclopedia of DNA Elements (ENCODE) project (Fiorentino et al., 2014). For example, intron 3 has also been shown to contain an enhancer which can interact with the *CACNA1C* transcription start site (Roussos et al., 2014) and is therefore likely to determine gene expression, although results are variable (Gershon et al., 2014; Eckart et al., 2016). Eckart and colleagues show that rs1006737 is an expression quantitative trait loci (eQTL) for *CACNA1C* expression and is associated with decreased expression of *CACNA1C* in the prefrontal cortex, cerebellum and superior temporal gyrus (Gershon et al., 2014; Roussos et al., 2014; Eckart et al., 2016), although another study which studied the consequences of

rs1006737 on *CACNA1C* expression in the dorsolateral prefrontal cortex showed increased levels (Bigos et al., 2010).

1.1.6 Rare deleterious mutations

Rare (less than 1 in 10,000) *de novo* or inherited mutations in coding sequences can have neutral or disruptive results. As these mutations are so rare, individuals are likely to be heterozygous, not homozygous, for the mutation. A large exome sequencing study of schizophrenia revealed that patients had an enriched number of disruptive mutations compared to controls; many of these mutations were within genes previously implicated in schizophrenia. This included a significant enrichment in a calcium ion channel gene-set (Purcell et al., 2014), representing many different calcium channel genes. The enrichment appeared to be at part driven by mutations within the $\alpha 1$ and $\alpha_2\delta$ subunits , with two mutations in *CACNA1C*. Both of these mutations were protein-truncating and likely to lead to loss-of-function protein. Mutations in other implicated calcium channel genes (*CACNA1B, CACNA1H, CACNA1S, CACNB4, CACNA2D1*) are also likely loss-of-function mutations, through a variety of mechanisms including premature stop codons and frame shifts (Purcell et al., 2014).

In another exome sequencing study focusing on ASD, mutations in calcium channel genes CACNA2D3 and CACNA1D have been implicated (De Rubeis et al., 2014). A whole genome sequencing study of ASD revealed a mutation in CACNA1C (R1522Q) in the proximal C-terminus in a proband with autism however, this was inconclusive as the mutation was also found in other unaffected family members (Jiang et al., 2013).

1.1.7 Single gene disorder (Timothy Syndrome (TS))

Monogenic psychiatric diseases are uncommon, but missense mutations in exon 8/8a of *CACNA1C* can cause an autosomal dominant genetic disorder called Timothy

Syndrome (TS). TS is a multisystem channelopathy characterised by symptoms such as long QT syndrome, syndactyly and craniofacial abnormalities (Dick et al., 2016). Patients with this disorder also present with autism spectrum disorder (ASD), developmental delay especially in language skills, reduced social skills and cognitive impairments (Barrett and Tsien, 2008). Timothy Syndrome dramatically reduces life expectancy but a rare case study when a sufferer survived to adulthood showed the development of bipolar disorder (Gershon et al., 2014)

There are two common types of TS characterised by mutation; TS1 (G406R in exon 8a) and TS2 (G406R or G402S in exon 8). Exon 8 and Exon 8a have a different expression profile in various tissues, meaning that the two types of TS have different symptoms. Exon 8a is expressed in a wide selection of tissues but at a lower distribution in the heart and brain and thus TS1 appears to be more compatible with survival. Exon 8 is expressed in a smaller subset of tissues but is highly expressed in the heart and, therefore, TS2 patients are far rarer than TS1 and present with a severe form of long QT syndrome (Krause et al., 2011).

Mutations in both exon 8 and 8a are gain-of-function mutations in *CACNA1C* that impair the function of Cav1.2 (Splawski et al., 2004). These mutations lead to prolonged depolarisation of the Cav1.2 channel, allowing excess calcium to enter the cell. This suggests that increases of calcium influx in excitable cells may contribute to a psychiatric phenotype.

1.1.8 Calcium signalling pathways in psychiatric disorders

Therefore, there is a substantial body of genetic studies that implicate *CACNA1C* in psychiatric disorders and, additionally, there is evidence that calcium signalling on a wider scale may be disrupted in a disease state. The calcium signalling pathway in neurons is responsible for regulating neuronal excitability, synaptic plasticity, cognition and information processing, which are features often impaired in psychiatric

disorders (Berridge, 2014). Thus, many studies have investigated the impact of psychiatric disorders on Ca^{2+} signalling. Ca^{2+} levels in the cerebrospinal fluid of BPD patients with mania have been reported to be altered (Jimerson et al., 1979), and lithium treatment has been revealed to alter calcium metabolism if taken chronically, suggesting an association between impaired Ca^{2+} signalling and BPD (Franks et al., 1982). This has led to LTCC antagonists, primarily verapamil, being utilised in the treatment of BPD, however evidence suggests that these drugs are not effective at treating mania (Cipriani et al., 2016).

A study which integrated GWAS data with genome-wide expression data from human post-mortem schizophrenic patients found an highly significant enrichment in ion channels and calcium signalling (Hertzberg et al., 2015), which further implicates calcium related processes in psychiatric disorder. Finally, calcium signalling through NMDAR and calcium channels is essential for synaptic plasticity (Moosmang et al., 2005). Synaptic plasticity has also emerged as being disrupted in both schizophrenia and BPD and may be a key pathogenic factor of the disease, considering its role in learning, memory and neural circuity (Hall et al., 2015; Forsyth and Lewis, 2017).

Both rare and common genetic variation findings appear to support a prominent role for calcium channels and calcium signalling in the pathophysiology of psychiatric disorders. Therefore, the structure and function of calcium channels are discussed in more detail below.

1.1.9 Voltage-gated calcium channels (VGCCs)

Neuronal electric activity, cell signalling and plasticity relies on several different voltage and ligand-gated ion channels that are permeable to various ions, including calcium (Ca^{2+}). Voltage-gated calcium channels (VGCCs) are a group of voltage-gated ion channels that serve as the key transducers of cell surface membrane potential changes into local intracellular calcium transients that initiate many different

downstream pathways (Figure 1.1) (Simms and Zamponi, 2014). These channels form a family including L-, T-, P/Q-, N- and R- type channels depending on the calcium current they produce (Table 1.2) (Catterall, 2011). In neurons, these channels are the primary mediators of calcium entry into neurons following depolarisation.

Under normal physiological circumstances, intracellular Ca^{2+} concentration rests at approximately 100nm. If VGCCs are activated and open, Ca^{2+} influxes in along the electrochemical gradient, causing intracellular Ca^{2+} levels to rise by 10 or 100 fold (Catterall, 2011). This increase in Ca^{2+} ions within the cells triggers a wide range of downstream processes, including activation of calcium-dependent enzymes such as calmodulin-dependent protein kinase II (CaMKII), neurotransmitter release and gene transcription (West et al., 2001; Tao et al., 2002) (Figure 1.1). This process is under strict regulation from intrinsic gating processes and signalling pathways that control channel activity and membrane trafficking (Simms and Zamponi, 2014). It may therefore not be surprising that dysregulation of these tightly controlled pathways and VGCCs themselves are associated with many types of both neurological and psychiatric disorders.

Table 1.2: Summary and comparison of different types of VGCCs (HVA = high voltage activated channels, LVA = low voltage activated channels).

Type	Gene	Channel	Auxillary Subunits	Physiology
L	CACNA1S CACNA1C CACNA1D CACNA1F	Cav1.1 Cav1.2 Cav1.3 Cav1.4	$\alpha_2\delta$, β, γ	HVA
P/Q	CACNA1A	Cav2.1	$\alpha_2\delta$, β, (γ)	HVA
N	CACNA1B	Cav2.2	$\alpha_2\delta$, β, (γ)	HVA
R	CACNA1E	Cav2.3	$\alpha_2\delta$, β, (γ)	HVA
T	CACNA1G CACNA1H CACNA1I	Cav3.1 Cav3.2 Cav3.3	none	LVA

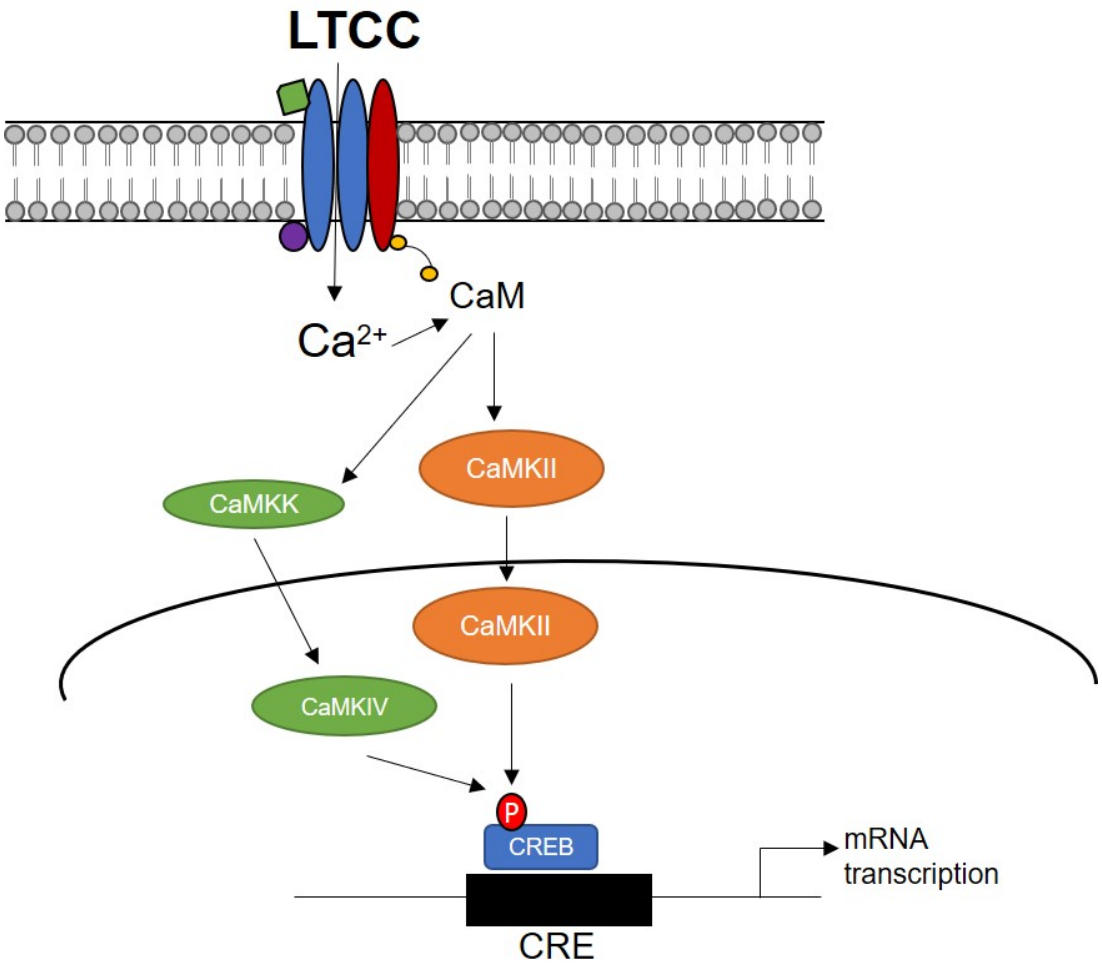

Figure 1.1: Calcium-activated signalling pathways that can contribute to regulation of gene expression. LTCCs consist of various subunits, including the pore-forming α1 subunit (blue ovals), see Figure 1.2. for detailed description of other subunits. When the neuronal membrane is depolarised, voltage-gated calcium channels open to allow Ca^{2+} to influx through the channel pore. Calcium activates calmodulin (CaM) (yellow circles) which can activate multiple signalling molecules including Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK). These molecules, either directly or indirectly, carry the Ca^{2+} signal to the nucleus and phosphorylates cAMP response element-binding protein (CREB). This allows it to bind to cAMP response elements (CRE) and stimulate expression of certain genes including *c-fos* and *BDNF*.

VGCCs can be low voltage-activated (LVA) channels or high voltage-activated (HVA) which, as the name suggests, is due to how much voltage is needed to activate them. Both LVA and HLA channels contain a pore-forming alpha Cavα1 subunit, but HVA channels have additional ancillary subunits: beta ($Ca_V\beta$), alpha-2-delta ($Ca_V\alpha_2\delta$) and gamma ($Ca_V\gamma$) (Figure 1.2) (Simms and Zamponi, 2014; Nanou and Catterall, 2018).

The $\alpha 1$ subunit forms the channel pore that allows calcium entry and determines the voltage sensitivity, kinetics, single-channel conductance and pharmacological properties of the channel. $Ca_V\alpha 1$ is structurally comprised of four major transmembrane domains separated by cytoplasmic linkers. Each transmembrane domain contains pore loop motif regions which consists of negatively charged amino acid residues to form a highly selective pore to allow Ca^{2+} entry (Ellinor et al., 1995) (Figure 1.2). The $\alpha 1$ subunit determines the subtype of calcium channel: Ca_V1, Ca_V2 or Ca_V3 (Table 1.2). The Ca_V1 channel encodes three neuronal L-type calcium channels (LTCCs), $Ca_V1.2$, 1.3 and 1.4, and a skeletal muscle-specific channel, $Ca_V1.1$. Ca_V2 channels are represented in P/Q-, N- and R calcium channels, whereas T-type channels consist of Ca_V3 channels (Catterall et al., 2005) (Table 1.2).

$Ca_V\beta$ interacts with $Ca_V\alpha 1$ at the intracellular loop between transmembrane domains I and II of the α1 subunit (Buraei and Yang, 2010). They contain guanylate kinase (GK) and SRC Homology 3 (SH3) domains (Kobayashi et al., 2007) and function to traffic the $\alpha 1$ subunit to the cell membrane. They also provide binding domains for other signalling proteins and regulate the gating properties of the channel.

The Cav$\alpha_2\delta$ subunit is attached to the extracellular portion of the plasma membrane by a glycosylphosphatidylinositol (GPI) anchor. They influence the trafficking and localisation of the channels (Dolphin, 2013) and increase the density of VGCCs at the plasma membrane (Davies et al., 2007). Finally, the $Ca_V\gamma$ subunit which was originally only detected in skeletal muscle has also been associated with neuronal VGCCs. This

subunit is the least studied out of the VGCC subunit complex, but may be associated with epilepsy and ataxia (Letts et al., 1998) and has been found to regulate post synaptic glutamate receptors (Andronache et al., 2007).

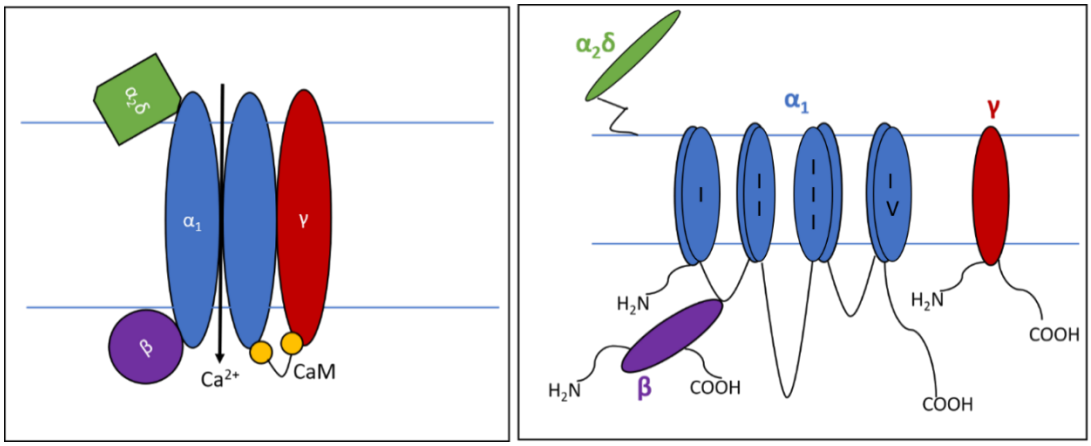

Figure 1.2: Schematic representation subunit structure of high voltage activated calcium channels. The α1 subunit (blue) is the pore forming subunit which is made up of 4 homologous domains (I-IV), each containing 6 transmembrane domains. The α1 subunit contains voltage-sensing properties and forms the binding sites for VGCC antagonists. The auxiliary subunits, $\alpha_2\delta$ (green), β (purple) and γ (red), function to enhance expression, traffic the channels to the membrane and modulate voltage dependence and gating kinetics of the channel. Calmodulin (CaM) (yellow) is associated with the COOH (C-terminus) and binds Ca^{2+} once it influxes. This causes CaM to reposition by interacting with the N-terminus to cause inactivation of the channel (calcium-dependent inactivation).

1.1.10 L-type voltage-gated calcium channels

LTCCs, which contain a Cav1 α1 subunit, require strong depolarisation to be activated and produce a slow and long-lasting Ca^{2+} influx. They are inactivated by conformational changes within the channel in response to prolonged depolarisation, in a process known as voltage-dependent inactivation (Stotz et al., 2004). These channels are also subject to calcium-dependent inactivation where Ca^{2+} influx binds to calmodulin, causing it to reposition beneath the pore, causing channel inactivation (Liu and Vogel, 2012). These modes of depolarisation and negative feedback keep Ca^{2+} tightly regulated within the cell, which is highly important considering its role in downstream signalling.

LTCCs are all sensitive to selective LTCC antagonists; organic compounds that block Ca^{2+} influx. These compounds include dihydropyridines, phenylalkylamines and benzothiazepines which are primarily used in the treatment of hypertension and cardiac problems (Godfraind, 2017). While all LTCCs are blocked by these antagonists, different subtypes of LTCC may be more sensitive to certain compounds. For example, $Ca_V1.2$ may have a greater sensitivity to dihydropyridines such as nimodipine than $Ca_V1.3$, although this does not allow for a reliable distinction in practice (Koschak et al., 2001; Tarabova et al., 2007).

LTCCS: function and gene expression

LTCCs are critical to physiological brain functioning due to their roles within neurons. For example, LTCCs associate with calcium-activated potassium channels (KCa1.1) to shape action potential repolarisation (Berkefeld and Fakler, 2008), indicting a role in moderating neuronal firing properties. However, most research has indicated roles for LTCCs in mediating downstream signalling pathways that result in gene transcription within the nucleus.

LTCCs have a prominent role in controlling gene expression through coupling membrane depolarisation with cAMP response element-binding protein (CREB)

phosphorylation via local Ca^{2+}/CaMKII signalling (Wheeler et al., 2012) (Figure 1.1). CREB can bind to a critical Ca^{2+} response element within brain derived neurotrophic factor (BDNF) to trigger its transcription (Tao et al., 1998, 2002) (Figure 1.1). This pathway, and particularly CREB and BDNF have been shown to be essential for learning and memory processes (Tao et al., 1998; West et al., 2001). Synaptic plasticity, which is thought to underlie learning and memory, can be modulated by LTCCs (Weisskopf et al., 1999; Degoulet et al., 2016). For example, LTCC antagonists reduce induction of long-term potentiation (LTP) in the CA1 of the rat hippocampus (Freir and Herron, 2003). Cav1.2 knockdown models have shown reduced CREB transcription and hippocampal LTP (Moosmang et al., 2005; Striessnig et al., 2006), implicating the important of these channels in gene expression and plasticity.

1.1.11 Calcium channel, L type, alpha 1c subunit (Cav1.2)

CACNA1C is located on chromosome 12p13 in humans, and in rats: 4q42. *CACNA1C* encodes $Ca_V1.2$ which is expressed in cardiac tissue, smooth muscle, endocrine cells and neurons (Catterall, 2011). $Ca_V1.2$ containing channels are activated by high voltages of more than -35mV and are inactivated slowly. They have a role in coupling neuronal activity to gene transcription and modulate dendritic processing (Catterall et al., 2005; Nanou and Catterall, 2018). $Ca_V1.2$ containing LTCCs make up over 80% of LTCCs within the brain (Sinnegger-Brauns et al., 2004, 2009) with differential expression throughout both cortical and subcortical regions as well as the cerebellum, including within the hippocampus, amygdala, nucleus accumbens and substantia nigra (Sinnegger-Brauns et al., 2009; Schlick et al., 2010; Striessnig et al., 2014). In neurons, Cav1.2 is located on primarily the cell membranes of cell bodies and on dendritic spines and shafts, predominately in post-synaptic regions (Tippens et al., 2008; Leitch et al., 2009; Nanou and Catterall, 2018). LTCCs are therefore positioned

for the initiation of signalling cascades from the synapse, vital for synaptic plasticity and gene expression.

Within the hippocampus, $Ca_V1.2$ is highly expressed within the dentate gyrus, with lower abundance within the cornu ammnis (CA) regions (Hell et al., 1993). $Ca_V1.2$ expression within the CA1 and CA3 of the hippocampus is likely dynamic during development; showing an increased expression during the early neonatal period and decreased expression by day 12 in rats (Kramer et al., 2012; Morton et al., 2013). This increased expression of $Ca_V1.2$ may be required to moderate the maturation of the GABAergic circuity; GABAergic chloride currents switch from excitatory to inhibitory in this period (Kramer et al., 2012). *CACNA1C* is present throughout life from the one-cell stage of development (Dedic et al., 2018), which also suggests suggesting roles for these channels in neurodevelopment.

1.1.12 Current animal models of *Cacna1c* dysfunction

Due to the consistency of findings of genetic variation in *CACNA1C* in psychiatric disorders, research on mouse models of *Cacna1c* dysfunction has been conducted to examine phenotypes relevant to mental illness. Most models have concentrated on reduced gene dosage. Some studies utilise a constitutive heterozygote model (*Cacna1c*$^{+/-}$) to study gene dosage effects as the homozygote model is embryonically lethal. However, other studies have utilised region-specific complete knockouts of *Cacna1c (Cacna1c*$^{-/-}$*)* driven by specific promotors to disentangle the neuronal contribution of this gene. Bader and colleagues (2011) developed a genetic mouse model based on TS2 (Bader et al., 2011). While both homozygote and heterozygote knockout of exon 8a were lethal, a heterozygote model that included an inverted neonmycin cassette was viable (TS2_neo) (Bader et al., 2011). An overview of the genetic *Cacna1c*/Cav1.2 mouse models and their associated phenotypes are presented in Table 1.3.

Cacna1c gene knockdown has been shown to result in decreased sociability in certain studies (Dedic et al., 2018; Kabitzke et al., 2018), but not all (Bader et al., 2011). TS mice models show increases in cue and contextual fear memory (Bader et al., 2011) however, other models of neuron specific knockdown show impairments in acquisition, consolidation or recall of contextual (McKinney et al., 2008; Temme et al., 2016) or auditory (Langwieser et al., 2010) fear conditioning paradigms. There is also evidence that *Cacna1c*$^{+/-}$ mice may display increased anxiety-like phenotypes (Dao et al., 2010; Bader et al., 2011; Lee et al., 2012; Dedic et al., 2018), although this is not consistent across all models (Moosmang et al., 2005; Bavley et al., 2017), which could be as a result of different anxiety tests. Interestingly, *Cacna1c* knockdown mice may have an increased resilience to depression (Dao et al., 2010; Bavley et al., 2017; Kabir et al., 2017; Dedic et al., 2018), although heterozygous deletion during development resulted in an increased susceptibility to chronic social defeat stress (Dedic et al., 2018). SNPs in *CACNA1C* in humans interact with trauma to predict depressive symptoms (Dedic et al., 2018), suggesting that depressive phenotypes may be conditional on environmental factors interacting with *CACNA1C*.

In cognitive based tests, TS models show increased perseverative behaviour in the Y maze (Bader et al., 2011) but no differences to wild-types in the T-maze (Kabitzke et al., 2018). Utilising the Morris water maze task, knockdown mice could learn the spatial task correctly (McKinney et al., 2008; White et al., 2008), but had profound deficit in spatial memory when tested 30 days later (White et al., 2008). Impairments were also seen when a more complex forms of the water maze were used (Moosmang et al., 2005; Temme et al., 2016). These results suggest that $Ca_V1.2$ is not necessary for basic cognitive tasks, but may be essential for more complex cognitive behaviours where allocentric representations are required.

The phenotypes observed in these models of *Cacna1c* dysfunction have implications for understanding how genetic variants can have an impact on underlying

impairments in psychiatric disorders. However, more research is required to clarify which biological pathways are principally affected.

Table 1.3: Overview of currently studied mouse models of Cacna1c dysfunction and their associated psychiatric and mood disorders

Study	Model	Phenotype
(Bader et al., 2011)	TS2_neo$^{+/-}$	↓ novelty induced locomotion ↓ sociability ↑ cued and contextual fear memory ↓ extinction of fear memory ↑ preservation in Y maze
(Kabitzke et al., 2018)	TS2_neo$^{+/-}$	↓ social-induced locomotion ↓ sociability
(Dao et al., 2010)	*Cacna1c*$^{+/-}$	↓ exploratory activity in females ↓ locomotion in females ↓ depressive phenotype ↑ anxiety in females
(Bader et al., 2011)	*Cacna1c*$^{+/-}$	↓ basal and novelty induced locomotion ↑ anxiety
(Bavley et al., 2017)	*Cacna1c*$^{+/-}$	↓ depressive phenotype
(Moosmang et al., 2005)	*Cacna1c*$^{-/-}$ (forebrain only)	↓ spatial discrimination
(McKinney et al., 2008)	*Cacna1c*$^{-/-}$ (forebrain excitatory neurons only)	No effect on contextual fear memory
(White et al., 2008)	*Cacna1c*$^{-/-}$ (forebrain only)	↓ long-term spatial memory
(Langwieser et al., 2010)	*Cacna1c*$^{-/-}$ (CNS only)	No effect on cued fear memory
(Lee et al., 2012)	*Cacna1c*$^{-/-}$ (prefrontal cortex only)	↑ anxiety
(Temme et al., 2016)	*Cacna1c*$^{-/-}$ (neurons only)	↓ context discrimination ↓ spatial memory (complex task) ↓ neurogenesis
(Lee et al., 2016)	*Cacna1c*$^{-/-}$ (forebrain only)	↓ neurogenesis
(Kabir et al., 2017)	*Cacna1c*$^{-/-}$ (prefrontal cortex only)	↓ depressive phenotype
(Dedic et al., 2018)	*Cacna1c*$^{+/-}$ (excitatory neurons only)	↓ sociability ↓ depressive phenotypes ↑ susceptibility to chronic social defeat stress ↑ anxiety

1.1.13 Conclusions

Genome-wide association studies have consistently demonstrated that variation in the gene *CACNA1C* increases risk for psychiatric disorders. *CACNA1C* encodes the $Ca_v1.2$ subunit of voltage-gated calcium channels (VGCCs), which themselves have been functionally implicated in a broad spectrum of neuropsychiatric syndrome. The collective impact of genetic variation within these channels has been the focus of much research to understand the consequences on behaviour and physiology, however much still remains to be determined in order to understand the underlying mechanisms that associate genetic variation with psychiatric disorders.

1.2 Environment factors in BPD and SCZ

1.2.1 Introduction

Whilst both BPD and SCZ are highly heritable, genetics do not account for 100% of risk for psychiatric disorders. Environmental factors, including various stressors, also have a strong effect on mental health and act alongside and with genes to contribute to increased risk for psychiatric disorders (Benros et al., 2011).

Environmental factors have been proposed to interact with high genetic risk in individuals predisposed to mental illness (Figure 1.3). For example, genetic background may lead to dysfunctional brain circuitry development which increases disease risk, and an environmental insult may further affect circuitry, inducing disease onset (Keshavan and Hogarty, 1999; Schmitt et al., 2014). There are many environmental factors that have been proposed as contributing to this increased risk: obstetric complications (Brown, 2012), urbanicity (Heinz et al., 2013) or cannabis use in adolescence (Schmitt et al., 2014) to name but a few. However, it is early life stress that consistently and convincingly has been shown to have lasting negative consequences on an individual's mental health and has been associated with multiple types of psychopathology and mood disorders (Martins *et al.*, 2011; Varese *et al.*, 2012; Schmitt *et al.*, 2014; Spies and Seedat, 2014; Morgan and Gayer-Anderson, 2016; Syed and Nemeroff, 2017).

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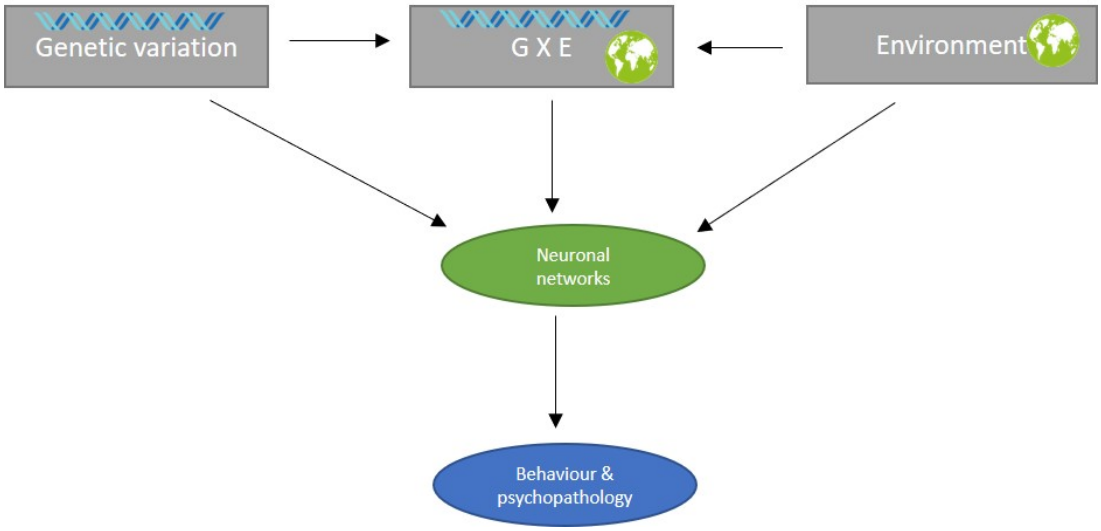

Figure 1.3: Environmental factors interact with risk genes to increase the risk for schizophrenia development. Both genetic variation and the environment can have effects on neuronal development, network activity and subsequent behaviour, these two factors can also interact together through gene x environment interactions to have similar, additive or different effects on neuronal functioning and psychopathology.

1.2.2 Early life stress

Children who experience severe stress, such as abuse, severe bullying or sufficient trauma, experience both physical and emotional harm. They may develop notions that may appear advantageous in their immediate environment, such as interpreting the world to be unpredictable, threatening and dangerous and moderating their behaviour as such, but these ideas can lead to lasting harm in future life (Syed and Nemeroff, 2017). This can have a dramatic effect on their neurodevelopment and can have long-lasting consequences. In fact, children exposed to extreme levels of stress are more likely to develop a myriad of psychopathology – including mood, anxiety and psychiatric disorders (Morgan and Gayer-Anderson, 2016). Thus, the impact of stress on a child's neurodevelopment is not only an important public health concern, but can also allude to the mechanisms to which these disorders manifest. Discovering how early life stress can shape neural and cognitive development is highly important to inform both prevention and intervention strategies.

There are many models that explore how early life stress can exert its effect. Upon encountering stress, the body can produce molecular changes that can lead to either vulnerability and resilience. It is multiple factors that determine how these interact and their final effect on the body and brain. The allostatic load model suggests an additive effect when chronic or repetitive bouts of extreme stress leads to many acute responses that add up to cause pathology (McEwen, 1998) . The reactive scope model extends this idea by predicting that excessive stress results in a misbalance between vulnerability and resilience which can go in either direction (Romero et al., 2009), whereas the 'match/mismatch hypothesis' suggests that early life stress provides coping mechanisms that protected individuals are without. These models suggest that early life programming can either facilitate resilience or vulnerability depending on other factors such as future experiences or genetic makeup (Howell and Sanchez, 2011).

1.2.3 The physiological stress response

In mammals, if stress or danger is encountered, there is a fast and co-ordinated response by the body's physiological stress response systems to increase available energy for the 'flight or fight' response. The system is called the hypothalamic-pituitary-adrenal (HPA) axis and it allows for the enhancement of threat detection and heightening of the immune response (van Bodegom et al., 2017).

The HPA axis has a step by step co-ordinated mechanism of action (Figure 1.4). When a stressor is encountered, the paraventricular nucleus of the hypothalamus releases corticotrophin-releasing hormone (CRH). CRH acts on the pituitary gland, which in turn releases adrenocorticotrophic hormone (ACTH) which acts on the adrenal gland. When the adrenal gland detects ACTH it releases glucocorticoids, primarily cortisol, the key stress hormone in humans (Stratakis and Chrousos, 1995). Cortisol has a negative feedback effect on the HPA axis for example, it binds to glucocorticoid (GR) and mineralcorticoid (MR) receptors in the hippocampus to inhibit further production of CRH from the hypothalamus (Jacobson and Sapolsky, 1991). Glucocorticoids bind to the GR and MR throughout the body which can interact with G-protein-coupled receptors (GPCRs) to influence intracellular signalling and ultimately have an effect of plasticity, behaviour and metabolism (Oakley and Cidlowski, 2013).

In adults, this system is highly important for health and survival and the effects fade when the stressor is removed. However, it is extremely sensitive to damage by prolonged or extreme levels of stress. Early life stress is associated with HPA axis dysregulation (Loman and Gunnar, 2010), and dysfunction of this axis has associated with the aetiology of many psychological disorders (Keller et al., 2006).

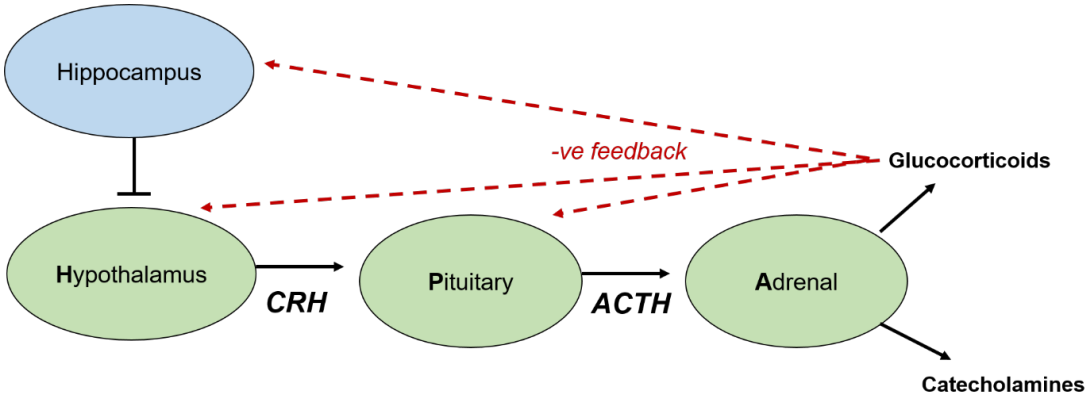

Figure 1.4: Overview of the HPA axis. When the body experiences stress, the hypothalamus is stimulated to produce corticotrophin-releasing hormone (CRH). CRH is transported to the anterior pituitary gland where it stimulates production of protein proopiomelanocortin (POMC). POMC is a precursor to adrenocorticotrophic hormone (ACTH) which is released to stimulate cells within the adrenal glands. The adrenal gland produces glucocorticoids such as cortisol which are vital for moderating the stress response. It has been shown that increased acute and chronic glucocorticoid levels in the hippocampus exerts a negative regulation of the HPA axis, particularly by interacting with the hypothalamus

1.2.4 Childhood maltreatment and psychiatric disorders

Children with histories of abuse have an increased risk of depression and anxiety in adult life and the severity of maltreatment often correlates with the severity of depressive or anxious behaviour (Gibb et al., 2003; Spinhoven et al., 2010; Rehan et al., 2017). There is also robust evidence to link early maltreatment to alcohol abuse (Rothman et al., 2008), suicidal behaviour (Turecki et al., 2012), schizophrenia (Sideli et al., 2012) and bipolar disorder (Aas et al., 2016). Therefore, there is clear evidence that childhood trauma is a risk factor for psychiatric and mood disorders across diagnostic boundaries.

Child abuse has been associated with altered HPA axis functioning in some children (Cicchetti and Rogosch, 2001) and the axis remains atypical in adulthood. Both hyperactivity and hypoactivity of the HPA axis have been reported in adults and is thought to represent varying forms and duration of childhood maltreatment; hyperactivity of the axis is thought to manifest in post-traumatic stress disorder (PTSD) symptoms in adulthood, whereas hypoactivity is associated with depression (McCrory et al., 2012). Childhood abuse in humans can also result in changes in subcortical brain structure; PTSD sufferers with a history of childhood abuse have smaller hippocampal volumes and abused children present with increased amygdala volume (Tottenham et al., 2010). Decreased cerebellum volume is also a key feature of childhood abuse due to its connections to the HPA axis and limbic axis (Gilbert et al., 2009). Volume decreases in the corpus callosum have also been reported in maltreated children (Gilbert et al., 2009), suggesting that abuse can influence brain white matter. In addition to these structural changes, functional magnetic resonance imaging (fMRI) studies have shown that maltreated children display increased amygdala response in threatening situations (Tottenham et al., 2011) and increased anterior cingulate cortex (ACC) activation during cognitive tasks (Mueller et al., 2010). Abused PTSD sufferers also exhibited decreased right hippocampal activity during a

verbal declarative memory task, which was associated with avoidance behaviour (Carrión et al., 2010). Physically abused children appear to be hyper-vigilant to potential social threat, which is demonstrated by increased brain activity when shown angry faces and an decreased ability to disengage from this stimuli when angry faces served as invalid cues (Pollak and Tolley-Schell, 2003).

1.2.5 Animal models of early life stress

Therefore, that early life stress has negative consequences on mental health in humans is well recognised in the literature. Consequently, animal models have been extensively utilised in order to understand the underlying pathways and neurobiology that lead to this vulnerability. Similarly to human studies, exposing rodents or primates to early trauma leads to profound alterations in the physiological stress system, behaviour and gene expression in adulthood. Animals have been used to model early life stress from within the prenatal environment to preadolescence, with functional consequences seen at each time point.

Prenatal and early postnatal stress

Foetal development is highly sensitive to influences from the external environment including stress felt by the mother (Seckl and Meaney, 2004; Kawamura et al., 2006). Animal models of prenatal stress are generated through exposing pregnant dams to either chronic stress paradigms, exogenous glucocorticoid administration or immune activation using polyinosinic:polycytidylic acid (poly I:C) injections (Weinstock, 2008, 2017). These methods have been associated with various consequences on the offspring such as increased risk of premature birth, HPA axis hyperactivity, increased fear and anxiety in adulthood and defaults in neuronal spine density within the hippocampus (Wadhwa et al., 1993; Jia et al., 2010; Wilson et al., 2013; Lilliecreutz et al., 2016; Weinstock, 2017).

Postnatally, brain development continues with neuronal circuits being actively grown, stabilised and pruned in accordance to both genetic and environmental cues (Novais et al., 2017). Rodent models of early postnatal stress mainly revolve around disturbing maternal care, which is highly important in development of pups. This normally involves maternal separation for up to 6 hours a day, early weaning (George et al., 2010) or encouraging poor care by limiting nesting material (Rice et al., 2008). These models present consistently with altered synaptic plasticty (Aisa et al., 2008), increased fear and anxiety (George et al., 2010; Vetulani, 2013) and reductions in cognitive ability (Diehl et al., 2014; Molet et al., 2016).

Prepubertal/juvenile stress

The early postnatal stress paradigms described above contribute to the majority of animal models of childhood adversity. However, it is also highly important to consider the postwearning to peripubertal period, which is the period this thesis will henceforth focus on. This is a sensitive developmental period that includes maturation of the amygdala, hippocampus and PFC as well as synaptic pruning throughout the brain and can induce long-lasting changes in behaviour and neurobiology as seen by various animal models and human studies (Albrecht et al., 2017). Different studies utilise different methodologies for modelling childhood adversity in animals with varying durations and severities of stress. This can lead to different behavioural outcomes associated with different disorder diagnoses, sometimes make it difficult to compare results.

The impact of prepubertal stress on behaviour

A singular stress paradigm resulted in increased anxiety, startle response (Bazak et al., 2009; Poulos et al., 2014) and sensitised fear leaning (Poulos et al., 2014) in adulthood. Paradigms lasting 3-7 days where the same stress is repeated everyday reported increased startle response (Avital and Richter-Levin, 2005; Jacobson-Pick et al., 2008), increases in depression-like behaviour (Lyttle et al., 2015) and

decreases in novel object recognition and working memory (Arcego et al., 2016). Anxiety levels seem to vary dependent on protocol with some studies reporting increased anxiety (Avital and Richter-Levin, 2005; Jacobson-Pick et al., 2008) and others showing no differences (Post et al., 2014; Arcego et al., 2016). This discrepancy may be due to the type of stress and anxiety, as the studies which showed increased anxiety utilised elevated platform as their mode of stress whereas those that didn't show any anxiety differences were stressed by social isolation. Variable stress for up to one week resulted in increased anxiety (Jacobson-Pick and Richter-Levin, 2010; Muller *et al.*, 2014; Brydges *et al.*, 2014), increased fear-induced ultrasonic vocalisations (Yee et al., 2012a) and decreased avoidance learning (Horovitz et al., 2014). In terms of learning and memory, variable stress for 3-7 days resulted in increased cue fear memory (Yee et al., 2012b), decreased contextual fear memory in males (Brydges et al., 2012) and increased spatial reference memory in females (Brydges *et al.*, 2014). Chronic stress also increased anxiety (Lukkes et al., 2009; Veenit et al., 2014) and depression-like behaviour (Eiland et al., 2012). Cued fear memory is also increased following chronic stress via a daily restraint (Eiland et al., 2012).

Accordingly, there is evidence that even a single severe stress experience in juvenility can induce a long-lasting effect on behaviour in adulthood. However, a short variable stress paradigm appears to produce the most robust alterations in behaviours such as increased anxiety, startle response and emotional memory formation.

The impact of prepubertal stress on gene expression

Various forms of early life stress (ELS) have shown that stress in early life can result in profound and long-lasting gene alterations. This is likely through alterations to the epigenome (Silberman et al., 2016), which can have a profound effect on gene expression. Epigenetics describes a collection of DNA chemical modifications and

histone proteins that affect the expression of genes without altering the DNA sequence itself (Figure 1.5). Of these modifications, DNA methylation is thought to be highly sensitive to environmental insults and external stimuli (Szyf, 2013).

Research has concentrated on the impact on genes involved in the HPA axis circuitry. For example, the *NR3C1* (nuclear receptor subfamily 3 group 3) gene which encodes the GR has been shown to be epigenetically modified in rodent models of early postnatal stress (Meaney, 2001; Meaney and Szyf, 2005). In prepubertal stress (PPS), no difference in *NR3C1* was observed in the hippocampus, however *NR3C2,* which encodes MR was increased in the hippocampus (Brydges *et al.* 2014). Protein studies, on the other hand, showed that PPS resulted in increased GR within the amygdala (Poulos et al., 2014). Early adversity and altered *NR3C1* methylation have also been reported in human subjects; in post-mortem brain tissue from abused suicide victims (McGowan et al., 2009) and also in white blood cells of adults with borderline personality disorder, BPD and major depressive disorder who experienced childhood abuse (Perroud et al., 2011, 2014).

However, expression alterations following PPS have also been shown in psychiatric risk genes. A model of prepubertal stress in mice revealed long-lasting changes in disrupted-in-schizophrenia I (DISC1), glycogen synthase kinase-3β and neuregulin 1 (Brydges *et al.* 2014) gene expression. Long PPS stress exposure (PND28-42) also resulted in increased mRNA expression of *NR1* (a subunit within the NMDAR) and decreased GAD67 gene expression in the amygdala in adulthood suggesting that PPS can lead to profound changes in the genes involved in excitatory and inhibitory neurotransmission. PPS in animal models has been seen to decrease brain-derived neurotrophic factor (*BDNF*) levels, leading to hippocampal neuronal atrophy (Bazak et al., 2009; Schmitt et al., 2014; Daskalakis et al., 2015). This is particularly interesting as human studies have shown decreased *BDNF* mRNA in post-mortem

tissue from a range of mood and psychiatric disorders including depression and schizophrenia (Ray et al., 2014). Additionally in humans studies, the well-researched *BDNF* val66met SNP significantly reduces *BDNF* activity and, while not directly associated with psychiatric disorders, has been shown to modify the effect that stressful life events has on the onset of depression (Kim et al., 2007; Elzinga et al., 2011; Brown et al., 2013).

In combination with these candidate gene studies, whole epigenome approaches have identified a large range of genes subject to epigenetic alterations following PPS. Labonté et al (2012) looked at promoter methylation in the hippocampus of human male subjects who had experienced severe child abuse compared to nonabused controls. It was found that 362 CpG sites were subject to differential methylation following stress; with genes involved in neuronal plasticity the most significantly differentially expressed (Labonté et al., 2012). Another study looked at the methylome of various ELS paradigms in different model systems (human, monkey and rat) and tissues (blood and prefrontal cortex) and elegantly identified a list of 30 genes that overlapped with all paradigms, including *CACNA1C* (Nieratschker et al., 2014). By analysing the promoter regions, they showed that, in humans, those who had a history of prenatal stress had a lower degree of methylation at the *CACNA1C* promoter. However, in adult rat PFC, prenatal stress signified more methylation at this promoter region (Nieratschker et al., 2014). This suggests that expression of this gene can be driven by methylation changes, and that experiencing stress in early life may affect methylation at promoter regions – driving or reducing expression of *CACNA1C.* This also suggests that stress may affect methylation differentially in the CNS vs peripheral tissues.

The interaction between ELS and *CACNA1C* has been further explored in a recent study that showed that individuals with the risk rs1006737 mediated allele showed a significant genotype by ELS interaction on the cortisol awakening response, a metric

that is associated with depression and anxiety (Klaus et al., 2018). This result was particularly evident in individuals that had experienced ELS before adolescence.

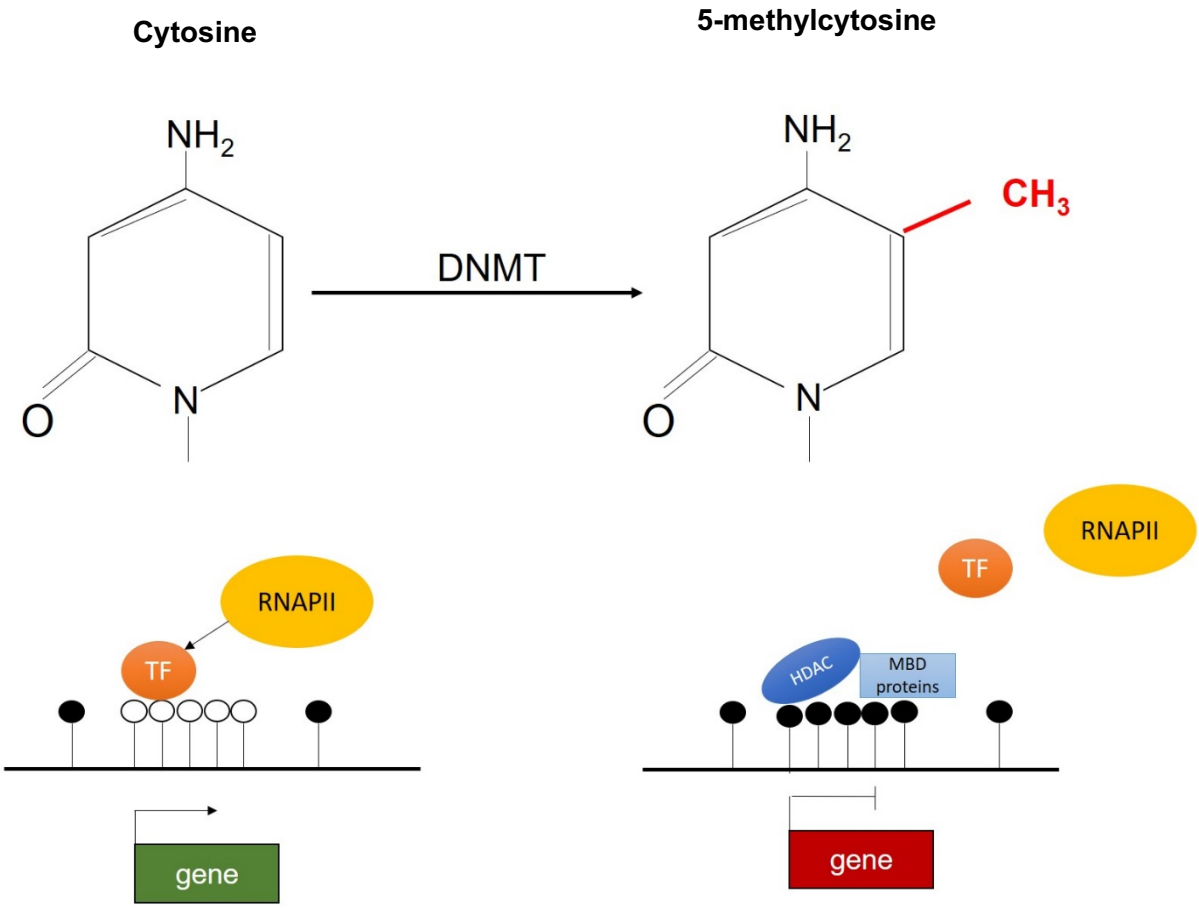

Figure 1.5: Schematic representation of the effect of DNA methylation on gene expression. Methylation can change the activity of a DNA segment by adding methyl groups to the DNA molecule. Left: When cytosine is unmethylated, there is an increased binding of transcription factors (TF) to gene promoters and enhancers. This results in the elevated transcription of active genes by the binding of RNA polymerase II (RNAPII). Right: When a methyl group is added to cytosine, it becomes 5-methylcytosine. This may lead to repressed gene expression by the binding of methyl-binding domain proteins (MBD proteins) which recruits HDACs (histone deacetylases) and other co-repressors to alter chromatic structure and inactivate genes.

1.2.6 Conclusions

Stress in early life can have a significant effect on behaviour, gene expression and HPA axis functioning in adulthood. Early life stress at various time points also increases the risk for mood and psychiatric disorders. Therefore, it is likely that genetic variation can interact with stress, or other environmental factors, to cause development of disorder and lead to a psychiatric phenotype.

1.3 The role of the hippocampus in psychiatric disorders

The hippocampus is located in the medial temporal lobe and is found in all mammalian species. It plays a key role in several forms of learning and memory and is essential for spatial navigation, as well as mediating hypothalamic function (Knierim, 2015). A plastic and vulnerable structure, the hippocampus is implicated in a variety of neurological and psychiatric disorders (Sala et al., 2004).

1.3.1 Hippocampal formation and function

Cut in coronal cross-sections, the hippocampus is a C-shaped structure that resembles ram's horns – this horned appearance is due to the subfield differences in densities of cell bodies and neuronal fibres (Knierim, 2015). The hippocampus is organised into subfields; *cornum ammonis* areas one (CA1), two (CA2) and three (CA3), as well as the dentate gyrus (DG) (Figure 1.6). The CA regions are formed of many different layers in clearly defined strata (Table 1.4).

The hippocampus is composed of a large number of specialised cell types, enabling highly refined electrophysiological behaviours. The human hippocampus contains approximately 10 million neurons, approximately 90% excitatory pyramidal cells and 10% GABAergic inhibitory neurons (West and Gundersen, 1990; Konradi et al., 2011a; Freund et al., 2013).

Table 1.4: The strata within the subregions of the hippocampus and the predominant cell types that make up each layer (green text indicates excitatory neurons, red indicates inhibitory)

Strata	Present in	Predominant cell types
Stratum oriens (SO)	CA1 CA3	Cell bodies of inhibitory cells Basal dendrites of pyramidal neurons
Stratum pyramidale (SP)	CA1 CA3	Cell bodies of pyramidal cells Cell bodies of inhibitory interneurons
Stratum lucidum (SL)	CA3	Mossy fibres
Stratum radiatum (SR)	CA1 CA3	Cell bodies of inhibitory interneurons Commissure and septal fibres Schaffer collateral fibres
Stratum lucunosum-moleculare (SLM)	CA1 CA3	Cell bodies of inhibitory interneurons Perforant path fibres
Molecular layer (ML)	DG	Perforant path fibres Dendrites from granule cells
Granule layer (GL)	DG	Cell bodies of granule cells Cell bodies of inhibitory interneurons
Polymorphic layer/Hilus (PML)	DG	Cell bodies of mossy cells Cell bodies of inhibitory interneurons

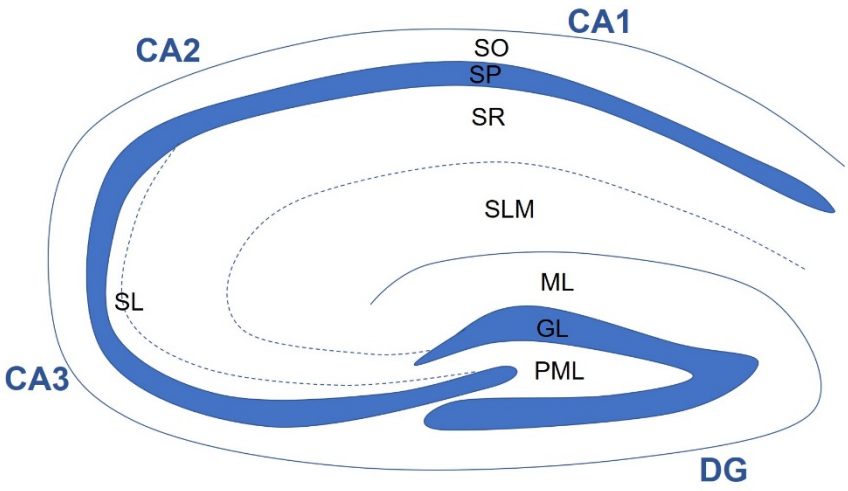

Figure 1.6: Schematic representation of the hippocampus with each subregion and strata labelled. CA1/2/3 = cornum ammonis, DG = dentate gyrus, SO = stratum oriens, SP = stratum pyramidale, SL = stratum lucidum, SLM = stratum lucunosum-moleculare, ML = molecular layer, GL = granule layer, PML = polymorphic layer/hilus

1.3.2 The hippocampal circuity

Hippocampal anatomical connectivity forms what is known as the 'trisynaptic loop' (Figure 1.7) where information is input to the dentate gyrus (DG) by the entorhinal cortex and is projected through the CA3 to the CA1 through multiple pathways, detailed in Figure 1.7.

