The role of the psychiatric risk-associated gene *Cacna1c* and sex in hippocampal dependent cognition

A thesis presented for the degree of

Doctor of Philosophy

By

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September 2024

Abstract

Psychotic and mood disorders such as schizophrenia, bipolar disorder and depression affect a substantial proportion of individuals and constitute a considerable degree of years lived with disability. Common risk single-nucleotide polymorphisms (SNPs) of the *CACNA1C* gene, which encodes the α1c subunit of L-type voltage gated calcium channels (LTCCs), have been robustly associated with all three disorders. Biological sex also has a major impact on risk for psychotic and mood disorders. However, the degree to which risk SNPs differentially impact sexes is not well understood. We have investigated the effects of low *Cacna1c* gene dosage and sex using contextual fear conditioning behaviours known to depend upon the hippocampus. Both *Cacna1c* heterozygosity and sex showed independent effects on hippocampal dependent associative fear learning by affecting the integration of previously learned information in contextual fear processing. *Cacna1c*$^{+/-}$ animals also showed increased expression of genes with functions related to neuroimmune processing and the brain extracellular matrix (ECM), and sex differences were seen in genes with functions related to epigenetic histone protein modifications, among others. *Cacna1c* heterozygosity also appears to affect males and females similarly, however, sex differences in the impact of low *Cacna1c* gene dosage were observed in both behavioural and (hypothalamic-pituitary-adrenal) HPA-axis response measures. These findings suggest that *CACNA1C* risk variants may confer increased risk for psychotic and mood disorders by affecting contextual associative fear learning processes mediated by the hippocampus, possibly via affecting neuroimmune, brain ECM and HPA-axis processes. Although interactions between sex and *Cacna1c* genotype were limited, the findings suggest that the neurobiological processes implicated in *CACNA1C* risk SNPs may be sexually dimorphic.

Declarations

This thesis is being submitted in partial fulfilment of the requirements for the degree of PhD.

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is it