The entorhinal cortex provides input from the cortex into the hippocampus, projecting predominately along the perforant pathway to the DG. The DG then projects to the CA3 via the mossy fibre pathway. Neurons within the CA3 project to the CA1 via the Schaffer Collateral pathway which projects back to the entorhinal cortex to complete the circuit (Figure 1.7). Thus, for many years, the hippocampal circuity was thought to be unidirectional (Benes, 1999). However, recent evidence has shown that the reality is much more complex; the entorhinal cortex projects also to the CA3 and CA1, and cells within the CA3 are also able to feedback to the DG via the mossy fibre pathway (Knierim, 2015).

Along the longitudinal axis, there are stark differences in connectivity. The posterior hippocampus in humans, which corresponds to the dorsal hippocampus in rodents, receives input from the medial entorhinal cortex and is associated with spatial cognition, whereas the primate anterior hippocampus, ventral in rodents, is highly connected to the amygdala and is predominately association with emotional memory and cognition (Lee et al., 2017).

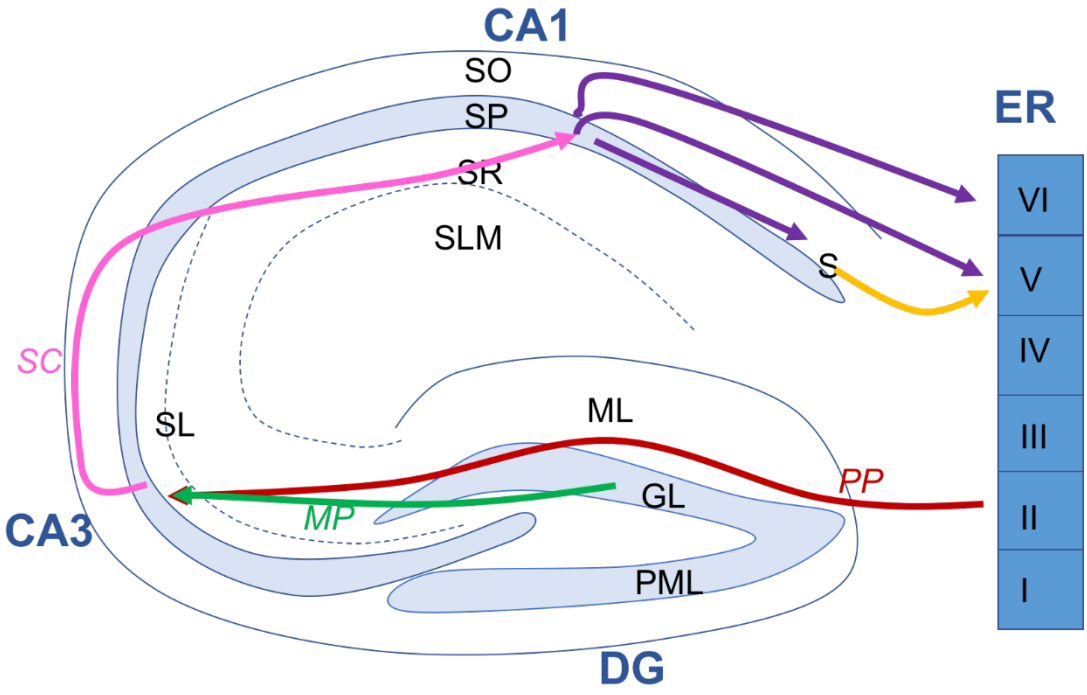

Figure 1.7: Simplified diagram showing the basic circuitry of the hippocampus, commonly termed the trisynaptic circuit. Layer II of the entorhinal cortex (ER) provides input to the granule cells of the dentate gyrus via the perforant pathway (PP, red). The granule cells project to the CA3 pyramidal cells via the mossy fibre pathway (MP, green). In turn, these CA3 pyramidal cells project their information to CA1 pyramidal cells via the schaffer collaterals (SC, pink). The CA1 pyramidal cells project to layers V and VI of the ER (purple) and also the subiculum (S, yellow).

1.3.3 The hippocampus in psychiatric disorders

The biological correlates of schizophrenia are highly variable across patients and are not fully understood, although grey matter reductions (Gur et al., 1999; Vita et al., 2012) are often reported. Structural magnetic resonance imaging studies have consistently shown decreased hippocampal volumes in first-episode schizophrenia patients (Steen et al., 2006; Vita and de Peri, 2007; Adriano et al., 2012; Arnold et al., 2015). Additionally, hippocampal structural abnormalities have been reported in first-degree relatives of schizophrenic probands (Heckers, 2001) as well as in asymptomatic individuals who had a high genetic risk for disorder (Witthaus et al., 2009). However, it should be noted that these decreases are comparatively low (approximately 4%) when looked at in reference to the dramatic reductions shown in neurodegenerative diseases (Nelson et al., 1998).

In addition to the structural changes reported, functional alterations have also been shown. Changes in hippocampal activity, particularly in the CA1, have been shown in schizophrenia (Schobel et al., 2009, 2013), which is evident at early stages (Schobel et al., 2009).Cerebral blood flow through the hippocampus is found to be elevated in psychosis, under basal and active conditions (Medoff et al., 2001). During episodic and recognition memory tasks, patients with schizophrenia display decreased hippocampal activation (Heckers et al., 1998; Weiss et al., 2004) compared to age-matched controls. Connections between the prefrontal cortex and hippocampus are also impaired in disorder (Bullmore et al., 1997; Rapoport et al., 2012) and animal models with perinatal hippocampal lesions demonstrate dysfunction of the prefrontal cortex (Lipska, 2004). This suggests that hippocampal impairments may have wide-spread implications in various brain regions thought to be involved in pathophysiology.

On a cellular level, Konradi and colleagues showed that whilst pyramidal cell numbers were unaltered in schizophrenic patients, decreased gene and protein expression

was observed in hippocampal interneurons. This suggests an imbalance between glutamatergic excitation and GABAergic inhibition within the hippocampus in schizophrenia (Harrison, 1999; Harrison and Weinberger, 2005; Lewis and Sweet, 2009; Gonzalez-Burgos et al., 2010; Konradi et al., 2011a).

Similarly to schizophrenia, reductions in hippocampal volume have been reported in BPD (Otten and Meeter, 2015), but this is not consistent across all studies (Frey et al., 2007). Functional imaging studies also showed abnormal hippocampal activation during memory and activation tasks (Frey et al., 2007) whereas post-mortem studies of BPD patients report abnormal glutamate and GABA transmission in the hippocampus.

1.3.4 Hippocampal functions in health and disorder

1.3.4.1. Memory and learning

The hippocampus has been extensively studied in both human populations and animal models to determine its function. Hippocampal physiology is unique and contains a great level of plasticity, which is important for learning and memory. The hippocampus has a high concentration of NMDARs, particularly in the CA1 (Cotman et al., 1987); the activity of these receptors are thought to underlie long-term potentiation (LTP), a process associated with learning and memory. The hippocampus has also been implicated in forming memory representations that underlie flexible cognition and social behaviour (Rubin et al., 2014), processes that are essential for a wide range of real-world scenarios: memory, imagination, decision-making, character judgements and social discourse. Memory generalisation, the ability to flexibly generalise memories from past events when encountering novel situations, is a process highly dependent on the hippocampus and impaired in schizophrenia (Shohamy et al., 2010). Interestingly, schizophrenic patients on antipsychotic medications showed improvements in memory generalisation, which suggests that medications may have a functional effect on the hippocampus.

1.3.4.2. Associative learning

Learning-related patterns of neural activity occur in the hippocampus during associative memory formation (Brasted et al., 2003). Aversive conditioning to investigate the molecular underpinnings of associative learning has been the focus of much study in the past century, typically through fear conditioning based on Pavlovian principles (Pavlov, 1927; Maren et al., 1997; Sah and Westbrook, 2008). Fear conditioning has proved highly beneficial for study of neurobiological mechanisms of learning and memory, as well as informing on emotional leaning and mood disorders. It provides a simple, reductive and tightly controlled behavioural paradigm that results in rapid leaning.

The mammalian fear circuity is common among many species; associating external stimuli with threat or safety is a key element of survival (Adolphs, 2013). Fear memories are not created and reside in a single anatomical locus, they are formed from interactions across a neural circuit (Kim and Jung, 2006). The amygdala is a critical site for fear conditioning - amygdala lesions result in abolishment of all conditioned fear responses to context and auditory cues (Helmstetter and Bellgowan, 1994; Maren et al., 1996). While a simple auditory fear circuit includes direct projection from the thalamus to the amygdala, more complex variants of fear conditioning such as contextual and trace fear conditioning require processing through the hippocampus (Kim and Jung, 2006) (Figure 1.8).

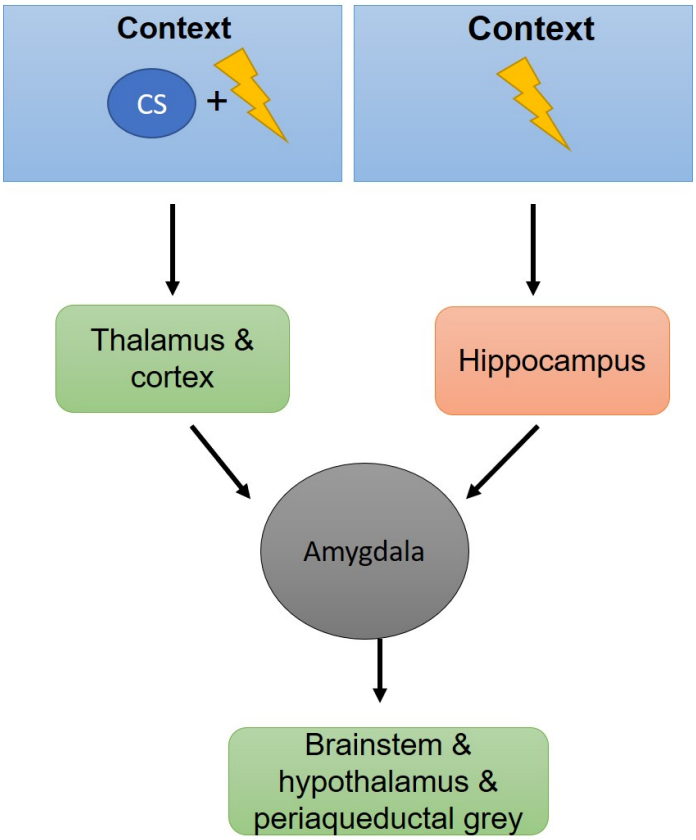

Figure 1.8: Simplified schematic of the neural circuity of fear conditioning. During simple auditory fear conditioning, the thalamus and cortex receive information about the conditioned stimulus (CS) (a light or tone) and unconditioned stimulus (US, represented here as a lightening bolt). This information is then relayed to the amygdala and eventually out to the brainstem and other regions to have various behavioural and neuroendocrine responses. In contextual fear conditioning, the hippocampus receives information about the CS-US association and outputs it for amygdala processing.

A basic Pavlovian fear conditioning experiment investigates how a subject can learn about the relationships between stimuli. For example, in the laboratory, a rodent is placed in a particular context (a box with particular odour, geometry and lighting), and after a few minutes, a brief stimuli such as a tone or noise is presented. This is known as the conditioned stimuli (CS), representing a previously neutral stimuli becoming associated with aversity conditional on its pairing with the footshock. At the termination of the CS, an aversive stimulus such as a footshock is presented, allowing for an association between the tone/noise cue and the aversive event to form. The footshock forms the unconditioned stimulus (US) (Maren, 2008; Curzon et al., 2009).

It is important to note that while it is clearly advantageous for an animal to associate cues that may predict predators, their survival is also dependent upon learning which environments are safe to facilitate reproduction and food gathering. Thus, it is important to see how animals respond to different contexts, which can also help inform on human fear and anxiety disorders (Steimer, 2002).

In 1972, Rescorla and Wagner presented a mathematical model intended to account for many elements of classical aversive conditioning such as acquisition, extinction, and conditioned inhibition, essentially by predicting the CS associative value (Rescorla and Wagner, 1972) (Equation 1).

$$\Delta V = \propto (\beta)(\lambda - V)$$

Equation 1: Rescorla and Wagner equation. V is the current CS associative state, alpha is the salience of the CS, beta is the US strength and lambda is the maximum associative value that can be conditioned to the CS under the experimental conditions.

This equation describes the 'prediction error' model, where learning is induced when experiences are unpredictable or challenge currently-held beliefs (Fletcher and Frith, 2009; Moore et al., 2011). Once CS-US events become associated, expectations are built up and become predictable. This model proposes that instability in this system could lead to the persistence of delusional beliefs in the face of conflicting evidence,

as seen in the positive symptoms of schizophrenia (Fletcher and Frith, 2009). Genomic evidence shows that the gene sets statistically associated with schizophrenia-associated copy number variants were those relating to 'associative learning' and 'cued and contextual conditioning behaviour' (Pocklington et al., 2015), suggesting that the biological mechanisms on which this model is based may be relevant to schizophrenia pathology.

1.3.4.3. Neurogenesis

Adult hippocampal neurogenesis describes the generation of adult-born functional granule cells within the subgranular zone (SGZ) of the dentate gyrus. This occurs through the amplification of neural progenitors and neuroblasts, followed by the integration of new neurons into the hippocampal circuitry (Figure 1.9). In this way, adult hippocampal neurogenesis is hypothesised to provide structural and functional plasticity in the tri-synaptic hippocampal circuit (Toda et al., 2018). While adult hippocampal neurogenesis has been confirmed in rodents and other mammals (Deng et al., 2010; Amrein, 2015), the extent of neurogenesis in humans has been controversial. Two recent publications published opposing results, revealing in one study that humans lack hippocampal neurogenesis (Sorrells et al., 2018), whereas another showed substantial neurogenesis that lasted throughout life (Boldrini et al., 2018). The latter study fits with previous literature investigating the rate of neurogenesis in humans (Kempermann et al., 1998; Spalding et al., 2013) however there are methodological issues that may conflict these results. The degree and existence of adult neurogenesis in humans is therefore still a subject of controversary that requires further large-scale experiments to investigate this phenomena.

Developmental process of hippocampal neurogenesis

Adult hippocampal neurogenesis is a complex multistep process that is necessary for the generation of new neurons from neural stem cells (NSCs). Neuronal precursor cells are known as type 1 cells that have low division rates and express markers of

neural stem cells. Adult neurogenesis progresses from these progenitors over the initial highly proliferative expansion stage to the survival and elimination stage (Kempermann et al., 2015) (Figure 1.9). The precursor stages expand the pool of cells that can differentiate into neurons and gives rise to intermediate progenitor cells with first a glial (type 2a), then neuronal (type 2b) and finally a migratory neuroblast-like phenotype (type 3). These now lineage-committed cells exit the cell cycle and express many markers of mature neurons. These neurons extend their dendrites towards the dentate gyrus molecular layer and spread their axons to CA3 (Deng et al., 2010; Kempermann et al., 2015). However, even at this stage, these neurons are not fully mature; they have different firing properties and lack glutamatergic input. They do, however, receive excitatory GABAergic synaptic input which results in neuronal depolarisation and appears to be vital for further development and maturation of the neurons. Eventually, adult-born neurons establish mature connections with the local network, receive glutamatergic synaptic inputs, whilst GABAergic input becomes inhibitory. These young, adult-born cells exhibit stronger synaptic plasticity than surrounding granule cells, implicating their importance in learning and memory processes (Götz and Huttner, 2005; Deng et al., 2010).

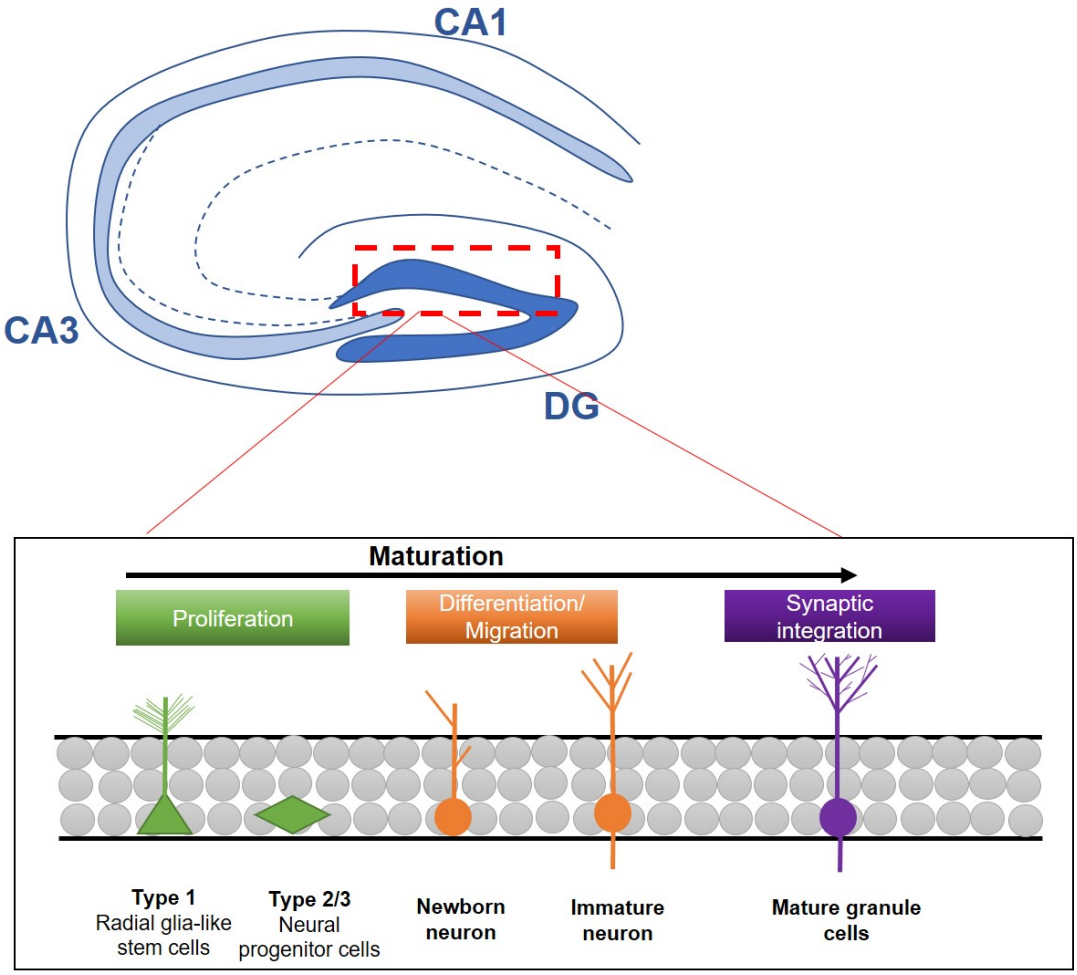

Figure 1.9: The developmental process of adult hippocampal neurogenesis that occurs within the dentate gyrus. Type 1 radial glia-like stem cells (adult neural stem cells) differentiate into neural progenitor cells. These cells develop into new-born neurons that process into mature granule cells that integrate into the hippocampal circuity.

As described, the hippocampus is a crucial structure for memory and associative learning. Therefore, whether hippocampal neurogenesis is influenced by, or itself influences, learning and memory processes has been the focus of many studies.

The rate of adult neurogenesis can be affected by both an animal's behaviour and environment. For example, voluntary wheel running and environmental enrichment can increase the rate of neurogenesis, potentially though stimulating the hippocampal neural network (van Praag et al., 1999a; Waddell and Shors, 2008; Deng et al., 2010). Hippocampus dependent tasks such as water maze tasks also increase the number of adult-born neurons (Gould et al., 1999; Leuner et al., 2004; Epp et al., 2007), however, one study reports that they have no effect on proliferation (Gould et al., 1999), suggesting a specific role for increasing survival of already proliferating and maturing neurons.

Trace eyeblink conditioning also promotes the long-term survival of adult-generated neurons (Leuner et al., 2004; Waddell and Shors, 2008). When neurogenesis is ablated, trace fear conditioning is disrupted, indicating that neurons born through adult neurogenesis are recruited through hippocampal dependent learning tasks (Shors et al., 2001). Furthermore, neurogenesis depletion disrupted working memory in the Morris Water Maze task (Snyder et al., 2005) and short-term memory in contextual fear conditioning (Winocur et al., 2006). Interestingly, it has also been seen that neural stimuli appear to preferentially activate adult-born granule cells, with an increased proportion of adult-born cells expressing the immediate early gene *Arc* (activity-regulated cytoskeleton-associated protein) in comparison to already existing granule cells when exposed to novelty (Ramirez-Amaya et al., 2006) or the Morris Water Maze (Kee et al., 2007; Deng et al., 2010). A recent meta-analysis concluded that the bulk of the literature also supports a role of hippocampal neurogenesis on behavioural pattern separation (França et al., 2017) in rodents. Computational and experimental evidence hypothese that granule cells in the dentate gyrus are

responsible for performing pattern separation on the overlapping representations arriving from the entorhinal cortex and projecting this signal onto the CA3 (Deng et al., 2010; Yassa and Stark, 2011) – processing highly similar inputs to less similar output firing patterns. This allows the hippocampus to discriminate among similar experiences (Yassa and Stark, 2011; Yau et al., 2015) and, thus, has a vital role in learning and memory. Adult-born neurons are thought to have a particular role in discriminating highly similar contexts (Nakashiba et al., 2012) and behaviour studies have shown that ablating hippocampal neurogenesis in mice impairs pattern separation (Yau et al., 2015), whereas increasing adult neurogenesis improves it. Adult neurogenesis is also implicated in the process of 'forgetting' old memories in order to store new memories and clear old ones – potentially through modification of the memory trace (Yau et al., 2015). Mice with impaired neurogenesis demonstrated increased freezing responses to a contextual fear retention test after two weeks (Feng et al., 2001), suggesting an impaired 'old' memory clearance.

Neurogenesis and psychiatric disorders

Psychiatric disorders, and in particular mood disorders, have been linked to alterations in adult neurogenesis (Apple et al., 2017). A range of psychotropic medications have been associated with increasing neurogenesis in rodent models (including SSRIs, selective SNRIs and tricyclic antidepressants) (Malberg, 2004). Additionally, ablation of SGZ neurogenesis prevents the positive effects of antidepressants on behaviour in mice (Santarelli et al., 2003). There has been extensive investigations into the role of adult neurogenesis in depression, which has been shown to have effects on both proliferation and survival on new-born neurons (Bowers and Jessberger, 2016). In schizophrenia, genes implicated in pathology such as disrupted-in-schizophrenia 1 *(DISC1*) and *BDNF* are associated with altered rates of neurogenesis and impaired hippocampus-dependent behaviours in mice (Schoenfeld and Cameron, 2015; Toth et al., 2016). Hippocampal post-mortem

studies on individuals with schizophrenia demonstrated a decrease in proliferating cells (Reif et al., 2006; Allen et al., 2016), however future studies in both humans and animal models would clarify any association between neurogenesis and schizophrenia .

1.3.5 Conclusions

Structural and functional changes have been observed in the hippocampus in individuals with neuropsychiatric disorders. The hippocampus plays a central role in the acquisition, consolidation and retrieval in learning and memory, particularly in contextual processing within associative learning. Adult hippocampal neurogenesis, which takes place exclusively in the sub granular layer of the dentate gyrus, is hypothesised to mediate some forms of learning and memory. Therefore, the hippocampus is a fascinating and relevant structure to examine for converging features of pathopsychological risk.

1.4 Experimental Plan

LTCCs are presented here as a critical convergence of genetic pathways implicated in psychiatric disorder. The consequences of early life stress are also described and suggested to interact with gene expression to allow for manifestation of mental illness, potentially through affecting the structure and function of the hippocampus.

In this thesis, with a focus of the impact on the hippocampus, I further the investigation of how genetic variation and environmental factors may interact together to increase risk of psychiatric disorder. My aims were as follows:

- Determine if prepubertal stress can regulate the expression of *Cacna1c* within the hippocampus
- Explore the effect of *Cacna1c* heterozygosity and prepubertal stress on the acquisition, consolidation and retrieval of various forms of aversive fear memory as a model of associative learning
- Investigate the impact of prepubertal stress and reduced gene dosage of *Cacna1c* on molecular substrates of the hippocampus: adult neurogenesis and GABAergic interneurons
- Observe whether an enriched environment can correct deficits in the hippocampus associated with *Cacna1c* heterozygosity.

Chapter 2: General Methods

2.1 Animals

2.1.1 Wild-type animals

Adult male Sprague Dawley rats (300-500g, Charles River, Margate, UK) were housed in groups of 2-4 in standard cages (38cm (W) x 56cm (L) x 22cm (H)) with ad libitum access to food (standard rat chow, RM1, Special Services Diet, Lillico, UK) and water. All animals were housed on a 12:12 hour light-dark cycles (light phase 8am-8pm). Cages were lined with wood shavings, a cardboard tube and wooden stick as basic enrichment. All animals were given a minimum of seven days from arrival before being used in experiments. Humidity was maintained at 45-60% and temperature between 19-21°C. At the end of the experiment, rats were sacrificed by Schedule 1, via a rising concentration of CO_2 in a home cage culling chamber (Clinipath Equipment Limited, Hull, UK) or by overdose of anaesthetic (Euthatal (200mg/ml), Merial, Harlow, UK). Experiments were conducted under licence PPL 30/3135 and PIL I98C8E6DF. All procedures were carried out in accordance with local ethics guidelines, the UK Home Office Animals Act 1986 and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

2.1.2 Prepubertal stressed animals

Twelve female and twelve male Lister Hooded rats (250-275g, Charles River, Margate, UK) were placed in male-female pairs in standard cages for breeding. Pairs were left alone until pregnancy was confirmed and pups were born, whereupon males were removed. At weaning (PND21), animals were housed in groups of 3-5 in standard, same-sex, same-litter cages. Food and water was provided ad libitum. Six litters out of twelve, at random, were assigned to be prepubertally stressed. The prepubertal stress protocol has been described previously (Brydges et al, 2014). On

postnatal days (PNDs) 25, 26 and 27 animals were removed from their home cages and taken to a designated room, away from the regular housing area, and subjected to variable short-term stressors. The first stressor, on PND 25, consisted of a 10-min swim stress in an opaque swim tank (25cm (H) x 34cm (D)), filled with approximately 6L of 25°C water. After the swim, animals were briefly towel-dried and then taken back to their home cage. On PND 26, animals were subjected to three sessions of 30 minute restraint stress, in plastic restraint tubes (15cm (L) X 5cm (D)). Each session was separated by 30 minute breaks in the home cages. On PND27, animals were exposed to elevated platform stress; they were placed on an elevated platform (15 x 15cm, 115cm high) for three sessions of 30 minutes, separated by 60 minute breaks in the home cages. For each litter stressed, another litter acted as control. These animals were briefly handled on PND 25-27, but otherwise left in their home cages. Both prepubertally stressed and control litters were then left in their home cages until adulthood (PND60). A maximum of three animals per litter were used for individual experiments and litter of origin was accounted for in all statistical analysis.

2.1.3 Enriched environment

Animals exposed to an enriched environment were housed in large cages (74cm (W) x 59cm (W) x 40cm (H) in groups of 6-7 (Figure 2.1). This larger group size allowed for increased social interaction. These cages were equipped with a moveable platform to give the rats another level to explore. They were also given access to objects such as plastic igloos (31440P), wooden sticks to gnaw and play with (710113P, 7116419P), rearrangeable tunnels (7114739P) and wooden balls (7116401P) (all Pets At Home, Cheshire, UK) to stimulate exploratory play and behaviour. These rats had access to food and water ad libitum. The objects were rearranged 3 times a week and different objects were placed into the cages every week for novelty.

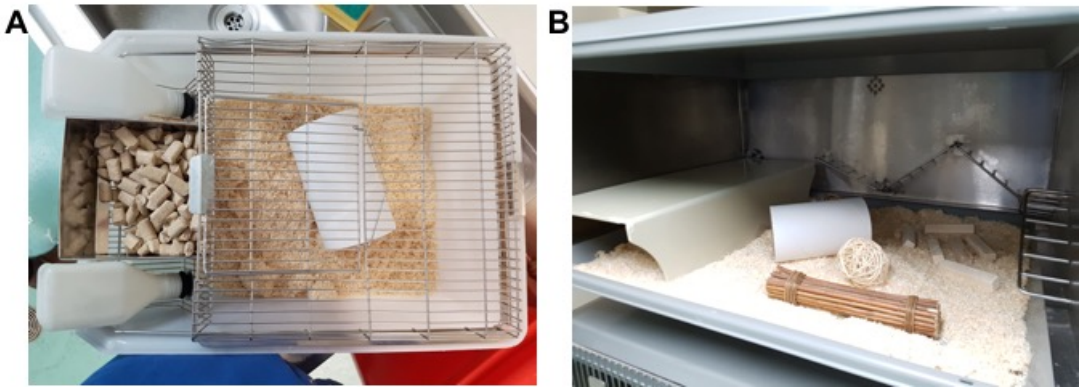

Figure 2.1: A = Standard housing (including cardboard tube not in photo). B = Enriched environment cages. Enriched environment cages were set up to include a moveable platform, toys and climbing frames with increased social interaction

2.1.4 Transgenic heterozygote *Cacna1c* knockout animals

Cacna1c heterozygote knock-out animals were created on a Sprague Dawley background (TGR16930, Sage Research Labs, Pennsylvania, USA) using Zinc Finger Nuclease (ZFN) technology. ZFN technology utilises DNA-binding proteins that create a double-stranded break in DNA at a specified location, stimulating natural DNA repair processes that can lead to mutations and/or deletions in the genome. The heterozygote rats created by ZFN have a 4bp deletion in exon 6 of *Cacna1c* (460649 bp – 460652 bp), resulting in a frame shift and subsequent early stop codon. Breeding of *Cacna1c*$^{+/-}$ rats resulted in litters of 4-16 with a Mendelian distribution of wild-type and heterozygote pups (homozygote deletion is embryonically lethal). *Cacna1c* heterozygote knock-out animals were shipped to Cardiff University in cohorts of 30-40 animals at a minimum age of 12 weeks.

2.1.5 Genotyping

Genotyping of heterozygote animals was performed both pre and post mortem. Initially, ear punches from all experimental animals were sent from Charles River for genotyping to allow for experimental setup. These genotypes were confirmed post-mortem by taking a small section of tail tissue. Qiagen DNeasy Blood and Tissue Kits (Qiagen, Manchester, UK) were used to extract genomic DNA (gDNA) as per the manufacturers standard protocol. Approximately 20mg tissue (0.6cm tail tissue or one single ear punch) was lysed at 56°C overnight in 180µl buffer ATL and 20µl proteinase K on a rocking platform. 200µl of buffer AL and 200µl of 96-100% ethanol were added to the sample before putting through a spin column at 8000rpm for 1 minute. Buffer AW1 was added the spin column as a first wash and spun through at 8000rpm for 1 minute. Buffer AW2 was then added and spun through at 8000rpm for 1 minute, before a final spin of 14,000 rpm for 3 minutes to dry the membrane. DNA was then eluted by adding 200µl of buffer AE to the membrane, incubating for 1 minute at room temperature and then centrifuged at 8000rpm for 1 minute. A NanoDrop

spectrophotometer (Thermo Fisher Scientific, Delaware, USA) was used to measure the concentration of DNA and purity. This extraction yielded approximately 20-40µg of DNA and was determined acceptable purity if the 260/280nm ratio was 1.8 or greater. DNA was stored at -20°C until required for qPCR.

Polymerase Chain Reaction

To determine a wild-type from a heterozygote animal, a multiplex reaction was set up. Two forward primers (24bp) were used; the first primer targeting a region upstream of the deletion (Chr 4: 216724640-216724663) and the second primer targeting the deleted region (Chr 4: 216724462-216724485) (Table 2.1). Therefore, the second primer was a heterozygote specific primer and would only bind if the 4bp was absent, i.e. in a heterozygote animal. Master mixes were made up to have a total of 24µl reaction mix per sample to be tested. 17.25µl distilled MilliQ H2O, 2.5µl 10 x PCR Buffer, 1µl of dNTPs, 0.25µl HotStarTaq DNA polymerase (Qiagen, Manchester, UK) and 1µl of each of the 3 primers made up the reaction mix. 2µl gDNA (0.4ug of DNA) was added to this to form the total sample for the multiplex reaction. Samples were run on a BioRad Thermal Cycler (T100^{TM} BioRad, Herts, UK), under the following conditions: 95°C for 600 seconds, followed by 40 cycles of 95°C for 40 seconds, 65°C for 40 seconds and 72°C for 60 seconds, with a fixed cycle of 72°C for 300 seconds. Samples were then cooled to 8°C.

Table 2.1: Primers used in genotyping to differentiate Cacna1c wild-types and heterozygote animals

Forward primer 1	5’ – AGCCTTTTATTGGGATGTGTCTCC – 3’
Forward primer 2	5’ – TCCTGAACTCCATCAGGCCA – 3’
Reverse primer	5’ – CTGATGAACGGTGGGTGCTTAC – 3’

Gel electrophoresis

To determine genotypes, samples were run on a 1.5% agarose gel made from 0.5 x Tris/Borate/EDTA (TBE) buffer (54g of Tris base, 27.5g boric acid and 20ml of 0.5M EDTA, pH 8.3). 5µl of ethidium bromide was used per 100ml agarose ran. 16µl of the resulting PCR samples (with 4µl DNA Gel Loading Dye (6X) (Thermo-Fisher Scientific, Delaware, USA) was loaded onto the gel and run at 120V for 30 minutes while the gel was submerged in 0.5µl TBE. Gels were visualised on a BioRad Gel Doc XR imager (BioRad, Herts, UK) (Figure 2.2). The presence of one band at 463bp or two bands at 463bp and 645bp were visually inspected to determine genotype. Heterozygote animals were determined by two bands, whereas wild-types were identified by the presence of one band.

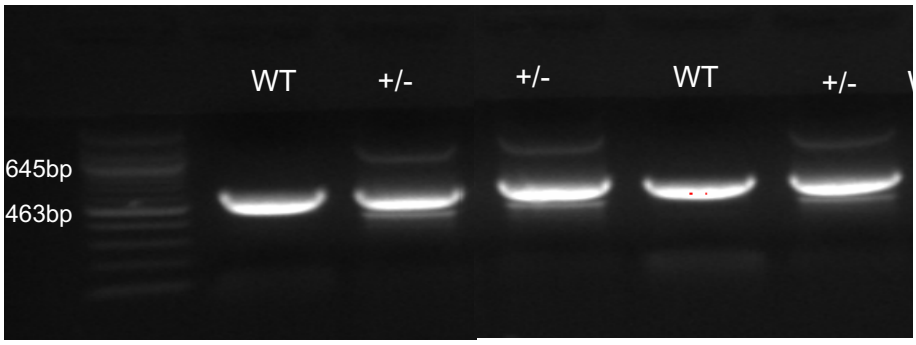

Figure 2.2: Representative image of genotyping results for Cacna1c wild-types (WT) and heterozygotes *(Cacna1c$^{+/-}$)*. *Cacna1c$^{+/-}$* animals were defined by presence of two bands at 463bp and 645bp whereas wild-types only had one band at 463bp.

2.1.6 5-Bromo-2'-deoxyuridine solution and injection

For neurogenesis analysis, rats were intraperitoneally injected with 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue that is incorporated into cellular DNA during S-phase. This allows selective marking of newly proliferating cells. BrdU (Sigma, Dorset, UK) was dissolved in 0.1M sterile PBS (pH 7.2) to 25mg/ml and stored at 4°C until needed. Rats were restrained and injected with a single bolus of BrdU (50mg/kg) by intraperitoneal injection, administered 6 hours before euthanasia. Rats were then returned to their home cage.

2.1.7 Perfusion

Fixation using post-mortem perfusion was performed to allow for rapid and uniform tissue preservation. The perfusion pump was set up and perfusion needle attached. Rats were sacrificed via intraperitoneal euthatal (200mg/ml) injection (Merial, Harlow, UK) and left in a holding cage until cessation of heartbeat. Rats were rapidly dissected to expose the heart and perfusion needle inserted into the protrusion of the left ventricle and clamped into place near the point of entry. The right atrium was cut with sharp scissors. The value was released to allow a slow, steady flow of 13-20 ml/min of 0.1M PBS ice-cold solution to flush through until the blood was cleared from the body. Following this, the perfusion solution was changed to ice cold 4% paraformaldehyde (PFA) (approximately 200-300ml per animal), which was allowed to flow at 20ml/min until spontaneous movement and lightened colour of the liver was observed (approximately 15-20 minutes). Perfusion was then stopped and brains rapidly dissected out. Brains were then transferred to fresh 4% PFA and immersion-fixed overnight at 4°C. Once fixation was complete, brains were placed in 30% sucrose to cryoprotect the tissues for 2-6 days.

2.1.8 Dissection

Animals not perfused were sacrificed by a rising concentration of a CO_2. Brains were rapidly removed and the hippocampus micro-dissected out before being flash frozen on dry ice. Dissected hippocampi were then stored at -80°C until needed.

2.2 Human samples

Post-mortem hippocampal tissue samples were obtained from the Edinburgh Brain Bank for cDNA extraction. Samples were from 8 males (39.4 +/- 7.4 years) with no history of early life trauma and 5 males (39.8 +/- 6.6 years) and 5 females (39.2 +/- 15.2 years) who reported early life stress. 0.03-0.04g of tissue was extracted per subject. All samples were obtained with ethical approval and with informed consent and were matched for post-mortem delay.

2.3 Behaviour

2.3.1 Auditory Fear Conditioning

All sessions were undertaken in two standard modular test chambers (32cm x 25.5 cm x 27 cm) for rats with sound-attenuating walls (Sandown Scientific, UK). Chambers had a grid floor consisting of 19 equally spaced stainless steel bars placed 1.6cm above the floor where 0.5mA shocks were delivered to. An aversive stimulator/scrambler controlled the shock given. Med-PC version IV research control and data acquisition system controlled the programmes sent to the boxes (Med Associated Inc., Vermont, USA). White noise at 75-86db formed the conditioned stimulus (CS). One test chamber contained lavender scent (Botanics Aromatherapy Pure Essential Oil, Boots, UK) and contained no light (an infrared bar was used to enable filming) (Context A), whereas the other test chamber contained fresh sawdust for scent, patterned walls (black stars on a white background) and lit by a house light (75W) (Context B) for the whole conditioning session (Figure 2.3A and B). Use of each context was counterbalanced across experimental group and time of day. Test chambers were thoroughly cleaned between each animal with 50% ethanol. Behaviour was digitally recorded from cameras (Fujifilm FE185C086HA-1 with Fish-eye Lense) positioned within the doors and viewed using VLC Media player for offline analysis.

2.3.1.1. Habituation

Once a day, for the three days prior to the conditioning sessions, animals were removed from their home cages and placed into transport boxes. They were transported to the testing room, left for five minutes in the room, briefly handled and returned to their home cages. This allowed them to habituate to the journey and the experimenter, reducing the confound of novelty on the experimental days.

2.3.1.2. Delay Conditioning

Animals were placed in one test chamber (Context A or B). Following a two minute baseline period, rats were given a 10 trial session of randomly presented CS-US pairings. CS was presented for 15 seconds and co-terminated with a 0.5mA, 0.5s footshock (unconditioned stimulus, US). The conditioning session lasted 53 minutes. Intertrial interval was 312s ± 62s (20%). Animals remained in the chambers for 2 minutes after the last pairing was presented, before being taken back to their home cages (Figure 2.3C).

2.3.1.3. Trace Conditioning

Animals were placed in one test chamber (A or B) and, following a two minute baseline period, given a 10 trial session of randomly presented CS-US pairings. CS was presented for 15s and 30s later a 0.5mA, 0.5s footshock was presented. The conditioning session lasted 58 minutes 29s. Intertrial interval was 312s ± 62s. Animals remained in the chambers for 2 minutes after the last pairing was presented, before being taken back to their home cages (Figure 2.3C).

2.3.1.4. Unpaired Conditioning

Animals were placed in one test chamber (A or B) and, following a two-minute baseline period, given 10 CS presentations and 10 0.5mA, 0.5s shocks in an explicitly unpaired manner. The conditioning session lasted 54 minutes 41 seconds. Any two stimuli were separated by an intertrial interval of 156 ± 31s. Animals remained in the chambers for 2 minutes after the last stimuli was presented, before being taken back to their home cages.

2.3.1.5. Context Recall

Twenty-four hours post-conditioning, rats were returned to the same conditioning chamber (CS) for a 10 minute recall session in the absence of both CS and US.

2.3.1.6. Cue Recall

Forty-eight hours ± 3 hours post-conditioning, rats were put into the opposite test chamber to their conditioned chamber (Context A rats go into Context B and vice versa). Context was further altered by offsetting the time of day tested by at least three hours and by placing a plastic sheet over the shock bars. These measures were undertaken to avoid generalised contextual representations. Animals experienced a 2 minute baseline period followed by a 6 minute CS presentation. Following CS cessation, animals were further monitored for 4 minutes (Post CS).

2.3.1.7. Analysis

Freezing behaviour was defined as complete immobility for 1s except for respiration movements. Animals were scored every 10 seconds with the experimenter blind to condition and genotype. The number of instances of freezing over the whole 10 or 12 minute recall sessions were recorded for each animal, as well as for separate pre and post US periods. The percentage freezing was calculated for each animal for each session, and by minute, and averaged across control and experimental groups. One-way and repeated measures ANOVA were conducted for each condition and genotype. All data was checked for normality and transformed if required via a Box-Cox transformation (One Way ANOVA) or a Greenhouse-Geisser correction (Repeated Measures). Post-hoc tests, where appropriate, where performed by Tukey Kramer HSD tests. Results were assumed to be significant if $P < 0.05$.

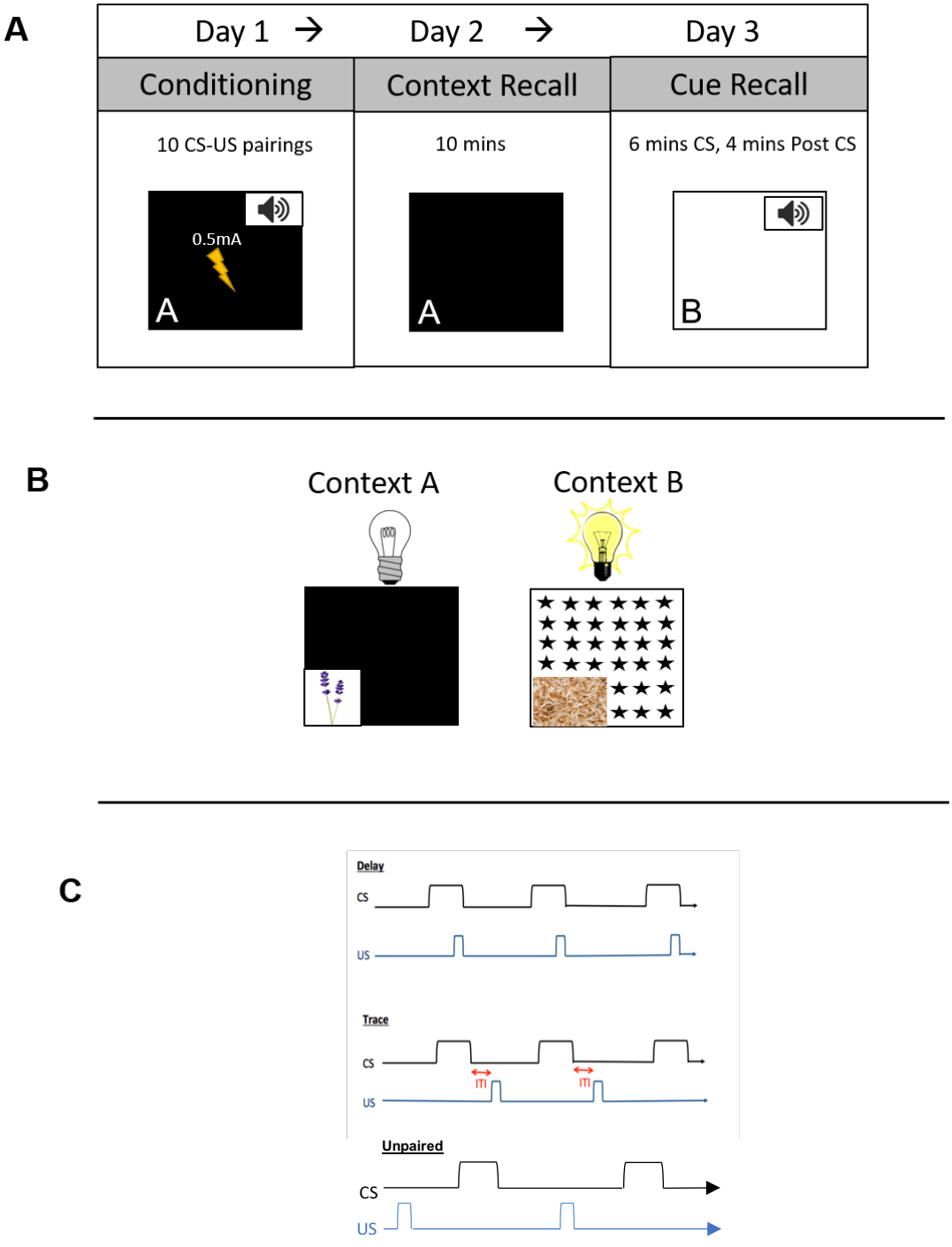

Figure 2.3: Experimental overview for auditory conditioning sessions. A = Rats were conditioned on Day 1 in Context A in either a delay, trace or unpaired paradigm. They then received a 10 minute Context Recall on Day 2 where they were returned to the same context as on Day 1 for 10 minutes. On Day 3 rats were placed into a novel context and given a Cue Recall. B = Two contexts were used to distinguish reactions to context and cue. Context A was a dark box with a lavender scent, whereas Context B was a light box with a starry background and sawdust scent. C = Delay and trace conditioning schematic. In delay conditioning, the CS terminates with the US. In trace conditioning however, the CS plays and the US is presented after a 30 second interval. In unpaired conditioning, the CS and US are explicitly unpaired. ITI = intertrial interval.

2.4 Laboratory Techniques

2.4.1 Quantitative Polymerase Chain Reaction (qPCR)

qPCR is a method that combines PCR amplification and detection for quantitative mRNA expression analysis. Fluorescent dyes are added to PCR products during thermal cycling and the subsequent fluorescent signal during the exponential phase of the reaction measured to give a read-out of gene expression.

2.4.1.1. RNA extraction

Hippocampi from flash-frozen brains were removed from the -80°C freezer and kept on dry ice. 20-30mg of tissue was used per sample and processed using the Qiagen RNeasy Kit (Qiagen, Manchester, UK), following the supplied protocol. 14.3M ß-Mercaptoethanol was added to supplied Buffer RLT (1:100) and 550µl of resulting solution added to the sample in a ribotube (MP Biomedicals, UK). The ribotube containing the sample was placed in a ribolyser (Bio-Rad Laboratories Inc., USA) and tissue homogenised using the fast prep homogeniser for 2 x 5 second blasts until the tissue was completely lysed. Samples were then centrifuged for 3 minutes at 14,000rpm for 3 minutes. Samples were then removed from the ribotube and placed into a standard 1.5ml Eppendorf, before then centrifuged again for 3 minutes at 14,000rpm to form the pellet. Once finished, the resulting supernatant was dissolved in 500µl 70% ethanol and 500µl of the supernatant/ethanol mix and loaded into a RNeasy spin column placed into a collection tube. The spin column and collection tube was centrifuged for 15 seconds at 10,000rpm. The flow through was discarded and this step repeated with the remaining 500µl supernatant. 250µl of buffer RLT + ß-Mercaptoethanol was then added to the pellet and centrifuged for three minutes at 10,000rpm. The supernatant was once again extracted, mixed with 250µl 70% ethanol and added to the same RNeasy spin column as before. The spin column was centrifuged for 15 seconds at 10,000 rpm and the flow through discarded. The spin

column was then incubated at room temperature for 5 minutes. Following this the spin column was washed by adding 700µl Buffer RW1 to the RNeasy spin column and centrifuging for 15 seconds at 10,000rpm and discarding flow through. 500µl Buffer RPE was then added, centrifuged for 15 seconds at 10,000rpm and flow through discarded. Another 500µl RPE was added to the spin column and the column centrifuged for five minutes at 10,000rpm to thoroughly wash the membrane. The spin column was then placed in a new collection tube and span at 14,000rpm for 1 minute to eliminate any possible carryover of Buffer RPE. The spin column was then placed into a 1.5ml collection tube. 30µl of RNase-free water (Ambion Life Technologies, UK) was added directly to the spin column membrane and incubated for 10 minutes at room temperature. To elute the RNA, the spin column was span at 10,000rpm for 1 minute. RNA content was measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Delaware, USA) to determine concentrations were a suitable level (between 100-10,000ng/ul). The A260/A280 value was used to determine purity, the value was required to be under 2.

2.4.1.2. DNAase treatment

RNA was DNAse treated to remove any DNA contamination using Ambion TURBO DNA-free™ Kit (ThermoFisher Scientific, Delaware, USA) in accordance with the supplied protocol. 0.1 volume of 10X TURBO DNase Buffer and 1ul TURBO DNase was added to each RNA sample and gently mixed. The samples were incubated at 37°C for 30 minutes. DNase Inactivation Reagent at 0.1 volume was then added to the sample and incubated for five minutes at room temperature, mixing frequently. The samples were then centrifuged at 13,000rpm for 1 minute and the pellet discarded.

2.4.1.3. cDNA synthesis

RNA was then used to create cDNA for analysis. 1.5µg RNA was added to RNA to cDNA Easy Premix (Random Hexamers) (Clontech Laboratories Inc., France) tubes.

Nuclease-Free water (Ambion Life Technologies, UK) was then added to make the total volume up to 20µl. Samples were then placed in a thermal cycler (Bio-Rad Laboratories S100, USA) and heated at 42°C for 75 minutes, following by 80°C for 15 minutes. Sample was then diluted 1:15 in nuclease-Free water and stored at -20°C.

2.4.1.4. Primer design

Primers were designed using the FASTA gene sequences from NCBI (http://ncbi.nlm.nih.gov) for both human and rat genes of interest. FASTA sequences were inputted to Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Probes were designed to be approximately 20 base-pairs long, have an AT:CG ration close to 50%, to span an exon-exon junction and a melting temperature around 60°C. Product length was required to be between 50-150 base-pairs. All designed probes were tested for homology elsewhere in the genome by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Ensembl was also used to ensure probes were targeting the required transcripts of the gene (http://www.ensembl.org/index.html). Probes were synthesised by Sigma-Aldrich (Dorset, UK). Upon arrival, they were diluted to 100µM in nuclease-free water. Aliquots of working solutions (10µm) were made up for each probe to minimise repetitive freeze-thaw cycles. Primers were then validated in terms of having both single-amplicon specificity and consistently high amplification efficiencies. In order to check for single-amplicon specifity, a default melting programme was run on a RT-PCR machine (Applied Biosciences) at the end of the cycling programme. Primers were said to be specific if dissociation curves were visualised as a single peak with no shoulders. Following this, a calibration curve was produced where a 1:5 serial dilution series was generated using a cDNA template of known concentration. qPCR was then run to generate a standard curve by first heating to 95°C for 10 minutes, followed by 45 cycles of 95°C (15 seconds) and 60°C (1 minute) to allow for primer

annealing and elongation. Finally in the melt curve stage, samples were heated to 55°C for one minute and 95°C for 15 seconds. The generated Ct values plotted against the initial amounts of input material on a semi-log10 plot. This data was fitted to a straight line and the gradient calculated. The gradient was inputted into the Life Technologies Efficiency calculator with the ideal gradient of -3.33 giving an amplification efficiency of 100%. Probes were considered valid at 90-110% efficiency. This is a necessary step as quantification calculations are dependent on amplification efficiencies of approximately 100%.

2.4.1.5. qPCR

96-well plates were loaded, each well containing a total of 15µl reaction mixture (1.9µl sterile RNAase free water, 0.3µl 10µM forward primer, 0.3µl 10µM backward primer, 7.5µl SYBR-Green SensiMix (Bioline) and 5µl cDNA). SYBR Green I dye is a fluorescent dye that binds to the minor groove of double stranded DNA. *Gapdh* and *Hprt* primers (Sigma) were used as housekeeping controls for rat cDNA, *GAPDH* and *UBC* were used as housekeeping genes for human cDNA. All results are normalised from these values. After loading, plates were span down at 3,000 rpm for approximately 10-20 seconds before being transferred to Real-Time PCR instrument (Applied Biosystems®). qPCR was then run as before: 95°C for 10 minutes (holding stage), followed by 45 cycles of 95°C (15 seconds) and 60°C (1 minute). Finally in the melt curve stage, samples were heated to 55°C for one minute followed by 95°C for 15 seconds.

2.4.1.6. Analysis

The output from the qPCR cycle is given in cycle threshold (Ct) values, essentially the number of cycles it took to detect a signal from the cDNA above that of background fluorescence. Ct values correlate negatively to the amount of nucleic acid in a sample. Threshold levels were set to coincide with the beginning of the

exponential phase. Quantitation was done using the comparative Ct method ($2^{-\Delta\Delta CT}$ method) to produce fold changes in between groups tested. This method involves subtracting the average housekeeping gene from the gene of interest ($\Delta C_T = C_{T\ target} - C_{T\ reference}$). The $\Delta\Delta C_T$ value is then calculated by subtracting the experimental group from the control group ($\Delta\Delta C_T = \Delta C_{T\ test} - \Delta C_{T\ control}$) and incorporating standard deviations into the fold change. Data was analysed using one-way ANOVAs. All data were checked for homogeneity of variance using Levene's Test and normality of distribution. If found to be outside normal distribution, results were transformed accordingly using the most appropriate method (Box Cox transformation or square root transformation) that resulted in data normalization. Litter was nested within the data as a random variable where appropriate. All data was analysed using JMP statistical software (JMP, SAS Institute, Cary, NC, USA).

2.4.2 *In-situ* hybridisation

In situ hybridisation is a useful method for quantifying localisation and expression levels of specific mRNA sequences by using radiolabelled strands of complementary nucleotide sequences.

2.4.2.1. DEPC treatment and slide preparation

1ml of diethylpyrocarbonate solution (DEPC, Sigma-Aldrich, Dorset, UK) was added to 1 litre of distilled water. The solution was shaken vigorously and left for 2-4 hours in a fume hood before being autoclaved and left to cool to room temperature. Poly-L-lysine was dissolved in DEPC treated water (1:10 dilution) and stored in 1ml aliquots before use. Twin frost microscope slides (VWR International, Leicester, UK) were dipped in a working solution of 2% Poly-L-lysine, air dried and stored at 4°C until required.

2.4.2.2. Sectioning and fixing of brain slices

Rats were killed by CO_2 inhalation and whole brains extracted rapidly. Brains were flash-frozen on dry ice and stored at -80°C. Coronal sections of 14µm were sliced on a cryostat (Leica Microsystems CM1860UV) at -20°C and mounted on poly-L-lysine coated slides. The arrangement of brain sections on each slide was designed in a pseudo-random manner so each slide had 1 slice from 6 brains, counterbalanced and without position bias for each experimental condition. 114 sections were taken from each brain. For each experiment, slides were processed in parallel for technical control. Slides were placed into glass slide racks and fixed in 4°C 4% paraformaldehyde (PFA) in phosphate buffer (PBS; 1.3M NaCl, 70Mm disodium phosphate, 30Mm monosodium phosphate, pH 7.2) solution for five minutes, before washing in PBS for one minute, followed by 70% ethanol for four minutes. All slides were then stored at 4°C submerged in 95% ethanol until required. Slides were left for at least two weeks before use to ensure complete delipidation.

2.4.2.3. Oligonucleotide probe design

For each gene of interest, single stranded oligonucleotide probes were designed to target the mRNA sequence. FASTA gene sequences were inputted into 'Oligo' - a MATLAB tool designed to produce potential oligonucleotide probes. Probes were designed to be 45 base pairs long to allow for sufficient cell penetration. Probes were also required to have an AT:CG ratio close to 1, with a maximum of 3 bases differences and maximum of 3 consecutive bases; these steps help prevent excessive secondary folding of the probe. All designed probes were tested for homology elsewhere in the genome by using BLAST. Probes (Sigma-Aldrich, Dorset, UK) were diluted to 1 ug/ul in phosphate buffer (PB, pH 7) and were further diluted in DEPC-treated water to 5 ng/ul to give a working solution.

2.4.2.4. 5'end ^{35}S labelling of oligonucleotides

Probes were radiolabelled using deoxyadenosine 5'- (α-thio) triphosphate [^{35}S] (dATP) (Perkin Elmer, MA, USA), which has a half life of 87 days. 1.5µl of ^{35}S-dATP radiation was added to 2ul of 5ng/µl oligonucleotide probe, along with 4.5µl DEPC treated water, 2.5µl terminal deoxynucleotidyl transferase buffer (Promega, WI, USA) and 1.5µl terminal deoxynucleotidyl transferase (Promega, WI, USA). The resulting solution was incubated at 35°C for an hour. 38µl DEPC water was then added to stop the enzymatic reaction. Probes were then purified using the Qiaquick Nucleotide Removal Kits (Qiagen, Manchester, UK) according to the manufacturers recommendation. Labelled probe was added (1:10) to buffer PNI in a spin column and spun at 6000rpm for one minute. The column was washed twice by adding 500ul Buffer PE and spinning for 6000rpm, discarding the flow through each time. Samples had a final spin at 13,000 rpm to remove any residual ethanol. Labelled oligonucleotide probe was then eluted by applying 50µl DEPC-treated water directly to the spin column membrane. Columns were incubated at room temperature for 1 minute and centrifuged at 13,000 rpm for 1 minute. 2µl of 1M dithiothreitol (DTT) was added to the probe - this reduces the thermal stability of the bonds, allowing hybridisation to be carried out at a lower temperature. The probe activity was measured by diluting (1:1000) a small sample in scintillation fluid (Perkin Elmer, MA, USA) and activity was measured using a Packard Tri-CARB 2100 TR liquid scintillation counter (Perkin, MA, USA). Labelling was considered acceptable if values were between 200,000-800,000 counts per minute (cpm).

2.4.2.5. In-situ hybridisation

For each experiment, two or three separate series of fixed brain slices were used to total at least 6 per experimental condition. For each brain, 2 slices were labelled with radioactive probe to determine total hybridisation levels and 1 slice was labelled with 'cold' probe to define the non-specific hybridisation level. Radiolabelled probe was

incubated with 2% 1M DTT and 100µl hybridisation buffer before being added to each slide. Hybridisation buffer (25ml deionised formamide, 10ml 20X saline-sodium citrate buffer (SSC) (3M NaCl, 0.3M sodium citrate),2.5ml 0.5M sodium phosphate, 0.5ml 0.1M sodium phosphate, 0.5ml 0.1M sodium pyrophosphate, 5ml 50X Denhardts solution, 2.5ml 4mg/ml acid-alkali hydrolysed salmon sperm DNA, 1 ml 5mg/ml polyadenylic acid, 50ul 120mg/ml heparin and 5g dextran sulphate (Sigma-Aldrich, Dorset, UK) was made up at least a week before experiment and stored in the dark at 4°C.

To determine the non-specific hybridisation level, unradiolabelled probe was added to radiolabelled probe (with HYB buffer and DTT) at a ratio of 8:1 and applied to the remaining slide. Slides with applied probes were sealed with a Parafilm coverstrip (Sigma-Aldrich, Dorset, UK), to ensure even probe coverage across all brain sections on each slide and to form the necessary matrix for ISH. All slides were sealed in airtight humidified plastic chambers and incubated at 42°C for at least 8 hours. Parafilm coverslips were removed by placing slides in 1X SSC at room temperature. Slides were then washed thoroughly in 1X SSC at 52°C for one hour followed by a 1 min 0.1X SSC wash and finally dehydrated for one minute in 70% ethanol and one minute in 95% ethanol.

2.4.2.6. Film development

Slides were left to air-dry before being transferred to Amersham rapid development cassettes (Thermo Fisher Scientific, Delaware, USA), along with a ^{14}C ladder slide (American Radiolabelled Chemicals, Saint Louis, USA) to allow for quantification. Carestream Biomax MR Film (Anachem, Luton, UK) was then placed in the same cassette, covering all slides. Films were exposed to slides for one week before being developed in a dark room using an automatic developer (Photon Imaging Systems, Swindon, UK).

2.4.2.7. Analysis

Following development, films were scanned (Epson Photo Scanner V330, Hertfordshire, UK) at high resolution and viewed in ImageJ (Version 1.51a, NIH, USA). A standard curve was created from the ^{14}C ladder and used to covert radiolabelled regions of interest into pixel intensity values. Five measurements were taken from each region of interest, from each hemisphere of each radiolabelled brain slice. Measurements were taken in parallel from the non-radiolabelled slide and subtracted from the radiolabelled measurements to account for non-specific binding of the probe. One-way ANOVAs were conducted to assess the effect of experimental condition on normalised density value of the probe within the region(s) of interest. Litter was nested in to experimental group as a random effect.

2.4.3 Western Blotting

Western blotting is a technique used to detect specific proteins within a tissue extract.

2.4.3.1. Tissue homogenisation

50mg of hippocampus tissue from flash-frozen hemibrains was washed briefly in chilled 0.1M PBS and transferred to a Dounce homogeniser. 500ul radioimmunoprecipitation assay (RIPA) buffer (150mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 50mM Tris, pH 8.0) with cOmplete protease inhibitor cocktail tablet (Roche, Sussex, UK) (1 tablet per 10ml) was also added to the homogeniser. The tissue was homogenised thoroughly, transferred to an Eppendorf and span for 20 minutes at 12,000g at 4°C. The supernatant was then transferred to a new Eppendorf and stored at -20°C.

2.4.3.2. Bicinchoninic (BCA) protein assay

The BCA protein assay is used to quantify the total protein in the homogenate. This assay combines the Cu^{2+} reduction to Cu^{1+} by protein in an alkaline environment which forms a light blue colour with a BCA-Cu^{1+} reaction to form a dark-purple colour.

This dark-purple colour has a strong linear absorbance at 562nm with increasing protein concentrations. A range of bovine serum albumin (BSA) standards (Sigma-Aldrich, Dorset, UK) from 0.125mg/ml – 2mg/ml were prepared with Milli-Q water and 10% RIPA buffer. 5µl of tissue homogenate from experimental samples were diluted in 45µl Milli-Q water. BCA Reagent A and B from the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Delaware, USA) were mixed at a 50:1 ratio to form the BCA working reagent. 1ml of BCA working reagent was added to 50ul of standards and samples, mixed well and all samples incubated at 37°C for 30 minutes. Standards and samples were poured into a clean cuvette and all absorbance readings were taken on a spectrophotometer at 562nm, all within 10 minutes. A cuvette filled with water was used to blank the spectrophotometer. The absorbance values for the standards were used to plot a standard curve of BSA concentration and the resulting equation of the line of best fit used to determine concentration of the samples.

2.4.3.3. Gel electrophoresis

2 x Bio-Rad Laemmli Sample Buffer (Bio-Rad Laboratories Ltd, Watford, UK) was added to ß-mercaptoethanol at a 20:1 ratio. Homogenate samples were diluted in RIPA buffer in order to load 15-25µg of protein per well. Samples were added to Laemmli buffer at a 1:1 ratio and this mixture was heated at 96°C for five minutes in order to denature the protein-protein interactions. Samples were then loaded onto a 7.5% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories Ltd, Watford UK), along with Precision Plus Protein™ Kaleidoscope Standards protein ladder for calibration. Samples were added so that groups were counter-balanced across the gel. Gels were run in Tris/Glycine/SDS running buffer at 85V for 20 minutes and then for a further hour at 115V. The protein was then transferred to a nitrocellulose membrane using the Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories Ltd, Watford, UK).

2.4.3.4. Antibody application

Membranes containing the transferred protein were washed in Tris-Buffered Saline (20mM Tris, 150mM NaCl, pH 7.6) with 0.1% Tween 20 (TBST). Membranes were then blocked in 5% milk or BSA for one hour at room temperature. Primary antibodies were diluted to appropriate concentrations in 5% milk or BSA and incubated with the membrane overnight at 4°C, with GAPDH acting as a loading control. Membranes were then subject to 3 x TBST washes for 10 minutes each before incubation with the appropriate fluorescent IRDye 680RD secondary antibodies at 1:15,000 dilution. After another series of TBST washes, membranes were imaged on Odyssey® CLx Imaging System (Li-COR, Germany) in the 700nm channel.

2.4.3.5. Analysis

Densitometric analysis of bands was performed using ImageLab 6.0 (https://imagej.nih.gov/ij/). The densities (with background subtracted) of the protein of interest were divided by the loading control densities for each sample to provide normalised values. Densities were then averaged by group. Results were analysed for normal distribution and homogeneity of variances and transformed if necessary. One-Way ANOVAs were then employed to determine any statistical effect, with litter nested as a random variable.

2.4.4 Immunohistochemistry

Immunohistochemistry uses antibodies to determine the tissue distribution of an antigen and is useful for observing protein expression in brain slices.

2.4.4.1. Hippocampal sectioning for immunohistochemistry

Perfused brains were extracted from sucrose solution and dissected to remove the prefrontal cortex and the cerebellum. The remaining brain was mounted, posterior side down, to a frozen cryostat chuck using Tissue-Tek® OCT solution. The chuck and attached tissue was left to freeze at -20°C overnight. The brain was then

sectioned coronally in 40um slices from co-ordinates obtained from Paxinos and Watson (2005): Bregma -2.04mm to -4.68mm (dorsal hippocampus) using a cryostact (Leica Microsystems CM1860UV). Sections were placed into a 12-well plastic plate (Sigma) into sterile 0.1M PBS to provide free-floating sections.

2.4.4.2. Immunohistochemical staining

For nuclear staining (e.g. BrdU), sections were first incubated at 37°C for 30 minutes in 2M hydrochloric acid to allow for DNA hydrolysis. Sections were then washed 5 times in PBS. Sections were blocked in blocking agent appropriate to antibody for two hours at room temperature. Primary antibodies were diluted in blocking solution to appropriate concentrations and allowed to bind sections overnight at 4°C. Sections were washed in 0.1M PBS (or blocking solution) at least three times and incubated with Alexa secondary antibodies for 2 hours at room temperature in the dark. Sections were washed with 0.1M PBS and incubated for ten minutes in the dark with DAPI stain (1:200) for nuclei staining. Sections were washed twice more in 0.1M PBS before being mounted on standard microscopy slides using Mowiol aqueous mounting medium and standard cover slips. Sections were imaged using an epifluorescent microscope (Leica DM6000B Upright Timelapse System with Leica Application Suite Advanced Fluorescence 3.0.0 build 8134 software, Leica Microsystems) at x20 magnification.

2.4.4.3. Analysis

For every animal, one in 12 sections throughout the brain region of interest were stained to be counted. Immunopositive cells were counted following the division of the hippocampus into distant regions: CA1, CA3 and DG. These regions were then also divided into their respective sublayers (Figure). A defined area was measured for each region of interest and the number of immunopositive cells quantified through visual counting, giving a cell count per mm^2. This analysis was performed using

ImageJ 6.0 software with the associated plugin 'Cell Counter' (https://imagej.nih.gov/ij/plugins/cell-counter.html). Each count represents the average of at least 6 individually stained hippocampi sections from each brain. Cell counts were then compared subject to genotype. Counts were checked for normality and homogeneity of variances (Levene's test) and transformed if appropriate. Two-factor ANOVA factorial models were conducted for each region to determine the effect of genotype in each sublayer of the hippocampal region (sublayer*genotype). If a significant interaction was found, post-hoc analysis was performed using Tukey Kramer analysis, taking multiple comparisons into account.

Chapter 3: The expression of *Cacna1c* following prepubertal stress

3.1 Introduction

Genetic variation and environmental factors are both implicated in increasing risk for psychiatric disorders. This has generated interest in the possibility that genes and the environment may interact to influence disease risk, either through a genetic factor influencing sensitivity to a particular environment, or, alternatively, an environmental factor leading to significant risk gene expression changes (Brydges *et al.*, 2014; Uher, 2014). This chapter investigates the effect of prepubertal stress (PPS) on the expression of the psychiatric risk gene *Cacna1c* and associated pathways.

3.1.1 Early life stress and gene-environment interactions

Stress in early life is highly associated with long-lasting changes in the brain that can affect multiple neuroendocrine and behavioural processes (Marco et al., 2011). Early life stress (ELS) includes physical, sexual and emotional abuse as well as physical and emotional neglect in early life, typically occurring before puberty. Multiple meta-analyses and systematic reviews in humans have found that all subtypes of ELS can predict the development of psychopathology in adults from SCZ to anxiety disorders (Carr et al., 2013) with overwhelming evidence that ELS can trigger, aggravate and maintain the occurrence of disorder.

As well as the environment, genetic risk factors are important contributors to risk for several psychiatric disorders and brain development. There have been increasing interest in examining whether established risk genes interact with recognised environmental components in order to dictate disorder risk. In primates, possessing

a loss-of-function polymorphism in the serotonin transporter gene (*5-HTT*) was found to influence anxiety and depressive phenotypes, an effect more pronounced when combined with maternal deprivation (Bennett et al., 2002). The high risk allele for the dopamine D2 receptor gene (*DRD2*) has also been largely associated with interacting with stress in humans – for example, children with the risk allele displayed greater extraversion when living in an alcoholic home in comparison to a non-alcoholic home (Ozkaragoz and Noble, 2000). *BDNF,* widely implicated in psychiatric disorder due to its crucial role in plasticity, neurogenesis and development in the brain, is reduced following stress in animal models (Schmidt and Duman, 2007) and in humans with depression. Abnormalities in BDNF signalling has also been implicated in neurological processes and behavioural changes in psychiatric disease (Ray et al., 2014). The val66met polymorphism results in reduced BDNF activity in humans and, although this genotype is not directly associated with mood disorders itself, has been seen to modify the effect that stressful life events had on the onset of depression (Kim et al., 2007; Brown et al., 2014).

3.1.2 LTCC expression in stressful conditions

Risk SNPs for *Cacna1c* have been shown to affect measures of anxiety and cognitive functioning which may suggest an increased susceptibility to the effects of adverse life events, such as early life stress. CACNA1C SNPs rs73248708 (residing in intron 3) and rs116625684 (intron 1) have been shown to interact with adult trauma to predict depressive symptoms in healthy humans in an African American cohort, which was a highly traumatised group (Dedic et al., 2018). The same authors also report that heterozygous deletion of Cacna1c during embryonic development in mice increases the susceptibility to chronic social defeat stress in adulthood (Dedic et al., 2018).

VGCCs are thought to play an important role in the development of adaptive changes associated with stress. After stress, corticosteroid levels rise, activating both GRs and

MRs. Corticosterone, through stress or *in vitro* glucocorticoid receptor activation, enhances L-type calcium currents in mouse CA1 pyramidal neurons (Kerr et al., 1992; Karst et al., 2002; Chameau et al., 2007). It is suggested that this increased calcium influx contributes to what is known as a spike-frequency adaptation – which is the decrease of neuronal firing rate to a stimulus of constant intensity (Joels and de Kloet, 1989; Kerr et al., 1989). Spike-frequency adaption could contribute to normalisation of CA1 activity following stress, however enhanced calcium activity can also be disadvantageous to the cells after long periods. Corticosterone does not alter the expression of $Ca_V1.2$ or $Ca_V1.3$ in the CA1, however does increase the expression of the auxiliary β4 subunit (Chameau et al., 2007). This may act to increase the surface expression of the channel. However it does not appear that corticosterone affects any calcium channel currents in the DG or have an effect on $Ca_V1.2$ mRNA expression (Van Gemert et al., 2009).

Acute and chronic restraint stress in rats resulted in an elevated density of L-type Ca^{2+} channels in the hippocampus, cortex and basolateral amygdala (Mamczarz and Vetulani, 1997; Maigaard et al., 2012). Nifedipine, a calcium channel blocker, given pre-restraint stress, blocked this increase and also prevented the stress-induced increase of locomotor activity. Interestingly electroconvulsive stimulations also reversed the stress-induced increase in $Ca_V1.2$ channels (Mamczarz and Vetulani, 1997). A recent study also reported that chronic stress led to a delayed increase in $Ca_V1.2$ protein in the prefrontal cortex, however no effect was seen in the hippocampus or amygdala (Bavley et al., 2017). *Cacna1c* mRNA levels in the nucleus accumbens were reduced in mice susceptible to the harmful effects of chronic social defeat stress; these animals also presented with social interaction and hedonic response impairments (Terrillion et al., 2017). However, to the best of our knowledge, there have not been any functional studies evaluating the effect of early life stress on *Cacna1c*. Therefore there is a body of evidence that suggests a link between stress

and $Ca_V1.2$/*Cacna1c*, although exactly how these two factors may interact has yet to be fully determined.

3.1.3 Stress-induced epigenetic changes in Cacna1c

CACNA1C may be susceptible to changes in expression following an environmental challenge via epigenetics, of which DNA methylation is the most studied and understood. Nieratschke et al (2014) compared methylation data from subjects who had experienced prenatal stress with controls in human and monkey peripheral tissue and rodent PFC (Nieratschker et al., 2014). They identified *CACNA1C* as one of only 30 genes whose methylation status was associated with prenatal stress in all tissues and species analysed. By analysing the promoter regions, they showed that, in humans, those who had a history of prenatal stress had a lower degree of methylation at the *CACNA1C* promoter within blood. However in adult rat PFC, prenatal stress signified more methylation at this promoter region (Nieratschker et al., 2014). This suggests that expression of this gene can be driven by methylation changes, and that experiencing stress in early life may affect methylation at promoter regions – driving or reducing expression of *CACNA1C.* This also suggests that stress may affect methylation differentially in the CNS vs peripheral tissues, and between different species.

Interestingly, *CACNA1C* methylation has been shown to be altered in bipolar disorder. Starnawska et al (2016) investigated six CpG sites within CG1 3 (a CpG island in intron 3) of *CACNA1C*. Five out of six of these sites showed significant hypermethylation in those with bipolar disorder (Starnawska et al., 2016). They also showed that this methylation could be driven by SNPs nearby the CpG sites, suggesting that the non-coding risk variants could drive a shift in DNA methylation in those with disorder (Starnawska et al., 2016). A recent pilot study looking at the link between *CACNA1C* and suicidality showed that people who had previously attempted suicide had altered methylation status in comparison to controls at 2 CpG sites within

the transcription factor binding site of *CACNA1C.* The methylation at these sites was positively correlated with fMRI signal intensity in the left thalamus after the subjects were shown triggering images (Kim et al., 2017). Therefore it is certainly possible that *CACNA1C* is subject to epigenetic regulation and this can have direct effects on expression.

This chapter looks to explore the effect of prepubertal stress on the expression of *Cacna1c* in the rat hippocampus in order to investigate the potential interaction between *Cacna1c* and early life stress.

3.2 Methods

3.2.1 Contributions

The prepubertal stress procedure in this experiment was performed by Dr N Brydges, cDNA synthesis was conducted by C Best and A Moon. All other analysis was conducted by A Moon.

3.2.2 Animals

Six litters of male and female Lister Hooded rats were subject to prepubertal stress (PPS) at PND 25-27 and housed in same-litter, same-sex standard housing cages as previously described (Figure 2.1) with ad libitum access to food and water. Six litters of male and female Lister Hooded rats acted as control animals. At PND 60, animals were sacrificed in home cages using a rising concentration of CO_2. One male and one female were taken from each litter (if available) for each molecular experiment (qPCR, ISH or WB). Brains were rapidly removed post-mortem and the hippocampus immediately micro-dissected before being flash frozen on dry-ice. No animals used in molecular analysis were subject to any behavioural manipulations prior to sacrifice.

3.2.3 Human samples

Post-mortem hippocampal tissue from individuals who had suffered early life trauma and controls were obtained with ethical approval and upon informed consent from the Edinburgh Brain Bank. 5 males and 5 females who had experienced early life stress were compared with 8 control males and were matched for post-mortem delay.

3.2.4 qPCR

Samples from nine control and nine PPS male rats and ten control and eight PPS female rats were used in this experiment. RNA was extracted from tissue using the Qiagen RNeasy Kit (Qiagen, Manchester, UK) and DNAse treated in accordance with the supplied protocols. RNA was then used to create cDNA for analysis using RNA

to cDNA Easy Premix (Clontech Laboratories Inc., France), heated at 42°C for 75 minutes, following by 80°C for 15 minutes. Sample was then diluted 1:15 in nuclease-Free water. 96-well plates were loaded, each well with 15ul reaction mixture (1.9µl sterile RNAase free water, 0.3µl 10µM forward primer, 0.3µl 10µM backward primer, 7.5µl SensiMix (Bioline) and 5µl cDNA). Primer sequences are supplied in Table 3.1. Expression of the gene of interest and housekeeping genes for each sample were included on the same plate. Plates were run on a Real-Time PCR instrument (Applied Biosystems®, UK) and ran at 95°C for 10 minutes, followed by 45 cycles of 15s at 95°C and 1 minute at 60°C. Finally, samples were heated to 55°C for 60s and 95°C for 15s. Results were quantified using the comparative Ct method ($2^{-\Delta\Delta Ct}$ method).

Table 3.1: Primer sequences used for qPCR analysis

Gene	Organism	Primer sequence
Gapdh	Rat	F: 5'-TCTCTGCTCCTCCCTGTTCT R: 5'- TACGGCCAAATCCGTTCACA
GAPDH	Human	F: 5'-CGCTCTCTGCTCCTCCTGTT R: 5'- CCATGGTGTCTGAGCGATGT
Hprt	Rat	F: 5'-TCCTCCTCAGACCGCTTTTC R: 5'- ATCACTAATCACGACGCTGGG
UBC	Human	F: 5'-CACTTGGTCCTGCGCTTGA R: 5'-TTATTGGGAATGCAACAACTTTAT
Cacna1c	Rat	F: 5'-ATGGTTCTTGTCAGCATGTTGCGG R: 5'-TGCAAATGTGGAACCGGTAAGTG
CACNA1C	Human	F: 5'-TGACTATTTTTGCCAATTGTGTGG R: 5'-GCGGAGGTAGGCATTGGG
Cacna1d	Rat	F: 5'-AAATCCAAGCTCAGATCGCACG R: 5'-CAAGTGGGCTGAGAACCTAGAC
Cacna1i	Rat	F: 5'-TGTAACCCGTGGTTCGAGTG R: 5'-GATCTTGCAACGGTCCGACA
Cacnb2	Rat	F: 5'-GGTGAAGCCACGCTCTGACT R: 5'-AGTCTGCCGAACCATAGGACAC
BDNF IX	Rat	F: 5'-GGTTATTTCATACTTCGGTTGC R: 5'-CCCATTCACGCTCTCCAG
BDNF IV	Rat	F: 5'-TAATACTCGCACGCCTTC R: 5'-ACCCACTTTCCCATTCAC
BDNF I	Rat	F: 5'-CTTTGGGGCAGACGAGAAAG R: 5'-ACCTGGTGGAACTCAGGGTCAG
BDNF II	Rat	F: 5'-CTAGCCACCGGGGTGGTGTAA R: 5'-TCACGTGCTCAAAAGTGTCAG
BDNF VI	Rat	F: 5'-ACCTGGTGGAACTCAGGG R: 5'-GCAGACGAGAAAGCGCAC

3.2.5 *In-situ* hybridisation

Nine control and nine male PPS rats were utilised for this experiment. Brains were dissected out post-mortem and flash frozen as whole brains on dry-ice. Brains were sliced in 14 µm coronal sections through the hippocampus and mounted on poly-L-lysine coated slides and fixed in 4% paraformaldehyde (PFA). Slides were then subject to ethanol dehydration and storage in 95% ethanol. The oligonucleotide probe designed to target *Cacna1c* is listed in Table 3.2. These probes were end-labelled with ^{35}S-ATP and hybridised to the tissue sections. For each individual rat, two slides were labelled with radioactive probe whilst one slide formed a 'non-specific' slide, where excess unlabelled probe was added at a ratio of 8:1 to define non-specific hybridisation of the probe to the tissue. Slides were incubated overnight at 42°C and then washed in successive SSC baths before being dehydrated in 70% and 95% ethanol. Slides were mounted together in a development cassette with radiographic film and left for 7 days before film development. mRNA expression, as measured by bound radiolabelled probe to the film, was quantified by densitometric analysis of the radiographic film.

Table 3.2: In-situ hybridisation probe sequence

Gene	***Accession number for FASTA seq***	***Nucleotide span***	***Probe sequence***
Cacna1c	NM_012517.2	3940-3896 (within 27th exon)	3' – TCGAAGTAGGTGGAGTTGACCACGTACC ACACTTTGTACTGGTGC – 5'

3.2.6 Western Blots

Seven control animals and six male PPS animals were used for the BDNF and Cacna1c experiments. Ten control animals and 9 female PPS animals were used for the BDNF experiment. Brains were removed post-mortem, the hippocampus dissected out and flash frozen. Hippocampal tissue was homogenised in RIPA buffer with protease inhibitors and protein concentrations determined using the bicinchoninic protein assay (BCA). Equal amounts of protein (15-25ug) per sample was mixed 1:1 with Laemmli Buffer (containing 5% ß-mercaptoethanol) and heated at 95°C for 5 minutes. Samples were loaded onto a 7.5% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories Ltd, UK) and ran at 85V for 20 minutes, followed by 120V for one hour. Protein was then transferred to a nitrocellulose membrane using Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories Ltd, Watford, UK). Membranes were blocked with 5% BSA (Cav1.2) or milk (BDNF). Primary antibodies were diluted in 5% BSA for anti-Cav1.2 (1:200, Merck, UK) and 5% milk for anti-BDNF (1:500, Icosagen, USA) (Table 3.3) and incubated with the membrane overnight at 4°C. Fluorescent secondary antibodies (1:15,000: IRDye 680RD; LiCor, Germany) were then allowed to bind at room temperature for 1 hour. Membranes were imaged on Odyssey® CLx Imaging System (Li-COR, Germany) in the 700nm channel. Protein expression was measured by densitometric analysis of the bands.

3.2.7 Statistics

Once quantified, results were analysed for homogeneity of variance (Levene's Test) and normality of distribution. If data was not normally distributed, Box-Cox transformations or square-root transformations were applied. For qPCR, One-Way ANOVAS were performed, with normalised Ct values for each gene forming the dependent variable and 'CON/PPS' group forming the independent variable (between subjects), with litter of origin nested as a random variable. For ISH, One-Way ANOVAS was performed on each region (CA1, CA3 and DG) separately, with

normalised intensity values as the dependent variable. CON/PPS group formed the independent variable, with litter of origin nested as a random variable. For western blot analysis, one-way ANOVAs were performed on normalised band intensity values (dependent factor), with CON/PPS group as the independent variable, with litter nested as a random variable. For each experiment where they were both used, males and females were analysed in separate ANOVA tests. All statistics were performed by JMP statistical software (JMP, SAS Institute, Cary, NC, USA).

Table 3.3: Primary antibodies utilised in western blot analysis

Target	***Host***	***Manufacturer***	***Cat. No***	***Dilution***
Cav1.2	Mouse (monoclonal)	Millipore	MAB13170	1:200
BDNF	Mouse (monoclonal)	Icosagen	329-100	1:500

3.3 Results

3.3.1 Cacna1c mRNA expression is altered in the rat hippocampus in adulthood following prepubertal stress in a sex-specific manner

Quantitative PCR was utilised to quantify the amount of *Cacna1c* mRNA in the whole hippocampus of PPS rats in comparison to control rats. *Cacna1c* mRNA was found to be significantly decreased in the hippocampus of PPS male rats (Group: $F_{(1, 17)}$ = 6.69, p = 0.019, One-Way ANOVA) (Figure 3.1). However there was no difference in *Cacna1c* expression in PPS female rats compared to control females (Group: $F_{(1, 17)}$ =0.18, p=0.39, One-Way ANOVA) (Figure 3.1). This suggests that *Cacna1c* is vulnerable to stress within the hippocampus in male rats, however its expression is preserved in female rats.

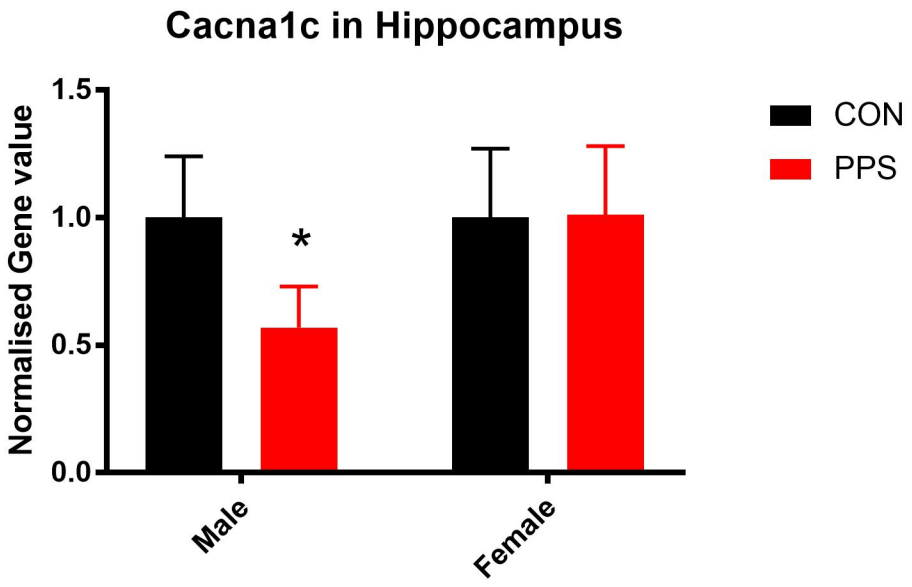

Figure 3.1: *Cacna1c* mRNA expression in control and PPS rats in the hippocampus. A significant 43% reduction was seen in the male PPS rats in comparison to male control rats. No differences were observed in female rats. Bars represent mean fold change for each group, error bars are SEM. Males: N = 9 CON, 9 PPS, Females = 10 CON, 8 PPS, * P < 0.05

3.3.2 The hippocampal defect in *Cacna1c* following PPS is specific to CA1 and CA3 subregions in male rats

This deficit in *Cacna1c* mRNA in the hippocampus as seen by qPCR suggests a general decrease within the hippocampal formation. However, this experiment did not determine which hippocampal fields may be contributing to this effect as qPCR samples were representative of the whole hippocampus. Thus, a different cohort of PPS male rats and their controls were used to set up an *in-situ* hybridisation experiment using a probe targeting *Cacna1c* mRNA. This revealed an 75% decrease in *Cacna1c* mRNA in the CA1 region (Group: $F_{(1, 17)} = 14.22$, $p = 0.002$, One-Way ANOVA), a 35% decrease in CA3 region (Group: $F_{(1, 17)} = 4.99$, $p = 0.040$, One-Way ANOVA), but no decrease was seen within the DG (Group: $F_{(1, 17)} = 0.05$, $p = 0.83$) One-Way ANOVA) (Figure 3.2).

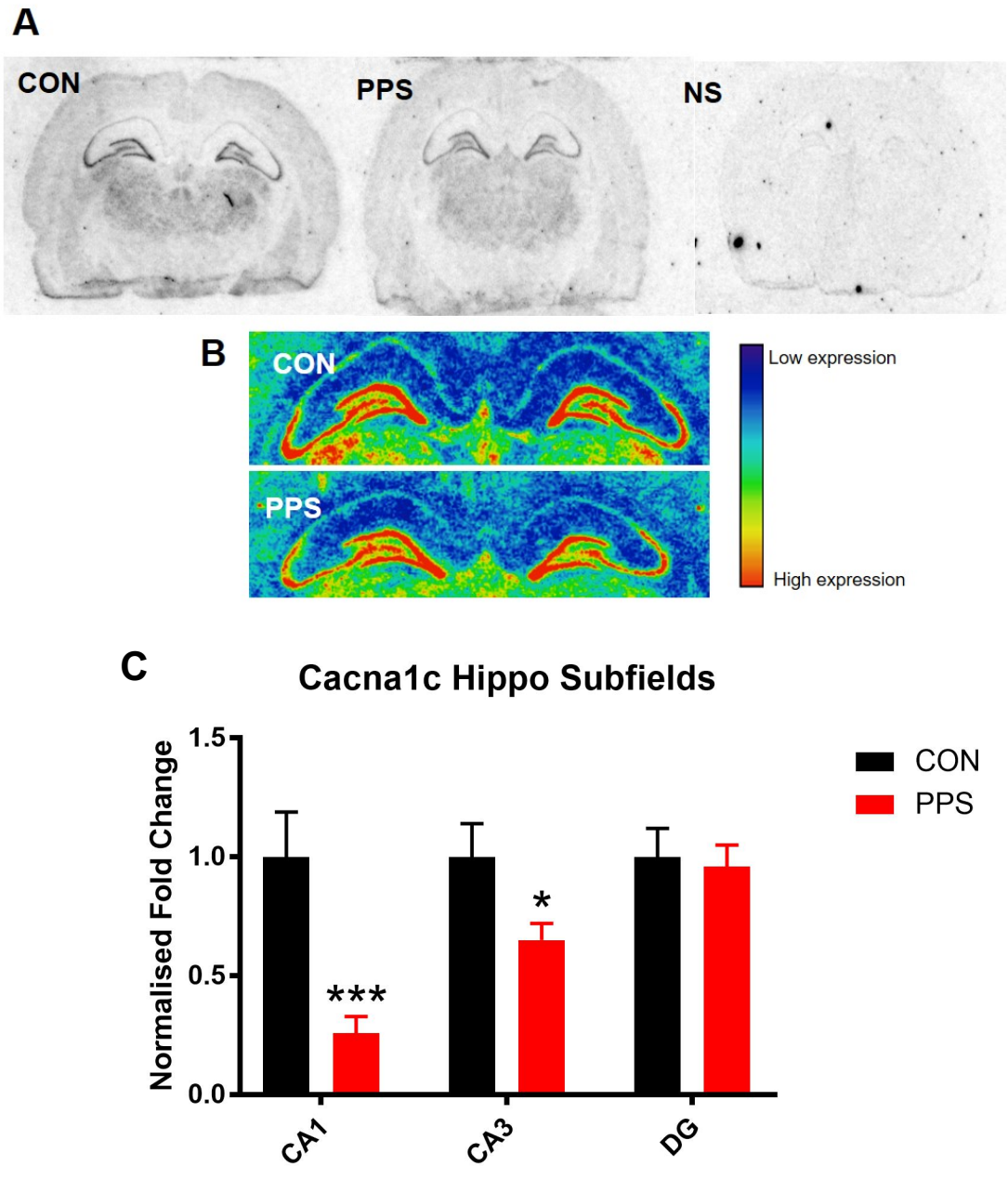

Figure 3.2: *Cacna1c* mRNA expression is significantly decreased in the CA1 and CA3 of PPS rats. A = representative radiographic film images of stressed and control rats and a non-specific slide to demonstrate background binding, B = heat map of the same radiographic film, showing the reduced expression of *Cacna1c*, C = quantification of in-situ hybridisation density values; bars represent mean density values, error bars are SEM. N = 9 CON, 9 PPS, * $p < 0.05$, ** $P < 0.01$

3.3.3 Cav1.2 protein levels following PPS show a trend to being decreased in the hippocampus in rats

Cacna1c is translated into Cav1.2 protein, thus Cav1.2 protein levels were analysed to determine if the decrease observed in mRNA was also maintained at protein level, and, therefore, may be functionally relevant. PPS rats had less, on average, Cav1.2 protein in the hippocampus which bordered on significant (Group: $F_{(1,12)} = 4.40$, $p = 0.060$, One-Way ANOVA) (Figure 3.3).

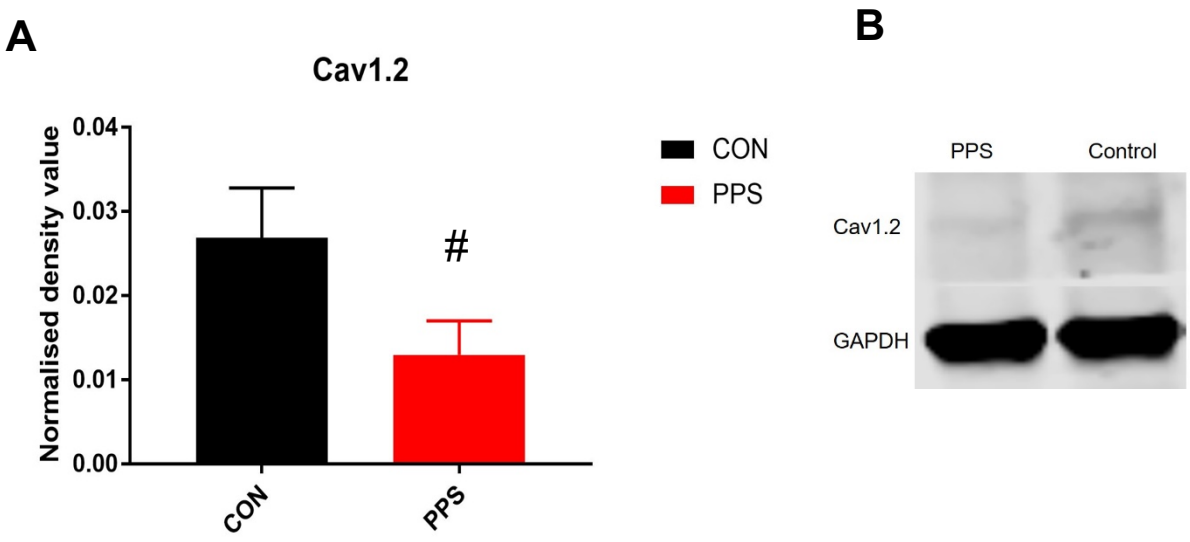

Figure 3.3: Cav1.2 protein trended to being decreased in the hippocampus of male PPS rats compared to non-stressed littermates. A = Quantification of western blot density bands; bars represent mean density values, error bars are SEM. B = representative western blot of Cav1.2 protein with GAPDH loading control. N = 7 CON, 6 PPS animals, # = p < 0.1.

3.3.4 The expression of *CACNA1C* in human cDNA stratified by early life stress

CACNA1C mRNA was assessed in humans using hippocampal post-mortem tissue from individuals that experienced early life stress and those that did not. There was no significant difference when comparing all controls with those who had experienced early trauma (Group: $F_{(1, 17)} = 1.74$, $p = 0.205$, One-Way ANOVA (CON males vs PPS males and females)) (Figure 3.4). However, it was noted that all controls were male, and my previous work suggests that there may be a sex-specific effect of *Cacna1c* dysfunction following stress in young life (Figure 3.4). Thus, the female subjects were removed from the analysis. This resulted in a trend to decreased *Cacna1c* in the hippocampus of humans subject to early life stress (Group: $F_{(1, 12)} = 3.26$, $p = 0.098$, One-Way ANOVA) (Figure 3.4). Whilst this result requires replication, it is consistent with the results seen in the rat model.

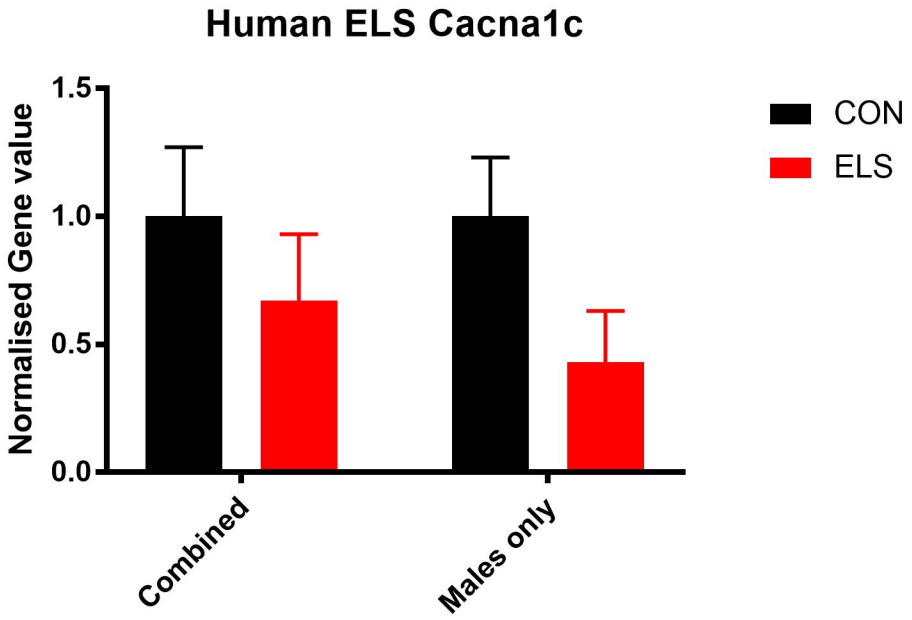

Figure 3.4: *CACNA1C* mRNA expression in hippocampal tissue from subjects who had experienced ELS compared to subjects with no childhood trauma. While there were no significant differences between the two groups, there was a trend to decreased *CACNA1C* in the hippocampus of male subjects. Bars represent mean fold change of *CACNA1C* for each group, error bars are SEM. N = 8 CON males, 5 ELS males, 5 ELS females.

3.3.5 The expression of other calcium channel risk genes following prepubertal stress in the rat hippocampus

In order to determine the specificity of these findings, other calcium channel risk genes *Cacna1d, Cacnb2 and Cacna1i* were assessed within the hippocampus of male PPS rats. *Cacna1d* encodes the Cav1.3 subunit of calcium channels and is often coexpressed with *Cacna1c* in cardiac and neural tissues. Therefore, the levels of *Cacna1d* were also examined to see if it was also affected, or to investigate any potential compensation. However there was no change in *Cacna1d* expression in the hippocampus of PPS rats in comparison to unstressed controls (Group: $F_{(1, 17)} = 0.37$, $p = 0.560$, One-Way ANOVA) (Figure 3.5A). Expression of *Cacna1i* (T-type channel alpha1 subunit) and *Cacnb2* (auxillary subunit β2), which have also been implicated in schizophrenia (Ripke et al., 2014; Li et al., 2017; Xie et al., 2018) were also analysed in the same samples as before. There was no difference found in *Cacna1i* (Group: $F_{(1, 17)} = 0.04$, $p = 0.845$, One-Way ANOVA) or *Cacn2b* (Group: $F_{(1, 17)} = 0.09$, $p = 0.769$, One-Way ANOVA) (Figure 3.5A), suggesting the deficit seen in *Cacna1c* following stress is specific to this subunit.

The expression of these risk genes was also investigated in female control and PPS rats. No alterations were observed in *Cacna1d* (Group: $F_{(1, 17)} = 0.37$, $p = 0.560$, One-Way ANOVA), *Cacn2b* (Group: $F_{(1, 17)} = 0.09$, $p = 0.769$, One-Way ANOVA) or *Cacna1i* (Group: $F_{(1, 17)} = 0.04$, $p = 0.846$, One-Way ANOVA) (Figure 3.5B).

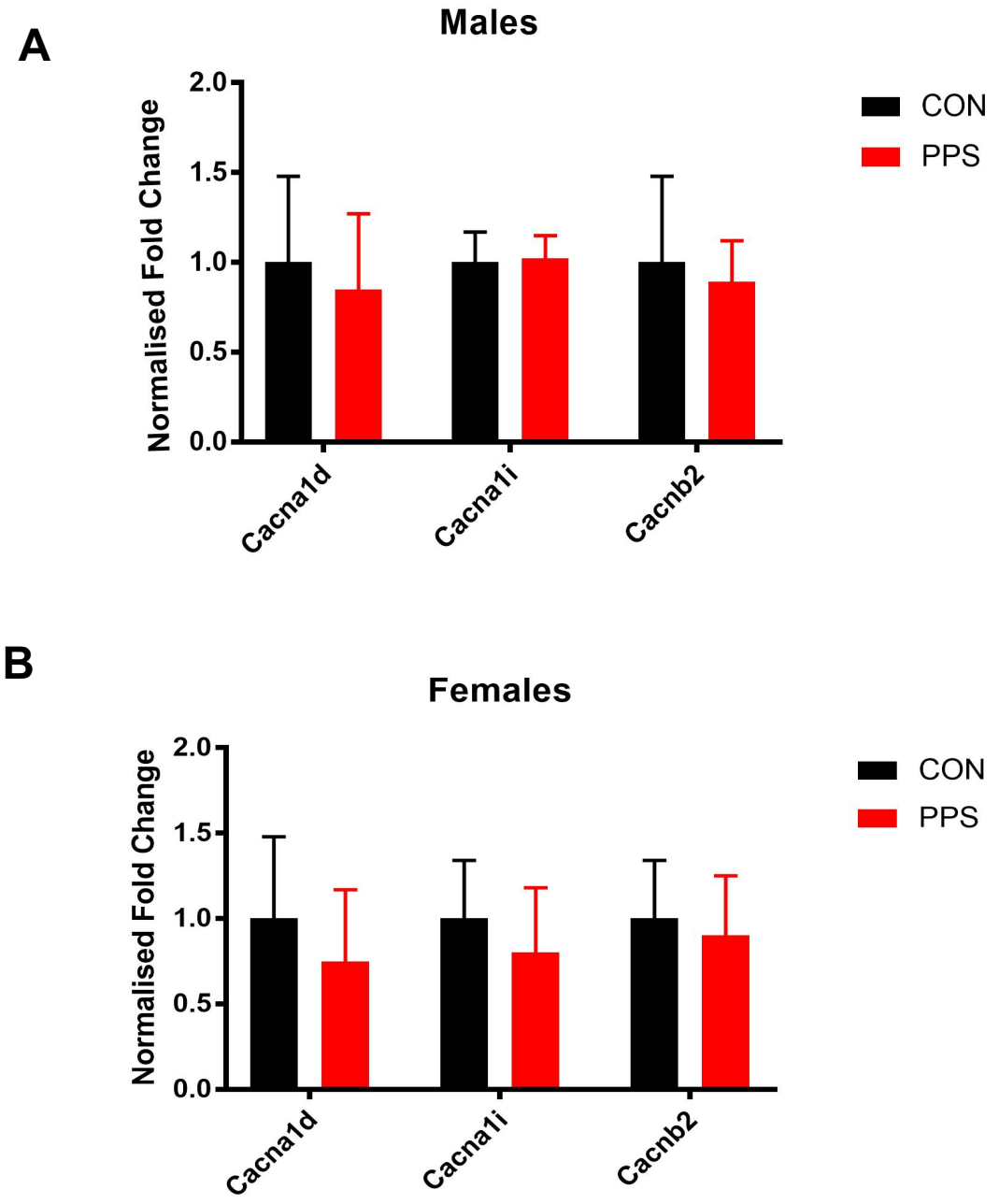

Figure 3.5: Cacna1d, Cacna1i and Cacnb2 mRNA expression in the hippocampus of both male and female PPS rats. There was no difference in any genes in either male or female PPS rats. Bars represent fold changes normalised to control animals, error bars are SEM, Males: N = 9 CON, 9 PPS, Females: N = 10 CON, 8 PPS.

3.3.6 BDNF expression following prepubertal stress in the hippocampus of rats

*BDNF i*s subject to transcriptional regulation by calcium influx via *Cacna1c* (West et al., 2001; Tao et al., 2002; Zheng et al., 2011). *BDNF* mRNA levels were analysed for variation in hippocampal expression in PPS rats and found to be significantly decreased (Group: $F_{(1,20)}$ =4.62, p = 0.045, One-Way ANOVA) in male rats (Figure 3.6A). This indicates a decrease in *BDNF* general expression, as these primers targeted exon IX, a common exon to all the variants of *BDNF*. Thus, exon-specific *BDNF* variants were also analysed. Transcripts I, II, IV and VI were chosen for analysis as the most abundant species in the rat hippocampus (Perovic et al., 2013). The *BDNF IV* exon containing transcript was significantly decreased in PPS male rats (Group: $F_{(1,20)}$ = 4.84, P = 0.040, One-Way ANOVA) (Figure 3.6A), however there was no significant different in *BDNF* transcripts containing exon I, II, VI.

In female rats, there was no difference in *BDNF IX* (Group: $F_{(1, 16)}$ = 0.376, p = 0.552, One-Way ANOVA) (Figure 3.6B). However when analysing each transcript variant, it was found that *BDNF VI* was significantly decreased in PPS rats (Group: $F_{(1, 7.136)}$ = 12.61, p = 0.009, One-Way ANOVA) (Figure 3.6B), but there was no significant difference in any other transcript.

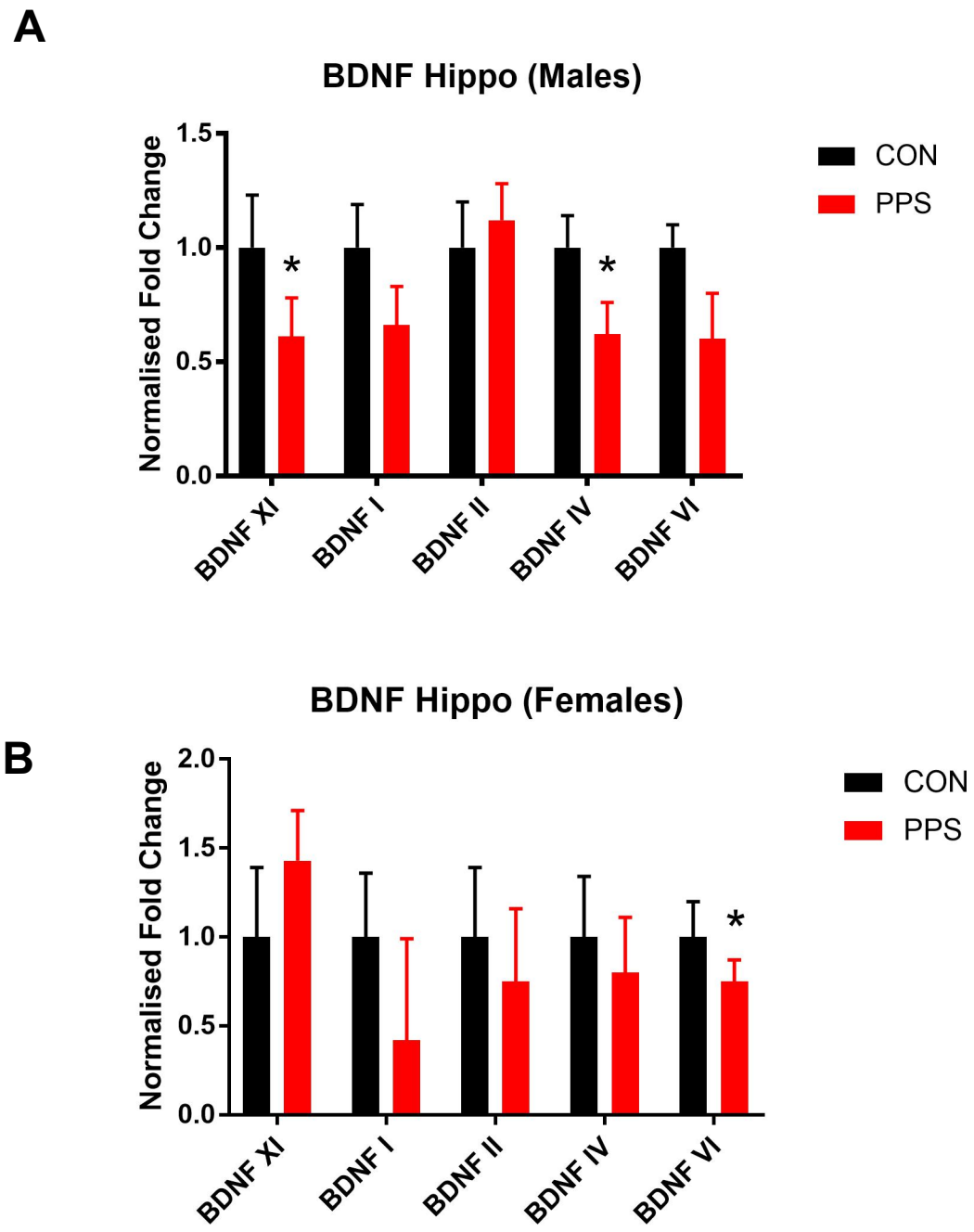

Figure 3.6: *BDNF* mRNA in control and PPS male and female rats. A = *BDNF* transcript mRNA expression in male rats within the hippocampus, revealing reduced expression of *BDNF IX* and IV, B = Hippocampal *BDNF VI* exon containing transcript mRNA is reduced in female rats. Bars represent mean fold changes of Ct values, error bars are SEM. Males: n = 9 CON, 9 PPS, Females: n = 10 CON, 8 PPS, * = p < 0.05.

3.3.7 Pro-BDNF and mature BDNF protein in the hippocampus of PPS rats

BDNF protein levels in the hippocampus were assessed in both the pro-BDNF and mature form by western blotting. Male PPS rats revealed no difference in pro-BDNF (Group: $F_{(1, 13)} = 0.972$, $p = 0.379$, One-Way ANOVA) or mature BDNF (Group: $F_{(1, 13)} = 0.006$, $p = 0.942$, One-Way ANOVA) (

Figure 3.7A and E). There was no difference in ratio of pro-BDNF:mature protein (Group: $F_{(1, 13)} = 1.372$, $p = 0.322$, One-Way ANOVA) (Figure 3.7C). However female PPS rats trended to an increased pro-BDNF protein (Group: $F_{(1, 19)} = 4.411$, $p = 0.084$) but no difference in mature protein (Group: $F_{(1, 19)} = 0.476$, $p = 0.570$, One-Way ANOVA) (

Figure 3.7B and E). There was a similar trend in ratio of pro-BDNF:mature protein (Group: $F_{(1, 19)} = 3.305$, $p = 0.087$, One-Way ANOVA) (Figure 3.7D).

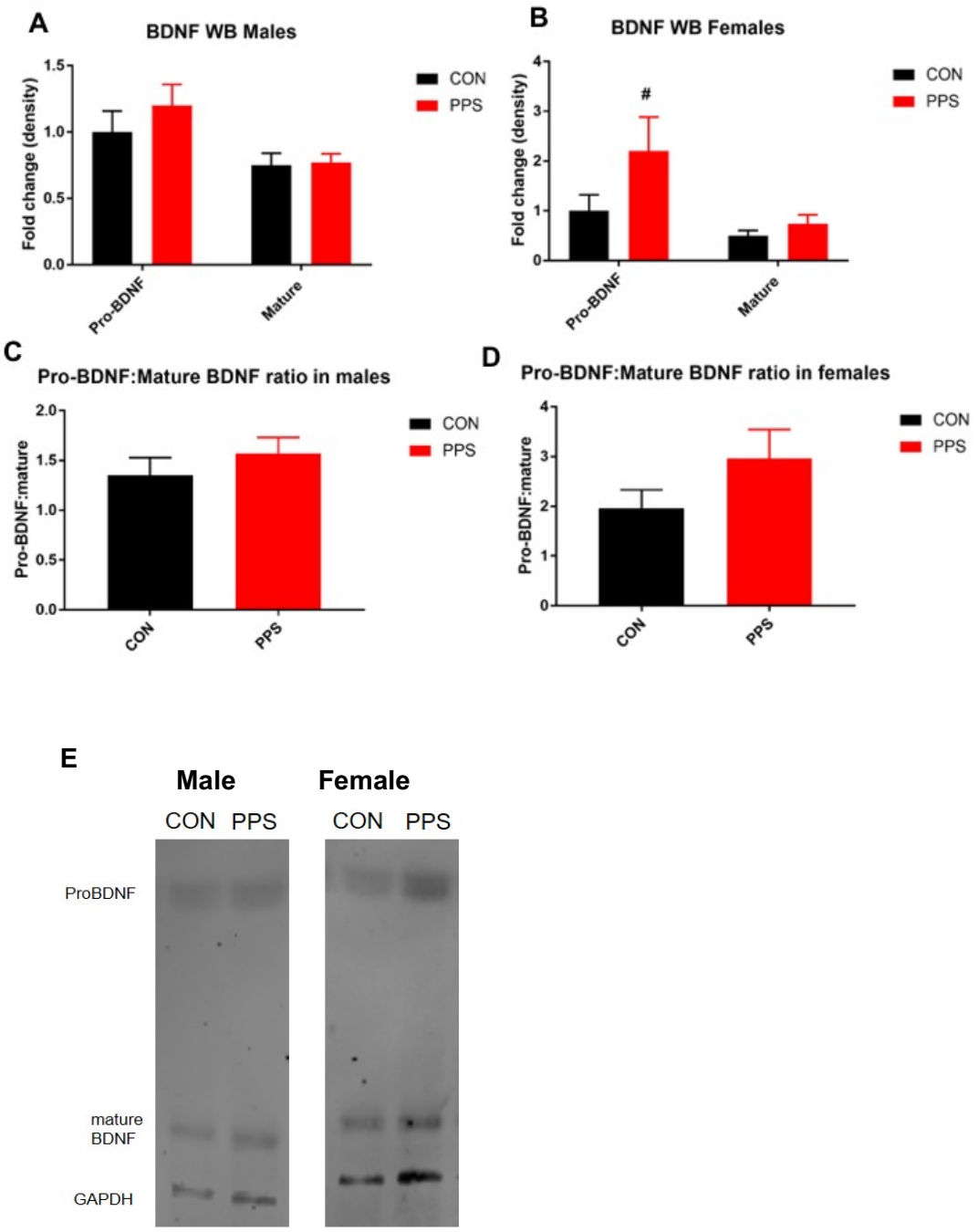

Figure 3.7: proBDNF and mature BDNF protein expression in the hippocampus of PPS rats. A-D = Graphical representations of proBDNF and BDNF expression in males (A and C) and females (B and D). proBDNF expression is increased in the hippocampus of female PPS rats, but there is no difference in the expression of the mature BDNF protein. Bars represent mean density values normalised to GAPDH, error bars are SEM. E = representative western blot of proBDNF and mature BDNF. Males: n = 7 CON, 6 PPS, Females: n = 10 CON, 9 PPS, # = $p < 0.1$.

3.4 Discussion

These experiments demonstrate that *Cacna1c* mRNA and $Ca_V1.2$ protein is significantly decreased in the hippocampus of PPS males, and this may have an impact on downstream pathways, demonstrated in this case by decreased *BDNF* expression.

3.4.1 Cacna1c/Cav1.2 expression following stress

The decrease in *Cacna1c* / $Ca_V1.2$ following PPS is consistent with recent evidence that this gene interacts with trauma and stress, and adds to the literature by showing that PPS has long lasting effects on *Cacna1c* expression that last into adulthood in rats. Dedic and colleagues showed that SNPs within *CACNA1C* predicted depressive symptoms when combined with adult trauma in humans, and in mice, *Cacna1c* heterozygosity increased stress vulnerability (Dedic et al., 2018). This reveals that *Cacna1c* and stress interact over species barriers. This cross-species relationship was also shown in this chapter; *Cacna1c* mRNA was decreased in both rats and humans who had experienced early life trauma (Figures 3.1 and 3.4). Although it is not currently clear as to whether the SNPs identified in Dedic's study lead to expression changes in *Cacna1c*, the current study adds to evidence that *Cacna1c* and stress interact to increase risk for psychiatric or mood disorders.

This study does apparently differ with *in vitro* work that revealed no $Ca_V1.2$ protein changes were apparent in the CA1 following acute corticosterone application (Chameau et al., 2007). This is likely due to the differences in applying stress hormones versus experiencing stress from the environment. Also importantly, our study looked at the consequences of variable stress in childhood on adulthood gene expression instead of an acute hit. This may suggest that the decreases in *Cacna1c* mRNA and protein expression is due to adaptive mechanisms, potentially through epigenetics, that occur over time and are long-lasting.

Also in contrast to our findings, other studies on rodents have shown stress is associated with increased $Ca_V1.2$ and *Cacna1c* in certain brain regions. Increased *Cacna1c* mRNA has been shown in the hippocampus and amygdala following chronic restraint stress (Maigaard et al., 2012) and chronic unpredictable stress increased $Ca_V1.2$ protein several days after stress in the prefrontal cortex (Bavley et al., 2017) and hippocampus (Yang et al., 2012). This increase in $Ca_V1.2$ in both brain regions was also associated with increases in depressive and anxiety-like behaviour, consistent with a stress-induced phenotype (Yang et al., 2012; Bavley et al., 2017).

It may therefore be surprising that we report a marked decrease in both mRNA and protein of $Ca_V1.2$ following PPS instead of an increase. However, this may be explained through the different stress procedures used and, importantly, the age at which the stress is given. The studies that revealed increased $Ca_V1.2$ were performed in adult rats and our studies were performed in the prepubertal stage. Early life stress can have a permanent and long-lasting effect on brain network development, particularly in childhood where the brain is vulnerable to environment experiences (Everson-Rose et al., 2003; Dich et al., 2015) and although adult trauma is still highly predictable of mood disorders, the effects are typically more transient (Chen and Baram, 2016). Therefore, the way that stress is experienced in childhood compared to adulthood is fundamentally different and may lead to different affected pathways and gene expression patterns. This is in line with epigenetic evidence that showed that adult rats who had experienced prenatal stress had increased methylation at the *Cacna1c* promoter, which is often associated with decreased gene expression (Nieratschker et al., 2014). Hypermethylation of a CpG island in intron 3 of *CACNA1C* has also been reported in bipolar disorder, suggesting that decreased *CACNA1C* expression may also be a feature of this disorder (Starnawska et al., 2016). Methylation analysis of *Cacna1c* following prepubertal stress would be a key future

study to determine if the expression changes seen are due to long-lasting epigenetic regulation.

There was no difference in mRNA expression between control and PPS female rats, suggesting a potential sexual dimorphism on the effect stress has on *Cacna1c* expression. Sexual dimorphism following stress is apparent in the literature, with the stress response and corresponding immune/inflammatory reaction being more robust in females, potentially through enhanced CRH neuron activation by oestrogen (Kirschbaum et al., 1996; Torpy et al., 1997). Gender is also an important consideration when studying psychiatric disease – women are almost approximately twice as likely to develop major depressive disorder than men, whereas men are more likely to suffer SCZ (Riecher-Rössler, 2017). Therefore, that the molecular correlates of stress and disorder are different between sexes is not surprising, and a subject that deserves more consideration in the literature.

3.4.2 BDNF dysregulation following stress

BDNF IX and *IV* mRNA was significantly decreased in the hippocampus of PPS male rats in comparison to littermate controls, whereas *BDNF VI* was decreased in female PPS rats. BDNF and its receptor TrkB have been found to be altered in hippocampal post-mortem tissue from schizophrenia patients (Takahashi et al., 2000) and, additionally, BDNF has been highlighted as a key molecule in mediating the effects of traditional anti-depressants and newer potential treatments for mood disorders such as ketamine (Björkholm and Monteggia, 2016). BDNF is also widely cited as a molecular substrate of stress, with both acute and chronic stress in animal models often resulting in altered BDNF expression (Schmidt and Duman, 2007; Shi et al., 2010; Eckert et al., 2017). Interestingly, BDNF exons IV and VI have previously been shown to be susceptible to methylation, resulting in corresponding decreases in BDNF expression, following prenatal stress (Boersma et al., 2013).

BDNF mRNA transcription is induced by neural and calcium activity; calcium influx through LTCCs or NMDARs increases *BDNF* mRNA for over 6 hours in cultured neurons (West et al., 2001; Tao et al., 2002). Calcium influx through LTCCs can activate many different transcriptional regulators including CREB and MeCP2 (Tao et al., 1998, 2002) which bind to the promoter of *BDNF IV* to control BDNF expression (West et al., 2001). Previous studies have found a reduction of *BDNF IX* expression in the hippocampus of forebrain $Ca_V1.2$ conditional knockout mice (Lee et al., 2016), which may be due to a lack of activation of transcriptional regulators. Therefore, the decrease in BDNF observed in male rats could potentially be a consequence of *Cacna1c* hippocampal reductions which leads to decreased calcium influx through LTCCs, affecting the transcription of BDNF. However, this is clearly not the only mechanism for stress-mediated disruption of *BDNF* transcription, as we also see a decrease in *BDNF VI* in PPS female rats, who do not show have any changes in *Cacna1c* or *Cacna1d* expression. This decrease could be mediated through alternative mechanisms such as other impairments in the Ca^{2+} signalling circuity or through epigenetic modifications (Zheng et al., 2012).

The dynamic gene structure of *BDNF* suggests a role for different transcripts in different cell types and following different stimuli. For example, *BDNF I* and *IV* are increased in the hippocampus and amygdala following fear conditioning and in the prefrontal cortex in fear memory extinction (Rattiner, 2004; Lubin et al., 2008; Zheng et al., 2012). Following acute restraint stress in adult rats, total *BDNF* mRNA levels in transcripts I-IV, VI and VII were reduced immediately and returned to control levels after 24 hours (Ieraci et al., 2015). It is therefore possible that an unpredictable stress paradigm experienced pre-puberty results in permanent downregulation of certain *BDNF* transcripts, potentially through epigenetic effects. The decrease in *BDNF IV* in male rats observed in the present study is intriguing as fear conditioning in males following prepubertal stress has been shown to be impaired, whereas fear

conditioning in females is intact (Brydges *et al.*, 2014). Given the importance of *BDNF* in memory consolidation, it could be suggested that disruption in this pathway following prepubertal stress has consequences on behaviour and fear memory processing.

Despite the changes in *BDNF* gene transcription, there does not appear to be any gross changes to either pro-BDNF or mature BDNF protein in PPS male rats; suggesting that while there may be changes at transcript level, overall BDNF protein is maintained at basal level. However, it is important to remember that BDNF is an activity-regulated protein (Bramham and Messaoudi, 2005) and thus observing BDNF protein changes after activity or stimulation may be necessary to pull out stress mediated differences. Female PPS rats did trend to having increased pro-BDNF levels (Figure 4.7B), the precursor to BDNF. ProBDNF, if not processed to BDNF, can bind to neurotrophin receptor p75 in the hippocampus and promote long-term depression whereas BDNF, through binding to TrkB receptors, typically promotes early LTP (Chao and Bothwell, 2002). In the hippocampus, increased conversion of proBDNF to BDNF has been reported in enriched environment protocols (Cao et al., 2014; Jha et al., 2016), whereas in conditions of chronic stress, the proBDNF-p75 pathway is upregulated, leading to depressive-like behaviours (Bai et al., 2016). Therefore, the increased pro-BDNF seen in female PPS rats could be indicative of stress-induced mood disturbances in this model, and behaviour tests to correlate this could interrogate this further.

3.4.3 Conclusion

This chapter demonstrates a sex-specific effect of prepubertal stress on *Cacna1c* / $Ca_V1.2$ and *BDNF* expression. Male rats who has been subject to PPS displayed a decreased *Cacna1c* mRNA and $Ca_V1.2$ protein in the hippocampus in adulthood, a difference that was partially replicated in a small sample of human subjects. This effect was specific to the Cav1.2 subunit of LTCCs as there was no evidence of

differences, or indeed compensation, by other calcium channel variants. Reduced *Cacna1c* was correlated with decreased *BDNF IV* and *IX* mRNA expression in the hippocampus, indicating that reductions in *Cacna1c* can have effects on downstream signalling and transcriptional mechanisms regulated by LTCCs.

Chapter 4: The effect of *Cacna1c* heterozygosity on auditory cued fear memory

4.1 Introduction

Altered associative learning has been implicated in the pathology of various psychiatric disorders (Miller, 1976; Diwadkar et al., 2008; Hall et al., 2009; Brambilla et al., 2011), particularly in the manifestation of positive symptoms. There is also genomic evidence that these learning processes are implicated in schizophrenia (Pocklington et al., 2015; Clifton et al., 2017). The results presented in this chapter utilise different auditory cued fear memory paradigms in order to investigate how associative learning may be altered in psychiatric disorders in the context of Cacna1c heterozygosity.

4.1.1 Auditory cued fear conditioning

Fear has an essential role in survival by enabling an individual to cope with threats. However fear can become maladaptive if they are not appropriate to a particular situation. Maladaptive fear systems have been shown in any psychiatric disorders such as schizophrenia and anxiety and thus are an important area of investigation. Fear learning in human and animal research is most often based on Pavlovian fear conditioning where a neutral conditioned stimulus (CS), such as a white noise or light is paired with an aversive unconditioned stimulus (US). This allows CS-US associations to form and evokes a conditioned response (CR) upon presentation of CS alone (Pavlov, 1927). There are several elements to the formation and expression of associative memory: acquisition of the memory, consolidation of the memory and retrieval upon recall. The hippocampus is integral for certain types of associative fear conditioning, but not for others (Table 4.1).

Table 4.1: The main brain structures implicated in different forms of fear conditioning. ACC = anterior cingulate cortex, PFC = prefrontal cortex. (See references Ewald et al, 2014, Kochli et al, 2015, Maren et al, 2016 and Bangesser et al, 2006)

Type of conditioning	Brain structures involved
Delay (cued)	Amygdala, ACC?, Insular cortex
Trace	Amygdala, Hippocampus, medial PFC, ACC, insular cortex
Unpaired/contextual	Amygdala, Hippocampus, medial PFC, ACC, Insular cortex

4.1.2 Delay fear conditioning

Delay conditioning is a form of associative learning where the CS and US are presented or co-terminate together so that a strong association between the two is formed (Nees et al., 2015). Research has shown that the main neural structures involved in the acquisition and consolidation of delay conditioning are the amygdala, the insular cortex and, potentially, the anterior cingulate cortex (ACC) (Ewald et al., 2014), however not all studies find a role for the ACC (Han et al., 2003). The insular cortex is thought to be required in fear conditioning to convey a cortical representation of fear to the amygdala (Phelps et al., 2001) and thus is part of the amygdala circuity of fear. Infusion of anisomycin into the basolateral amygdala (BLA) before delay conditioning disrupts freezing to both tone and context in both rats and mice (Kwapis et al., 2011; Raybuck and Lattal, 2014; Kochli et al., 2015). The lateral nucleus of the amygdala is thought to be particularly important in the delay fear response (Pape and Pare, 2010), which is seen when post-training lesions of the lateral, but not basal nucleus, resulted in disrupted freezing to context (Kwapis et al., 2011; Kochli et al., 2015). This suggests the amygdala plays a critical role in delay associative fear learning; receiving both unimodal and multimodal sensory information and projecting out to various other circuits to coordinate a fear response. Additionally, it is likely that the catecholaminergic system is important within the ceruleo-cortical projections for mediating fear in delay conditioning; noradrenaline depletion in this region resulted in increased fear memory to both context and cue (Selden et al., 1991b).

4.1.3 Trace fear conditioning

Hippocampal lesions have no effect on delay conditioning (Bangasser et al., 2006). However, in trace conditioning, the hippocampus has a far more important role. Trace fear conditioning is similar to delay conditioning, however the CS and US are separated by a temporal gap, called the trace interval, between CS cessation and US onset. This means that a memory 'trace' is required to learn CS-US associations.

Contextual cues ordinarily occupy the trace interval and as the interval increases in duration, there ought to be less conditioning to the CS and greater conditioning to the background stimuli (Marlin, 1981). The main brain structures required for the formation of intact trace conditioning are, like in delay conditioning, the amygdala (Kochli et al., 2015), insular cortex (Ewald et al., 2014), the ACC (Han et al. 2003) but also and importantly, the hippocampus (Bangasser et al., 2006).

Similarly to delay conditioning, the noradrenaline system is important for appropriate responding to trace fear conditioning. Cortical noradrenaline depletion resulted in reduced fear conditioning to the CS but increased conditioning to the context (Selden et al., 1990), whereas amygdala-specific depletion of noradrenaline and dopamine only caused impaired CS fear memory (Selden et al., 1991a). Hippocampal lesions, however showed no impairment of CS conditioning but impairments of contextual fear memory, suggesting a complementary role for hippocampal and amygdala catecholamine networks in the formation of trace memory (Selden et al., 1991a).

Indeed, several studies have shown that rodents with lesions of the hippocampus were unable to acquire trace conditioning (Bangasser et al. 2006; Beylin et al. 2001; McEchron, Tseng, and Disterhoft 2000; McEchron et al. 1998). The hippocampus has been suggested to be required to overcome the CS-US discontiguity within trace conditioning (Bangasser et al., 2006), although how this is done is still under debate. One theory suggests that increased firing of hippocampal neurons throughout the trace interval that ends upon US occurrence could represent the CS through a relay of neuronal activity (Rodriguez and Levy 2001; Solomon et al. 1986; McEchron, Tseng, and Disterhoft 2003). Another theory postulates that the hippocampus uses contextual information to form a context within the trace interval that bridges the CS and US (Quinn et al., 2002; Bangasser et al., 2006) – making the temporal gap a form of contextual representation itself. Finally, there is a theory that the hippocampus is involved in task difficulty and is critically involved when the association is difficult to

learn (Beylin et al., 2001; Bangasser et al., 2006), thus is engaged in trace conditioning and not delay. For example, it was shown that rats could acquire correct trace conditioning without an intact hippocampus if the stimuli have already been associated together suggesting that the role of the hippocampus in conditioning is to bind stimuli together that are spatially or temporally distinct (Beylin et al., 2001). This type of complex learning may involve the utilisation of adult hippocampal neurogenesis and thus may explain why depletion of adult neurogenesis results in disrupted trace conditioning (Shors, 2004a).

4.1.4 Contextual fear conditioning

The hippocampus is also critical in the related process of contextual fear conditioning, where no tone forms the CS and the US is simply experienced within a distinct context. Therefore, animals learn to associate the context (CS) with the US (footshock), and on return to the context will demonstrate a freezing response. In order to acquire contextual fear learning, animals first need to encode the elements of a context to form a representation of the space (Maren et al., 2013), which is highly important to support task-relevant information against unnecessary information. Due to the important role that the hippocampus has in spatial and episodic memory (Burgess et al., 2002; Rolls et al., 2002; Ekstrom and Ranganath, 2017), it is thought to be essential for the formation of these context representations and thus contextual fear associative learning itself. In trace fear conditioning, a context representation during the trace interval is thought to be integral for acquiring trace conditioning, and therefore contextual fear conditioning and trace conditioning share many molecular and behavioural features. Additionally, Rescorla and Wagner's model teaches us that even simple CS-US associations, such as in delay conditioning, have a contextual element and thus all classical conditioning is a case of compound conditioning where we have to consider context (Rescorla and Wagner, 1972).

Dorsal hippocampal lesions produce deficits in freezing behaviour (Phillips and LeDoux, 1992; Ji and Maren, 2007; Tronson et al., 2012), particularly if given very soon after conditioning. It is suggested that these deficits are due to a problem in encoding the contextual representation rather than in forming the CS-US association as pre-exposing the rats to the context eliminates the effect of hippocampal lesions on freezing behaviour (Young et al., 1994). Interestingly, pre-training lesions do not consistently result in impaired fear conditioning (Maren et al., 1997), suggesting that other brain areas are also important for contextual learning. The amygdala is also critical for the processing of contextual fear (Kochli et al., 2015), as is the ACC (Ewald et al., 2014) and the medial prefrontal cortex (Gilmartin and Helmstetter, 2010). Muscimol infusions to the medial prefrontal cortex resulted in impaired trace and contextual, but not delay, fear conditioning, suggesting that along with the hippocampus, the mPFC may also play a role in the formation of contextual representations in these types of conditioning (Gilmartin and Helmstetter, 2010).

4.1.5 Associative learning in psychiatric disorder

Enrichment of copy number variants in gene sets associated with abnormal associative learning, including contextual and cued conditioning (Pocklington et al., 2015), suggests that patients with psychiatric disorders may have deficits in acquiring correct learning behaviour. The large body of genetic evidence supporting the notion that synaptic plasticity and function is impaired in schizophrenia cases may underlie these deficits (Hall et al., 2009, 2015).

Human studies of delay fear conditioning have shown increased heart rate and skin conductance reactivity (SCR) to CS in PTSD patients in comparison to controls (Orr et al., 2000), with corresponding amygdala hyperactivation in response to CS (Bremner et al., 2005). Depressive patients also show increased SCR following delay fear conditioning (Nissen et al., 2010). In SCZ, patients displayed abnormal hippocampal activity during contextual fear conditioning, as well as inappropriately

elevated fear to a 'safe' context in comparison to healthy controls (Holt et al., 2012). There does not appear to be any well-powered studies that look at trace fear conditioning in psychiatric populations, however a mouse model of the schizophrenia risk gene TCF4 presented with deficits in trace fear memory (Brzózka and Rossner, 2013).

Fear conditioning studies are difficult and few in human populations. Eyeblink paradigms, which regard pairing a CS with a puff of air to the cornea, resulting in increased blinking following CS presentation, are often used as a means of examining associative learning in human populations. This paradigm does not map perfectly onto the fear circuity that is used in rodent studies (it is heavily reliant on the cerebellum), however does engage several other brain areas involved in fear conditioning such as the hippocampus and amygdala (Christian, 2003). In schizophrenia, many studies have examined delay eyeblink conditioning, with the majority reporting decreased conditioned responses in schizophrenics (see review Kent *et al.*, 2015), however two studies reported increased conditioned responses (Spain, 1966; Sears et al, 2000). In bipolar disorder, decreased conditioned responses to a delay eyeblink task are seen with the most pronounced deficits seen in those with 'mixed mood' rather than manic/depressive (Bolbecker et al., 2009) whereas autistic individuals display quicker learning but reduced latency of response (Sears et al., 1994; Welsh and Oristaglio, 2016). In trace eyeblink conditioning, no differences in conditioned responses were seen in SCZ, although this was confounded by increased spontaneous blinking. However SCZ patients showed increased responding to CS in comparison to controls (Marenco et al., 2003). Autistic children show normal conditioned responses during a trace eyeblink paradigm, however display a quicker conditioned response onset (Welsh and Oristaglio, 2016).

4.1.6 LTCCs and fear conditioning

LTCCs have been implicated in synaptic plasticity, learning and memory (Moosmang et al., 2005), including fear memory; it has been shown that Cav1.2 levels are increased in the amygdala following fear conditioning (Shinnick-Gallagher et al., 2003). Systemic LTCCs inhibitors do not impair acquisition or consolidation of cued fear learning, but they do produce deficits in extinction (Cain et al., 2002) and latent inhibition (Barad, 2004). However, infusion of LTCC inhibitors directly to the basolateral amygdala has been shown to impair consolidation and extinction of cued fear learning (Bauer et al., 2002) and deletion of Cav1.2 in the ACC results in decreased observational fear learning, where unconditioned mice develop freezing behaviour by observing conditioned mice receiving foot shocks (Jeon et al., 2010). Animals with neuronal specific knockout of *Cacan1c*$^{-/-}$ show no impairments in acquisition, consolidation or recall of auditory (Langwieser et al., 2010) or contextual (Temme et al., 2016) fear conditioning paradigms. A mouse with *Cacna1c* completely knocked out in forebrain excitatory neurons also maintained successful consolidation and extinction of conditioned fear (McKinney et al., 2008). However, studies using a Timothy syndrome mouse model show that these mice can acquire the memory of cued fear conditioning correctly and to the same extent as wild-types, however demonstrate increased freezing in context and cue recalls, as well as reduced extinction (Bader et al., 2011). The authors suggest that this is due to an enhanced perseverance of both tone and context memory. This is interesting as other repetitive behaviours have been noted in this model by increased marble burying (Bader et al., 2011).

In this chapter, I measured the response of *Cacna1c*+/- rats to delay, trace and unpaired fear in order to investigate the role of Cav1.2 in the consolidation of fear memory within these paradigms.

4.2 Methods

4.2.1 Contributions

All work in this chapter performed by A Moon

4.2.2 Wild-type animals

Conditioning

General methodology used for trace, delay and unpaired auditory cued fear conditioning is described at length in General Methods and Figure 2.3. 24 behaviourally naïve adult (PND 60-70) male Sprague Dawley rats (300-400g) underwent a one hour conditioning session in one of two novel but distinct contexts (Context A and B). Rats were randomly assigned to receive delay, trace or unpaired conditioning (each rat only received one type of conditioning). Within this session, they were exposed to 10 presentations of a 15s white noise and 10 footshocks (0.5mA for 0.5s) in various combinations. In delay conditioned rats (n = 8), the footshock and white noise co-terminated, whereas in trace conditioning (n = 8), the footshock occurred 30 seconds following white noise cessation. In unpaired conditioning (n = 8), the footshocks and white noise presentations were explicitly unpaired and presented in an unpredictable order. Rats were then returned to their home cages.

Recall

Associative learning for both context and cue fear memory were then assessed. Contextual fear memory was investigated by placing the rat within the original context for 10 minutes, 24 hours later (Context Recall). Cued fear memory was assessed by exposing the rat to a different context 48 hours later for 2 minutes (Novel) and then presenting the white noise for 6 minutes, followed by a post-CS period of 4 minutes (Cue Recall) (Figure 2.3). Freezing behaviour was recorded and assessed for the whole of both recall sessions as a record of fear memory. Freezing was defined as a complete lack of movement in the animal, except respiration.

Analysis and statistics

Freezing behaviour was analysed as a percentage (%) of time within a session that the animal froze. Freezing percentages were checked for normality of distribution and homogeneity of variances (Levene's Test) and transformed by Box-Cox transformations if required. Delay, trace and unpaired groups were first analysed separately by Repeated Measures (RM) ANOVA with 'Freezing %' forming the dependent variable and the recall session ('Context Recall', 'Cue Recall' and 'Post CS') forming the within-subjects independent variable to assess how each group reacted to each recall. Following this, One-Way ANOVAs within each recall session were conducted, with 'Freezing %' forming the dependent factor and 'Group' (Delay, Trace, Unpaired) forming the between-subjects independent factor to determine if different groups reacted differently to each recall. Post-hoc tests in the form of Tukey Kramer Honestly Significant Difference (HSD) were performed where a significant interaction was found, unless variances were unequal, in which case Games Howell Tests were used.

4.2.3 *Cacna1c*$^{+/-}$ rats

Behaviourally naive adult (PND60-70) *Cacna1c*$^{+/-}$ rats and their wild-type littermates were fear conditioned in a delay (n = 7 per genotype), trace (n = 8 per genotype) and unpaired (n = 7 per genotype) manner as detailed above. Context Recall, Cue Recall and Post CS were tested as before.

Analysis and statistics

Acquisition of fear conditioning for each type of conditioning was assessed by a Two-Way Repeated Measures (RM) ANOVA with 'Freezing %' as the dependent variable and 'Genotype' (between subjects factor) and 'Session' (Baseline to Post US) (within subjects factor) forming the independent variables. Context Recall was analysed by One-Way ANOVAs (or Welch's Test if variances were unequal) with the 'Total

Freezing %' across the whole session forming the dependent variable and 'Genotype' forming the independent variable. Additionally, 2-way Repeated Measures ANOVA were also conducted across each the whole Context Recall session to determine any inter-session variation with 'Freezing %' per minute forming the dependent variable and 'Genotype' (between subjects) and 'Minute' (within subjects) forming the independent variables. Within Cue Recall, 2-Way Repeated Measures ANOVAs were conducted with 'Session' (Novel Baseline, First 3 minute CS, Last 3 minute and Post-CS) forming the within-subjects factor and 'Genotype' forming the between subjects factors. Post-hoc tests in the form of Tukey Kramer Honestly Significant Difference (HSD) were performed where a significant interaction was found, unless variances were unequal, in which case Games Howell Tests were used.

4.3 Results

4.3.1 Generating context and cue specific fear memory using trace, delay and unpaired paradigms.

The degree of freezing that delay, trace and unpaired conditioning elicited in different recalls was analysed. Animals froze to a different extent depending on recall (Context, Cue or Post CS) in the delay group ($F_{(2,12)}$ = 30.180, p < 0.001, RM ANOVA). Post-hoc analysis reported that delay conditioned animals froze significantly more to 'Cue Recall' than during 'Context Recall' (p<0.001, Tukey Kramer HSD) and within the 'Post-CS' period (p <0.001, Tukey Kramer HSD), suggesting that delay animals have strong CS-US associative learning, but little contextual fear memory (Figure 4.1).

In the trace group animals also froze differently to each recall ($F_{(2, 45)}$ = 3.352, p = 0.044, RM ANOVA) (Figure 4.1). Trace conditioned animals freeze similarly to 'Context Recall' and 'Cue Recall' (p = 0.998,Tukey Kramer HSD), but trended to increased freezing in the 'Post-CS' period (p = 0.060, Tukey Kramer HSD). The unpaired group also displayed different freezing behaviour depending on recall session ($F_{(2, 12)}$ = 10.093, p < 0.001, RM ANOVA) (Figure 4.1). Unpaired however showed increased freezing within 'Context Recall' (p < 0.001, Tukey Kramer HSD) and the 'Post-CS' period (p = 0.006, Tukey Kramer HSD) compared to 'Cue Recall' (Figure 4.1). This shows that each cue presented determines the amount of freezing, revealing specific associations have been learnt by the different groups.

During Context Recall, there was a significant effect of 'Group' ($F_{(2, 29.08)}$ = 8.374, p < 0.001, Welch's Test). Unpaired and delay groups showed significantly different freezing behaviour (p = 0.001, Games-Howell Test), whereas trace and delay (p = 0.299, Games-Howell Test) and trace and unpaired (p = 0.072, Games-Howell Test) show similar freezing.

There was also a significant effect of 'Group' during Cue Recall ($F_{(2, 27.88)}$ = 51.748, p = <0.001, Welch's Test). Delay animals froze significantly more than unpaired animals (p = <0.001, Games-Howell Test) and trace (p = <0.001, Games-Howell Test) animals. Animals in the trace group also trended to freezing more than unpaired animals (p = 0.099, Games-Howell Test).

In the Post-CS period, there was also a significant effect of 'Group' ($F_{2, 45)}$ = 6.569, p = 0.003, One-Way ANOVA). Delay animals froze less than trace animals (p = 0.003, Tukey-HSD Test) and unpaired animals (p = 0.033, Tukey HSD Test). However trace and unpaired groups froze to a similar extent (p = 0.629, Tukey HSD Test).

To summarise, different types of CS-US combinations resulted in different patterns of freezing over sessions within group and between groups (Table 4.2).

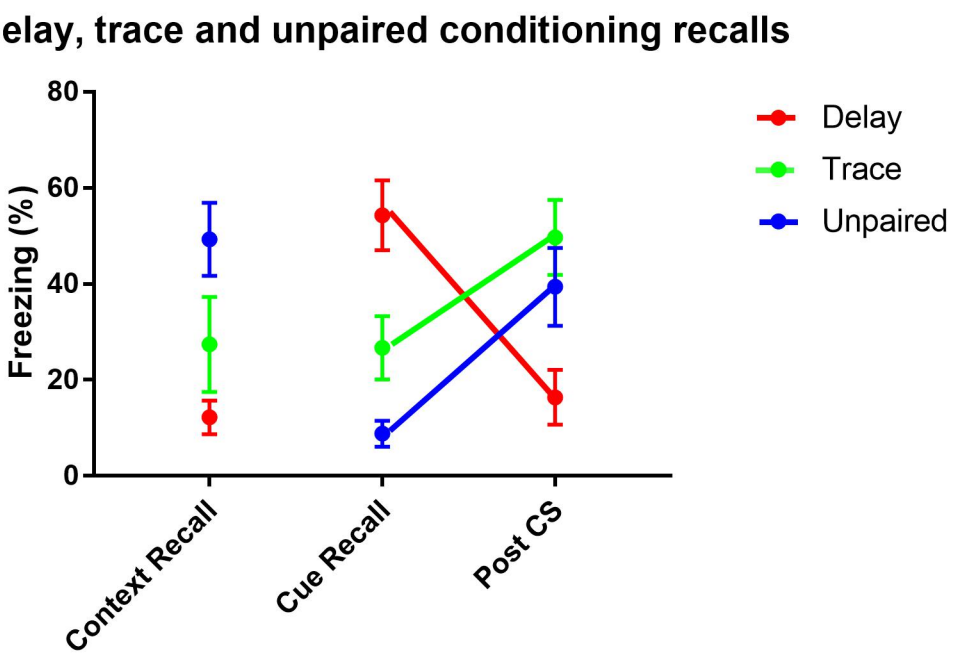

Figure 4.1: Freezing behaviour in wild-types rats following delay, trace and unpaired auditory fear conditioning when presented with different cues. Rats experiencing delay fear conditioning displayed low freezing levels to context but froze significantly more to CS. Trace conditioned rats displayed most freezing during the post-CS period, whereas unpaired rats froze more to context in comparison to CS. Points represent mean % freezing over the whole session, error bars are SEM, n = 16 per group)

Table 4.2: Summary of the degree of freezing seen for each recall session, dependent on auditory fear conditioning paradigms experienced

	Context	CS	Post CS
Delay	Low	High	Low
Trace	Moderate/High	Moderate	High
Unpaired	High	Low	High

4.3.2 Investigating the effect of *Cacna1c* knockdown on contextual and cued fear memory

4.3.2.1. Cacna1c heterozygosity causes an increased contextual fear memory following delay auditory fear conditioning

In this paradigm, foot shocks elicited a robust freezing response in both *Cacna1c*$^{+/-}$ and wildtypes (Session: $F_{(1,12)} = 217.472$, $p < 0.001$, 2-Way RM ANOVA; Figure 4.2A). There was no difference between genotypes (Genotype: $F_{(1, 12)} = 0.010$, $P = 0.923$, 2-Way RM ANOVA) or any evidence of an interaction (Genotype*Session: $F_{(1, 12)} = 0.32$, $P = 0.876$, 2-Way RM ANOVA), indicating that a reduction in *Cacna1c* does not affect the acquisition of delay fear memory (Figure 4.2A).

Cacna1c$^{+/-}$ and WT animal freezing behaviour was compared across each recall session and showed significantly different total freezing % behaviour at Context Recall ($F_{(1, 12)} = 6.86$, $P = 0.028$, One-Way ANOVA) (Figure 4.2A and B). When the context session was broken down by minute, there was no difference across minutes (Minute: $F_{(9,4)} = 3.954$, $p = 0.099$, 2-Way RM ANOVA), a significant effect of genotype (Genotype: $F_{(1,12)} = 5.764$, $p = 0.034$, 2-Way RM ANOVA) but no interaction (Genotype*Minute: $F_{(9,4)} = 0.423$, $p = 0.870$, 2-Way RM ANOVA), suggesting that freezing across Context Recall was apparent across the whole session (Figure 4.2B).

There was no effect of genotype during Cue Recall (Genotype: $F_{(1,12)} = 1.481$, $p = 0.247$, 2 way RM ANOVA) (Figure 4.2A and C) and no session*genotype interaction (Genotype*Session: $F_{(1,12)} = 1.73$, $p = 0.136$, 2 way RM ANOVA) (Figure 4.2C).

There was no freezing behaviour observed in either group during baseline recordings in either context (Genotype: $F_{(1,12)} = 0$, $P = 1$ for both baseline sessions, One-Way ANOVA), confirming these animals do not show abnormal behaviour prior to conditioning, with differences confined to Context Recall following training. These results suggest that *Cacna1c* heterozygosity results in different associative learning of delay fear conditioning, or potentially, a deficit in context discrimination.

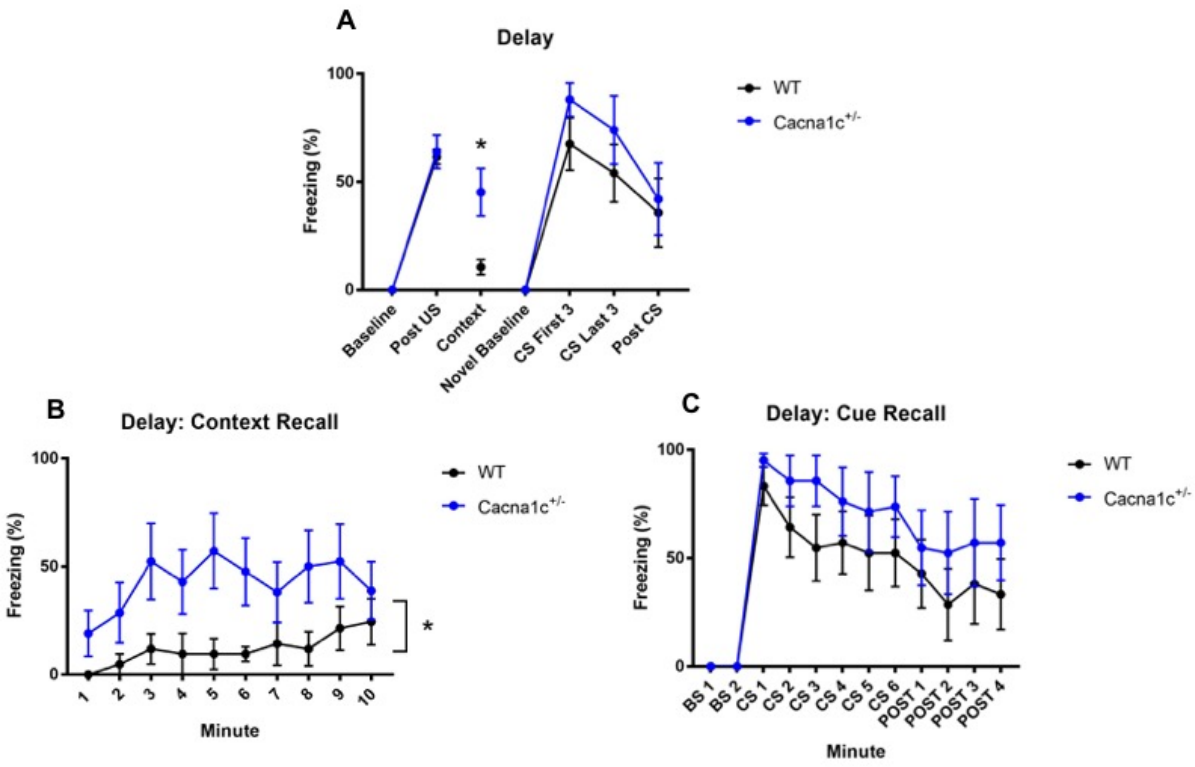

Figure 4.2: Delay conditioning in *Cacna1c*$^{+/-}$ rats. A = Overview of freezing behaviour. *Cacna1c*$^{+/-}$ rats show normal baseline and conditioned freezing, as well as maintained freezing to cue but a deficit in Context Recall. B = Delay conditioned *Cacna1c*$^{+/-}$ rats display increased freezing to context in comparison to wild-types. C = Cue Recall showed similar freezing behaviour between heterozygotes and wild type rats. Data points represent mean % freezing per group for each session or minute of session, error bars are SEM. *Cacna1c*$^{+/-}$ vs wild-type groups, n = 7 per genotype, * = $p < 0.05$. (BS = baseline, CS= conditioned stimulus presentation, POST = post-CS).

4.3.2.2. Trace conditioned Cacna1c$^{+/-}$ rats show deficits in cued recall but not contextual recall of fear memory

Trace conditioned *Cacna1c*$^{+/-}$ and wild-types animals showed the expected increase in freezing following exposure to the US, with increased freezing observed immediately following shocks (Session: $F_{(1, 14)} = 859.3$, $P < 0.001$, 2-Way RM ANOVA) (Figure 4.3A). There was no effect of genotype (Genotype: $F_{(1, 14)} = 0.833$, $P = 0.377$, 2-Way RM ANOVA) or any evidence of an interaction (Session*Genotype: $F_{(1, 14)} = 0.018$, $P = 0.929$, 2-Way RM ANOVA), indicating that a reduction in *Cacna1c* does not affect the acquisition of trace fear memory.

Cacna1c$^{+/-}$ and wild-types animals showed similar freezing behaviour during Context Recall (Figure 4.3A and B) (Genotype: $F_{(1,14)} = 0.075$, $P = 0.788$, One-way ANOVA), indicating they can consolidate contextual cues appropriately.

Cacna1c$^{+/-}$ animals have increased freezing at Cue Recall, in comparison to wild-types. Across Cue Recall, there is a significant effect of session (Novel Baseline, First 3 minute CS, Last 3 minute CS, Post CS) (Session: $F_{(2,13)} = 27.94$, $p < 0.001$, 2-Way RM ANOVA) (Figure 4.3C), trend to genotype effect (Genotype: $F_{(1,14)} = 3.281$, $p = 0.092$, 2-Way RM ANOVA) and significant session*genotype interaction (Session*Genotype: $F_{(2, 13)} = 3.870$, $p = 0.048$, 2-Way RM ANOVA). Post-hoc tests revealed that there was no difference at novel baseline ($p = 0.130$, Tukey Kramer HSD) or Post CS ($P = 0.568$, Tukey Kramer HSD), however during CS presentation, *Cacna1c*$^{+/-}$ froze significantly more within the first 3 minutes ($p = 0.003$, Tukey Kramer HSD) and last 3 minutes ($p = 0.029$, Tukey Kramer HSD) (Figure 4.3A and C).

To determine if perception of fear of the auditory cue between genotypes that may be driving this effect (i.e. they find the noise fearful itself), freezing behaviour immediately following the first CS presentation on the conditioning day was analysed. No freezing behaviour was observed in either *Cacna1c*$^{+/-}$ rats or wildtypes (Genotype: $F_{(1, 14)} = 0$,

P = 1, One-Way ANOVA), suggesting that the CS is not enough to drive a fear response without the associated footshock.

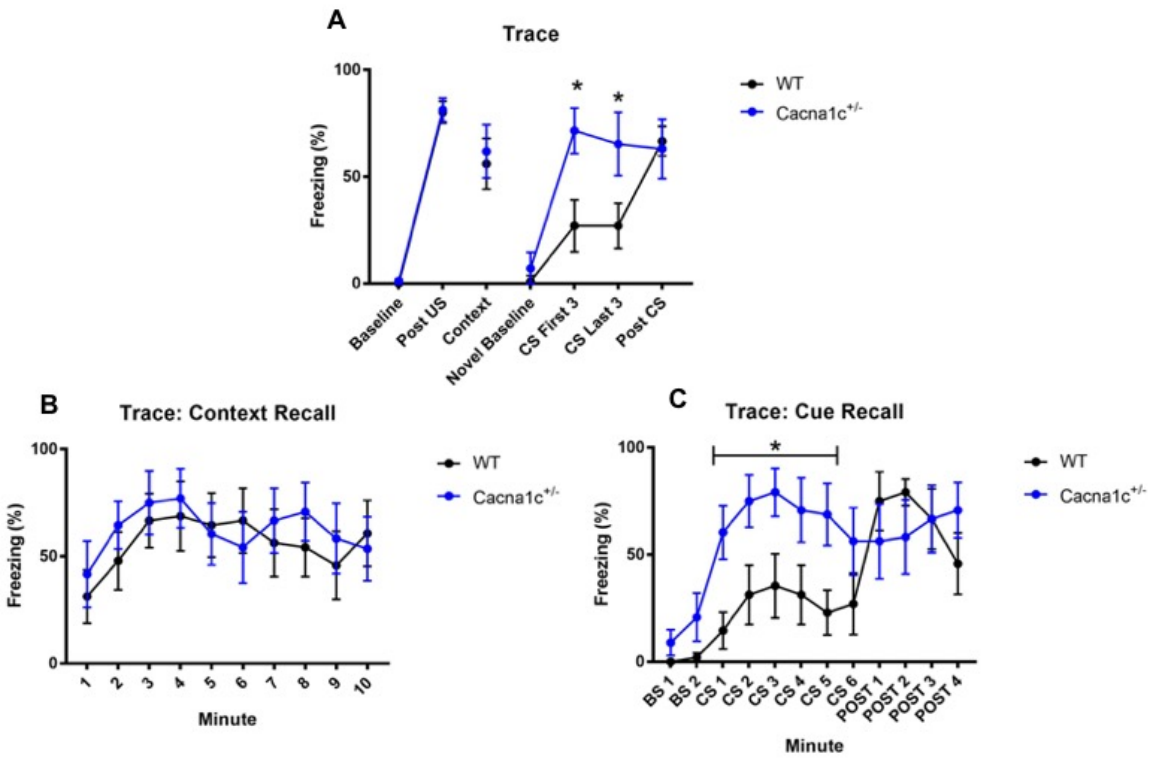

Figure 4.3: Trace conditioning in *Cacna1c*$^{+/-}$ rats. A = *Cacna1c*$^{+/-}$ show normal freezing behaviour during the conditioning session and Context Recall, however display increased freezing during CS presentation in a novel context. B = During context recall, *Cacna1c*$^{+/-}$ rats show similar freezing behaviour to wild-types. C = *Cacna1c*$^{+/-}$ rats demonstrate increased freezing during CS presentation within Cue Recall, however showed similar freezing behaviour to wild-types during novel baseline and post-CS. Data points represent mean % freezing, error bars are SEM. *Cacna1c*$^{+/-}$ vs wild-type groups, n = 8 per genotype, * = $p < 0.05$. (BS = baseline, CS= conditioned stimulus presentation, POST = post-CS)

4.3.2.3. *Unpaired auditory fear conditioning results in increased contextual and cued fear memory*

Unpaired conditioned *Cacna1c*$^{+/-}$ and wildtype rats displayed greatly increased freezing following footshock in comparison to baseline (Session: $F_{(1,12)}$= 2097.143, p < 0.001, RM ANOVA), however there was no difference between genotypes (Genotype: $F_{(1,12)}$ = 1.702, p = 0.217, 2-Way RM ANOVA) or an interaction (Session*Genotype: $F_{(1,12)}$ = 1.209, p = 0.348, 2-Way RM ANOVA suggested that both genotypes can acquire unpaired fear memory (Figure 4.4A).

However, *Cacna1c*$^{+/-}$ rats displayed increased fear memory as displayed by increased freezing in Context Recall (Genotype: $F_{(1,12)}$ = 5.030, p = 0.045, One-Way ANOVA) (Figure 4.4A and B). When the context session was broken down by minute, there was no difference across minutes (Minute: $F_{(9,4)}$= 1.924, p = 0.237, 2-Way RM ANOVA), a significant effect of genotype (Genotype: $F_{(1,12)}$ = 5.432, p = 0.021, 2-Way RM ANOVA) but no interaction (Minute*Genotype: $F_{(9,4)}$ = 0.768, p = 0.872, 2-Way RM ANOVA), suggesting that freezing across Context Recall was apparent across the whole session (Figure 4.4B).

During Cue Recall, there was a significant effect of session (Novel Baseline, First 3 minute CS, Last 3 minute CS and Post CS) (Session: $F_{(3,11)}$ = 6.632, p = 0.013, 2-Way RM ANOVA) and genotype (Genotype: $F_{(1, 12)}$ = 6.900, p = 0.022, 2-Way RM ANOVA) but no significant interaction (Genotype*Session: $F_{(3,11)}$= 0.576, p = 0.578, RM ANOVA). Post-hoc analysis revealed that there was a significant increase in freezing between genotypes during the First 3 minutes of CS presentation (p = 0.002, Tukey Kramer HSD) and the Last 3 minutes of CS presentation (p = 0.023, Tukey Kramer HSD) (Figure 4.4A and C). This suggests an increased fear memory in *Cacna1c*$^{+/-}$ rats.

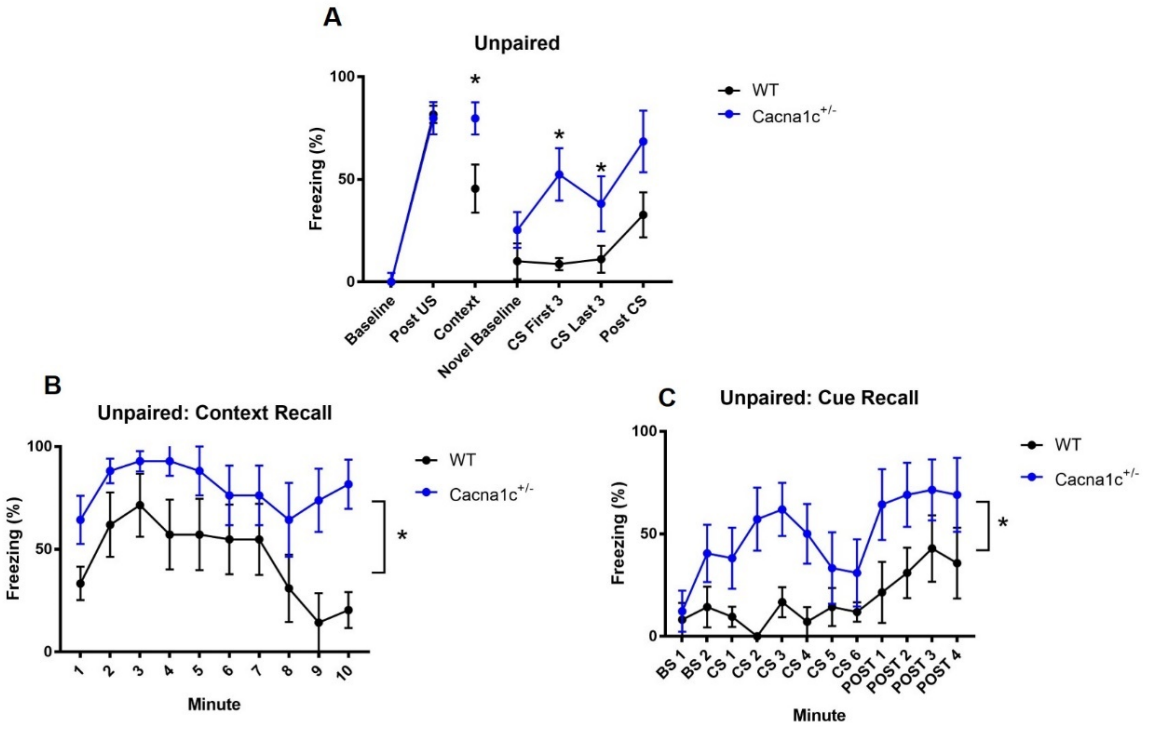

Figure 4.4: Unpaired fear conditioning in *Cacna1c*$^{+/-}$ rats. A = *Cacna1c*$^{+/-}$ rats show similar freezing behaviour during the conditioning session but show increased fear memory in both recall sessions. B = *Cacna1c*$^{+/-}$ knock out animals show increased freezing at Context Recall over that of wild-type behaviour. C = During Cue Recall, *Cacna1c*$^{+/-}$ rats also show increased freezing during CS presentation, no deficit in cued recall at baseline and a trend towards increased freezing post-CS. Data points represent mean % freezing, error bars are SEM. *Cacna1c*$^{+/-}$ vs wild-type groups, n = 7/genotype, * = $p < 0.05$. (BS = baseline, CS= conditioned stimulus presentation, POST = post-CS).

4.4 Discussion

Genetic knockdown of *Cacna1c* had significant effects on all cued and contextual fear conditioning paradigms tested. Following delay fear conditioning, *Cacna1c*$^{+/-}$ rats showed increased fear responses to context, whereas in trace fear conditioning, they showed increased freezing to cue presentation. Unpaired conditioning resulted in increased fear responses to both context and cue recalls.

Acquisition of fear memory was unaffected in *Cacna1c*$^{+/-}$ rats with all rats showed robust freezing post-US to the same degree as the wild-types, showing that they can acquire the memory without difficulty. This is in agreement with previous studies of Cav1.2 deletion in mouse models; neuronal specific knockouts of *Cacna1c*$^{-/-}$ showing correct acquisition of auditory (Langwieser et al., 2010) and contextual (Temme et al., 2016) fear conditioning.

In delay fear conditioning, while acquisition and cue recall responses to fear memory are left intact in *Cacna1c*$^{+/-}$ compared to wild-types, there is evidence of increased freezing during contextual recall. As seen at cue recall, the CS-US association has been learnt, but the *Cacna1c*$^{+/-}$ rats also appear to display generalised fear to the context. This suggests the calcium influx through Cav1.2 is necessary for the consolidation of contextual cues in relation to auditory fear conditioning. This proposes a role for Cav1.2 beyond the simple consolidation of a CS-US association, but within CS associations in the absence of a US. Typically the basolateral amygdala is seen as the primary site for delay conditioning with little hippocampal involvement, however it may be proposed that in *Cacna1c*$^{+/-}$ rats there is increased hippocampal activity in order to form an inappropriate contextual representation.

In trace conditioning, *Cacna1c*$^{+/-}$ rats displayed increased freezing to the CS in cue recall. Furthermore, in unpaired conditioning, which mostly represents contextual conditioning, increased fear responses were seen at both context and cue

presentation. This suggests that all cues, including the auditory CS and the context are being processed differently. For example, in trace conditioning, while the wild-types learn that the footshock is separated from the white noise by 30 seconds, *Cacna1c*$^{+/-}$ rats do not appear to learn this temporal rule and just learn the CS-US association by itself, compounded by contextual information. It could be suggested that the observed increase in freezing behaviour is due to a generalised anxiety following fear conditioning, however there is no difference in freezing behaviour between wild-types and heterozygotes within a novel context at baseline, indicating that there likely isn't increased anxiety in these animals.

These results suggest that Cav1.2 has a critical role in cue/context discrimination and determining what is the correct association to make as *Cacna1c* heterozygote rats appear to react to all context and cue presentations to a similar degree, without discriminating based on learnt CS-US associations. This agrees with Temme et al (2016), who showed significant context discrimination deficits in their *Cacna1c*$^{-/-}$ neuronal knockout mouse (Temme et al., 2016). However Temme et al (2016) also reported preserved contextual fear conditioning in this model. *Cacna1c*$^{+/-}$ rats appear to lose specificity of their fear response so that, either the correct CS-US association has not been learnt, or cannot be retrieved. This points to a form of overgeneralisation of fear memory with the *Cacna1c* heterozygotes attributing fear to all aspects of conditioning, not just the predictive rule, regardless of spatial or temporal information.

The ability to generalise learning across stimuli has an evolutionary advantage as natural stimuli are unlikely to occur in the exact same form twice. However, the ability to discriminate between different stimuli to gain specificity is also important to limit inappropriate behaviour responses. This subtle balance between generalisation and specificity is vital in associative learning. Overgeneralisation of a fear response is common in generalised anxiety disorder, obsessive compulsive disorder or stress disorders such as post-traumatic stress disorder (PTSD). Anxiety is also a key feature

of schizophrenia (Hall, 2017). Individuals with PTSD often experience generalisation rather than specificity towards memory encoding, resulting in memories of traumatic events being vulnerable to be triggered by many different stimuli, including neutral stimuli that are minimally related to the traumatic event or not normally sufficient to trigger recall (Anastasides et al., 2015). Like SCZ and BPD, PTSD has a high heritability and a recent study has implicated a *CACNA1C* SNP (rs1990322) in the development of PTSD in traumatised police officers and replicated this finding in another study of traumatised children (Krzyzewska et al., 2018). The hippocampus has also been linked to fear generalisation as it involves events encoded as complex multimodal representations (Dunsmoor and Paz, 2015). Lesions of the dorsal hippocampus result in less generalisation and better cue discrimination to delay fear conditioning when presented with a novel tone (Quinn et al., 2009). It may therefore be suggested that in the *Cacna1c*$^{+/-}$ model, rats can correctly learn both delay and trace fear conditioning protocols, however may present with overgeneralisation of fear responses to both cue and context. Presenting the *Cacna1c*$^{+/-}$ rats with a novel auditory stimulus in a novel context would be a useful experiment to fully investigate generalisation of CS cues in this model.

An alternative explanation is that the increased conditioned responses may be due to alterations in context representations. Context processing refers to the use of prior contextual representations to allow for the adaptive control of behaviour and selection of appropriate behaviour (Reilly et al, 2016). Context processing is responsible for the selection of information required for processing and memorizing as well as inhibition of task-irrelevant information. It has been argued that disruptions in cognition and inhibition in schizophrenia may be explained by deficient context processing (McClure et al., 2008). Therefore, if *Cacna1c*$^{+/-}$ rats are not forming correct contextual representations during acquisition of learning, this will dramatically affect their responses during recalls. In a reward test, patients with schizophrenia show less

neural adaption to reward contexts, which results in imprecise representations of the reward (Kirschner 2016).

The results presented in this chapter are not consistent with LTCC antagonists given systemically to mice, where no differences in freezing response were seen in either cued or contextual fear conditioning (Cain et al., 2002). However, this could be explained by the robustness of the paradigm, most studies utilise a single shock presentation whereas this study used 10 CS-US pairings to ensure associative learning in the trace conditioning animals. It also could be explained by off-target effects of the LTCC inhibitors due to the systematic mode of delivery or by inadequate dosing in the brain, particularly in the hippocampus and amygdala where processing of fear memory is thought to occur. Conversely, Bader et al (2011) also showed increased freezing to both context and cue recalls in their Timothy Syndrome model, as well as reduced extinction; which they suggest is due to enhanced persistence of both tone and context memory (Bader et al., 2011).

The results presented in this chapter could not be conclusively stated to be due to enhanced persistence of tone and context memory as the deficits were specific to a particular recall, however there may be an element of persistence of contextual cues leading to the increased fear memory seen. Extinction is a form of inhibitory leaning and therefore is useful to gauge persistent fear memories in animal models. LTCC antagonism prior to extinction in mice leads to perseverance of freezing behaviour in extinction in both cued and contextual conditioning (Cain et al., 2002). Extinction was not investigated in this chapter as this paradigm was too robust (10 CS-US presentations) to illicit extinction within a reasonable time frame; however the literature does seem to suggest a role for Cav1.2 in extinction, which may elude to a persistence of fear memory that is represented by increased recall freezing in this study.

4.4.1 Conclusions

These results demonstrate a selective role of Cav1.2 in specific aspects of associative learning. *Cacna1c*$^{+/-}$ rats show increased freezing in contextual recall following delay conditioning, in cued recall following trace conditioning and in both recalls following unpaired/contextual conditioning. This indicates a role for Cav1.2 in the formation of specific CS-US associations and buffering against non associative cues that may led to overgeneralisation of the fear response, as well as relevant contextual representations.

Chapter 5: Auditory cued fear conditioning in prepubertally stressed rats

5.1 Introduction

Early life trauma has been associated with altered associative learning in both animal models and human studies, which can have a considerable effect on their cognitive functions and social behaviour in later life (Bouffard-Bouchard *et al.* 2017; Krugers *et al.* 2017). Early life stress is also highly implicated in risk of mood and anxiety disorders in adulthood (Syed and Nemeroff 2017) which are highly associated with altered associative learning (Otto et al., 2014). *Cacna1c* heterozygosity showed profound changes in certain elements of cued fear conditioning (Chapter 4). We also showed *Cacna1c* expression in adulthood was reduced following prepubertal stress (PPS) (Chapter 3). Thus here delay, trace and auditory fear conditioning were investigated in the PPS rat model to determine if similar patterns were observed to *Cacna1c* heterozygosity.

5.1.1 Associative learning following early life stress

Many studies indicate that learning, especially associative learning, is directly affected by the caregiving and environment that an organism experiences in early life (Hanson et al., 2017; Sheridan et al., 2018). Early life stress can have a large impact on the structure of the hippocampus and amygdala, as well as neural circuits within these structures, regions that undergo significant postnatal development (Jabès et al., 2011; Chareyron et al., 2012). These regions are central to processes vital for associative learning such as emotional processing, executive functions, including working memory, and attention (Pechtel and Pizzagalli, 2011). Thus brain circuits vulnerable to early trauma include those thought to be important for associative

learning and the impact of early trauma during this period can persist many years after early life stress has ended (Pechtel and Pizzagalli, 2011).

Previous studies have shown that adolescents exposed to childhood trauma show deficits in associative learning compared to controls, which was correlated with increased behavioural problems (Bouffard-bouchard et al., 2017). In a reward task where participants had to choose between two stimuli that had different rewards and different probabilities of winning, maltreated children responded the same way no matter what the reward, whereas non-abused children responded more quickly when the probability of reward increased – suggesting a learning deficit to positive stimuli and insensitivity to changes in value (Guyer et al., 2006; Weller and Fisher, 2013). Women who had been sexually abused in childhood showed lower accuracy in tasks that required responses to rely on previously rewarded information and increased activation in the anterior cingulate cortex (Pechtel and Pizzagalli, 2011). Adolescent girls who had experienced early trauma also displayed deficient inhibitory control processes (Mueller et al., 2010). Hanson et al (2017) suggest that these learning deficits are due to maltreated individuals not correctly using reward-based information from the environment, making early decisions in the learning process without fully forming associations and not correctly learning predictable relationships – as if rewards occurred at random (Bouffard-bouchard et al., 2017).

5.1.2 Animal models

Studies in animal models have further investigated the relationship between associative learning and early life adversity. In animal models, adverse caregiving is consistently associated with emotional and cognitive dysfunction in adulthood (Kosten et al., 2012). Meta-analyses revealed that postnatal stress consistently results in enhanced performance in inhibitory learning tasks and impairments in aversive conditioning (Kosten et al., 2012). Contextual fear conditioning in adulthood following maternal deprivation or early handling in rats appears to repeatably

decrease fear responses as measured by conditioned freezing response to the context exposure (Lehmann et al., 1999; Meerlo et al., 2001; Toda et al., 2014; Zalosnik et al., 2014). However, there is also evidence that contextual memory can be enhanced following postnatal stress if learning occurs under 'stressful' conditions (Champagne et al., 2008). Cued fear conditioning also shows a similar pattern of reductions in fear responses following early postnatal stress (Madruga et al., 2006; Stevenson et al., 2009; Chocyk et al., 2014), however some studies report no effect of stress on cued fear responses (Pryce et al., 2003; Mathieu et al., 2011). These disparities are likely due to the changes in postnatal stress protocol such as the number of days the pups were left without their mother and how early post-birth the deprivation/handling occurred. Maternal separation also has been reported to result in impaired recognition memory in males but not females. This corresponded with decreased cross-frequency coupling within prefrontal-hippocampus networks, suggesting a dysregulated communication between these two regions (Reincke and Hanganu-Opatz, 2017).

Prepubertal stress, occurring later in life than early postnatal stress models, has also been similarly shown to result in impairments in associative learning in adulthood, suggesting that the neural and behavioural networks required for processing associations are still developing throughout infancy. Male rats subject to a 3 day variable stress protocol at PND 25-28 showed decreased freezing to a contextual fear protocol in adulthood, although females appeared to have in-tact associative aversive learning (Brydges *et al.*, 2014). Additionally, both male and female rats demonstrated decreased contextual fear freezing responses following daily footshocks between PND14-18 (Hiraide et al., 2012); whereas daily footshocks between PND21-25 impaired extinction in adulthood following contextual fear conditioning (Matsumoto et al., 2008). A 3 day repeated stress of fox odour and elevated platform at PND 28-30 resulted in increased freezing in males, decreased freezing in young females and

impaired extinction (Toledo-Rodriguez and Sandi, 2007). Therefore, it appears that following PPS there is an impairment in contextual fear conditioning in adulthood, although females appear to be more resistant.

Conversely, it has also been demonstrated that PPS enhances delay cued fear conditioning. PND 27-29 stress resulted in increased freezing to cue at recall and had increased aversive 22-kHz ultrasonic vocalisations (Yee et al., 2012a).Tsoory and colleagues found that prepubertal stress resulted in enhanced delay cued fear conditioning and impaired avoidance learning, alongside increased expression of neural cell adhesion molecule L1 throughout the limbic system, including the amygdala and the hippocampus in adulthood (Tsoory et al., 2010). Members of the immunoglobulin family including L1 have been implicated in synaptic changes underlying learning and memory processes (Tsoory et al., 2010), demonstrating that prepubertal stress may have a long-lasting effect on elements of synaptic plasticity, that manifest in behaviour deficits.

5.1.3 Trace conditioning following stressful conditions

Cacna1c heterozygote rats showed a deficit in cue recall of trace conditioning (Chapter 4), however, there is a significant gap in the literature in regards to trace conditioning (where the CS and US are separated by a temporal interval) following early life stress. The majority of studies examining stress in cued fear conditioning use the standard delay conditioning protocol, where the CS-US co-terminate. However, there are a small number of studies that evaluate the effect of acute stress in a trace conditioning protocol. Acute stress given 24 hours before a trace eyeblink conditioning paradigm resulted in enhanced responses in males and reduced responses in females, particularly when tested in proestrus (Wood et al., 2001; Shors, 2004b). Similar results were seen in males for delay eye blink conditioning but not unpaired blink conditioning (Shors and Servatius, 1997; Shors, 2001). This increased response was not apparent if stress was experienced during conditioning (Shors,

2001). Interestingly, acute stress impairs conditioning in female rats subject to the same conditioning paradigm (Wood and Shors, 1998), highlighting the importance of reproductive hormones in the effects of stress on associative conditioning (Shors, 2004b).

In a healthy cohort of humans, a model was set up to evaluate both delay and trace conditioning within the same task, so that a CS-US combination that co-terminated was presented alongside a different CS-US combination that were separated by 3 seconds. When males were subject to an acute stress, delay conditioned responses were increased during recall 24 hour later, whereas trace conditioned responses were reduced (Vogel et al., 2015). This is thought to occur through a mineralocorticoid (MR) dependent mechanism where MR allows for stress-induced shifts between multiple memory systems, probably to allow for adaptive behaviour in stressful situations. These results support that, following stress, individuals shift towards cognitively less demanding systems, shown by the dominance of delay over trace conditioning in this study. However, when acute stress was followed by a single-cue trace conditioning eyeblink task, stress resulted in increased conditioned responses during acquisition, but there was no difference between the stressed and unstressed subjects (Duncko et al., 2007).

Thus there is a plethora of evidence that suggests that early trauma can have a large impact on associative fear learning. PPS appears to impair contextual fear conditioning, whereas it also enhances delay cued fear conditioning, suggesting that there is a differential impact on different neuronal circuits following ELS. Males also appear to be more likely to present with associative learning deficits following PPS, suggesting that sex may also be a risk factors.

There is also evidence that prepubertal stress affects other associative learning paradigms; it is associated with altered performance in the Water Maze (Avital and Richter-Levin 2005; Brydges *et al.* 2014).

This chapter investigates the effect of prepubertal stress on delay, trace and unpaired auditory fear conditioning in adulthood to investigate how early life stress can impact on neural and behavioural networks long-term.

5.2 Methods

For detailed methods of generating the PPS model and the aversive conditioning protocol, see General Methods.

5.2.1 Contributions

The practical procedures in this experiment were conducted by Dr N Brydges. All the data scoring, assessment and analysis was conducted by A Moon.

5.2.2 Prepubertal stress

On PND 25-27, bred Lister-Hooded rats litters were randomly allocated to experimental groups: prepubertal stress and control. Stress groups experienced a short-term unpredictable stress protocol. On PND 25, animals were given a 10 minute swim stress. PND 26 included a restraint stress in restraint tubes for 3 sessions of 30 minutes, separated by 30 minutes in their home cage and PND27 an elevated platform stress for 3 sessions of 30 minutes, separated by 60 minutes in their home cage. Animals were then returned to their home cages and left undisturbed until PND60. Control animals were left undisturbed from birth to adulthood.

5.2.3 Conditioning

All rats were behaviourally naïve and only subject to one form of conditioning. 36 control male rats and 31 male PPS rats were used for trace, delay and unpaired aversive conditioning, with sample sizes of 10-13 per group. Once a day for three days before conditioning, animals were habituated to transport and handling by taking them to the testing room and briefly handling. Conditioning and recall sessions were as previously described in General Methods, Chapter 4 and Figure 2.3. Briefly, rats were exposed to 10 presentations of a 15s CS (white noise) paired with a US (0.5mA, 0.5s footshock) within Context A or B. In delay conditioning, CS and US co-terminated with an ITI of 312s +/- 62s. In trace conditioning, CS and US were separated by 30s

(ITI: 312s +/- 62s), whereas in unpaired conditioning, CS and US were explicitly unpaired (ITI: 156s +/- 31s).

Twenty four hours following conditioning, animals were returned to the context in which they were conditioned for 10 minutes to assess contextual fear responses (Context Recall). 48 hours following conditioning, rats were placed into a second context (Novel Baseline) for 12 minutes and received a 6 minute CS presentation beginning 2 minute after the start of the recall session (Cue Recall).

5.2.4 Analysis

Freezing behaviour was analysed across all recall sessions by an observer blind to group and expressed as a percentage of immobility. These percentages were subject to checks for homogeneity of variances (Levene's Test) and normality of distribution and transformed (Box-Cox) if appropriate. 2-way Repeated Measures (RM) ANOVAs were used to analyse acquisition of fear conditioning, with independent variables: 'Group' (CON/PPS) (between factors) and 'Session' (within subjects) (Baseline to Post US) and 'Freezing %' as the dependent variable. Post CS, Post US and Context Recall sessions were analysed initially by One-Way ANOVAs with 'Group' as the independent variable and the dependent variable representing the total % of freezing across the total session. Cue Recall was analysed by a 2-way RM ANOVA with 'Session' (Novel Baseline, First 3 minute CS, Last 3 minute CS, Post CS) forming the within-subjects factor and 'Genotype' the between subjects factor.

2-way Repeated Measures ANOVAs were also conducted within Post CS, Post US and Context Recall sessions to determine any effects within the session. In this case, % freezing per minute formed the dependent variable and 'Group' formed the between subjects factor and 'Minute/Shock Number/Interval Number' formed the within subjects independent variables. If differences were found following 2-way RM ANOVAs, Tukey-Kramer HSD was used for post-hoc analysis, unless variances were

different, whereabout Games Howell tests were used. For all analysis, litter was nested as a random variable.

5.3 Results

5.3.1 PPS animals demonstrate an increased freezing to context in early stages of recall following delay conditioning

Both PPS and control animals show an increase in freezing behaviour from baseline to post-shock (Session: $F_{(1, 21)} = 788.051$, $p < 0.001$, 2-Way RM ANOVA), with no difference between PPS and control groups (Group: $F_{(1, 21)} = 0.021$, $p = 0.886$, 2-Way RM ANOVA) and no significant interaction (Group*Session: Group: $F_{(1, 21)} = 0.861$, $p = 0.656$, 2-Way RM ANOVA. This shows that PPS does not affect the response to the stimuli or affect correct acquisition of delay fear memory (Figure 5.1A and B).

Freezing behaviour of PPS and control animals were compared across each recall session. There was no overall significant difference in conditioned freezing during Context Recall (Group: $F_{(1, 8.593)} = 2.408$, $p = 0.157$, One-Way ANOVA), however when analysed by minute, there is an effect of group (Group: $F_{(1, 21)} = 5.200$, $p = 0.033$), 'Minute' (Minute: $F_{(1, 21)} = 3.900$, $p = 0.023$) and a significant interaction between group*minute (Group*Minute: $F_{(1, 21)} = 4.862$, $p = 0.031$) (Figure 5.1C). Post-hoc tests show that this was specific to 2 minutes ($p = 0.046$, Tukey-Kramer HSD) and 3 minutes ($p = 0.039$, Tukey-Kramer HSD).

For Cue Recall, there was a significant effect of session (Session: $F_{(1, 21)} = 14.567$, $p = 0.001$) but no effect of group (Group: $F_{(1, 21)} = 0.022$, $p = 0.885$, 2-way RM ANOVA) or any interaction (Session*Group: $F_{(1, 21)} = 1.087$, $p = 0.436$) (Figure 5.1D).

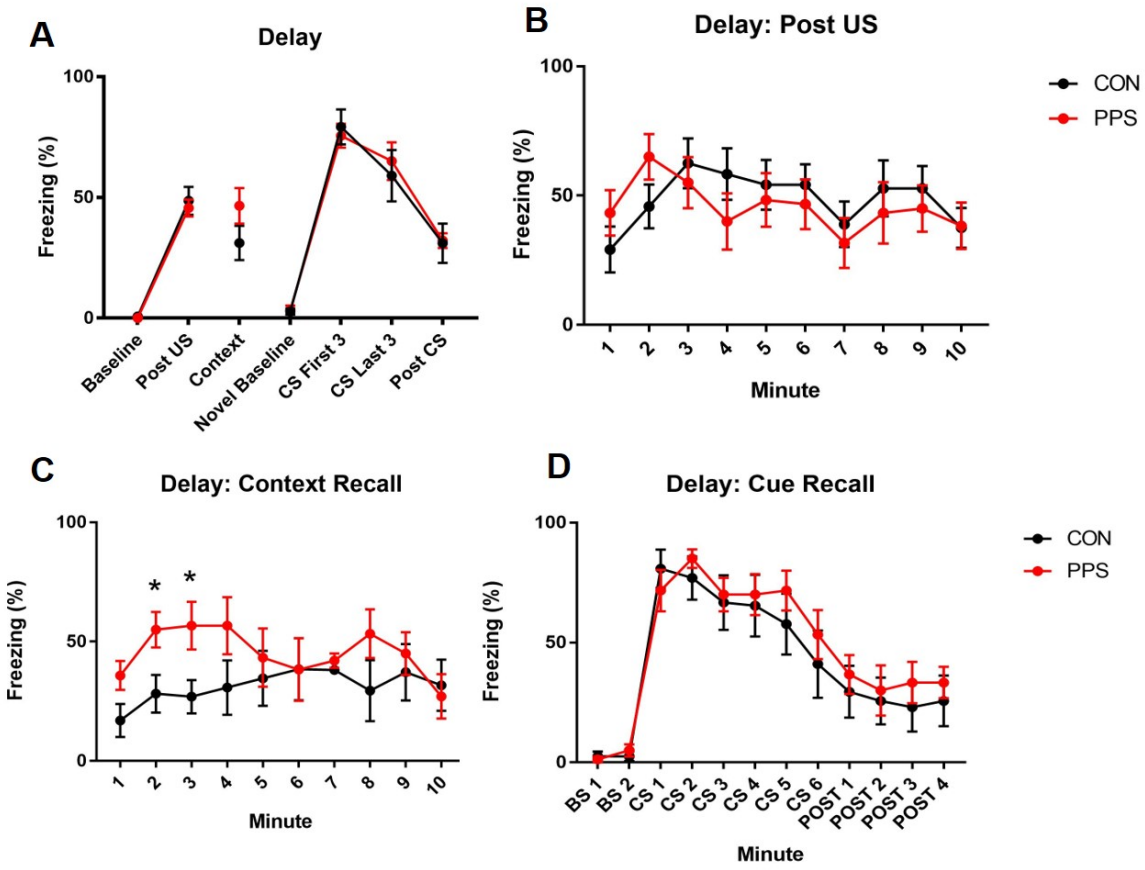

Figure 5.1: Delay conditioning in PPS and control rats. A = Mean freezing responses across conditioning and recall trials reveal no overall effect of PPS on behaviour. B = There were no differences in Post US freezing between PPS and controls. C = Context Recall 24hr after conditioning showed that PPS resulted in increased freezing to the conditioned context in the first five minutes of the session. D = There was no differences between the PPS and control groups in freezing behaviour in the Cued Recall 48hr after conditioning. Data points represent mean % freezing behaviour, error bars are SEM. PPS compared to Control (CON) groups, n = 13 CON, 10 PPS, * = $p < 0.05$. (BS = baseline, CS= conditioned stimulus presentation, POST = post-CS).

5.3.2 PPS animals show encoding deficits in trace fear conditioning

When acquisition of freezing behaviour was analysed, there was a significant effect of session (Session: $F_{(1, 22)} = 279.454$, $p < 0.001$, 2-Way RM ANOVA) showing that with both rats froze more post-US than at baseline but there was also an effect of group (Group: $F_{(1, 22)} = 5.579$, $p = 0.028$, 2-Way RM ANOVA) and a significant interaction (Session*Group: $F_{(1, 22)} = 8.672$, $p = 0.012$, 2-Way RM ANOVA). Post-hoc tests revealed that PPS rats froze significantly less than controls during the Post-US period ($p = 0.018$, Tukey Kramer HSD) (Figure 5.2A and C). This was seen for each shock presentation as there was no effect of shock number (Shock Number: $F_{(9, 14)} = 1.919$, $p = 0.132$, 2-Way RM ANOVA), but there was a significant effect of PPS (Group: $F_{(1,22)} = 7.827$, $p = 0.011$, 2-Way RM ANOVA, Figure 5.2C). Therefore, freezing responses for the 30 second 'trace' gap (after the CS, but before the shock) was analysed to investigate if there was a potential encoding deficit. PPS animals froze less overall within the trace interval (Group: $F_{(1, 11.12)} = 10.901$, $p = 0.007$, One-way ANOVA), suggesting that they have a potential deficit in learning the CS-US association (Figure 5.2A). When analysed over the conditioning session, there was no effect of trace interval (Trace Interval: $F_{(1,22)} = 1.459$, $p = 0.342$, 2-Way RM ANOVA) but a significant effect of PPS (Group: $F_{(1, 22)} = 10.855$, $p = 0.003$, 2-Way RM ANOVA), indicating that this was observed after each CS within the session.

During Context Recall, there was no difference between PPS and control rats (Group: $F_{(1, 9.741)} = 0.199$, $p = 0.665$, One-Way ANOVA) when the total freezing responses data was analysed by session (Figure 5.2A) nor when analysed minute by minute within session (Minute: $F_{(9, 14)} = 1.017$, $p = 0.324$, Group: $F_{(9, 14)} = 0.896$, $p = 0.234$, 2-Way RM ANOVA) (Figure 5.2D).

For Cue Recall, there was a significant effect of session (Session: $F_{(1,22)} = 11.345$, $p = 0.004$), and a trend to effect of PPS (Group: $F_{(1,22)} = 3.070$, $p = 0.051$, 2-Way RM ANOVA) and a significant interaction (Session*Group: $F_{(1,22)} = 3.472$, $p = 0.047$, 2-

Way RM ANOVA) (Figure 5.2A and E). Post-hoc analysis revealed that PPS rats froze less than controls during the first 3 minutes of CS presentation ($p = 0.047$, Tukey-Kramer HSD) (Figure 5.2E).

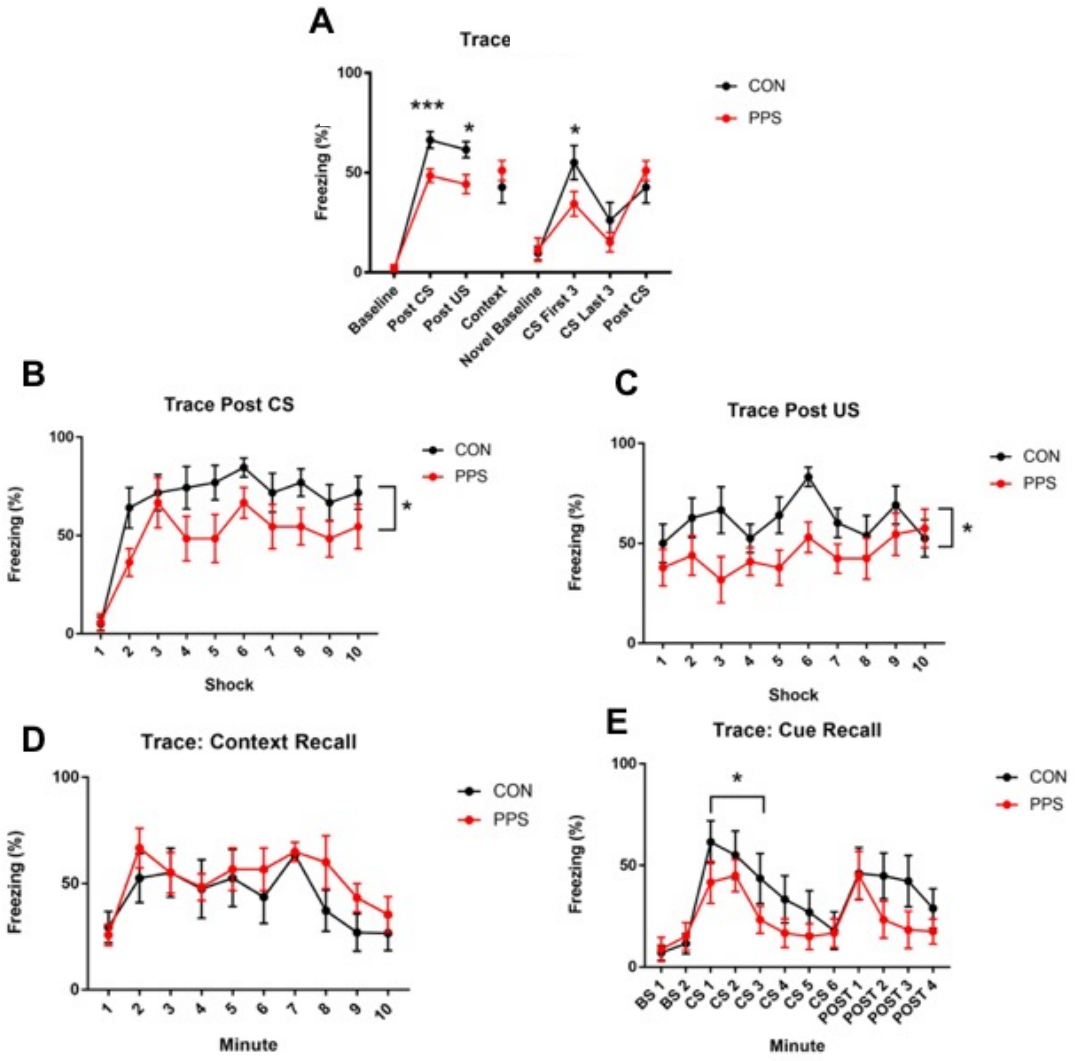

Figure 5.2: Trace conditioning in PPS and control rats. A = The PPS rats show reduced freezing behaviour during conditioning and decreased fear behaviours during CS but not context presentation in recall. B = During conditioning the PPS rats show less freezing in the 30s interval following CS presentation compared to control littermates. C = PPS rats freeze less than controls following footshock (US) in the conditioning trial. D = No difference in freezing responses were evident at Context Recall between PPS and controls when assessed per minute. E = PPS rats show a trend for reduced freezing during cue presentation in Cue Recall with a reduced CR measured in the first 3 minutes of CS presentation. Data is mean % freezing per group for each session or minute of the session, error bars are SEM. PPS versus Control (CON) groups, n = 13 CON, 11 PPS, * = p <0.05, ** = p <0.01. (BS = baseline, CS= conditioned stimulus presentation, POST = post-CS).

Unpaired conditioning is not affected by PPS

Following shock presentation in unpaired conditioning, both PPS and control rats showed an increase in freezing (Session: $F_{(1, 21)}$ = 248.926, p < 0.001, 2-Way RM ANOVA) but PPS had no effect (Group: $F_{(1, 21)}$ = 0.013, p = 0.909, 2-Way RM ANOVA) and there was no evidence of an interaction (Session*Group: Group: $F_{(1, 21)}$ = 0.143, p = 0.756) (Figure 5.3A and B) on acquisition of conditioning.

Freezing did not alter between control and PPS rats in Context Recall (Group: $F_{(1, 10.66)}$ = 0.574, p = 0.465, One-Way ANOVA) (Figure 5.3C). There was no difference between PPS and control groups across any minute of recall (Group: $F_{(1, 21)}$ = 0.596, p = 0.449, Minute: : $F_{(1, 21)}$ = 0.596, p = 0.449, 2-Way RM ANOVA) and no interaction (Group*Minute: $F_{(1, 21)}$ = 0.418, p = 0.765).

Similarly, there was no effect of PPS within Cue Recall (Group: $F_{(1, 21)}$ = 1.55, p = 0.226, Session: $F_{(1, 21)}$ = 8.675, p = 0.002, 2-Way RM ANOVA) (Figure 5.3D) and no evidence of an interaction (Group*Session: $F_{(1, 21)}$ = 8.675, p = 0.002)

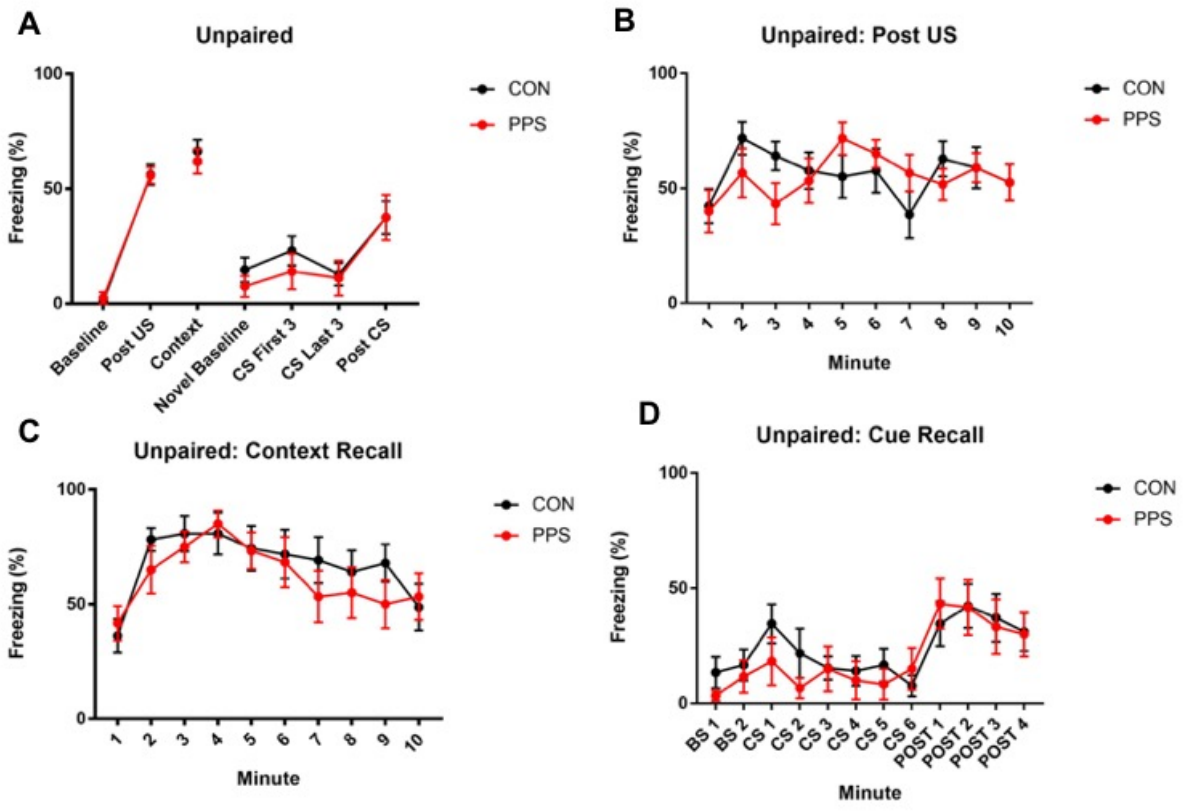

Figure 5.3: Unpaired conditioning in PPS and control rats A = overview of all sessions, showing that freezing responses do not differ between PPS and control littermates. B = Post US responses did not differ between PPS and Control rats. C = Fear responses were maintained following PPS in Context Recall, C = Cue Recall also revealed no differences of fear responses following PPS. Data points represent mean % freezing across group, errors bars are SEM. PPS vs Control (CON) groups, n = 13 CON, 10 PPS. (BS = baseline, CS= conditioned stimulus presentation, POST = post-CS).

5.4 Discussion

Male PPS rats show a deficit in trace conditioning, displaying an impaired conditioning to cue, with some evidence that this may, in part, be due to an encoding impairment within the conditioning session. There was no difference in fear responses to unpaired conditioning between PPS and control littermates. PPS rats also show intact delay conditioning and cue recall, with a possible enhancement of freezing during context recall.

The disrupted trace conditioning seen following PPS suggests that at a neurobiological level there may be an underlying deficit in hippocampal function within the wider fear circuity because trace conditioning critically depends on this region, whereas delay and unpaired conditioning do not (Quinn et al., 2002; Bangasser et al., 2006). Trace conditioning is typically seen as a more 'difficult' learning paradigm than delay conditioning due to the interval between CS and US (Beylin et al., 2001; Bangasser et al., 2006). Thus the potential encoding deficit seen in trace conditioned PPS rats may be revealing a deficit in learning complex or weaker associations, specifically when the hippocampus needs to form a temporal representation between two associated events. The dorsal hippocampus in rodents supports context encoding, short-term contextual memories and trace fear conditioning (Phillips and LeDoux, 1992; Beylin et al., 2001) suggesting that PPS may result in a hippocampal deficit in processing trace fear.

Children exposed to early life adversity display poor encoding of contexts paired with angry faces, reduced hippocampal activation during this encoding and increased hippocampal-ventrolateral PFC connections (Lambert et al., 2017). Lambert et al (2017) also showed that context encoding, including hippocampal activation and context memory, did not change after 8 years old and persisted into later life (Lambert et al., 2017). Therefore, early life stress may affect context encoding, however in the

current study this encoding deficit is specific to trace conditioning and is not apparent in delay or unpaired paradigms. This may reflect the difficulty of the task or other mechanisms specific to trace memory encoding.

Human studies have revealed that acute stress causes a decrease in freezing to trace conditioned cues, whilst increasing responses to delay conditioned cues (Vogel et al., 2015), suggesting that stress causes a shift to amygdala-dependent forms of learning. Vogel et al (2015) also showed that this was likely mediated through MRs (Vogel et al., 2015). PPS has been shown to increase MR expression in the hippocampus of adult animals (Brydges *et al.*, 2014) which is correlated with increased anxiety-like behaviour. MR-activation also rapidly increases neural excitability in the hippocampus and amygdala in rodents (Shors, 2004b). Thus differences in MR:GR ratios may mediate these stress-induced differences in learning and lead to differences in anxiety and fear related behaviours.

However, stress can have wide-ranging effects throughout the limbic system outside that of corticosteroid receptor manipulations. These mechanistic effects can have impacts on the neural systems implicated in associative learning. *BDNF* gene expression has an important role in certain forms of fear conditioning and, as discussed in Chapter 3, is decreased in PPS in males. *BDNF* is increased in the CA1 of the hippocampus after acquisition of contextual fear conditioning (Hall et al., 2000; Kirtley and Thomas, 2010) and is necessary for the consolidation of fear memory (Lee et al., 2004). *BDNF* expression is also increased in the amygdala following cued fear conditioning (Rattiner, 2004) and $BDNF^{+/-}$ rats have impaired cued delay fear conditioning (Harris et al., 2016), suggesting that BDNF signalling in the amygdala is required for acquisition of cued conditioned fear. The reduced *BDNF* expression seen following PPS may be affecting the consolidation of fear memory, although if this was correct it may be expected that we would see differences between PPS and control rats in unpaired conditioning also, as it is a type of contextual conditioning.

The catecholamine system may also have a role in mediating some of the effects seen in this study. Cortical noradrenaline depleted rats have reduced trace fear conditioning to the CS but increased conditioning to the context (Selden et al., 1990). The impaired fear conditioning to explicit cues was correlated with corticosterone levels in rats; there is a wide-range of evidence that PPS results in altered corticosterone levels (Albrecht et al., 2017). Noradrenaline strengthens synaptic contacts in the CA1 of the hippocampus (Katsuki et al., 1997) and the strength of a memory has been correlated with amygdala concentration of noradrenaline (McIntyre et al., 2002). Investigations into how noradrenaline, and the dopamine system in general, are disrupted in this PPS model would give insight into the mechanisms behind their behaviour.

The increase in contextual freezing following delay fear conditioning is different to the result previously seen after PPS where decreased contextual fear responses were reported (Brydges *et al.*, 2014). This can be explained by the different behavioural tasks used in the two studies to measure contextual fear. The original study used a single CS-US presentation where context was the CS. However, the present study used 10 CS-US presentations where a discrete cue (white noise) formed the CS, suggesting that animals may respond differently to multiple trials than a single trial. Also, contextual fear conditioning relies on the hippocampus whereas delay conditioning is thought to primarily depend on the amygdala (Selden et al., 1991a; Phillips and LeDoux, 1992). Therefore, the enhanced contextual freezing in this current study may be a result of increased control of the conditioned responses by the context or context-CS associations, potential due to greater activation of the hippocampus following PPS. This suggests that fear responses following different paradigms may be processed by different neuronal circuits and thus manifest in opposing behavioural outcomes.

5.4.1 Conclusions

This chapter reveals PPS in rats causes a disruption to trace auditory fear conditioning in adulthood. PPS decreases fear responses to CS following trace conditioning and increases fear to context within a delay paradigm. This joins the existing literature that details a role for early life stress on fear memory encoding and retrieval and investigates the effect of PPS on trace conditioning for the first time. The results highlight how PPS has an effect on the hippocampus to alter the way trace memories are processed, demonstrating how PPS can have a profound effect on the hippocampus. However, why not all hippocampal tasks, such as unpaired conditioning, are affected is currently unknown, although may be due to the multiple cues presented in the task.

Chapter 6: The role of *Cacna1c* and stress in the rate of adult hippocampal neurogenesis

6.1 Introduction

Psychiatric disorders, and in particular mood disorders, have been linked to alterations in adult hippocampal neurogenesis (Anacker and Hen, 2017; Apple et al., 2017). It has also been shown that a range of psychotropic medications have been associated with increasing hippocampal neurogenesis in rodent models (including SSRIs, selective SNRIs and tricyclic antidepressants) (Malberg, 2004), and ablation of hippocampal neurogenesis also prevents the positive effects of antidepressants on behaviour in mice (Santarelli et al., 2003). This evidence suggests that hippocampal neurogenesis in adulthood may have a role in the manifestation of psychiatric disorders and the treatment response. The results presented in this chapter investigate the effect of *Cacna1c* heterozygosity and juvenile stress on neurogenesis occurring in the dentate gyrus in adulthood.

6.1.1 Adult hippocampal neurogenesis

In the mammalian adult hippocampus, it is generally accepted that there is constant generation of new neurons which are incorporated into the hippocampal circuity (Altman and Das, 1965; Kaplan and Hinds, 1977; Cameron and Mckay, 2001; Apple et al., 2017). This multi-step process occurs within the dentate gyrus where precursor cells reside in the subgranular zone. This forms a microenvironment for the development of new neurons within a vascular niche which eventually integrate into the dentate gyrus as granule cells (Deng et al., 2010). Granule cells are the primary excitatory cells of the dentate gyrus which receive input from the entorhinal cortex

and project excitatory input to the CA3 via mossy fibres (Deng et al., 2010) and thus have an important role in how the hippocampus processes stimuli. Adult hippocampal neurogenesis has been suggested to have a critical role in hippocampus-dependent forms of learning and memory in animal studies such as pattern separation, complex water maze tasks and trace conditioning (Gould et al., 1999; Leuner et al., 2004; Epp et al., 2007; Waddell and Shors, 2008; Deng et al., 2010).

In the adult rat, there is a high degree of neurogenesis in the dentate gyrus – estimates suggest thousands per day, many of which differentiate into functional neurons (Cameron and Mckay, 2001; Leuner et al., 2004). In humans, there appears to be a lower turnover of neurons, with some studies finding hundreds of new neurons are added to the dentate gyrus daily, while some studies find much fewer (Spalding et al., 2013). Controversially, a recent study did not find evidence that robust adult neurogenesis occurred in the human hippocampus past childhood when staining for DCX+/PSA-NCAM+ cells (Sorrells et al., 2018) although this may be due to different methodologies as another recent study found that adult neurogenesis was present and stable in humans during aging using the same markers (Boldrini et al, 2018).

6.1.2 Neurogenesis markers

Identification of adult-born granule can be done via different approaches. Injection of synthetic nucleotide analogues (e.g. 5'bromodeoxyuridine (BrdU)) and subsequent immunohistochemical analysis has been widely used and characterised as a neurogenesis detection technique. BrdU is incorporated into a cell during cell division and thus forms a marker of cell proliferation (Götz and Huttner, 2005). A concern of this technique is that incorporation of BrdU into the DNA strand may influence cell-cycle kinetics and cell viability and thus other endogenous cell-cycle proteins such as Ki-67 which is expressed during S phase (Kuhn et al., 2016) . BrdU labelling also cannot distinguish progression through the cell cycle or subsequent cell cycle re-

entries. More mature neurons can be identified by the presence of mature neuronal markers in cells such as polysialilated neural-cell-adhesion molecule (PSA-NCAM) or doublecortin (DCX) (Kuhn et al., 2016). DCX, a microtubule-associated protein, is expressed in young migratory neurons and is commonly utilised to mark the differentiation phase of neurogenesis (Von Bohlen Und Halbach, 2011).

6.1.3 Dorsal and ventral hippocampal neurogenesis

There is an evolving view that the dorsal and ventral sections, along the longitudinal axis, of the hippocampus have different functions. The dorsal (or posterior in humans) hippocampus has a key role in cognition and learning, whereas the ventral (or anterior in humans) hippocampus is more involved in stress and emotional behaviours (Fanselow and Dong, 2010). In humans, the anterior hippocampus is smaller in patients with untreated depression (Boldrini et al., 2009) and, in non-human primates, increased metabolism is seen within the anterior hippocampus of individuals with increased anxiety (O'Leary and Cryan, 2014; Anacker and Hen, 2017).

Adult neurogenesis occurs along the entire dorsal-ventral axis of the hippocampus (Kheirbek and Hen, 2011). However, in rodents, the dorsal hippocampus has been reported to contain increased immature neurons, whilst the number of mature adult-born neurons are higher in the ventral hippocampus, suggesting that overall, more adult-born cells maybe added to the ventral dentate gyrus (Tanti et al., 2012; Anacker and Hen, 2017). In addition, adult-born granule cells within the dorsal and ventral hippocampus may be functionally distinct and have different roles once mature. This is important as it has been shown that antidepressants appear to target ventral hippocampal neurogenesis over dorsal (Banasr et al., 2006; Boldrini et al., 2009). Additionally, in rodents, enrichment promoted neurogenesis specifically within the dorsal dentate gyrus whereas unpredictable chronic stress decreased neurogenesis

restrictively within the ventral hippocampus (Tanti et al., 2012). Therefore, in this study dorsal and ventral hippocampal neurogenesis were analysed separately.

6.1.4 Hippocampal neurogenesis and LTCCs

GABA inputs depolarise neural progenitor cells and evoke calcium influx through voltage-gated calcium channels (Tozuka et al., 2005). This contributes to downstream signalling pathways which leads to transcription of genes involved in proliferation, differentiation and survival of adult-born neurons (Spitzer et al., 1994; West et al., 2001; Kang et al., 2016). LTCCs have been particularly implicated in the process of adult neurogenesis; Deisseroth and colleagues (2004) showed that Cav1.2 and Cav1.3 agonists and antagonists upregulated and downregulated adult neurogenesis respectively in cultured neurons derived from neural progenitors in the hippocampus (Deisseroth et al., 2004). In neural progenitor cells taken from mouse brain cortex, nifedipine, a LTCC antagonist, reduced the rate of neurogenesis, whereas a LTCC agonist upregulated neurogenesis (D'Ascenzo et al., 2006). It has been reported that LTCCs are particularly important in the later stages of adult neurogenesis, within a late differentiation stage – at day 9 following induction, LTCC agonists increased survival of immature neurons, whereas antagonists decreased survival (Teh et al., 2014).

In genetic models, a *Cacna1c* $^{-/-}$ forebrain knockout mouse and a neuron-specific *Cacna1c* $^{-/-}$ deletion mouse model both show decreases in immature neurons (Table 6.1). In the forebrain-Cav1.2 knockout, this was attributed to increased cell death of young neurons, correlated with decreased BDNF levels (Lee et al., 2016), which agrees with the cellular work (Teh et al., 2014). However, in a pan-neuronal *Cacna1c* deletion mouse model, marked decreases in cell proliferation were seen which may also contribute to decreased numbers of immature neurons (Temme et al., 2016). Völkening et al (2017) deleted *Cacna1c* on Type 1 cells and reported decreased

proliferation and immature neuron production (Völkening et al., 2017) (Table 6.1). These mice also showed deficits in cue discrimination in a pattern separation paradigm – a type of learning thought to require intact hippocampal neurogenesis (Völkening et al., 2017).

Table 6.1: Summary of adult hippocampal neurogenesis altercations observed in Cacna1c knockdown models

	Model	Proliferation rate	Immature neurons	Cell survival	Dentate gyrus size
Lee et al, 2016	Forebrain-Cav1.2 CkO (-/-)	n.d	↓	↓	n.d
Temme et al, 2016	Neuron-specific deletion of Cav1.2 CkO (-/-)	↓	↓		n.d
Völkening et al, 2017	$Tg^{GLAST\text{-}CreERT2}$ /$Cacna1c^{fl/fl}$ /RCE:loxP mice (Mouse model with Cacna1c-deficient Type-1 cells)	↓	↓		

6.1.5 The effect of stress on hippocampal neurogenesis

There is a wealth of evidence to suggest that stress, and glucocorticoids, can affect adult neurogenesis in several different species (Egeland et al., 2015). Exposing rodents to an acute or chronic stressor such as predator odour results in decreased cell proliferation and decreased survival of immature neurons (Mirescu and Gould, 2006; Egeland et al., 2015) and rodents exposed to chronic unpredictable mild stress show depressive-like behaviour and an impaired ability to cope with stress (Mineur et al., 2007). Interestingly, this depressive phenotype is not seen when neurogenesis is ablated in the absence of stress, suggesting that neurogenesis reduction leads to depressive behaviour through impairing stress modulation. It has been shown that depleting neurogenesis and then exposing a mouse to stress causes an increased stress hormone response and decreased negative feedback on the HPA axis (Snyder et al., 2011). Adult neurogenesis is also thought to be involved in determining if events are stressful or not and whether a stress response is required, potentially through pattern separation. A loss of this ability, potentially through decreased adult neurogenesis, may lead to several previous negative experiences and thus overgeneralisation of novel experiences as negative (Egeland et al., 2015).

There have been many suggested mechanisms for how stress has its impact on adult neurogenesis including: alterations in glucocorticoid signalling mechanisms, cytokines or neurotrophic factors. Glucocorticoids, acting through glucocorticoid receptors (GRs), can influence every stage of neurogenesis by altering gene transcription of pathways known to regulate neurogenesis (such as transforming growth factor-B-SMAD2-SMAD3 pathway or the Hedgehog pathway), modifying the expression and phosphorylation of GRs on neuronal progenitors (Anacker et al., 2013) or through interacting with transcription factor proteins such as NF-κB and CREB to modulate adult neurogenesis under stress (Datson et al., 2012). Cytokines, which are also released in stressful situations alongside stress hormones, can also

have pro-inflammatory effects on the brain that impacts on neurogenesis, for example IL-1B and IL-6 are reported to be increased in the hippocampus following chronic stress which correlates with decreased cell proliferation and neurogenesis (Koo and Duman, 2008). Neurotrophic factors such as BDNF, demonstrated to be susceptive to stress, have a key role in growth and survival of neurons and thus have an impact on neurogenesis (Schmidt and Duman, 2007).

Furthermore, a recent study has directly shown that activity of adult-born granule cells in the ventral dentate gyrus are required to protect from stress-induced anxiety-like behaviours. The ventral dentate gyrus was shown to contain a stress-susceptible cell population that are inhibited by adult-born granule cells and this protects against the negative effects of a chronic social defeat paradigm (Anacker et al., 2018). Thus, there is a clear interaction between stress and neurogenesis, of which we are starting to tease out the causative mechanisms.

Early life environment can strongly affect adult neurogenesis (Loi et al., 2014). Prenatal stress, given in the second trimester, appears to result in a marked reduction in cell proliferation and differentiation within neurogenesis in offspring once they reach adulthood (Lemaire et al., 2006; Mandyam et al., 2008; Rayen et al., 2011). Another study that looked at prenatal stress in the first trimester also demonstrated a reduction in DCX in male and female rats (Madhyastha et al., 2013). Effects of early postnatal stress, by maternal separation and/or deprivation, are much more varied. In males, one study reported increased DCX (Oomen et al., 2009) at PND21, but when rats were tested at 10 weeks of age they demonstrated decreased proliferation and differentiation, suggesting this initial increase in DCX was a transient effect (Oomen et al., 2010). Other studies also reported reductions in cell proliferation in males following maternal separation (Mirescu et al., 2004; Hulshof et al., 2011), however another study reported no effects (Kumar et al., 2011). There may also be sex specific effects in the impact of early postnatal stress on neurogenesis; Oomen and

colleagues (2009) reported decreased DCX in stressed females at PND21 with no effect on proliferation (Oomen et al., 2009). However, at 10 weeks old, female rats exposed to maternal deprivation presented no differences in neurogenic markers, however there was a significant reduction in DG granule cell number and density (Oomen et al., 2011). Changes in neurogenesis are also seen when stress is given later in infancy; when male mice were subject to ten days of the social defeat test within the pre-pubertal phase, they displayed decreased cell proliferation in the dentate gyrus as measured by BrdU administration, both 1 day after the last stress session and also 4 weeks later, suggesting that this decreased proliferation may be a long-term consequence (Mouri et al., 2018). Restraint stress given in adolescence (PND 30-52) also resulted in decreased neurogenesis in adulthood in female rats only, with a small increase in cell survival seen in males (Barha et al., 2011). Interestingly, another study reported that neurogenesis in the prepubertal period is vital for normal social development in female mice (Wei et al., 2011), demonstrating the importance of neurogenesis for corresponding behaviour.

This chapter details the effect of both Cacna1c heterozygosity and PPS on neurogenic markers in the dentate gyrus. In regard to the PPS paradigm, work within our group has previously shown a decrease in BrdU in the ventral hippocampus of these rats, and thus this chapter only investigates the impact of stress on DCX levels.

6.2 Methods

6.2.1 Contribution

It should be acknowledged that Dr N Brydges performed the stress procedure, perfusion and sectioning for the PPS animals. A. Moon completed all other analysis and all work on the Cacna1c heterozygote model.

6.2.2 *Cacna1c*$^{+/-}$ rats

Sixteen Sprague Dawley behaviourally naïve male rats (Charles River), 8 wild-type and 8 *Cacna1c*$^{+/-}$ litter mates were housed in mixed genotype groups of 2-4. All animals were handled and tail-marked prior to experiments proceeding. A second cohort of 6 wild-types and 10 *Cacna1c*$^{+/-}$ male behaviourally naïve rats were also housed in the same fashion for ventral hippocampus analysis.

6.2.3 PPS animals

Eighteen Lister Hooded male rats (bred from Charles River Lister Hooded pairs), nine control and nine were subjected to a 3 day variable juvenile stress protocol at PND25-27, were housed in same-litter groups of 2-5 with ab libitum access to food and water. All animals were handled and tail-marked prior to experimentation, but behaviourally naïve.

6.2.4 BrdU injection and perfusion

On day of experimentation (PND 60-70 for all animals) rats were manually restrained and administrated with a single intraperitoneal injection of 50mg/kg BrdU. 6 hours following BrdU injection rats were euthanised via intraperitoneal injection of Euthatal (200mg/ml) and transcardially perfused with 4% paraformaldehyde (PFA) (Figure 6.1A).

6.2.5 Sectioning

Following perfusion, brains were rapidly dissected and post-fixed overnight in 4% PFA. Brains were then cryoprotected in 30% sucrose for 6 days and then sectioned coronally on a cryostat to produce 40µm sections of the dorsal hippocampus (Bregma -2.04 to -5.04mm) or ventral hippocampus (Bregma -5.16 to -6.48mm) and transferred to a plastic 12-well plate (Sigma-Aldrich, Dorset, UK).

6.2.6 Fluorescent imaging

Sections were washed between each step for 3 x 20 minutes in 0.1M PBS (pH 7.4). One in every 12 sections throughout the dentate gyrus was denatured at 37°C in 2M hydrochloric acid for 30mins, followed by incubation in blocking solution (1% Triton-X100 and 10% donkey serum in 0.1M PBS) for two hours. Primary antibodies (Table 6.2) were diluted in 0.1% Triton-X and 0.2% donkey serum in PBS to appropriate concentrations (BrdU: 1:500, DCX: 1:100) and allowed to bind to sections overnight at 4°C. Sections were incubated for two hours in the dark with donkey anti-goat Alexa Fluor 647 and donkey anti-rat Alexa Fluor 555 (Life Technologies, UK). Sections were then exposed to DAPI stain (1:1000 diluted in 0.1M PBS) for ten minutes, washed twice in 0.1M PBS and transferred to slides where they were mounted on standard microscopy slides. The dentate gyri from each section were imaged on an epifluorescent microscope (Leica DM6000B Upright Timelapse System with Leica Application Suite Advanced Fluorescence 3.0.0 build 8134 software, Leica Microsystems) at x20 magnification.

6.2.7 Analysis

Immunopositive cells were counted throughout the whole subgranular layer of the dentate gyrus (1 in 12 sections per animal). The area of the individual supra- or infrapyramidal blade was measured and BrdU/DCX stained cells were manually counted by eye. The total cells per section were divided by the area of the DG to give

a cell count per mm^2. Cell counts for the 'suprapyramidal', 'infrapyramidal' blades were analysed separately, as well as the total cell count for both blades. Cell counts were checked for normality and homogeneity of variances (Levene's test) and transformed if appropriate by Box-Cox transformations. One-way ANOVAs were then conducted to determine the effect of genotype/stress on cell counts in the DG, with cell count forming the dependent variable and 'Genotype'/'Group' forming the between subjects factor. For stressed animals, litter was nested within group as a random variable to account for litter of origin.

Table 6.2: Primary antibodies utilised in for neurogenic analysis

Target	Host	Manufacturer	Catalogue no	Dilution
BrdU	Rt (monoclonal)	Bio-Rad	OBT0030	1:500
DCX (1)	Gt (polyclonal)	Santa Cruz (discontinued)	SC8066	1:100

6.3 Results

6.3.1 The effect of *Cacna1c* heterozygosity on adult hippocampal neurogenesis

6.3.1.1. Dorsal hippocampus

There was a significant decrease in BrdU immunopositive cells in *Cacna1c*$^{+/-}$ rats in comparison to wild-types throughout the dorsal dentate gryus (Genotype: $F_{(1, 15)}$ = 12.098, p = 0.004, One-Way ANOVA) (Figure 6.1, Figure 6.2B). This difference was noted in both the suprapyramidal blade (Genotype: $F_{(1, 15)}$ = 11.913, p = 0.004, One-Way ANOVA) and the infrapyramidal blade (Genotype: $F_{(1, 15)}$ = 12.709, p = 0.004, One-Way ANOVA) (Figure 6.2B). This suggests that *Cacna1c*$^{+/-}$ rats demonstrate a decrease in cell proliferation. However, there were no differences seen in doublecortin, the marker of immature neurons in the dorsal hippocampus (Genotype: $F_{(1, 15)}$ = 1.050, p = 0.324, One-Way ANOVA) (Figure 6.2). When separated into the suprapyramidal and infrapyramidal blade, there was also no difference seen (Supra; $F_{(1, 15)}$ = 0.339, p = 0.570, Infra; $F_{(1, 15)}$ = 2.646, p = 0.127, One-Way ANOVA) (Figure 6.2).

6.3.1.2. Ventral hippocampus

Neurogenesis was also investigated in the ventral hippocampus. There was an overall trend to a decrease in BrdU immunopositive cells in *Cacna1c*$^{+/-}$ rats throughout the ventral dentate gyrus (Genotype: $F_{(1, 15)}$ = 3.014, p = 0.093, One-Way ANOVA). There was a significant decrease in the suprapyramidal blade (Genotype: $F_{1, 15)}$ = 4.542, p = 0.042, One-Way ANOVA), but not the infrapyramidal blade (Genotype: $F_{(1, 15)}$ = 0.820, p = 0.373, One-Way ANOVA). There was no significant difference in doublecortin labelled cells in the ventral hippocampus (Genotype: $F_{(1, 15)}$ = 1.484, p = 0.251, One-Way ANOVA), in either blade (Supra; $F_{(1,15)}$ = 2.668, p = 0.133, Infra; $F_{(1,15)}$ = 0.326, p = 0.581, One-Way ANOVA).

6.3.1.3. Dentate gyrus thickness

There was no difference seen in the thickness of the granule layer in either the dorsal (Genotype: $F_{(1, 14)} = 0.086$, $p = 0.774$, One-Way ANOVA) or the ventral hippocampus (Genotype: $F_{(1, 11)} = 1.162$, $p = 0.301$, One-Way ANOVA).

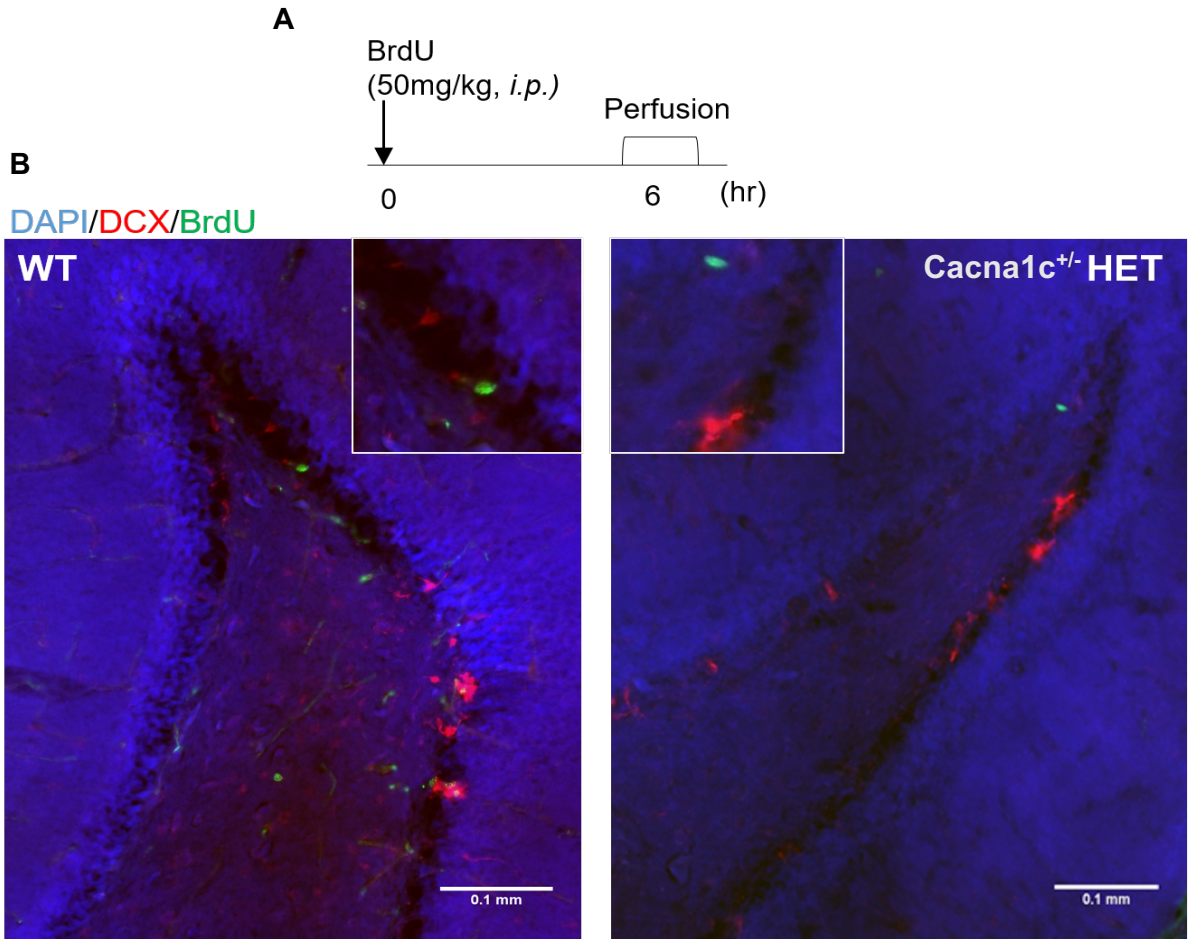

Figure 6.1: Neurogenesis analysis of *Cacna1c*$^{+/-}$ rats compared to wild-types. A: Schematic of the timeline of BrdU injection and perfusion, B = representative immunohistochemical images of wild-type and *Cacna1c*$^{+/-}$ animals with stained cells (red = DCX, green = BrdU), n = 8 WT, 8 *Cacna1c*$^{+/-}$.

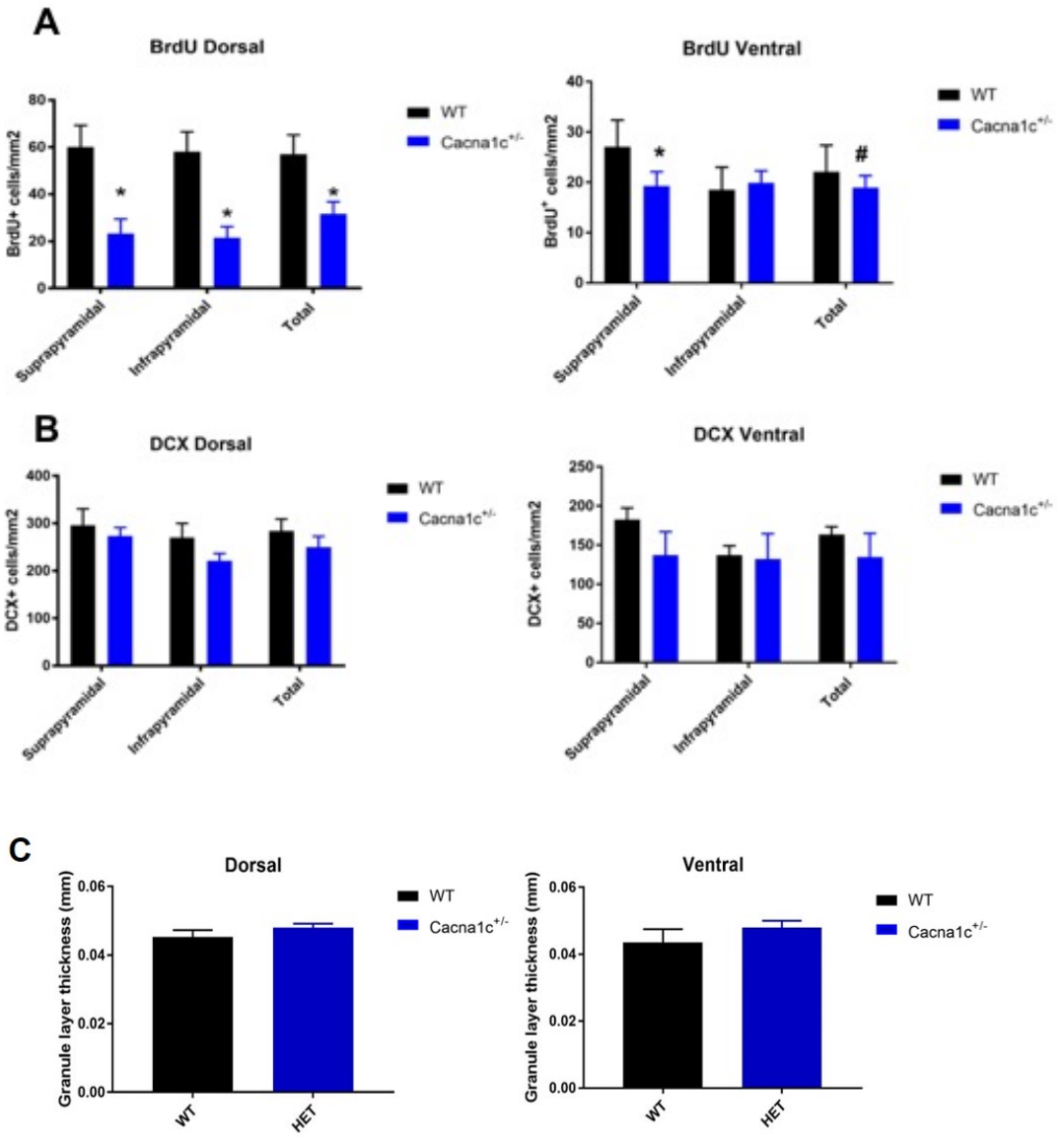

Figure 6.2: A and B = Graphical representations of BrdU and DCX counts in the dorsal and ventral hippocampus of *Cacna1c*$^{+/-}$ and wild-type rats. Bars represent mean cell counts/mm^{2}, error bars are SEM. C = graphs of granule cell layer thickness in the dorsal and ventral hippocampus. Bars represent mean granule layer thickness, error bars are SEM. WT compared to *Cacna1c*$^{+/-}$, n = 8 WT, 8 *Cacna1c*$^{+/-}$ (dorsal), 6 WT, 10 Cacna1c$^{+/-}$ (ventral), $p < 0.05$, # = $p < 0.1$.

6.3.2 Adult hippocampal neurogenesis and the consequences of PPS

6.3.2.1. Dorsal hippocampus

No significant difference was seen between control and stressed adults in the dorsal hippocampus (Group: $F_{(1, 9.29)} = 1.536$, $p = 0.254$, One-Way ANOVA) (Figure 6.3A and 6.4A). When each blade was analysed separately, there was no difference in the suprapyramidal blade (Group: $F_{(1, 9.29)} = 2.597$, $p = 0.147$, One-Way ANOVA) or in the infrapyramidal blade (Group: $F_{(1, 9.29)} = 1.607$, $p = 0.240$, One-Way ANOVA) (Figure 6.4A).

6.3.2.2. Ventral hippocampus

DCX^+ cells were significantly increased in the ventral hippocampus of prepubertally stressed animals (Group: $F_{(1, 9.29)} = 14.206$, $p = 0.004$, One-Way ANOVA) (Figure 6.3B, Figure 6.4A). This was true for both the suprapyramidal blade (Group: $F_{(1, 9.29)} = 14.774$, $p = 0.003$, One-Way ANOVA) and the infrapyramidal blade (Group: $F_{(1, 9.29)} = 15.341$, $p = 0.023$, One-Way ANOVA) (Figure 6.4A).

6.3.2.3. Dentate gyrus morphology

In the dorsal hippocampus, there was no difference in the granular layer thickness in the dorsal hippocampus (Group: $F_{(1, 9.29)} = 0.047$, $p = 0.835$, One-Way ANOVA) or the ventral hippocampus (Group: $F_{(1, 9.29)} = 0.201$, $p = 0.672$, One-Way ANOVA) (Figure 6.4B).

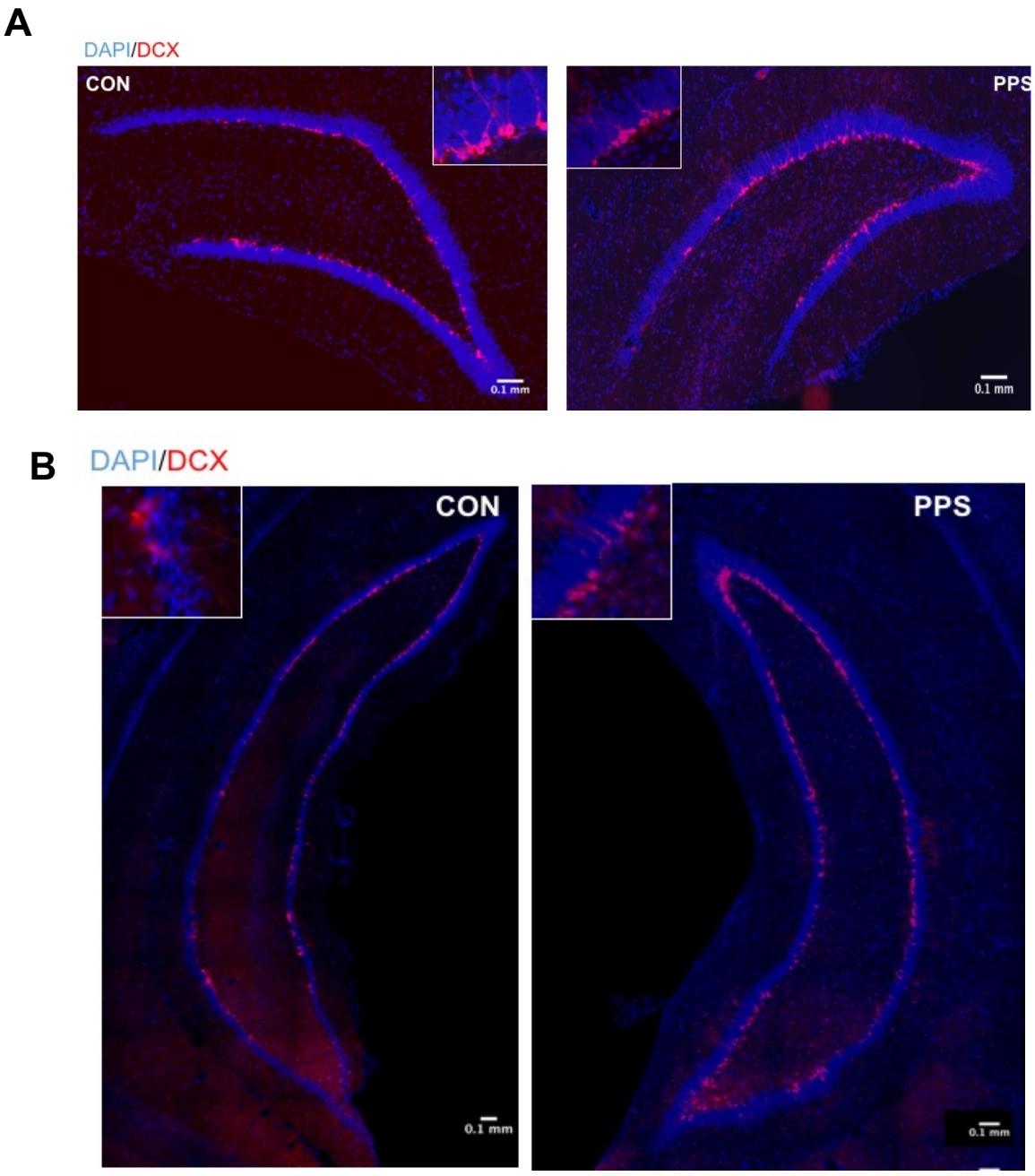

Figure 6.3: DCX counts in PPS rats. A = immunohistochemical images of DCX cell counts in the dorsal hippocampus in control and PPS rats, B = immunohistochemical images of DCX counts of PPS and control rats in the ventral hippocampus, n = 9 CON, 9 PPS. Scale bar = 0.1mm.

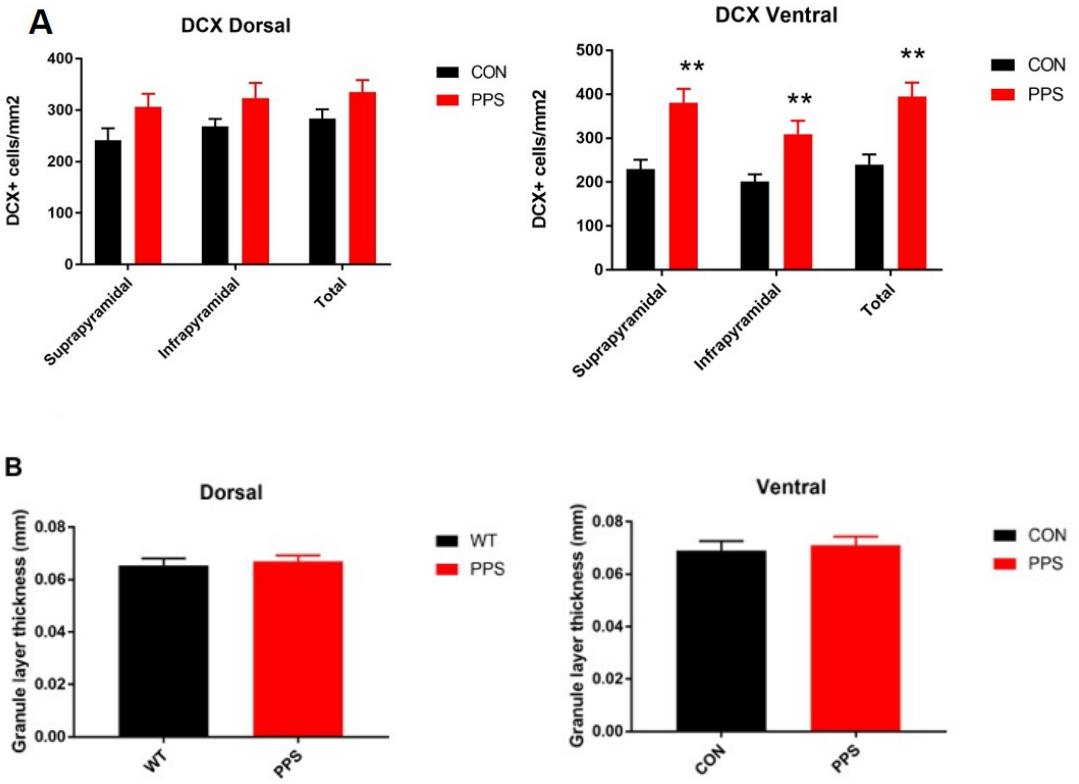

Figure 6.4: A = DCX cell counts in both the dorsal and ventral hippocampus of control and PPS animals, displaying increased DCX$^+$ stained cells in PPS rats. Bars represent mean cell counts per group, error bars are SEM. B = bar charts displaying the granule layer thickness of the dentate gyrus in the dorsal and ventral hippocampus. Bars represent average thickness per group, error bars are SEM. Control (CON) vs PPS, n = 9 CON/9 PPS, ** = p < 0.01.

6.4 Discussion

Adult hippocampal neurogenesis is a process that is thought to be highly important for hippocampal-dependent learning, memory and the stress response. In the above studies, it was seen that both *Cacna1c* heterozygosity and prepubertal stress resulted in altered DG neurogenesis. *Cacna1c*$^{+/-}$ rats demonstrated decreased cell proliferation as measured by a short-term single pulse BrdU injection throughout the whole hippocampus, whereas stressed rats presented with increased immature neurons in the ventral hippocampus.

6.4.1 Cav1.2 and neurogenesis

These results support the existing literature that details a role for LTCCs in the modulation of adult hippocampal neurogenesis (Table 6.1). Neural progenitor cells sense Ca^{2+} influx through LTCCs that occurs in response to excitatory stimuli (Deisseroth et al., 2004). This influx results in proneuronal gene expression and drives the conversion of progenitors into mature neurons, as well as increasing cell survival (Deisseroth et al., 2004; D'Ascenzo et al., 2006). Previous studies on *Cacna1c* deletion mouse models have showed a profound effect on cell survival and proliferation. The current study utilized a novel heterozygote rat model of *Cacna1c* deficiency to show that adult neurogenesis, at least at the proliferative stage, following Cav1.2 disruption is impaired across species. This is particularly important due to the apparent involvement of neurogenesis in learning and memory; the physiological systems involved with cognition have been extensively studied in the rat. Rates of neurogenesis have also been shown to be different between mice and rats; rats appear to have a faster rate of maturation, decreased cell death and were more likely to be activated during learning than in mice (Snyder et al., 2009). These profound differences demonstrate how important it is to examine phenotypes across species boundaries.

It is important to consider that Cav1.3 genetic elimination results in an even more severe phenotype, with altered adult hippocampal neurogenesis and reductions in the overall size of the dentate gyrus (Marschallinger et al., 2015), indicating that both Cav1.3 and Cav1.2 are required for intact neurogenesis. It has previously been suggested that Cav1.3 has a more profound effect of neurogenesis because it is expressed in immature progenitor cells whereas Cav1.2 is only expressed in more mature neurons (Marschallinger et al., 2015; Lee et al., 2016). However *in vitro* work has demonstrated the presence of Cav1.2 in progenitor cells (Teh et al., 2014) and has revealed that deletion of *Cacna1c* in type 1 cells resulted in decreased proliferation and enhanced differentiation into astrocytes (Völkening et al., 2017), culminating in decreased numbers of new-born progenitors and mature neurons. However acute treatment of neural progenitor cells with calcium antagonists and agonists did not appear to effect proliferation in the manner seen in deletion models (D'Ascenzo et al., 2006; Teh et al., 2014). This may suggest that the effect on neurogenesis through deletions of Cav1.2 and Cav1.3 is a non-autonomous effect; potentially through BDNF production. LTCC signalling has been linked to *BDNF* transcription in hippocampal neurons (Ghosh et al., 1994) and a mouse model with a forebrain conditional deletion of Cav1.2 presented with deficient neurogenesis, which was correlated with decreased *BDNF* levels (Lee et al., 2016). BDNF is thought to support neurogenesis by activating TrkB receptors and subsequent activation of MAP and PI3 kinase pathways that promote proliferation and survival of progenitors (Barnabé-Heider and Miller, 2003). It is possible that Cav1.2 heterozygosity results in decreased *BDNF* transcription which in turn results in reduced proliferation of type 1 cells.

This study did not find any differences in DCX, a marker of immature neurons, between heterozygotes and wild-types. This is in contrast to previous studies (Lee et al., 2016; Temme et al., 2016) which found decreases in immature cell markers as

well, showing that survival of progenitors is also reduced. However, it should be noted that these studies were performed in mice, and thus this could be reflective of species differences, and these studies utilised a forebrain and neuronal homozygote deletion of *Cacna1c* respectively. It may be suggested that heterozygote deletion is not sufficient to result in net difference in neurogenesis, or that the homozygote models are not representative of physiological compensatory mechanisms. *In vitro* work also has shown that application of LTCC antagonists and agonists has no effect on proliferation or early survival, but when applied at the late differentiation stage, had a significant effect on the rate of neurogenesis (Teh et al., 2014). Therefore, it may be surprising that we do not see an impact on DCX within our model. However, this may be due to many reasons. For example, the hippocampus may compensate for the lack of cell proliferation by increasing the survival rates of the neural progenitors that do mature. Ordinarily, programmed cell death (PCD) plays an important role in the regulation of adult neurogenesis and the integration of neurons into the hippocampal circuity (Ryu et al., 2016). Approximately 30-70% of newly born cells are eliminated within the first month, depending on the animal's condition and experience. Elimination of erroneous cells is a major function of PCD in the adult brain and is vital for efficient establishment of synaptic connections and full integration into the dentate gyrus (Ryu et al., 2016). It has been shown that endoplasmic reticulum-mitochondrial Ca^{2+} promotes the commitment of neural stem cells to cell death (Rowland and Voeltz, 2012) and Ca^{2+} entry through LTCC have been shown to promote apoptosis in adrenal medulla chromaffin cells (Cano-Abad et al., 2001), suggesting there may be a role for Ca^{2+} influx in PCD within the dentate gyrus.

Also, while being highly valuable as a predictive tool of neurogenesis, doublecortin is only one marker of immature neurons, and there have been reports of a pool of doublecortin-containing cells within other regions in the hippocampus that are uncoupled from adult neurogenesis (Kremer et al., 2013). Validation of this result

using additional markers of immature neurons such as PSA-NCAM (Seki, 2002) would be advantageous in this case.

6.4.2 Prepubertal stress and neurogenesis

Early postnatal life stress has been consistently shown to produce significant alterations in the adult hippocampus in rodents, which has been correlated with impaired synaptic plasticity and decreased BDNF expression (Egeland et al., 2015). However, little is known about the effect of PPS on neurogenesis, and no other studies have looked at the long-term consequences of PPS on neurogenesis. This study shows that there is increased survival of immature neurons following PPS as measured by DCX in the ventral hippocampus. This is somewhat surprising as decreased proliferation has been seen in this model (Brydges, unpublished data), suggesting that the hippocampus might compensate for this decreased proliferation by increasing adult-born cell survival in the ventral hippocampus. Studies looking at the effects of early life stress on adult neurogenesis have overwhelmingly suggested that stress resulted in a decreased proliferation and survival of adult born cells, however a slight increase in cell survival in male rats following stress in adolescence has been demonstrated (Barha et al., 2011), in agreement with the results seen in this chapter. This may suggest that when stress is given later in infancy and adolescence, it has a differing effect to prenatal or early postnatal stress, demonstrating the importance of timing in early life stress models.

Increased cell survival within adult neurogenesis is often thought to have positive effects on the hippocampus, brain and subsequent behavior. Increased adult neurogenesis has been shown to be increased by exercise, antidepressant use and learning (Epp et al., 2007; Fabel et al., 2009; Anacker and Hen, 2017) in both the dorsal and ventral hippocampus. However increased neurogenesis may not always result in improved function. For example, there are many behaviours, such as spatial

learning and certain working memory tasks (Scharfman and Hen, 2007) that are not improved by increased neurogenesis, and in fact, animals without any adult-born neurons may perform better at working memory paradigm (Scharfman and Hen, 2007). Increased neurogenesis has also been associated with stress susceptibility (Levone et al., 2015); when primates are housed in stressful conditions, they display increased neurogenesis and learning improvements (Lyons et al., 2010). Furthermore, mice who were seen to be susceptible to developing social avoidance behaviour following social defeat stress had increased cell survival of adult-born neurons 4 weeks later (Lagace et al., 2010). Therefore, the increased cell survival following PPS may be associated with an increased susceptibility to the negative effects of stress, which could be explored with future behaviour experiments.

The specific increase in DCX cell counts was seen in the ventral hippocampus not the dorsal hippocampus. Studies that have investigated regional specific changes along the septo-temporal axis of the hippocampus have shown that chronic mild stress results in decreased cell proliferation and neurogenesis in the ventral hippocampus in both mice and rats (Jayatissa et al., 2009; Tanti et al., 2012, 2013). The ventral hippocampus is involved in the control of emotional behaviour and has been shown to be susceptible to stress (Tanti et al., 2012), due to its many connections with the limbic system. Therefore, the results presented in this chapter contribute to the idea that the ventral hippocampus might be more vulnerable to the effects of stress.

6.4.3 Conclusions

The results presented in this chapter add to the current literature that indicates a role for Cav1.2 in adult hippocampal neurogenesis. However, unlike results seen in mouse and cell models, the consequence of reduced *Cacna1c* dosage was confined to cell proliferation as marked by BrdU. This suggests that there are mechanisms in place

to increase cell survival in immature neurons, a hypothesis that could be investigated by analysis of apoptotic markers. Prepubertal stress also has an impact on adult hippocampal neurogenesis at the ventral pole, resulting in increased immature neurons in adulthood. This demonstrates that both genetic and environmental risk factors for psychiatric factors can have an effect on adult neurogenesis and represents a potential pathway convergence.

Chapter 7: The expression of parvalbumin containing interneurons in the hippocampus of *Cacna1c*$^{+/-}$ rats and PPS rats

7.1 Introduction

GABAergic interneurons are imperative for physiological brain function and cognition. They are critical for maintaining the correct excitatory-inhibitory balance, primarily through their regulation of the activity of the principal excitatory cells. However their functions are wide ranging within the nervous system. Parvalbumin (PV) positive interneurons have been found to be abnormal in the brains of schizophrenic patients (Beasley and Reynolds, 1997; Konradi et al., 2011a), although the mechanism and the consequences of their dysfunction is still to be fully eluded. This chapter investigates the expression of PV and GAD67 expression in the hippocampus of *Cacna1c*$^{+/-}$ rats to determine if LTCC signalling pathways may contribute to PV dysfunction in psychiatric disorders.

7.1.1 GABAergic interneurons

The main microcircuits in the cortical and subcortical regions of the brain involve excitatory glutamatergic pyramidal cells and inhibitory GABAergic interneurons (Figure 8.1) (Marín, 2012). While pyramidal cells transmit information across the brain, interneurons provide inhibitory inputs, synchronise oscillations and contribute to local neuron assemblies (Klausberger and Somogyi, 2008). GABAergic interneurons are considered to be responsible for controlling hyperexcitability in the brain and deficits in the GABAergic system are associated with pathological levels of excitability (Dichter and Ayala, 1987). Multiple animal models and studies in humans

have shown that deficits in excitatory-inhibitory balance may be present in many psychiatric conditions (Marín, 2012).

There are many ways to classify GABAergic interneurons, including morphology or distribution of axonal plexus (Errington, 2014). Interneurons can also be classified biochemically, by their calcium-binding proteins and neuropeptides present in the cell bodies and axon terminals (Marín, 2012). This includes parvalbumin (PV), cholecystokinin (CCK), somatostatin (SOM), calretinin and neuropeptide Y containing interneurons (Pelkey et al., 2017).

7.1.2 PV^+ interneurons

PV^+ interneurons express parvalbumin, a high affinity calcium-binding albumin protein, and account for approximately 40% of all GABAergic interneurons in rodents (Rudy et al., 2011). PV^+ interneurons, which include chandelier (axo-axonic) and basket cells, are fast-spiking interneurons known to play essential roles in the regulation of network oscillations and synchrony. They play an important role in feedback and feedforward inhibition as their inhibitory terminals synapse onto the cell body and axon initial segment of their target neurons (Figure 7.1) (Muller and Remy, 2014; Pelkey et al., 2017). PV^+ interneurons can convert an excitatory input signal into an inhibitory output signal within a millisecond. This is due to their polarity – they possess weakly excitable dendrites to sample activity in the surrounding networks and highly excitable axons to convert analogue neurochemical signals to digital, which are then quickly propagated to a large number of target cells (Szabadics et al., 2006; Bartos and Elgueta, 2012). This process is highly dependent on the coupling of Ca^{2+} channels and release sensors at PV^+ interneuron output synapses (Horn and Nicoll, 2018). PV^+ interneurons are also necessary for the production of gamma (30-80Hz) oscillations, which enables information processing and circuit noise reduction (Sohal et al., 2009). The exact mechanisms for this aren't clear but may involve

feedback loops from excitatory neurons onto PV interneurons or oscillatory behaviour within PV neuronal networks (Nakazawa et al., 2012).

PV^+ interneurons and their associated circuits are vital for the processing of information related to working memory, learning, social behaviour and sensory perception (Steullet et al., 2017). These interneurons also have a key role in controlling hippocampal neurogenesis. Song et al showed that proliferating hippocampal neural precursors receive GABA inputs from PV^+ interneurons which correlated with survival of progenitors. High activity from PV^+ interneurons leads to survival of proliferating precursors whereas low activity leads to cell death (Song et al., 2013).

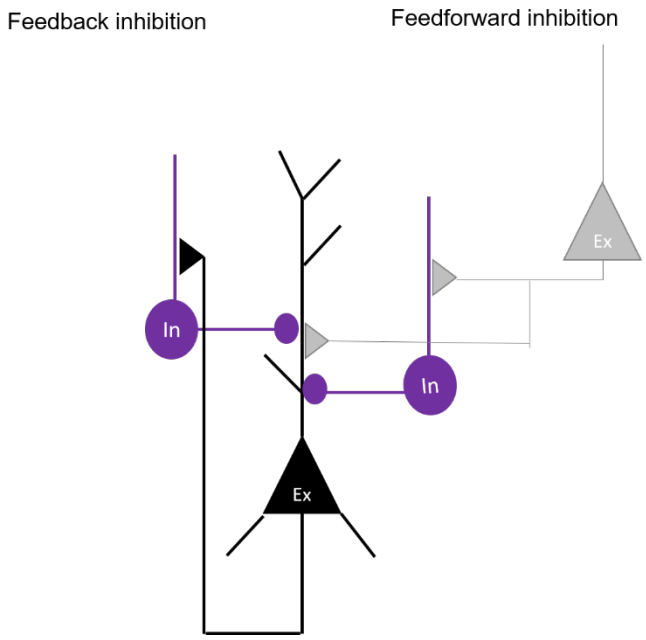

Figure 7.1: A simplified diagram to demonstrate the importance of GABAergic interneurons (purple) in excitation-inhibition balance in the brain. Feedback inhibition shown on the left demonstrates how a glutamatergic excitatory pyramidal neuron excites an interneuron which releases a GABAergic signal to inhibit the pyramidal neuron. Feedforward inhibition shown on the right indicates how an excitatory pyramidal neuron can excite inhibitory interneuron which in turn inhibit a group of postsynaptic excitatory neurons outside of the initial excitatory neurons area (Muller and Remy (2014))

7.1.3 PV and psychiatric disorders

Abnormalities in GABAergic interneurons, including PV$^+$ interneurons, has been reported in mood and psychiatric disorders such as depression, bipolar disorder and, most notably, schizophrenia in various brain areas (Sanacora et al., 1999; Heckers et al., 2002; Zhang and Reynolds, 2002; Hossein Fatemi et al., 2005; Torrey et al., 2005).

Alterations in PV$^+$ interneurons have been found in post-mortem brains of psychiatric disorder sufferers. PV$^+$ interneurons in the dorsolateral prefrontal cortex of adults with schizophrenia show a decreased expression of GAD67 (Akbarian et al., 1995; Hashimoto et al., 2003) which may interfere with the inhibitory properties of the cell. Reductions in PV mRNA itself have also been seen in the prefrontal cortex (Lewis and Gonzalez-Burgos, 2006). A reduced density of PV$^+$ neurons has also been reported in the all hippocampal subfields (Zhang and Reynolds, 2002; Torrey et al., 2005; Konradi et al., 2011a) and the parahippocampal region (Wang et al., 2011) in schizophrenia. Gamma frequency oscillations, which depend upon PV$^+$ interneuron function, also are abnormal in the brains of schizophrenia sufferers (Haenschel et al., 2009; Uhlhaas and Singer, 2010; Williams and Boksa, 2010), however whether this is due to impairments in PV$^+$ function or as a secondary effect from other neurotransmitter systems is not clear.

It may be suggested that changes in GABAergic interneurons in schizophrenia may be secondary to N-methyl-D-aspartate receptor (NMDAR) dysfunction. The NMDAR-hypofunction or glutamatergic hypothesis of schizophrenia arose from the finding that NMDAR antagonists such as ketamine or phencyclidine (PCP) are able to induce the psychotic and cognitive symptoms characteristic of schizophrenia in healthy humans and worsen symptoms of patients with schizophrenia (Javitt and Zukin, 1991; Krystal et al., 1994; Newcomer et al., 1999; Moghaddam and Javitt, 2012). It has been suggested that GABAergic interneurons may be part of this mechanism; hippocampal

GABAergic interneurons are more sensitive to NMDAR antagonists than pyramidal neurons are (Grunze et al., 1996). Therefore, the excitation induced by NMDAR antagonists may actually be due to inhibition of fast-spiking interneurons, thus disinhibiting excitatory neurons (Homayoun and Moghaddam, 2007). The finding that repeated exposure to NMDAR antagonists decreases PV and GAD67 expression in GABAergic interneurons (Keilhoff et al., 2004; Rujescu et al., 2006) adds more validation to this hypothesis. Furthermore, heterozygous deletion of NR1, a subunit of the NMDAR, in GABAergic interneurons in mice resulted in schizophrenia-like symptoms after adolescence (Belforte et al., 2010).

Reductions in GAD67 and PV^+ neurons have also been reported in the hippocampus and parahippocampus of bipolar disorder patients (Heckers et al., 2002; Konradi et al., 2011b; Wang et al., 2011). The volume of nonpyramidal neurons has also been shown to be decreased in the CA2 of both bipolar disorder and schizophrenia (Benes et al., 1998). It has been proposed that the decreased PV^+ interneuron density in bipolar disorder may suggest that the GABAergic disinhibition mechanism that may underlie schizophrenia may also be present in bipolar disorder (Konradi et al., 2011b). The fact that gamma oscillations are also disturbed in bipolar disorder adds extra evidence to this hypothesis (Liu et al., 2012).

7.1.4 PV and LTCCs

PV and Cav1.2 have been shown to be co-localised in the mouse hippocampus (Xu et al., 2007), suggesting that PV interneurons contain LTCCs. Cohen et al identified a novel Cav1-CaMK-CaM pathway in PV^+ interneurons that results in CREB phosphorylation, gene transcription and dendritic branching following activity which may influence PV and GAD67 expression within interneurons (Cohen et al., 2016). PV and GAD67 expression is dependent on learning and experience potentially through this Cav1 mediated pathway; fear conditioning results in increased PV and

GAD67 expression in basket cells which enhances memory consolidation and synaptic plasticity (Donato et al., 2013). A recent study has also shown that excitatory hippocampal pyramidal neurons can regulate PV^+ interneuron synapses by neuronal activity, through LTCC signalling (Horn and Nicoll, 2018). This indicates the important of LTCC signalling in activity-dependent GAD67 and PV expression changes and function.

Removal of extracellular or intracellular Ca^{2+} in spinal cord cultures of Xenopus results in a decreased number of GABA-expressing cells (Spitzer et al., 1993). In mice, LTCCs regulate the development of ventral hippocampal PV^+ interneurons (Jiang and Swann, 2005). The application of L-type calcium channel agonists and antagonists to ventral hippocampal slice cultures accelerate and suppress respectively the growth of PV^+ interneurons, as well as the expression of GABA synthetic enzymes (Jiang and Swann, 2005). Hence, LTCC may be necessary for the development of PV^+ interneurons and therefore, Cacna1c heterozygosity may be suggested to alter PV^+ cell levels.

7.1.5 PV and stress

Dysfunction in PV^+ cells has also been reported in the hippocampus and cortex following prenatal stress such as poly I:C (Meyer et al., 2008; Ducharme et al., 2012) or LPS injection (Jenkins et al., 2009) in animal models. Postnatal stress in the form of both acute and chronic isolation rearing (Harte et al., 2007) also resulted in decreased PV immunoreactive cells in the hippocampus (Filipović et al., 2013). Interestingly, fragmented maternal care straight after birth resulted in earlier development of PV^+ interneurons, which are normally a late developing class of neurons within the brain (Bath et al., 2016). Maternal separation on the other end resulted in decreased PV levels at adolescence within the prefrontal cortex (Holland et al., 2014), suggesting that early life stress has a differential effect on PV levels

throughout life and in different brain regions. These studies show that PV immunoreactivity and PV^+ interneurons are sensitive to neurodevelopmental and environment insults.

This chapter investigates PV and GAD67 protein expression in the hippocampus of *Cacna1c*$^{+/-}$ rats to determine if Cav1.2 LTCCs are necessary for expression of PV^+ interneurons *in vivo*. PV is also investigated in rats exposed to PPS to determine if stress at this time of life can affect PV expression.

7.2 Methods

7.2.1 Contributions

The stress procedure, perfusion and sectioning for the PPS animals was conducted by Dr N Brydges. All work on the *Cacna1c*$^{+/-}$ model and all staining and analysis was conducted by A Moon.

7.2.2 Animals

8 adult *Cacna1c*$^{+/-}$ and 8 wild-type Sprague Dawley behaviourally naïve male rats (300-400g) were housed in mixed genotype groups with ad libitum access to food and water. 12 Lister-Hooded adult PPS exposed rats and 9 adult control behaviourally naïve littermates were housed similarly. All rats were sacrificed via intraperitoneal Euthatal (200mg/ml) injection at PND 60-70 and transcardially perfused with 4% PFA. Brains were cryoprotected in 30% sucrose for 2-6 days.

7.2.3 Hippocampal sectioning

Brains were sectioned coronally in 40um slices throughout the hippocampus using a cryostat (Leica Microsystems CM1860UV) and stored in 0.1 M PBS. For every animal, one in 12 sections were stained throughout the hippocampus.

7.2.4 PV staining

Sections were blocked using 1% Triton-X and 10% donkey serum in 0.1 M PBS for 2 hours at room temperature. PV primary antibody (1:1000) (Table 7.1) was diluted in 0.1% Triton-X and 0.2% donkey serum (in 0.1 M PBS) and incubated with sections overnight at 4°C. Sections were washed in in 0.1 M PBS (0.1% Triton-X and 0.2% donkey serum) at least three times.

7.2.5 GAD67 staining

Sections were blocked in 5% donkey serum, 2.5% bovine serum albumin (BSA) and 0.2% Triton-X in 0.1M PBS for 2.5 hours at room temperature. GAD67 primary antibody (1:500) (Table 7.1) was diluted in the blocking solution and applied to sections for 48 hours at 4°C. Sections were washed in blocking solution.

Both GAD67 and PV stained sections were incubated with donkey anti-mouse Alexa Fluor™ 647 (Thermo Fisher Scientific, UK) diluted 1:1000 in 0.1M PBS for 2 hours at room temperature. Sections were washed with 0.1M PBS twice and incubated for ten minutes in the dark with DAPI stain (1:1000) for nuclei straining, before being washed in 0.1M PBS three more times. Sections were mounted on standard microscopy slides and imaged using an epifluorescent microscope (Leica DM6000B Upright Timelapse System with Leica Application Suite Advanced Fluorescence 3.0.0 build 8134 software, Leica Microsystems) at x20 magnification.

7.2.6 Analysis

Immunopositive cells were counted by manual visual counting. Cells were counted throughout the entire hippocampus within each region (CA1, CA3 and DG), and within each individual strata of that region. Each count represents the average of at least 8 individually stained hippocampi sections from each animal. The area of each region/strata was measured using ImageJ software and number of cells divided by this area to give a representative cell density (cells/mm^2). Cell densities were checked for normality of distribution and homogeneity (Levene's Test) and transformed by Box-Cox transformations if appropriate. Firstly, 2-way ANOVAs were performed, with 'cell count' forming the dependent variable and 'sublayer' (within subjects factor) and 'genotype' or 'group' (between subjects factors) comprising the independent variables. If significant differences were found, post-hoc analysis using Tukey Kramer HSD was performed, to take multiple comparisons into account. In the PPS model,

where there was an *a priori* reason to suggest that there may be a difference within a certain sublayer, One-Way ANOVAs were performed with 'cell count' forming the dependent factor and 'Group' forming the between subjects independent factor.

Table 7.1: Primary antibodies used in immunohistochemical analysis

Target	Host	Manufacturer	Catalogue no	Dilution
PV	Ms (monoclonal)	Sigma	P3088	1:1000
GAD67	Ms (monoclonal)	Millipore	MAB5406	1:500

7.3 Results

7.3.1 Parvalbumin expression in the hippocampus of *Cacna1c*$^{+/-}$ rats and wild-types

PV^+ was differentially distributed across sublayers (Sublayer: $F_{(8, 123)} = 134.87$, $p < 0.001$, 2-Way ANOVA) and was significantly affected by genotype (Genotype: $F_{(1, 123)} = 3.99$, $p = 0.048$, 2-Way ANOVA ANOVA). There was a trend to a significant interaction between the area and genotype (Genotype*Session: $F_{(8, 123)} = 1.78$, $p = 0.08$, 2-Way ANOVA). Post-hoc analysis was therefore conducted.

Sublayers

The stratum pyramidale in both the CA1 and CA3 had significantly increased PV^+ cell counts over all other sublayers ($p < 0.001$, all layers, Tukey Kramer HSD), with significantly more PV^+ cells in the CA1 ($p < 0.001$) (Figure 7.2). Within the DG, the granular layer had increased PV^+ cell counts in comparison to the CA1 and CA3 stratum radiatum (CA1: $p = 0.002$, CA3: $p = 0.018$) and the DG molecular layer ($p <0.001$), decreased cell counts in comparison to the pyramidale layers ($p < 0.001$), however a similar PV^+ cell counts all other layers. All other sublayers had similar PV^+ cell counts to each other.

Genotype

Within the CA1, there was a trend to decreased PV^+ cell counts in heterozygotes in the striatum oriens, however this was not significant when correcting for multiple comparisons ($p = 0.088$, Tukey-Kramer post hoc) (Figure 7.3A). Post-hoc Tukey-Kramer analysis did not reveal any other significant differences between genotypes within the CA1 subregions. There were also no significant genotype effects on PV^+ cell counts within the CA3 (Figure 7.3B), suggesting that while the amount of parvalbumin-positive interneurons differ between the strata, there is no difference in the number between heterozygotes and wildtypes.

However, within the dentate gyrus, *Cacna1c*$^{+/-}$ animals showed a decrease of PV^+ cell counts in the granular level of the dentate gyrus ($p = 0.007$, Tukey-Kramer HSD) (Figure 7.3C and D). This was specific to the cell dense granular layer as there was no difference between genotypes in interneurons in the molecular layer ($p = 0.548$, Tukey-Kramer HSD) or within the hilus ($p = 0.111$, Tukey Kramer HSD). This suggests that there is specific deficit of parvalbumin containing interneurons within the granular layer of the DG.

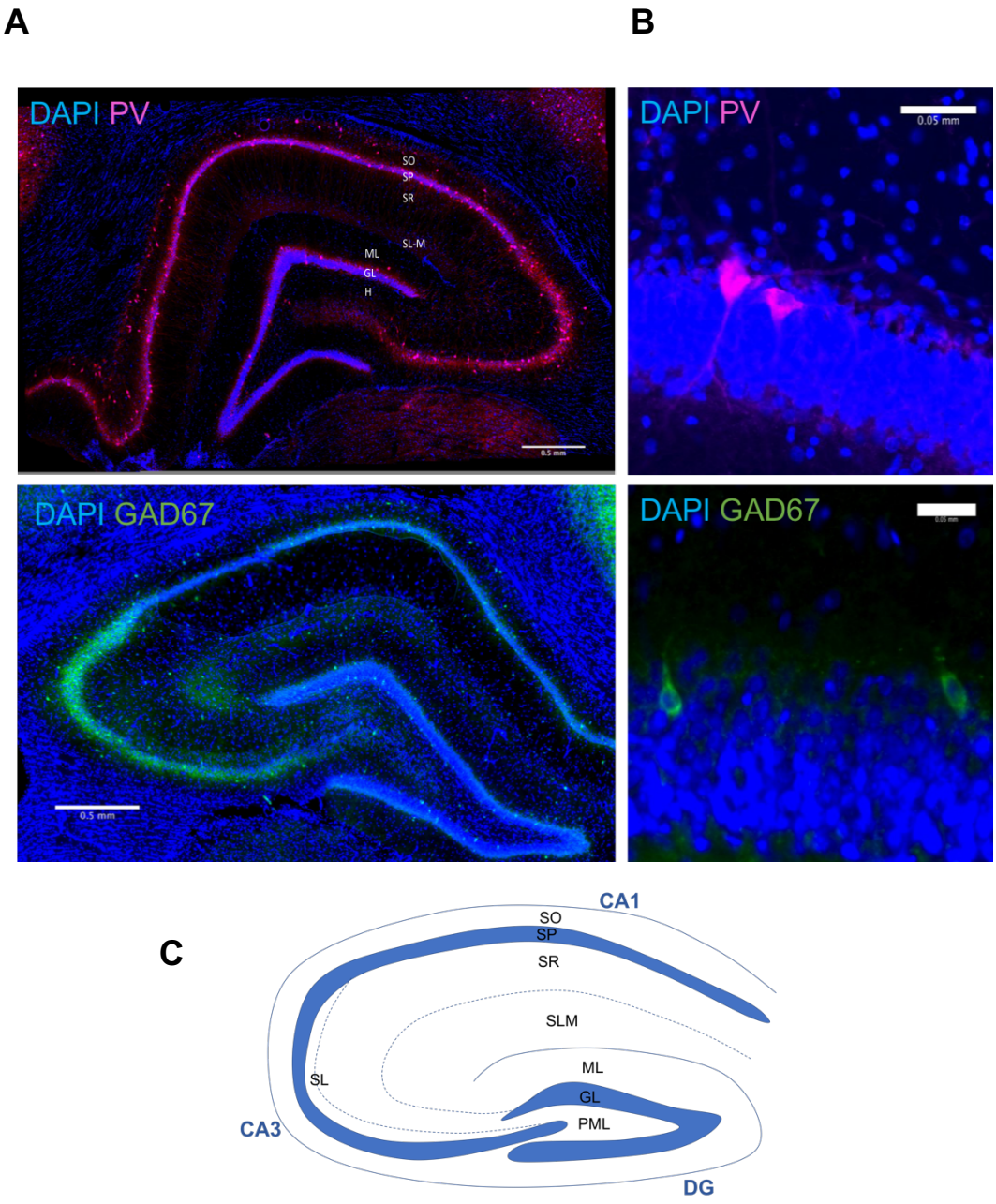

Figure 7.2: A and B = Representative fluorescent immunohistochemistry images demonstrating the distribution of PV^{+} and GABAergic interneurons in all regions of the hippocampus. C = Schematic of the subregions and strata that make up the hippocampus. Immunopositive cells were counted according to each strata. DAPI = 4', 6-diamidino-2-phenylindole, PV = parvalbumin, GAD67 = glutamate decarboxylase 67, CA1/3 = Cornu Ammonis, SO = stratum oriens, SP = stratum pyramidale, SR = stratum radiatum, SLM = stratum lacunsum-moleculare, ML = molecular layer, GL = granular layer, PML = polymorphic layer.

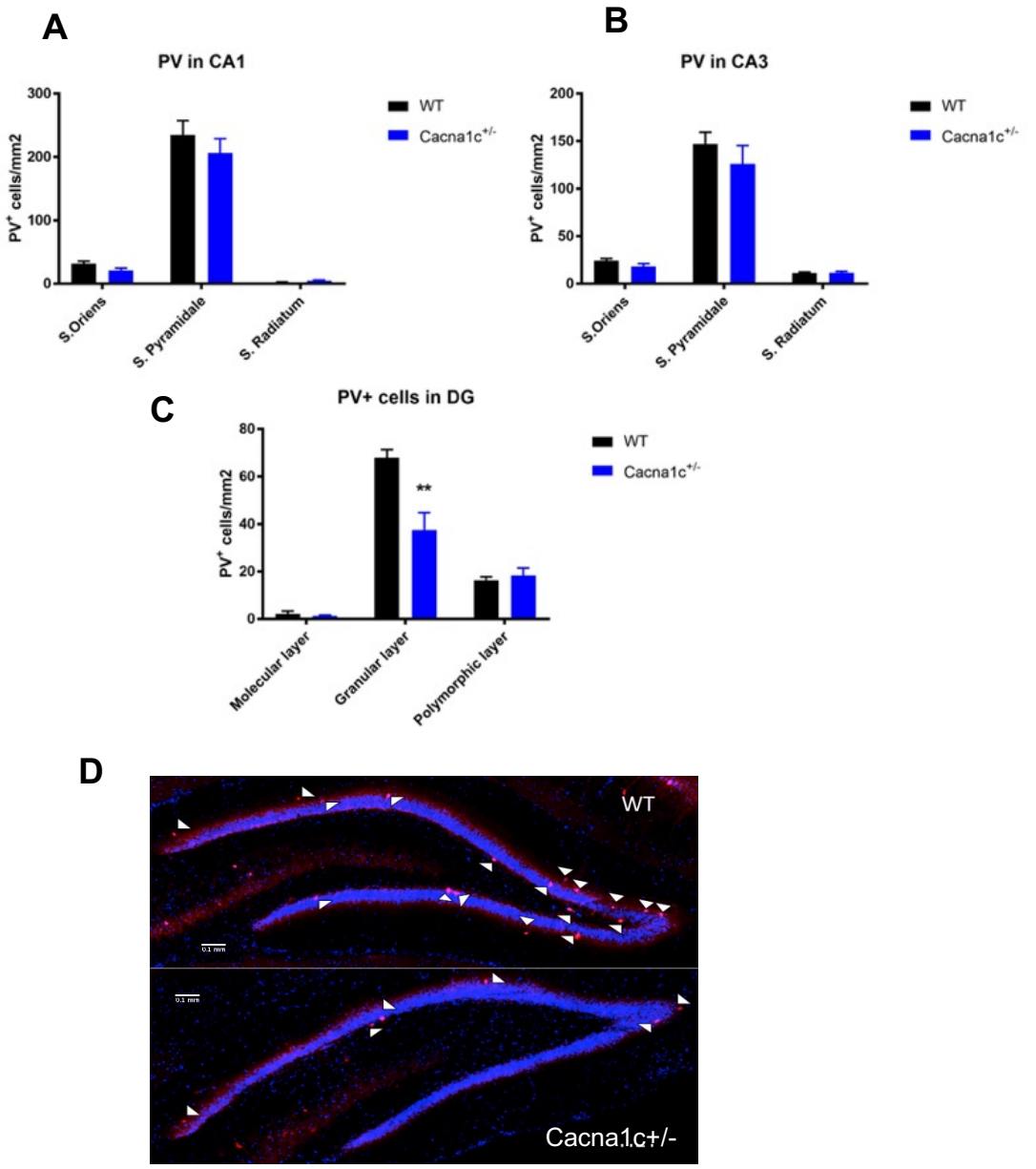

Figure 7.3 A-C = Bar charts representing the distribution of PV within the subregions of the CA1, CA3 and DG of the hippocampus in *Cacna1c*+/- and wild-types. PV immunopositive cells are decreased in the granular layer of the dentate gyrus of *Cacna1c*+/-. Bars represent average cell count per mm2 per genotype, error bars are SEM. D: Representative immunohistochemical image of PV immunofluorescence in the DG in *Cacna1c*+/- and wild-type rats, white triangles indicate PV+ cells. Wild-type compared to *Cacna1c*+/-, n = 8 per genotype, ** = $p < 0.01$. Scale bar = 0.1mm.

7.3.2 The effect of Cacna1c heterozygosity on GAD67 expression in the hippocampus

GAD67 marks GABAergic interneurons, including the subclass that includes parvalbumin. Analysing GAD67 expression alongside PV allows us to determine if only parvalbumin protein expression within interneurons is altered, or if GABA levels may also be affected within the interneurons. Sublayer cell count of GAD67 was significantly different (Sublayer: $F_{(11, 156)} = 49.93$, $p < 0.001$, 2-Way ANOVA). There was also very strong trend of genotype affecting $GAD67^+$ cell count (Genotype: $F_{(1,156)} = 3.75$, $p = 0.055$, 2-Way ANOVA) and a significant interaction between hippocampus sublayer and genotype (Sublayer*Genotype: $F_{(11, 156)} = 2.85$, $p = 0.002$, 2-Way ANOVA).

Sublayer

Both CA1 and CA3 stratum pyramidale sublayers had significantly more $GAD67^+$ cell staining than any other layer ($p < 0.001$, all layers, Tukey Kramer post hoc), with more in the CA1 ($p = 0.001$, Tukey Kramer post hoc) (Figure 7.2). CA3 stratum lacunsum-moleculare had an increased $GAD67^+$ cell count to its corresponding CA1 sublayer ($p < 0.001$, Tukey Kramer post hoc) as well as all other sublayers apart from stratum pyramidale ($p < 0.001$, all sublayers, Tukey Kramer post hoc). The molecular layer of the DG had a decreased $GAD67^+$ cell count in comparison to the CA3 stratum lucidum ($p < 0.001$, Tukey Kramer post hoc) and granular layer of the DG ($p = 0.002$, Tukey Kramer post hoc). The DG granular layer also had an increased $GAD67^+$ cell count in comparison to the CA1 stratum radiatum ($p < 0.001$, Tukey Kramer post hoc). All other sublayers had statistically similar expression to each other.

Genotype

There was a trend to a decreased $GAD67^+$ cell count in stratum oriens of the CA1 in *Cacna1c*$^{+/-}$ rats ($p = 0.09$, Tukey Kramer post hoc) after multiple comparison

corrections (Figure 7.4A). There were no other genotype differences in other CA1 sublayers. Within the CA3, there was a trend to an increase of GAD67$^+$ cell count in *Cacna1c*$^{+/-}$ rats within the stratum lacunosum-moleculare (GAD67: $p = 0.066$, Tukey Kramer post hoc), a region without enough PV cells for analysis. In *Cacna1c*$^{+/-}$ rats, there was a trend to a decreased GAD67$^+$ cell count within the stratum lucidum ($p = 0.060$, Tukey Kramer post hoc). There were no other significant effects on GAD67$^+$ cell counts within the CA3 (Figure 7.4B).

Within the dentate gyrus, there was a decrease in GAD67$^+$ cell counts within the granular layer ($p = 0.019$, Tukey Kramer post hoc) (Figure 7.4C and D).

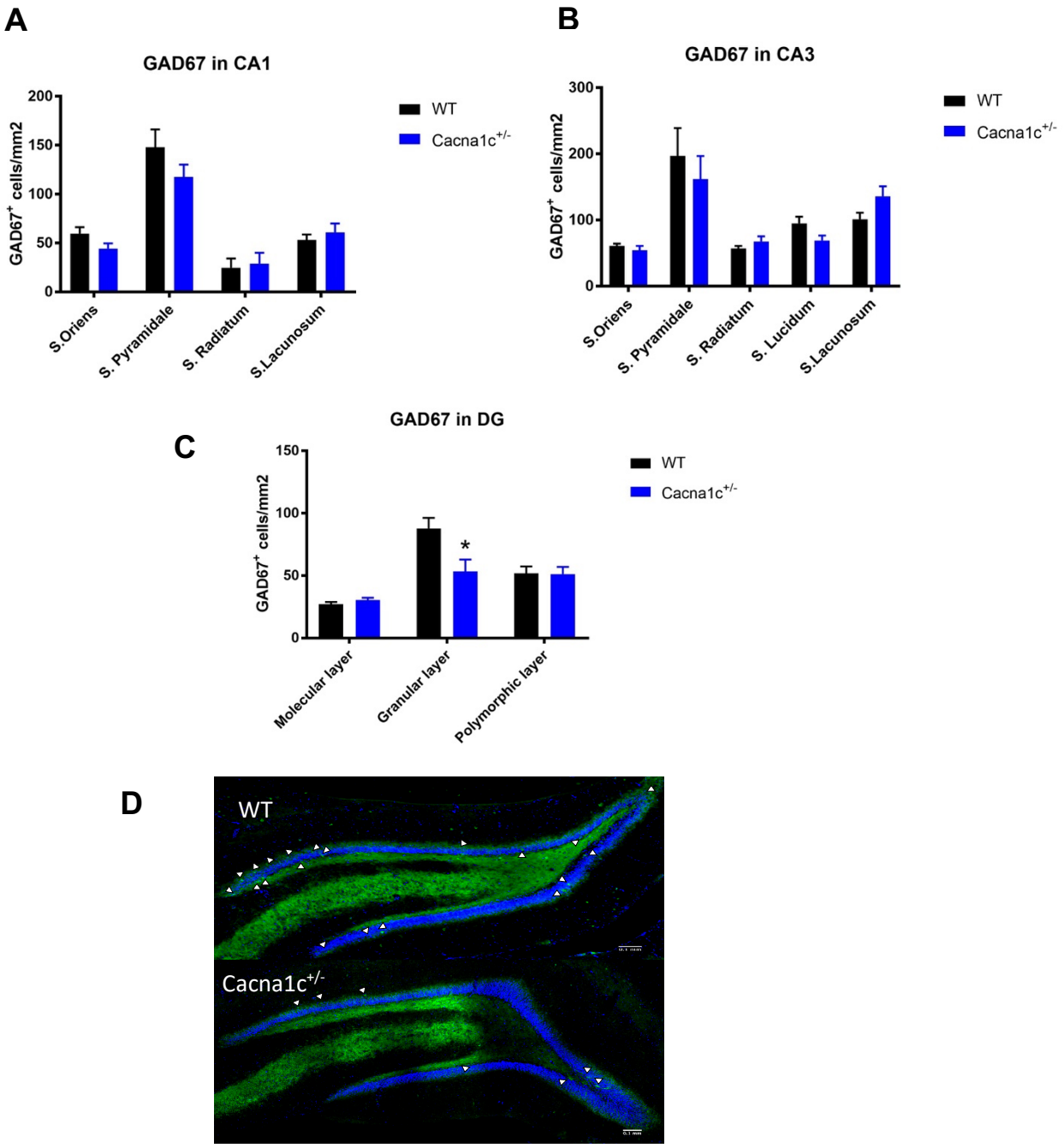

Figure 7.4: A-C = Bar charts representing the distribution of GAD67 within the hippocampus of wild-type and *Cacna1c*$^{+/-}$ rats. Bars represent average cell count per mm^2 per genotype, error bars are SEM. D = Representative immunofluorescent images demonstrating the distribution of GAD67^{+} cells in the dentate gyrus for both genotype, white triangles indicate GAD67^{+} cells in the granular layer of the DG. Wild-types compared to *Cacna1c*$^{+/-}$, n = 8 per genotype, * = $p < 0.05$. Scale bars = 0.1mm

7.3.3 PV changes in the PPS model

Previous analysis in this model has revealed an increase in $GAD67^+$ cell count in the hilus of the dentate gyrus PPS rats (Brydges, unpublished data), thus PV^+ cell count was analysed throughout the hippocampus to see if this was reflected in PV^+ interneurons.

Whole model analysis revealed a significant effect of area on PV^+ cell count in the hippocampus of both control and PPS rats (Sublayer: $F_{(5, 102.4)} = 148.715$, $p < 0.001$, ANOVA), but no effect of stress (Group: $F_{(1, 5.16)} = 0.597$, $p = 0.474$) and no area*stress interaction (Sublayer*Group: $F_{(5, 102.4)} = 0.865$, $p = 0.507$ (Figure 7.5). Therefore, while there was differential PV expression between sublayers of the hippocampus, PPS does not seem to affect expression.

In the hilus, where GAD67 changes have been seen, there was no difference of PV^+ cell count between PPS and controls (Group: $F_{(1, 8.813)} = 0.065$, $p = 0.804$, One-Way ANOVA). The granular layer, where PV^+ and $GAD67^+$ cell count was decreased in the *Cacna1c*$^{+/-}$ rats, also showed no significant difference (Group: $F_{(1, 8.549)} = 1.780$, $p = 0.217$, One-Way ANOVA) (Figure 7.5).

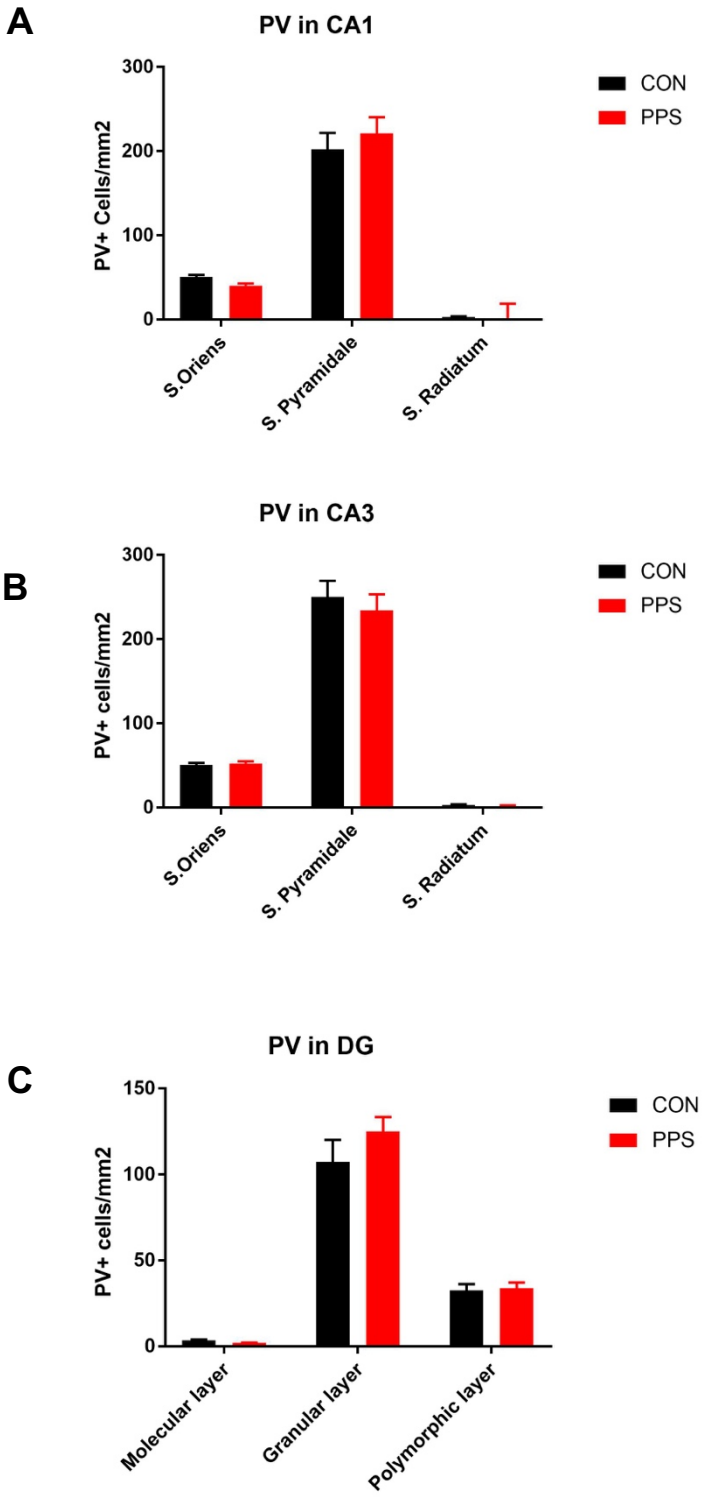

Figure 7.5: There is no difference in PV+ immunopositive cells throughout the hippocampus between PPS and control rats in the CA1 (A), CA3 (B) or DG (C). Bars represent average cell count/mm^2 of PV cells in both PPS and control rats, error bars are SEM. CON compared to PPS, n = 9 CON/12 PPS, * = $p < 0.05$.

7.4 Discussion

This study provides evidence that PV^+ and $GAD67^+$ cells were reduced in the granular layer of the dentate gyrus of *Cacna1c*$^{+/-}$ rats. This appears to be a specific deficit as no differences were seen throughout the rest of the hippocampus, in either the CA1 or CA3, or the rest of the DG. These results add to the existing literature describing an association between psychiatric disease risk and hippocampal interneuron dysfunction. However, there were no differences found in either PV or GAD67 expression following PPS.

LTCCs have previously been shown in ventral hippocampal slice cultures to determine PV^+ interneuron development, with LTCC antagonists reducing the amount of PV^+ cells produced (Jiang and Swann, 2005). The results presented in this chapter agree with this finding, in that *Cacna1c* heterozygosity, resulting in lower Cav1.2 dosage, also decreased PV expression in the DG. However, Jiang and Swann suggest that there is a much larger contribution of Cav1.3 than Cav1.2 to this phenomenon (Jiang and Swann, 2005). We show here that Cav1.2 reduction can have a profound effect on PV^+ interneurons within certain hippocampal areas without alterations in Cav1.3. The exact mechanism as to why decreased LTCC, and subsequent decreased Ca^{2+} influx, has such an impact of PV expression has not been fully eluded. Interestingly, while BDNF acts on some interneurons to promote their growth and maturation, PV expressing interneurons appear to be unresponsive to BDNF (Marty et al., 1996), suggesting a BDNF-independent mechanism is responsible for the *Cacna1c*$^{+/-}$ induced PV dysfunction. Potentially, the Cav1-CaMK-CaM pathway demonstrated in PV^+ interneurons that responds to activity to drive gene expression and dendritic branching (Cohen et al., 2016) may be deficient in *Cacna1c*$^{+/-}$ rats and result in decreased PV and GAD67 expression within interneurons.

Decreased PV and GAD67 immunoreactivity was demonstrated only in the granular layer of the dentate gyrus of *Cacna1c*$^{+/-}$ rats, although trends are evident in CA1 stratum oriens and other CA3 regions. This selective deficit in this region may be suggestive of general dentate gyrus dysfunction in the *Cacna1c*$^{+/-}$ model. The dentate gyrus is a critical structure within the hippocampal formation which supports encoding of multiple sensory inputs, memory based pattern separation and spatial contexts (Kesner, 2017). Some of these functions may be supported by neurogenesis. Therefore, selective deficits in this region could have large impacts on cognition and behaviour. Zhang and Reynolds reported a decrease in PV$^+$ immuno-reactive neurons in the dentate gyrus also, however they also demonstrated similar reductions throughout other hippocampal subfields (Zhang and Reynolds, 2002). Konradi and colleagues also show alterations in PV$^+$ expression throughout CA subfields in both schizophrenia and BPD, however they did not analyse any DG regions (Konradi et al., 2011a, 2011b).

Impairments in PV$^+$ interneurons has been linked to various behaviour abnormities such as spatial working memory (Murray et al., 2011) and social behaviour (Holland et al., 2014), but how PV$^+$ interneuron impairment in various subregions of the brain influences cognition and behaviour is still under investigation. However, as the dentate gyrus is a critical region for memory and learning, a recent study used DREADD (Designer receptors exclusively activated by designer drugs) to activate PV$^+$ interneurons in the dentate gyrus (Zou et al., 2016). They showed activation of these interneurons resulted in decreased anxiety, impaired social interaction to novelty and facilitated fear extinction, while cued and contextual fear memory was maintained (Zou et al., 2016). This suggests a critical role for PV$^+$ interneurons in the formation of inhibitory memory to conditioned fear, as well as lowering anxiety. Mouse models of low gene dosage of *Cacna1c*$^-$ have presented with increased anxiety (Dao et al., 2010; Bader et al., 2011; Lee et al., 2012; Dedic et al., 2018) and a model of

Timothy Syndrome demonstrated significantly reduced extinction of fear memory (Bader et al., 2011). Therefore decreased PV+ interneurons, as seen in the *Cacna1c*$^{+/-}$ model, could have influences on the behaviour and cognition of these animals.

PV and GAD67 cell count following PPS

This is, as far as we are aware, the first study to investigate the long-term impact of PPS on PV$^+$ cells within the hippocampus. However, both prenatal stress and early postnatal stress have been shown to result in the dysfunction of PV$^+$ interneurons within the hippocampus (Harte et al., 2007; Meyer et al., 2008; Filipović et al., 2013; Holland et al., 2014). Additionally, previous research has also indicated that the hippocampal GABAergic PV$^+$ interneurons appear to represent a vulnerable population to the effects of chronic stress (Czeh et al., 2005; Zaletel et al., 2016; Rossetti et al., 2018), with one study utilising using a combination of immunohistochemistry and quantitative stereological techniques to show that 5 weeks of stress in tree shews resulted in large reductions of PV+ neurons in the CA2, CA3 and DG (Czeh et al., 2005). This has been suggested to be mediated through dysregulated redox mechanisms (Cabungcal et al., 2013; Rossetti et al., 2018). It may therefore be unexpected that we do not see decrease PV expression in the hippocampus of PPS rats. This may be explained by the fact that PPS occurs over a much shorter time scale in comparison to standard chronic stress techniques and therefore may have less of a damaging effect on the PV$^+$ interneuron population. It may also suggest that PPS occurs at a time period if an organism's life that allows it to recover fully from acute insults on the GABAergic system, unlike the sensitive prenatal and early postnatal environment. The evidence for oxidative stress and redox mechanisms causing the decreased PV+ cells following early life insults (Cabungcal et al., 2013; Rossetti et al., 2018) may also suggest that PPS does not cause sufficient oxidative stress for these processes to occur, however, this is purely

speculative at present. Further research would be necessary to establish the impact of PPS on GABAergic interneurons in the hippocampus, taking into account the age that stress is given, any sex-specific effects and potential resilience mechanisms that may come into play.

There are many classes of interneurons in the brain, with PV^+ interneurons contributing to 40% of the total interneurons in the rodent hippocampus and less in humans (Pelkey et al., 2017). Somatostatin (SOM) and calretinin expressing interneurons make up the vast majority of non-PV^+ interneurons within the rat brain. SOM^+ interneurons have been suggested to be neuroprotective within the brain; when they are knocked out, excitotoxicity arises (Rajput et al., 2011). Alterations in SOM^+ interneurons have been shown to be decreased in several brain areas in schizophrenia, including the dorsolateral prefrontal cortex (Morris et al., 2008; Guillozet-Bongaarts et al., 2014) and the hippocampus (Konradi et al., 2011a). SOM^+ interneurons have also been reported to be decreased in bipolar disorder brains within the hippocampus (Konradi et al., 2011b). Therefore, despite being not as well replicated as PV^+ alterations in psychiatric disorders, SOM^+ interneurons may also be an interesting and valid target to examine in models of disorder. Calretinin$^+$ interneurons, which make up a smaller number of interneurons in the rat cortex and subcortex than PV^+ and SOM^+ cells, do not appear to be altered in disorder (Lewis et al., 2012; Brisch et al., 2015), although they are less studied than their more highly expressed counterparts. Therefore it may be possible that multiple types of GABAergic interneurons are decreased in the hippocampus of *Cacna1c*$^{+/-}$ rats, or other types of interneurons altered in the PPS model, and so further markers of interneuron subtypes should be investigated.

Furthermore, as mentioned, parvalbumin is expressed in both axo-axonic (chandelier) cells (AAC) and basket cells (Hu et al., 2014). AAC cells in the dentate gyrus reside in the granular cell layer and extend their dendrites towards the

hippocampal fissure. AAC terminals also target axon initial segments of postsynaptic targets in the hilus and CA3, allowing for control over spike generation from principal cells (Buhl et al., 1994; Pelkey et al., 2017). The cell bodies of PV^+ basket cells within the DG tend to localise within the granular layer also, particularly at the hilar-granular layer border, and innervate the perisomatic regions of post-synaptic excitatory cells (Kullmann, 2011). The methods used in the current study did not have the resolution to differentiate between basket cells and AAC, however it may be interesting to see if the PV^+ deficit in *Cacna1c*$^{+/-}$ rats was specific to a particular type of interneuron.

An important distinction to make when examining interneuron deficits in psychiatric disorders is that immunoreactivity and mRNA expression within neurons in different to total interneuron number themselves. This study did not utilize stereological techniques in order to determine if interneurons themselves were present, or if simply PV and GAD67 protein expression was lost. This is important to consider, particularly as PV expression within interneurons has been shown to be plastic and dependent on activity and experience (Donato et al., 2013). However, we can conclude that it is likely that PV^+ hippocampal interneurons within the granular layer of the dentate gyrus in *Cacna1c*$^{+/-}$ rats are abnormal at least and may be decreased all together.

Finally, it is unlikely that this deficit is confined to the hippocampus. There have been many previous reports of abnormal cortical interneuron function, including PV^+ reductions in schizophrenia (Gonzalez-Burgos and Lewis, 2008; Hashimoto et al., 2008). PV^+ interneuron reduction in the prefrontal cortex has even been suggested to be responsible for impaired working memory in schizophrenia (Volk and Lewis, 2010). It would also be interesting to look in other cortical areas in PPS to see if interneuron dysfunction is present outside of the hippocampus.

7.4.1 Conclusions

These results show a specific decrease in PV and GAD67 immuno-positive cells within the granular layer of the dentate gyrus, a subregion of the hippocampus, in *Cacna1c* heterozygote rats. This extends the already compelling evidence for impaired GABAergic inhibition within the hippocampus in schizophrenia and bipolar disorder. However, there was no effect on PV or GAD67 expression following PPS, suggesting that this may not be a consistent phenotype across models. Further investigation would be essential to examine any functional consequences of this phenotype within *Cacna1c*$^{+/-}$ rats and what it means for the excitatory-inhibitory balance within this model.

Chapter 8:Environmental enrichment in *Cacna1c*+/- rats

8.1 Introduction

The positive effects of environmental enrichment on the brain and its function have been the subject of interest since the 1940s when Hebb reported anecdotally that rats raised at his home had behavioural improvements over rats housed at the laboratory (Hebb, 1947). Enriching a rodents environment has been shown to elicit cellular, molecular and behavioural changes such as altering dendritic arborisation and having a profound effect on hippocampal-dependent cognition and adult neurogenesis (Clemenson et al., 2015; van Praag et al., 2000). This chapter focuses on the effect of an enriched environment on Cacna1c$^{+/-}$ rats, specifically to determine if complex environmental stimulation could correct the deficit seen in adult neurogenesis and trace fear conditioning.

8.1.1 Environment enrichment

The exact protocol and definition of an enriched environment (EE) differs between studies but tends to include housing in larger cages with increased toys and tunnels and in larger groups for more complex social interaction. In addition, running wheels are often added to give an opportunity for increased physical activity, however exercise is often studied independently of simple enrichment. Therefore, EE is made up of many components such as social, sensory, physical and spatial stimulation that all combine to produce observed effects. Rodents exposed to EE display increased brain sizes, altered behaviour and altered neurotransmitter levels (Rosenzweig et al., 1962; La Torre, 1968; Manosevitz and Joel, 1973) whilst in humans, enrichment protocols such as memory training and exercise can also lead to improved learning and memory (Aberg et al., 2009; Woollett and Maguire, 2011; Herting and Nagel,

2012). Conversely, stress (Vogel and Schwabe, 2016) and sensory deprivation (Proulx et al., 2014) have the opposite effect. Enrichment protocols also appear to result in increased gliogenesis, dendritic branching and synaptic formation throughout the brain, particularly within the cortex (van Praag et al., 2000).

8.1.2 The genetics of environmental enrichment

Some studies have taken a genetic approach to the effects of EE and consistently shown that genes involved in neuronal structure, activity, neurotransmission and synaptic plasticity are induced in EE (Rampon et al., 2000; Lee et al., 2013; Hüttenrauch et al., 2016). Differential gene expression is seen throughout the brain, with gene expression in the hippocampus most profoundly altered (Rampon et al., 2000; van Praag et al., 2000). Candidate studies looking at BDNF (Rossi et al., 2006; Kuzumaki et al., 2011) and VEGF (Cao et al., 2004), which are both induced by EE and voluntary exercise, and have an impact on neurogenesis (van Praag et al., 2000), suggest a functional role for these genes in mediating the effects of EE on the rate of neurogenesis and learning.

There is also some suggestion that EE may have beneficial effects on genetic conditions, for example, a mouse model with the N-methyl-D-aspartate (NMDA) receptor 1 subunit deleted in the hippocampus presents with deficits in spatial memory, neurogenesis and contextual fear memory (Rampon et al., 2000). Two months of EE resulted in improved memory and increased CA1 synaptic and spine density in these mice. Contextual and cued fear memory was also enhanced in both control and genetic knock-outs in this study (Rampon et al., 2000). EE has also been shown to improve anxiety phenotypes on genetic models of Alzheimer's disease (Pietropaolo et al., 2014) and delay the onset of motor associated symptoms in a mouse model of Huntington's Disease (Van Dellen et al., 2000).

8.1.3 LTCC and EE

There is no direct evidence currently that EE can result in LTCC expression changes and electrophysiological studies have shown that EE induced LTP that is correlated with increased learning is NMDAR-dependent and LTCC-independent (Stein et al., 2016). However, LTCC expression is associated with expression changes following stress (Bavley et al., 2017; Terrillion et al., 2017) and therefore may be influenced by positive environment alterations also, although this is purely speculative at present. EE is also seen to alter the expression of BDNF (Rossi et al., 2006; Sun et al., 2010), whose expression can be driven by LTCC-mediated Ca^{2+} influx (West et al., 2001) and is important for both hippocampal neurogenesis and behaviour.

8.1.4 Environment enrichment and neurogenesis

EE is consistently reported to increase the rate of adult neurogenesis, particular when combined with voluntary physical exercise (Clemenson et al., 2015; van Praag et al., 2000). The benefits of running and exercise on brain function in rodents are well-documented and access to a running wheel has been shown to dramatically increase hippocampal neurogenesis (Rossi et al., 2006; van Praag et al., 1999; Henriette van Praag et al., 1999; Vivar et al., 2012), even without additional enrichment. This may suggest that it is exercise driving the increased neurogenesis following EE. However, it is important to not discount the effect of EE alone. A number of studies have found that housing in EE without a running wheel is enough to elicit changes in adult neurogenesis (Clemenson et al., 2015; Freund et al., 2013; Kronenberg et al., 2003; Steiner et al., 2008; van Praag et al., 1999). EE alone appears to affect cell survival predominately whereas exercise increases proliferation and net survival (Fabel et al., 2009; van Praag et al., 1999), suggesting that these two protocols have different mechanistic effects on adult neurogenesis.

The increase of neurogenesis following EE without a running wheel may be explained by increased exploration of the more complex environment. Mice housed in EE were monitored for 'roaming entropy' as a measure of explorative behaviour; those mice who explored more had increased neurogenesis (Freund et al., 2013). Of course, having larger cages means that rodents housed within EE have the opportunity for increased physical activity regardless of a provided running wheel. It is likely that physical activity and EE have an additive effect on each other in terms of impact on neurogenesis. Fabel and colleagues showed that 10 days of wheel running followed by 35 days of EE yielded 30% more adult-born neurons than either running or EE in isolation (which themselves both increased neurogenesis above baseline (Fabel et al., 2009).

8.1.5 Environment enrichment and learning

EE has been shown to enhance learning and memory function across a range of tasks. Spatial memory performance is increased following EE housing in comparison to standard housing when measured by the Morris Water Maze (Pacteau et al., 1989; Kempermann et al., 1998; van Praag et al., 2000) or T-maze tasks (Birch et al., 2013). In aged rats, where neurogenesis is naturally decreased, a 10 week EE exposure resulted in increased spatial learning and memory (Speisman et al., 2013) suggesting that EE increased the ability of aged rats to rapidly acquire and retrieve spatial information, potentially though increased neurogenesis. Recognition memory, as measured by the novel object recognition task, has also been reported to be increased following 3 weeks of EE (Leger et al., 2012; Birch et al., 2013), which was correlated with increased neurogenesis and nerve growth factor concentration. EE has also been shown to increase contextual discrimination (Barbelivien et al., 2006; Clemenson et al., 2015) and working memory (Birch et al., 2013; Lee et al., 2013).

In fear conditioning, EE has been shown to improve contextual fear memory (Rampon et al., 2000; Duffy et al., 2001; Barbelivien et al., 2006; Miu et al., 2006) and, in some studies, also enhance cued fear memory (Rampon et al., 2000; Tang et al., 2001) but not all (Duffy et al., 2001). Barbelivien et al (2006) showed that in trace fear conditioning, EE resulted in an increased freezing to context, but decreased freezing to discrete cue, suggesting an enhanced learning about contextual cues (Barbelivien et al., 2006). EE has also been shown to facilitate contextual fear memory extinction when rodents were exposed to EE before fear training (Hegde et al., 2017). This was correlated by reductions in theta rhythm oscillations between the hippocampus, amygdala and infralimbic prefrontal cortex (Hegde et al., 2017).

This chapter investigates the influence of EE on Cacna1c$^{+/-}$ rats in terms of correcting their deficits in trace fear memory and adult neurogenesis.

8.2 Methods

8.2.1 Contributions

All work in this chapter was performed by A Moon.

8.2.2 Animals

12 wild-type and 22 *Cacna1c*$^{+/-}$ male adult (PND60+) rats (all behaviourally naïve prior) were randomly assigned to either standard or enriched housing. 11 *Cacna1c*$^{+/-}$ rats and 6 wild-type rats were housed in groups of 2-3 in standard cages (38cm (W) x 56 cm (L) x 22 cm (H)) with ab libitum access to food and drink. Standard cages were provided with minimal enrichment: a cardboard tube and chew sticks (Figure 8.1A). 11 *Cacna1c*$^{+/-}$ rats and 6 wild-type rats were housed in 'enriched' housing in groups of 5-7, in large cages (74 cm (W) x 59 cm (L) x 40 cm (H)) with a moveable platform that allowed for another level for the cage (Figure 8.1B) for six weeks (Figure 8.1D). Exploratory behaviour was encouraged by giving access to several cardboard and plastic tubes, toys, plastic shapes, wooden balls and chew sticks (Figure 8.1C). These toys and objects were rearranged 3 times a week and replaced once a week to maintain novelty. Animals were allowed access to food and water ad libitum. 3 animals had to be removed from the experiment due to the fighting within the enriched environment (2 wild-types, 1 heterozygote).

8.2.3 Behaviour

All animals housed in both standard and enriched environments underwent trace auditory fear conditioning as described in previous chapters (Chapters 2, 4 and 5). Animals were habituated for three days. On the conditioning day, animals were subject to a trace fear conditioning paradigm (10 presentations of CS-US pairings where a 15s white noise formed the CS and a 0.5mA, 0.5s footshock formed the US). CS and US were separated by a 30s trace interval. 24 hour following conditioning, rats were put into a different context than they were conditioned in. Animals

experienced a two minute baseline period, a 6 minute white noise CS presentation and finally a 4 minute post-CS phase. Freezing behaviour was recorded and analysed in 10s bins by video analysis with the experimenter blind to genotype and housing type.

8.2.4 Neurogenesis analysis

Two weeks after behaviour was conducted (Figure 8.1D), rats were restrained and given a single intraperitoneal BrdU (50mg/kg) injection. Six hours later, rats were euthanised via Euthatal injection (200mg/ml) and transcardially perfused with 4% PFA (Figure 8.4A). Following perfusion, brains were rapidly dissected and placed in fresh 4% PFA for overnight post-fixation. Brains were transferred to 30% sucrose for 72 hours to allow for cyroprotection. Brains were then sliced into 40µm coronal sections on a cryostat (Leica Microsystems CM1860UV) (Bregma -2.04 to -6.48mm) and transferred to a plastic 12-well plate and stored at 4°C.

Immunohistochemistry

One in every 12 sections throughout the dentate gyrus was denatured for 30 minutes at 37°C in 2M hydrochloric acid. Sections were then thoroughly washed in 0.1M PBS followed by a 2hr incubation in blocking solution (1% Triton-X100 and 10% donkey serum in 0.1M PBS). Primary antibodies (anti-rat BrdU: 1:500 and anti-guinea-pig DCX: 1:5000 (Table 8.1)) were diluted in 0.1% Triton-X100 and 0.2% donkey serum (in 0.1M PBS) were then added to sections and left to bind overnight at 4°C. Sections were then washed and incubated with secondary antibodies (donkey anti-rat Alexa Fluor 555 and donkey anti-guinea-pig Alexa Fluor 647 (1:1000 diluted in 0.1M PBS)) for 2 hours in the dark at room temperature. Following more 0.1M PBS washes, sections were incubated with a DAPI stain (1:1000 in 0.1 M PBS for ten minutes, washed and mounted on standard slides. Each hippocampi were then imaged on an epifluorescent microscope at x20 magnification. Cells were counted manually and

dentate gyrus size taken into account to give a cell count per mm^2 (see General Methods).

Statistics and analysis

Behaviour

For the trace conditioning paradigm, as there was only one recall session in this experiment, a 3-way Repeated Measures ANOVA was conducted over the whole experiment from baseline measures to Post CS, with 'Freezing %' (following tests for normality and homogeneity of variances) forming the dependent variable. 'Genotype' and 'Housing' formed the independent 'between subjects' factors whilst 'session' formed an independent 'within subjects' factor. Acquisition of fear memory was measured by using another 3-way Repeated Measures ANOVA to compare Baseline to Post US sessions (independent variable) with 'Freezing %' (dependent variable); 'Genotype' and 'Housing' formed the independent 'between subjects' factor. To analyse responses within the recall session, a 3-way Repeated Measure ANOVA compared 'Freezing %' (dependent factor) with session ('Novel Baseline' 'Cue Recall' and 'Post CS' (within-subjects factor)) and 'Genotype' and 'Housing' (between-subjects factors). If significant interactions were found, post-hoc Tukey Kramer HSD tests were performed.

Neurogenesis analysis

For neurogenesis analysis, cell counts/mm^2 were analysed for normality of distribution and homogeneity of variances and transformed if appropriate (Box-Cox). A 3-way ANOVA was set up; cell count/mm^2 formed the dependent variable whilst 'Genotype' 'Housing' and 'Dorsal/Ventral' formed the 'between-subjects' independent variables.

Table 8.1: Primary antibodies utilised in immunohistochemical analysis

Target	Host	Manufacturer	Catalogue no	Dilution
BrdU	Rt (monoclonal)	Bio-Rad	OBT0030	1:500
DCX	Gp (polyclonal)	Millipore	AB2253	1:5000

Figure 8.1: Timeline and set up of enriched housing compared to standard housing. A = standard housing (including cardboard tube not seen in photo), B = enriched environment; a large cage with a moveable platform, toys and climbing frames on the walls. C = other toys that were swapped into the enrichment cages throughout the 6 week period. D = Timeline of enrichment, behaviour, BrdU injection and perfusion.

8.3 Results

8.3.1 Enriched housing does not correct the trace conditioning deficit in *Cacna1c*$^{+/-}$ rats

Repeated measures ANOVA across all sessions showed a significant effect of session (Session: $F_{(4, 24)} = 180.593$, $p < 0.001$, 3-way RM ANOVA) (Figure 8.2A). There was a trend to a significant effect of genotype on freezing behaviour (Genotype: $F_{(1, 27)} = 3.7306$, $p = 0.0640$), no effect of housing (Housing: $F_{(1, 27)} = 0.254$, $P = 0.619$) and no significant interaction (session*genotype*housing: $F_{(4, 24)} = 1.132$, $p = 0.365$).

WT and *Cacna1c*$^{+/-}$ rats in both standard and enriched environments froze significantly more following each footshock than at baseline (Session: $F_{(1, 27)} = 422.738$, $p < 0.001$, 3-way RM ANOVA) (Figure 8.2A and B). There was no effect of genotype (Genotype: $F_{(1, 27)} = 0.071$, $p = 0.792$) , however there was a significant effect of housing (Housing: $F_{(1, 27)} = 4.3359$, $p = 0.047$) which interacted with session (Session*Housing: $F_{(1, 27)} = 4.2108$, $p = 0.050$) but not genotype (Housing*Genotype: $F_{(1, 27)} = 0.478$, $p = 0.495$). Post-hoc analysis revealed that this was specific to 'Post-US' ($p = 0.0484$, Tukey Kramer HSD) with EE animals freezing more than SE animals (Figure 8.2C).

A model was set up to investigate the effect of housing, genotype and session within the recall session on freezing behaviour in both WT and heterozygote rats and was seen to be significant (Housing*Genotype*Session: $F_{(1, 4, 19)} = 18.38$, $p < 0.001$, 3 way RM ANOVA) (Figure 8.2A and D). Repeated measures ANOVA showed a significant effect of the session (Session: $F_{(2, 26)} = 63.589$, $p < 0.001$, 3 way RM ANOVA), indicating that freezing behaviour differed between baseline, CS and post-CS periods. There was a significant effect of genotype (Genotype: $F_{(1, 27)} = 7.198$, $p = 0.008$) on freezing behaviour and a significant interaction between genotype and CS presentation (Genotype*Session: $F_{(1, 27)} = 3.610$, $p = 0.008$). However there was no

effect of housing on freezing behaviour (Housing: $F_{(1, 27)} = 0.324$, $p = 0.570$), no interaction between housing and part of the session (Housing*Session: $F_{(2, 26)} = 0.704$, $p = 0.590$) and no 3 way interaction between genotype, housing and session part (Genotype*Housing*Session: $F_{(2, 26)} = 0.622$, $p = 0.647$).

Post-hoc tests showed that the genotypes displayed significantly different freezing behaviour during the CS presentation ($p = 0.003$, Tukey Kramer HSD), but no other part of the recall (Figure 8.2A and D).

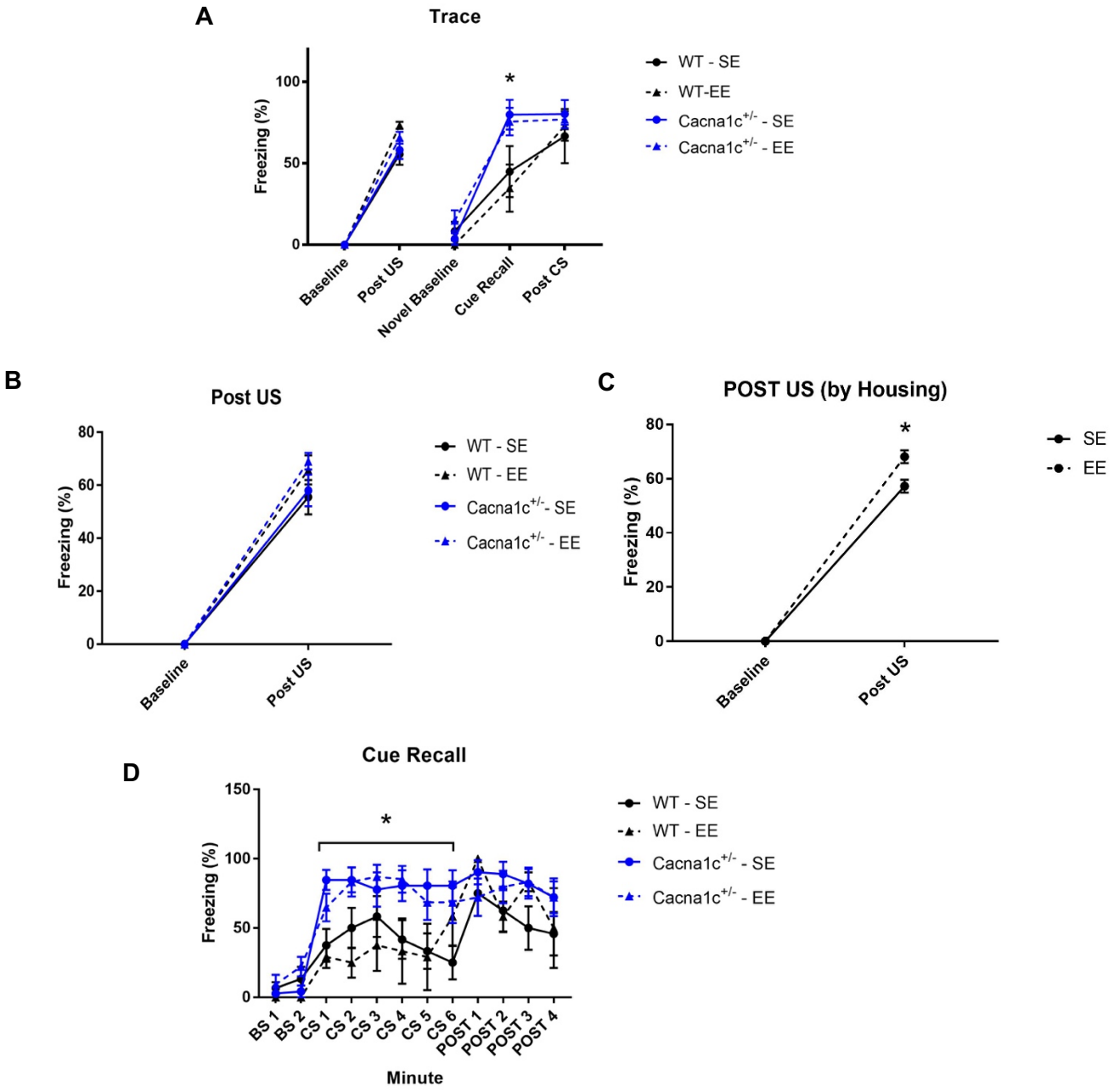

Figure 8.2: Trace conditioning in SE and EE *Cacna1c*$^{+/-}$ rats and wild-types. A = Both SE and EE *Cacna1c*$^{+/-}$ and wild-type rats display similar freezing behaviour during the conditioning session, however *Cacna1c*$^{+/-}$ rats have enhanced fear memory in response to CS, which is not corrected by EE. B/C = EE housed rats display increased fear following footshock in the conditioning session, which was not subject to genotype. D = within Cue Recall, both SE and EE *Cacna1c*$^{+/-}$ rats show increased fear behaviour, but similar freezing at baseline and Post CS. Results are presented as mean freezing % per group, error bars are SEM. *Cacna1c*$^{+/-}$ EE vs *Cacna1c*$^{+/-}$ SE vs WT EE vs WT SE, n = 11 *Cacna1c*$^{+/-}$, 6 wild-types in SE, 10 *Cacna1c*$^{+/-}$, 4 wild-types in EE, * = $p < 0.05$.

8.3.2 Impairments in adult neurogenesis in *Cacna1c*$^{+/-}$ are not corrected by enriched housing

BrdU

A model was set up to analyse the effect of genotype and housing on BrdU cell count and revealed a trend (Genotype*Housing*Dorsal/Ventral = $F_{(1, 27)}$ = 2.362, p = 0.081, 3-Way ANOVA) (Figure 8.3A and Figure 8.4). There was a significant effect of genotype (Genotype: $F_{(1, 27)}$ = 6.878, p=0.011, 3 way ANOVA), but no effect of housing (Housing: $F_{(1,27)}$ = 0.464, p = 0.498, 3-Way ANOVA) or an interaction between genotype and housing (Genotype*Housing: $F_{(1, 27)}$ = 0.290, p = 0.592, 3-Way ANOVA). There was no significant effect of dorsal or ventral hippocampus (Dorsal/Ventral: $F_{(1,27)}$ = 2.652, p = 0.109, 3-Way ANOVA) and no interaction with genotype (Dorsal/Ventral*Genotype = $F_{(1,27)}$ = 1.965 p = 0.236) or housing (Dorsal/Ventral*Housing = $_{(1,27)}$ = 1.054 p = 0.327) suggesting that this effect was throughout the span of the hippocampus (Figure 8.3A).

Doublecortin

The effect of genotype and housing on DCX cell count was analysed by a 3 way ANOVA and revealed no significant effect (Genotype*Housing*Dorsal/Ventral: $F_{(1, 27)}$ = 0.618, p = 0.609, 3 way ANOVA) (Figure 8.3B and Figure 8.4). There was no effect of genotype (Genotype: $F_{(1,27)}$ = 0.707, p = 0.408, 3 way ANOVA) or housing (Housing: $F_{(1, 27)}$ = 0.906, p = 0.350, 3 way ANOVA) on doublecortin cell count in the hippocampus. There was no significant interaction between genotype and housing either (Genotype*Housing: $F_{(1,27)}$ = 0.016, p = 0.900, 3 way ANOVA). There was no significant difference between the dorsal and ventral hippocampus (Dorsal/Ventral: $F_{(1,27)}$ = 0.199, p = 0.657, 3 way ANOVA) or any interactions with genotype or housing (Figure 8.3B).

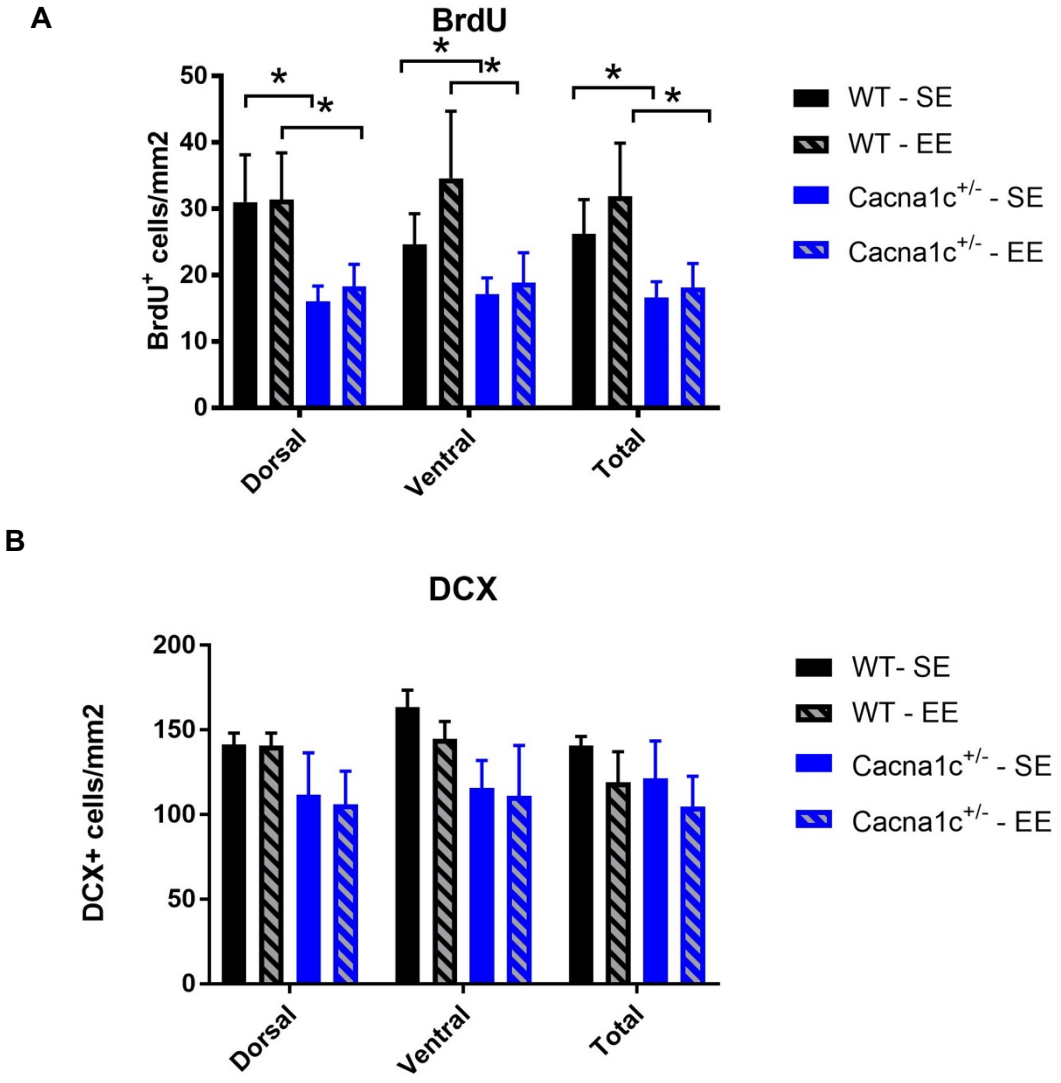

Figure 8.3: A = EE does not correct the cell proliferation in adult neurogenesis seen in Cacna1c$^{+/-}$ rats in both the dorsal and ventral hippocampus. B = DCX^{+} cells are also not different between genotype and environment throughout the hippocampus. Bars represent cell counts per mm^2 on average by group, error bars are SEM. *Cacna1c*$^{+/-}$ EE vs *Cacna1c*$^{+/-}$ SE vs WT EE vs WT SE, n = 11 *Cacna1c*$^{+/-}$, 6 wild-types in SE, 10 *Cacna1c*$^{+/-}$, 4 wild-types in EE, * = p < 0.05.

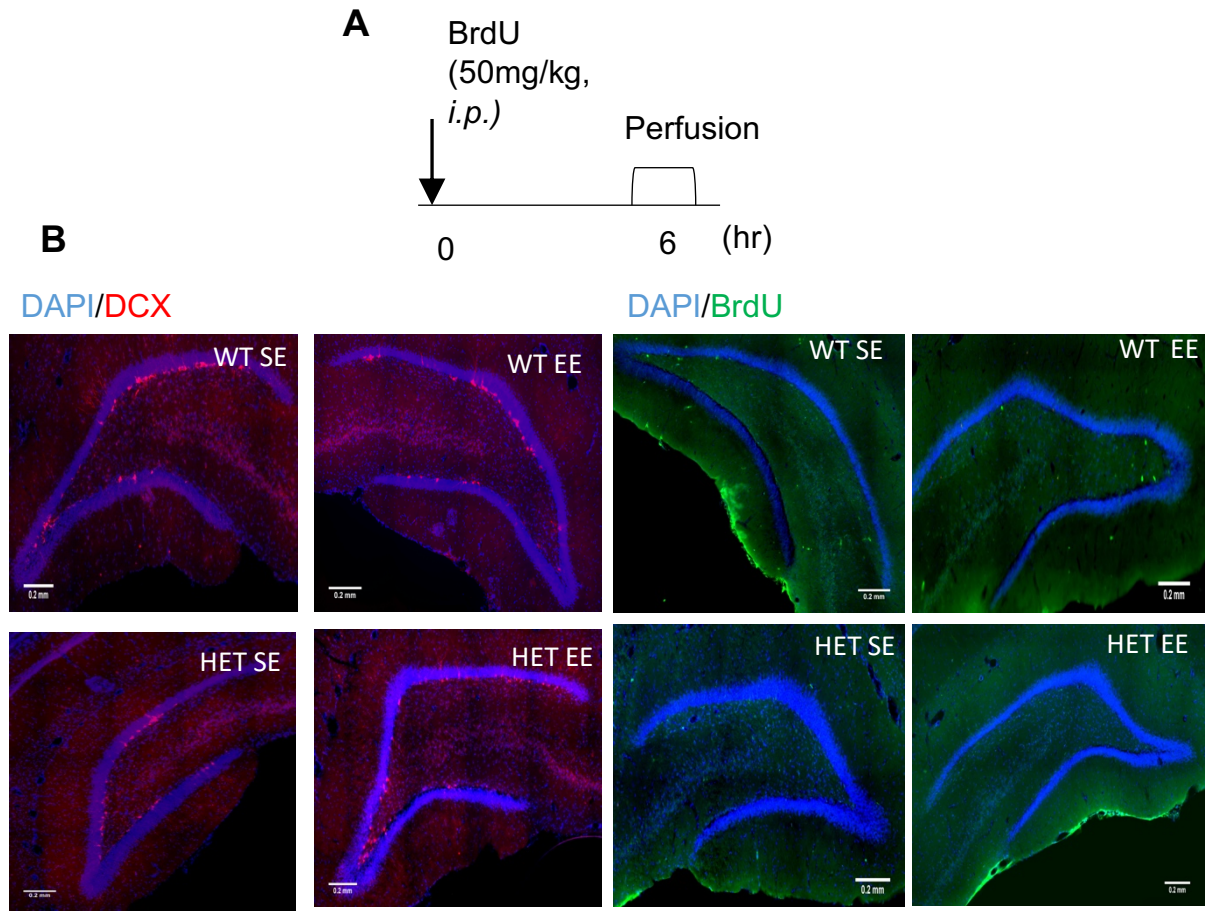

Figure 8.4: A: Timeline of BrdU administration and perfusion. B: Representative immunofluorescent images of BrdU and DCX stained cells within both genotypes and environmental condition. Scale bar = 0.1mm.

8.4 Discussion

The present findings replicate the results of previous chapters, where *Cacna1c*$^{+/-}$ rats demonstrate increased freezing during CS presentation of cue recall following trace fear conditioning. These same animals display decreased cell proliferation during adult neurogenesis as measured by BrdU in the dentate gyrus. These deficits persisted in animals which had been subject to 6 weeks of EE, demonstrating that simple social and spatial enrichment is not sufficient to correct these particular behavioural and molecular defects in the *Cacna1c*$^{+/-}$ model.

Both standard and enriched environment *Cacna1c*$^{+/-}$ rats froze significantly more than their wild-type equivalents to CS presentation, suggesting an enhanced or overgeneralised fear memory as discussed in Chapter 4. EE therefore had no impact on this trace fear memory deficit. This contrasts with the findings of Barbelivien and colleagues, who showed that EE had a significant impact on trace fear conditioning; EE resulted in a decreased freezing to discrete cues (Barbelivien et al., 2006) and increasing contextual freezing. Furthermore, in a voluntary wheel running experiment, running mice froze significantly more to the training context 48hrs after trace conditioning. This study did not look at a cue recall, but did note that there was no difference in freezing between runners and control mice during cue presentation during conditioning (Kohman et al., 2012), suggesting that both groups react similarly to the discrete cue. In the present experiment, EE does not appear to alter cued fear processing following trace conditioning in *Cacna1c*$^{+/-}$ rats. This could be because the fear memory deficit induced by genetic knock out of *Cacna1c* may be too severe for EE to significantly correct, or that EE only has a beneficial impact on selective hippocampal processes such as acquisition of contextual information, but not retrieval of cued trace fear memory. The latter conclusion is backed up by the fact that there was significant effect of housing on post-US freezing within the conditioning session,

with EE rats of both genotypes freezing more following footshock than standard housed rats, displaying an increased acquisition of fear memory. It should also be noted that Barbelivien et al used female rats in their study (Barbelivien et al., 2006) whereas the present results only utilised males, suggesting potential sex-differences in how EE affects behaviour.

Neurogenesis in *Cacna1c*$^{+/-}$ rats following EE

This study also showed no differences in neurogenesis following EE in either genotype. This may be surprising as there is a plethora of literature to describe the effects of EE on increasing the rate of neurogenesis (Clemenson et al., 2015), however the impact on neurogenesis following EE without aerobic exercise is more variable than with exercise. The current EE study did not include use of a running wheel because we aimed to examine if EE could have an effect on the examined parameters independent of the positive effects of exercise. Results from studies that examined if exercise was the critical variable in EE for enhancing neurogenesis determined that aerobic exercise such as running was the vital component (Kobilo et al., 2011; Mustroph et al., 2012), although both of these studies were in mice, not rats. Rats housed in EE without running however have shown increases in neurogenesis in both young and aged rats (Speisman et al., 2013), although in this study, toys were rotated daily and rats were only exposed to the EE for 2-3hrs a day. Therefore, the lack of neurogenesis increases seen in the current study may be explained by habituation to the EE and their toys which resulted in decreased exploratory behaviour over the 6 week exposure. Future work investigating both hippocampal neurogenesis and behaviour in the *Cacna1c*$^{+/-}$ model should concentrate on either increasing novelty in the EE or providing rats with a running wheel to determine the contribution of aerobic exercise to EE-induced improvements in these parameters.

There is also evidence that hippocampal neurogenesis is required for learning a trace conditioning task (Shors et al., 2001; Achanta et al., 2009), which may also explain why no behavioural improvements were seen on the trace conditioning task. However, increasing neurogenesis by wheel running was not sufficient to enhance performance on a trace conditioning paradigm (Kohman et al., 2012), showing that it is improvement for future research to determine which behavioural tasks are improved by enhancing neurogenesis to understand how important hippocampal neurogenesis is for behavioural outputs.

There were several limitations to the EE paradigm utilised in this study. For example, different time points of giving enrichment to rats, for example during adolescence or during youth, were not investigated, and it may be suggested that giving enrichment at a younger age may be more beneficial to shaping an animals behavioural and molecular response to EE. However, there is a convincing literature that demonstrates that enrichment can improve both memory and plasticity in adult, and even aged, rodents (Green et al., 1983; Rosenzweig and Bennett, 1996; Bennett et al., 2006; Harburger et al., 2007), potentially even above that of younger animals (Harburger et al., 2007). In the current study rats had 6 weeks of enrichment, which may not have been long enough for deficits to be reversed. There is no standardised length of time for EE in animal studies, however some studies, particularly those conducted on more aged rats, use more chronic EE exposure such as a year, suggesting that a longer period of EE may be beneficial.

Importantly, the response of animals to EE is at least partly reliant on their background strain, for example EE has been shown to increase exploratory behaviour in Berkeley S1 rats (Renner, 1987) but have no effect in Sprague Dawley rats (Bruel-Jungerman et al., 2005), the background strain of *Cacna1c*$^{+/-}$ rats. However, Konkle et al show that Sprague Dawley rats do respond to EE in terms of various depressive-like behaviours (Konkle et al., 2010). Another variable to note about the present study is

that 3 animals had to be removed from the EE cages and excluded from the study due to fighting. This may have resulted in a stressor to the other animals in the EE cage and reduced the effect of enrichment.

8.4.1 Conclusions

EE is an useful and interesting experimental paradigm that is subject to many variables: age, sex and strain of the animals and the influence of exercise and novelty. This chapter shows that EE alone is not sufficient to correct the trace conditioning and neurogenic deficits observed in *Cacna1c*$^{+/-}$ male rats. However, the results presented provide a framework for potential future experiments investigating the impact of aerobic exercise of *Cacna1c*$^{+/-}$ animals, in combination and without EE.

Chapter 9: General Discussion

9.1 Introduction

Various genomic and functional investigations have implicated *CACNA1C*, and LTCCs more generally, in psychiatric disorders. Calcium signalling plays a vital role in synaptic plasticity, learning and memory; processes often gone awry in mental illness. In recent years, the role that risk associated genetic variants may play in pathology has been the subject of investigation, with the hope of identifying biological pathways underlying mechanisms of disease. In order to do this, it is important to consider the impact of not only genetic variation but also the influence of the environment, including early life stress, which has itself seen strongly and consistently linked to the development of both mood and psychiatric disorders. If both genetic and environmental factors converge on, or interact together to impact on, particular pathways, it could inform on the molecular mechanism of disease that could indicate potential novel targets for treatment.

In this chapter, I summarise the main findings from each results chapter presented in this thesis and consider the potential implications of these findings. I will also discuss the limitations of the work presented and how these results can be used to inform future directions of study.

9.2 Summary of findings

Chapter 3: The effect of prepubertal stress (PPS) on Cacna1c expression in the hippocampus

- Hippocampal expression of *Cacna1c, Cacna1d, Cacna1i* and *Cacnb2*, all VGCC associated genes that have been implicated in schizophrenia and BPD, were compared in both male and female PPS and control rats.

- Using both *in-situ* hybridisation and quantitative PCR, I show that *Cacna1c* mRNA expression was decreased in the CA1 and CA3 of the hippocampus of PPS male rats. Western blot analysis showed that this was reflected by a trend to a decrease of Cav1.2 protein expression. A small cohort of human subjects who had suffered early life stress also demonstrate a trend to decreased *CACNA1C* in males.
- In the same group of PPS rats, *BDNF* expression, which is moderated by Ca^{2+} influx through LTCCs, was shown to be reduced, specifically the activity regulated exon IV and IX isoforms, in male rats only. BDNF protein expression however was unchanged in males, however the pro-BDNF protein was increased in female rats, with no change in mature form BDNF.
- Therefore, PPS resulted in down-regulation of *Cacna1c* and *BDNF* expression in the hippocampus of rats in a sex-specific manner.

Chapter 4: Auditory fear conditioning in Cacna1c heterozygote rats

- Delay, trace and unpaired auditory fear conditioning was performed on *Cacna1c*$^{+/-}$ and their wild-type littermates.
- In delay conditioning, *Cacna1c*$^{+/-}$ rats displayed increased contextual fear memory recall, with no differences in fear memory acquisition or cued fear memory.
- In trace conditioning, increased cued fear memory recall was displayed in heterozygote rats with no differences in acquisition or contextual recall.
- The unpaired conditioning paradigm resulted in increased contextual and cued fear memory recall, with no difference in acquisition.
- In summary, *Cacna1c* heterozygosity has no effect on acquisition of fear memory but can affect recall of both contextual and cued recall, depending on conditioning paradigm.

Chapter 5: The impact of PPS on auditory fear conditioning

- The same delay, trace and unpaired paradigms utilised in Chapter 4 were used to test the effect of PPS on auditory fear conditioning.
- Increased fear was noted during the first four minutes of contextual recall in PPS rats following delay conditioning. Acquisition of delay fear memory and cued recall was maintained.
- Trace conditioned PPS rats demonstrated decreased acquisition of fear memory and a trend to decreased cued recall.
- Unpaired conditioning, acquisition or recall was not affected by PPS.

Chapter 6: Adult hippocampal neurogenesis in *Cacna1c*$^{+/-}$ and PPS rats

- Adult hippocampal neurogenesis was investigated by analysing BrdU, a marker of cell proliferation, and DCX, which marks immature neurons, in *Cacna1c*$^{+/-}$ rats and PPS rats.
- In *Cacna1c*$^{+/-}$ rats, BrdU^{+} cells were decreased 6 hours after injection, suggesting a decrease in cell proliferation. However, there was no difference in the number of DCX^{+} cells.
- In PPS rats, DCX^{+} cells were increased in the ventral hippocampus only, suggesting an increase in immature neurons.

Chapter 7: The effect of reduced gene dosage of Cacna1c and PPS on parvalbumin positive GABAergic interneurons within the hippocampus

- PV and GAD67 expression within the hippocampus of *Cacna1c*$^{+/-}$ rats was analysed by immunohistochemistry. *Cacna1c*$^{+/-}$ rats displayed a region-specific decrease in PV and GAD67 expression in the granular layer of the dentate gyrus. No differences were observed in the CA1 or CA3.
- PV was also analysed throughout the hippocampus of PPS rats but no alterations were seen in any sub-region.

- Therefore, reduced gene dosage in *Cacna1c* results in abnormal interneurons in the dentate gyrus granular layer.

Chapter 8: The impact of environmental enrichment in *Cacna1c*$^{+/-}$ rats

- Six weeks of environment enrichment was provided to *Cacna1c*$^{+/-}$ rats in the form of increased social interaction, novel toys, large home cages with structural features to encourage play and increased physical activity.
- The deficit in cued fear memory recall following trace fear conditioning was replicated but not corrected or improved by environment enrichment.
- Similarly, when hippocampal neurogenesis was investigated, BrdU^{+} cells were decreased in *Cacna1c* heterozygote rats regardless of housing. Therefore, environment enrichment appears to have no impact of these observed deficits in *Cacna1c*$^{+/-}$ rats.

This thesis examined the consequence of reduced gene dosage of *Cacna1c* and prepubertal stress on the hippocampus. The results presented contribute to the large body of evidence that suggests both genetic and environmental factors associated with psychiatric disease alter the structure and function of the hippocampus.

9.3 Do Cacna1c and PPS interact to increase risk for psychiatric disorder phenotypes?

This thesis did not set out to prove conclusively that *Cacna1c* and PPS form a gene x environment interaction that ultimately causes schizophrenia or BPD pathology. In order to evaluate this would require a much larger experiment where *Cacna1c*$^{+/-}$ and wild-types rats are both subject to PPS and control conditions (See Future Directions). However, the results presented do indicate that both genetic variation in *Cacna1c* and PPS have profound and long-lasting effects on the hippocampus, and that the expression changes of PPS on *Cacna1c* expression (Chapter 3) do point to a possible

gene x environment interaction. This may suggest that the combined insult of *Cacna1c* genetic variation and PPS may have an additive effect on each other, as suggested by the dual-hit hypothesis of schizophrenia, and informs any future gene x environment studies that the hippocampus may be a region to examine. This states that an early insult, such as increased genetic risk, disrupts brain development in such a way that increases vulnerability to a second 'hit' such as early life stress. In other words, *Cacna1c* genetic variation and later stress can interact together to push an individual over a particular threshold for disease manifestation (Figure 9.1). Therefore, it may suggest that genetic variation and environment insults converge on the LTCC signalling pathway in order to increase risk for psychiatric disorders.

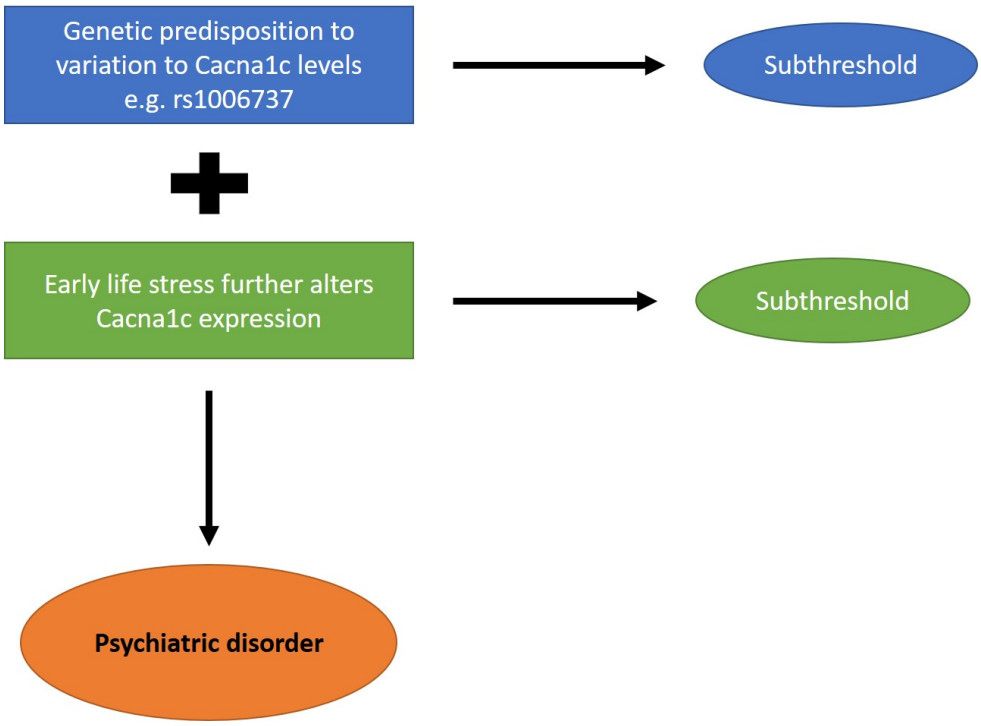

Figure 9.1: Schematic of a potential 'dual-hit' that could contribute to the development of psychiatric disorders such as schizophrenia. An early insult such as genetic variation in one or several risk genes may contribute to altered brain development that leads to an increased risk for disorder. A second 'hit' such as early life stress may further alter brain development that alone may be subthreshold for disorder, but combined with the earlier insult contribute to psychiatric disorder onset.

PPS resulted in decreased *Cacna1c* in the CA1 and CA3 of the hippocampus of male rats. To our knowledge, this is the first study to look at the effect of early life stress on *Cacna1c* expression in adulthood, adding to the existing literature showing that both acute and chronic stress can have an impact on LTCC expression in the hippocampus, amygdala and prefrontal cortex (Bavley et al., 2017; Terrillion et al., 2017; Dedic et al., 2018). In humans, there is some evidence that SNPs in *CACNA1C* interact with trauma to predict depressive symptoms (Dedic et al., 2018), although whether these SNPs, or other *CACNA1C* risk alleles indicated in illness (Table 1.1), can cause differential gene expression in the brain following early life stress is not currently known. Of course, it should be remembered that depression and psychiatric disorders are highly heterogeneous and *CACNA1C* may associate with these disorders significantly more in individuals who have experienced stress or experienced other environmental factors. The evidence for the potential interaction between *CACNA1C* and stress in disorder highlights the importance of controlling for environmental factors in genetic studies.

Both *Cacna1c*$^{+/-}$ rats and PPS rats display altered delay and trace auditory fear conditioning. In previous studies investigating the role of LTCCs in fear memory, application of LTCC antagonists had no effect on acquisition and consolidation of fear memories (Cain et al., 2002; Busquet et al., 2008), although the involvement of LTCCs in a multiple cue presentation delay paradigm as utilised here has not been previously investigated. The results presented in Chapter 4 indicate a select role for Cav1.2 in the appropriate formation of specific CS-US associations within multiple presentation auditory fear conditioning paradigms. *Cacna1c*$^{+/-}$ rats appear to form strong associations to both the context and CS regardless of the timing of CS-US pairings, suggesting a specific role of Cav1.2 and subsequent Ca^{2+} influx in identifying appropriate fear responses in relation to prediction error. Associative learning via the Rescorla-Wagner model states that learning is dependent on how 'surprised' the

animal was by the stimuli presented (Rescorla and Wagner, 1972). Therefore *Cacna1c*$^{+/-}$ rats may be experiencing deficits in learning and predicting the specific associations between CS and US and thus attribute fear to the whole conditioning session, i.e. the whole session is salient. This implies that Cav1.2 has a role in the inhibition of inappropriate aversive associations to both context and cue.

PPS has been previously associated with enhanced delay conditioned fear memory (Tsoory et al., 2010; Yee et al., 2012a), however no prior studies have looked at the impact of PPS on trace conditioning. Following delay fear conditioning, both *Cacna1c* heterozygosity and PPS cause an increase in contextual fear recall memory, as demonstrated by increased freezing over their respective control groups when they were returned to the conditioning context. Decreased *Cacna1c* expression in PPS rats may therefore, in part, mediate this contextual fear deficit, or, it could be possible that reduced gene dosage of *Cacna1c* and PPS cause increased contextual freezing following delay conditioning via similar mechanistic pathways yet to be eluded. Delay conditioning is generally considered to be amygdala-dependent with little dependence on the hippocampus. However, the hippocampus is critical for the formation of contextual representations (Ji and Maren, 2007), suggesting that in *Cacna1c*$^{+/-}$ and PPS rats, delay conditioning is processed within the hippocampus as well as the amygdala, indicating increased communication between these two brain areas within the fear circuity. However, it is unlikely that reduced *Cacna1c* is responsible for all auditory fear deficits seen in the PPS model as different responses are seen following trace and unpaired conditioning.

In trace fear conditioning, *Cacna1c*$^{+/-}$ rats display increased freezing during cued recall, whereas PPS rats appear to display decreased fear responses to CS and US within the conditioning session as well as a trend to decreased fear within cued recall. Therefore, it appears that these two models display different freezing behaviour to their respective control groups, but process trace fear conditioning quite differently. It

is likely that a similar mechanism to what is observed within delay conditioning is also present in the trace conditioned group within *Cacna1c*$^{+/-}$ rats, in that the reduced *Cacna1c* results in a lack of formation of specific CS-US associations resulting in an enhanced representation of the CS as fearful, instead of being a neutral or safety signal. In PPS rats, the decreased fear memory seen in conditioning and recall suggests a deficit in encoding the complex trace conditioning relationship. Hence, both PPS and *Cacna1c* heterozygotes appear to learn and process trace associative fear memory in different ways in both each other and their control groups. In unpaired conditioning, PPS rats show no deficits, however reduced *Cacna1c* appears to result in impairments in both contextual and cued memory. This indicates a profound difference in how *Cacna1c*$^{+/-}$ rats are processing contextual representations and their relationships to aversive stimuli; processes that are maintained in the PPS model.

Therefore, *Cacna1c* heterozygosity clearly results in increased fear across multiple paradigms, although whether this is a deficit of encoding during conditioning or a deficit within retrieval is not clear from the current results. This indicates dysfunction in multiple regions within fear circuitry, including the hippocampus and amygdala. In PPS however, the results are slightly more complex, as would be expected with an environment insult where several biological pathways are affected. PPS results in a different pattern of fear depending on timing and presentation of CS-US pairings. It is interesting that unpaired conditioning in this model was unaffected, suggesting that contextual fear conditioning is intact, in contrast to previous studies (Hiraide et al., 2012; Brydges et al., 2014d) and indicating that hippocampal functions are maintained. However, there are clear deficits in encoding in trace conditioning and in the formation of contextual representations in delay conditioning following PPS, suggesting that, similarly to results seen in *Cacna1c*$^{+/-}$ rats, the hippocampus and amygdala are both processing fear differently than in control animals. However, in PPS, the hippocampus may be more resilient and deficits only arise when complex

learning, such as trace conditioning, is required. It may be proposed therefore that *Cacna1c*$^{+/-}$ rats present with a broader phenotype of fear circuity dysfunction in auditory fear conditioning, whereas PPS rats can process contextual representations during unpairing conditioning but have a more specific deficit of hippocampal-amygdala communication within aspects of delay and trace fear conditioning.

9.4 Neurogenesis and trace conditioning

Trace fear conditioning has been associated with adult neurogenesis. Studies have shown that ablating neurogenesis results in impaired trace fear conditioning (Shors, 2001; Seo et al., 2015) and trace fear conditioning itself increases survival of newly born neurons (Leuner et al., 2004; Waddell and Shors, 2008). Neurogenesis ablation has been shown to increase contextual fear memory in trace conditioned rats alongside elevated non-associative anxiety; proposing that neurogenesis is important for supporting trace conditioning but also buffering against increased generalised fear and anxiety that may result from fear conditioning (Seo et al., 2015).

I show that *Cacna1c*$^{+/-}$ rats display increased fear memory to cue, which may be explained by over-generalised fear (Chapter 4) as well as downregulated cell proliferation in adult neurogenesis (Chapter 6). Therefore, the decreased rate of neurogenesis may contribute, at least in part, to the trace fear conditioning deficit seen in the heterozygote rats. However, it should be noted that the trace conditioning deficit reported by Seo and colleagues was seen following doublecortin ablation (Seo et al., 2015) whilst I show that *Cacna1c*$^{+/-}$ rats have intact doublecortin-stained cells (Chapter 6). There is no current evidence that immature neurons are altered in the *Cacna1c*$^{+/-}$ rat model, as opposed to the mouse models of reduced gene dosage of *Cacna1c* where doublecortin deficits are seen (Lee et al., 2016; Temme et al., 2016). Also, ablating neurogenesis had no effect on delay conditioning (Seo et al., 2015) whereas *Cacna1c*$^{+/-}$ rats are impaired in both delay and trace conditioning. Therefore,

even if altered neurogenesis does contribute to deficits in trace conditioning it is certainly not the only mechanism driving the increased fear responses to auditory fear conditioning in this model.

Intriguingly, PPS rats show a deficit in encoding during the conditioning phase of trace conditioning and trended to decrease freezing to cue during recall (Chapter 5), and also showed increased doublecortin stained cells (Chapter 6). While most studies discuss the negative consequences of decreased neurogenesis, the effects of increased neurogenesis are less researched (Scharfman and Hen, 2007), most likely as it is difficult to increase neurogenesis in a reliably quantitative way (enrichment and exercise protocols are mostly used). It may be hypothesised that increased neurogenesis following early life stress can lead to a degree of fear resilience. In other words, PPS may lead to a higher threshold of fear so that aversive conditioning is less fearful, resulting in decreased fear responses. This may be mediated in part by neurogenesis; increased hippocampal neurogenesis has been shown to affect both susceptibility and resilience in models of stress depending on species, strain and type of stressor (Levone et al., 2015; Anacker et al., 2018). There is variation in the literature as to whether neurogenesis ablated animals show increased susceptibility to stress-induced depressive phenotypes (Snyder et al., 2011; Mateus-Pinheiro et al., 2013) or not (Jayatissa et al., 2009), but the impact of increased neurogenesis on behaviour is much less researched. A recent study showed that activity of neurons born from adult neurogenesis within the ventral dentate gyrus was required to protect against stress-induced anxiety and social behaviour defects (Anacker et al., 2018), suggesting that increased anxiety in this region as indicated by increased doublecortin cells may have a protective effect on fear conditioning.

In the case of both models reported herein, these observations are only correlative and in order to further investigate any link between neurogenesis and trace conditioning, more causative studies, such as reducing neurogenesis in the PPS

model by irradiation methods or increasing neurogenesis in the *Cacna1c*$^{+/-}$ model by enrichment or exercise, would be necessary to establish a causative mechanism.

9.5 PV and neurogenesis

Adult hippocampal neurogenesis takes place exclusively within the subgranular zone of the dentate gyrus, whilst the deficit in PV^{+} interneurons observed in the *Cacna1c*$^{+/-}$ rats was confined to the granular zone of the dentate gyrus (Chapter 7). Therefore, a question could be asked as to if it were possible that the decrease in PV may be responsible for the cell proliferation deficit seen in *Cacna1c*$^{+/-}$rats? It has been shown that PV^{+} interneurons that reside in the dentate gyrus can regulate the quiescence of neural stem cells and their progeny through GABA signalling (Song et al., 2013). Dentate PV^{+} interneurons are depolarised by long-range GABA signalling from the medial septum and provide immature GABAergic synaptic input to proliferating neural precursors, affecting the rate of adult neurogenesis (Bao et al., 2017). PV^{+} interneurons partake in a diametric regulation of hippocampal neurogenesis where PV^{+} interneuron activation suppresses neural stem cell activation and promotes progenitor survival and development (Song et al., 2013). Therefore, the decreased PV^{+} interneurons observed in the *Cacna1c*$^{+/-}$ rat model should result in disinhibition of neural stem cell activation and increased proliferation. However, *Cacna1c*$^{+/-}$ rats display decrease cell proliferation as measured by BrdU (Chapters 6 and 8), suggesting that this is unlikely to be due to a PV interneuron-mediated mechanism. However, simply measuring the amount of PV within GABAergic interneurons does not reveal if and when these interneurons are activated. Activating PV^{+} interneurons has been seen to have large effects on behaviour and neurogenesis (Song et al., 2013; Zou et al., 2016); further investigations should focus on the activation status of the PV^{+} neurons within the *Cacna1c*$^{+/-}$ model.

The underlying pathways for decreased neurogenesis in *Cacna1c*$^{+/-}$ models (Table 6.1) is thus yet to be eluded, but could include altered neurotrophic factors, microglia or neurotransmitters. Lee et al (2016) suggest that decreased BDNF was responsible for the deficits in their *Cacna1c*$^{-/-}$ conditional knockout mouse (Lee et al., 2016), however BDNF expression is reported unchanged in the hippocampus in the *Cacna1c*$^{+/-}$ rat model (Sykes, unpublished data). Further investigations should focus on the mechanism of altered neurogenesis in reduced gene dosage *Cacna1c* models, as it has now become a consistently reported phenotype (Table 6.1).

9.6 PV and learning

GABAergic inhibitory interneurons are responsible for mediating many behaviours including working memory, spatial memory and cognition, primarily by controlling the excitatory-inhibitory balance in key brain areas such as the prefrontal cortex, striatum and hippocampus (Letzkus et al., 2015). Inhibition has an important role in driving certain behaviours; it ensures precise firing of pyramidal cells and projection neurons, regulates the processing of specific inputs and generates network oscillations which allows for information processing and communication between different brain areas. This allows for selective and discriminative responses to external stimuli – a key factor in learning and memory. Therefore, both inhibition and disinhibition have important roles in these processes.

In fear conditioning, several genes associated with the GABA pathway are downregulated including GAD65, GAD67 and several GABA type A receptor subunits, which may be necessary to facilitate learning (Heldt and Ressler, 2007). In the basolateral amygdala, PV^{+} interneurons are excited in response to the CS (cue) and act to inhibit SOM^{+} interneurons, which, in turn, disinhibit excitatory neurons to enhance auditory responses, promoting cue-shock associations (Wolff et al., 2014). However in response to US, such as an aversive footshock, PV^{+} and SOM^{+}

interneurons are strongly inhibited by US presentation, leading to disinhibition of principal excitatory cells, increasing their activity and enhancing associative learning (Wolff et al., 2014). In the hippocampus, inactivation of SOM^+ interneurons impairs contextual fear memory; suggesting that increased inhibition is required to exclude the US from the context. However, PV^+ inactivation had no effect (Lovett-Barron et al., 2014). During recall, PV^+ interneurons in the dorso-medial PFC are inhibited when mice show fear, suggesting that disinhibition is also necessary for memory expression (Wolff et al., 2014).

Taking these datas together, it appears that disinhibition via PV^+ and SOM^+ interneurons mediates behavioural learning by increasing excitability of principal cells, boosting their responses to the CS and inducing synaptic plasticity. Therefore, the reduced PV^+ interneurons seen in the *Cacna1c*$^{+/-}$ rat model may mediate their response to auditory fear conditioning. Indeed, the increased fear displayed to context and cue suggests a lack of inhibitory learning. If during CS and US presentation there are less PV^+ interneurons to disinhibit principal cells, it may mean that cue-shock associations are not learnt correctly and are processed differently, resulting in different responses within recall.

In mice with the GABA type A receptor knocked out, trace fear conditioning was enhanced in a similar manner to what is observed with the *Cacna1c*$^{+/-}$ model, suggesting reduced inhibition may facilitate increased learning of this paradigm (Martin et al., 2010). However, the deficit in PV^+ interneurons in *Cacna1c*$^{+/-}$ was very specific to the dentate gyrus in the hippocampus; when PV^+ interneurons were activated specifically in the dentate gyrus, there were no differences in cued or contextual fear memory, although extinction was increased (Zou et al., 2016). Investigating as to whether this PV deficit is seen within the amygdala and the prefrontal cortex may offer more insights as to how inhibitory mechanisms are affected in the context of *Cacna1c* heterozygosity. It is also important to note that the

evidence for decreased PV within the dentate gyrus is not indicative of a deficit in activation of the interneurons. Further studies to determine this would be highly valuable in determining the role of inhibition and disinhibition within the *Cacna1c*$^{+/-}$ model and how it affects their response to auditory fear conditioning.

9.7 Limitations

9.7.1 Models

It is highly important to emphasise that there is no complete 'animal model of psychiatric illness'. Schizophrenia and bipolar disorder are both highly heterogeneous and complex disorders that we cannot fully model in animals. Single gene knockout models, such as the *Cacna1c*$^{+/-}$ rat, and singular environmental models, such as PPS, do not take into account the complex polygenic background in which these insults can contribute risk. Having said this, the models we use to interrogate the impact of functional genetic variants can be used to understand parts of the underlying biology that can guide our understanding to how alterations in that gene, or pathway, may contribute to specific symptoms. Likewise, examining the consequences of stress can contribute to identifying molecular processes that may also lead to pathology. Once we have an increased understanding as to why a particular gene or environmental factor provides an increased risk over others, we can start to identify novel treatment targets for human psychiatric illness.

Many of the experiments presented in this body of work utilised a chronic genetic knockdown rat model of *Cacna1c* which has many advantages over pharmacological LTCC antagonism, such as specificity to the target gene and a reduction of off-target effects. However, there are still disadvantages to using these models. For example, it is possible that the prenatal or postnatal environment provided by a mutant or wildtype mother to offspring could affect the development of the offspring. This is particularly important considering the potential interaction of *CACNA1C* and early life

stress, as shown in this thesis. To combat this, one could utilise a conditional knockout, however a vital role for *Cacna1c* in neurodevelopment has been shown, where deletion has different consequences at different ages, showing the importance of modelling *Cacna1c* heterozygosity from birth (Dedic et al., 2018). In addition, it is not fully determined as to whether a knock-down model of *Cacna1c* is the most appropriate model for investigating the impact of genetic variation of this gene. Genetic literature suggests that gain-of-function phenotypes may also be relevant to disease (Bigos et al., 2010; Heyes et al., 2015) and therefore increased gene dosage should also be investigated in the context of *Cacna1c* genetic variation.

The PPS model used in this thesis has been validated before and shown to have dramatic effects on the adult animal (Brydges *et al.* 2014; Albrecht *et al.* 2017). However due to the consistent timing and duration of stress, it cannot account for the modelling of all PPS in humans which varies between individuals and can have different degrees of intensity and length. Early life stress models are time-consuming experiments, meaning that it was not possible to investigate different time points of giving stress within the confines of this thesis. Also, while the PPS paradigm was well-controlled between litters, the social stress that may have been experienced within the home cages was unknown, although animals were carefully monitored for signs of excessive fighting.

9.7.2 Behaviour

Linking animal behaviours to human phenotypes is imprecise and complex, as several aspects of psychopathology, and the symptoms associated with it, are classed as ‘human’ traits, particularly considering cognition. This is an important distinction to make when considering the translational aspect of functional animal work.

Within this thesis, I focus on the impact of *Cacna1c* variation and PPS on associative learning in the form of aversive auditory fear conditioning. Associative learning has been shown to be impaired in clinical studies of schizophrenic patients (Miller, 1976; Diwadkar et al., 2008; Holt et al., 2012), animal models of psychiatric disorders (Nakajima, 2004; Dickinson, 2012) and more recently in genetics studies (Hall et al., 2009; Pocklington et al., 2015; Clifton et al., 2017). Thus associative learning has been seen for many years as a robust paradigm to investigate how psychiatric symptoms such as psychosis and delusions arise (Rescorla and Wagner, 1972; Fletcher and Frith, 2009). However, it is well known that other cognitive domains are also impaired in schizophrenia and bipolar disorder, for example, working memory, episodic memory, executive functions and attention (Bowie and Harvey, 2006). In fact, schizophrenia in particular is associated with impairments across a wide-range of cognitive impairments, ranging from mildly affected to severely affected, with most patients demonstrating at least moderate cognitive impairments. Therefore, it should be acknowledged that the focus on associative learning presented here does not negate the importance of other cognitive domains implicated in schizophrenia, and other neurodevelopmental disorders.

9.7.3 Gene and protein expression

Several aspects of this work relies on the detection of changes in genes or proteins within the hippocampus using techniques such as qPCR and immunohistochemistry. While variables were kept constant over experiments, certain factors cannot be fully controlled while utilising these techniques. For example, while all the tissue was taken at ‘basal’ level, that is without the influence of behavioural experiments, we cannot know what the animals were experiencing in their home cages prior to sacrifice. For example, expression of certain transcripts of BDNF and PV are known to be activity dependent (Bramham and Messaoudi, 2005; Liu et al., 2006; Zheng et al., 2011; Donato et al., 2013), and thus, although animals were sacrificed at similar times in

the day, there may be certain differences in activity between different cages. The number of animals used for each experiment is also fairly small which may not be enough to pick up smaller expression changes as current tools available within the laboratory are designed principally to pick up larger expression changes. However, increasing the number of animals within a study is not always desired, for example, introducing variability into the environment and genetic background can mask effects. Also, it is important to take into account ethical concerns and the higher stringency in statistics that would be required.

9.8 Future experiments

The results presented in this thesis build a foundation for the investigation of a possible gene x environment interaction concerning *CACNA1C* and early life stress. Gene x environment (G x E) implies that a particular phenotype is caused by synergistic interaction between genes and environment, where the effect of one is conditional upon another. A genetic factor can influence the sensitivity of the individual to a particular environment exposure, or the environment may impact or epigenetically modify DNA sequences themselves. The first reported G x E interaction for a psychiatric disorder was a functional polymorphism in the gene encoding catechol-O-methyltransferase (COMT), an enzyme which metabolises dopamine. Caspi and colleagues found that those carrying a SNP that substitutes methionine (Met) for valine (Val), resulting in a less efficient COMT enzyme, had a reduced risk of developing schizophrenia following adolescent cannabis (Caspi et al., 2005). This finding, despite considerable disparity in the literature in terms of replication, opened the door for the exploration of gene-environment interactions in psychiatric disorders; for example, a polymorphism in the promoter of SLC6A4, which encodes the serotonin transporter, has been seen to moderate the effects of childhood maltreatment on persistent depression (Barnett and Smoller, 2009; Brown et al.,

2013). The association of *CACNA1C* with trauma (Dedic et al., 2018) suggests that this is another potential G x E interaction that requires further investigation.

This experiment could be instigated by subjecting both *Cacna1c*$^{+/-}$ rats and their wild-type littermates to PPS (alongside a non-stressed control group) and carefully examining chosen behaviours and biochemical parameters, as detailed in Figure 9.2. It would be particularly interesting to see how a dual-hit of risk gene variation and stress affects the processing of delay and trace conditioning, and the influence on neurogenesis.

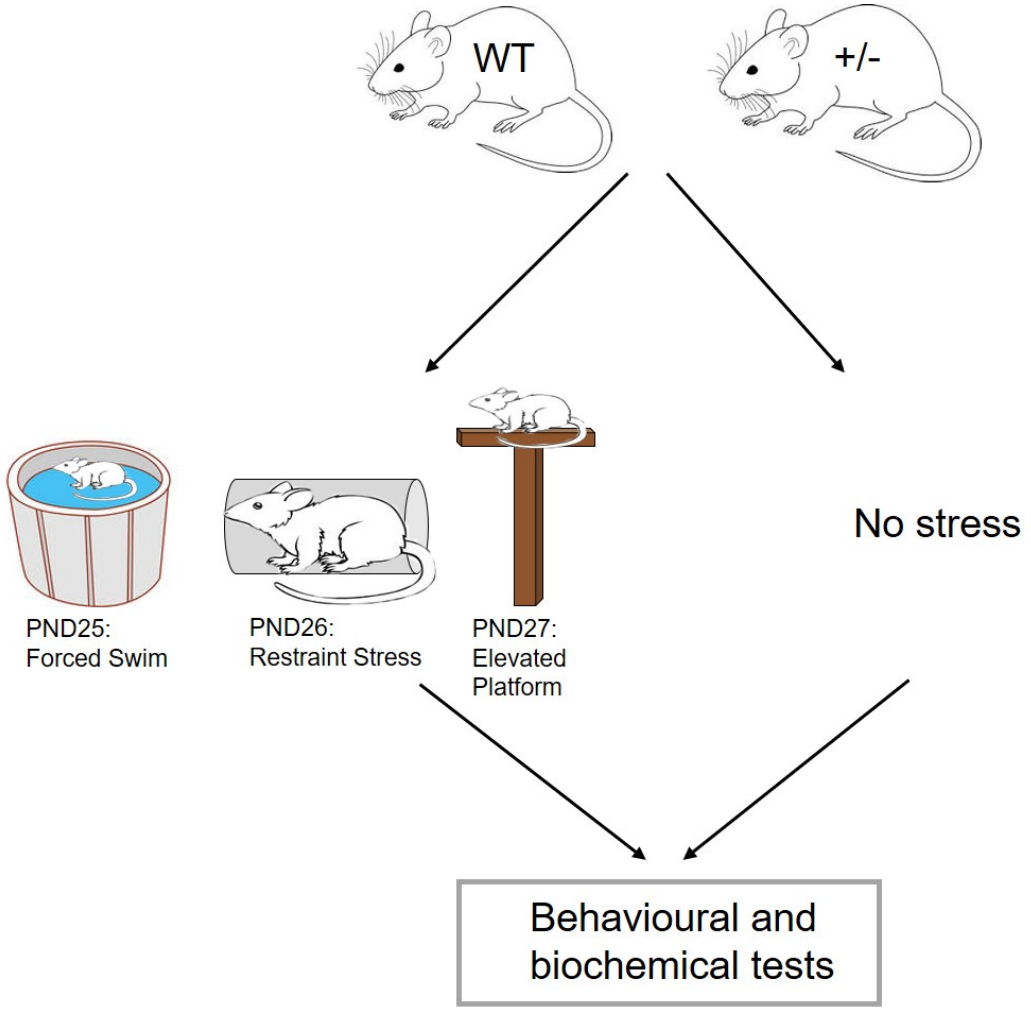

Figure 9.2: Gene x environment experiment concerning *Cacna1c*$^{+/-}$ and PPS. Both wild-type and heterozygote rats would be subject to PPS between PND25-27 whilst another group of wild-type and heterozygote rats would act as controls. Behavioural and molecular analysis could then be completed to assess how *Cacna1c* and PPS may interact to cause phenotypes associated with psychiatric disease.

To compliment this work, it would also be interesting to investigate the mechanisms as to how PPS decreases *Cacna1c* expression in the hippocampus through examining epigenetic modifications of the *Cacna1c* gene, particularly within promoter or enhancer regions. The methylation status of *CACNA1C* has been shown to be altered in bipolar disorder (Starnawska et al., 2016) and thus it would be prudent to begin with the examination of methylation changes of CpG sites, potentially through using techniques such as pyrosequencing. Additionally, further investigation of the effect of enriched environment on *Cacna1c*$^{+/-}$ and PPS rats, by the inclusion of running wheels for physical exercise, might provide insight as to how the environment can shape *Cacna1c* expression and interact with relevant phenotypes.

The reduction of PV and GAD67 in the dentate gyrus alludes to the fact there the excitatory-inhibitory balance in *Cacna1c*$^{+/-}$ may be disrupted, which could be influencing behaviour. Electrophysiological studies would be paramount to investigating this in more detail, within the hippocampus and also within other brain areas such as the prefrontal cortex and amygdala. Investigation of other subtypes of interneurons such as SOM^{+} interneurons should also be conducted. Furthermore, it would be beneficial to perform stereological experiments within the hippocampus to determine if the number of inhibitory interneurons are reduced, or PV and GAD67 are decreased within intact interneurons.

Finally, the results presented in this thesis contribute to the expansive literature that highlights the role of the hippocampus in the pathophysiology of psychiatric and mood disorders (Knable et al., 2004; Konradi et al., 2011a; Brydges et al., 2014d; Knierim, 2015). It may be argued that multiple genetic and environmental insults converge on the hippocampus, causing progressive damage and leading to the development of psychiatric symptoms. However, it is clear that other brain areas are impacted in psychiatric disorders such as the prefrontal cortex, basal ganglia and amygdala which were beyond the scope of this thesis. Further investigations should however consider

the impact of genetic variation and environment stress on multiple brain areas, as well as the influence on the communication between them that is vital for many cognitive processes.

9.9 Conclusions

Cacna1c and stress may interact to increase risk for psychiatric disorders, potentially by converging on the hippocampus. The current findings demonstrate that *Cacna1c* has a specific role in aspects of associative learning, particularly the specificity of associations made, whilst PPS also impairs trace fear conditioning. Cav1.2 is also shown to have a role in parvalbumin expression and adult neurogenesis within the dentate gyrus.

Deficits seen in both the *Cacna1c*$^{+/-}$ and PPS animals models are indicative of potential psychopathologies that underlie schizophrenia, bipolar disorder and other psychiatric disorders. Further investigation of both models, and importantly, on the potential interaction between PPS and *Cacna1c* would be vitally important to understand the biological mechanisms involved, and potentially highlight these pathways as targets for novel treatments.

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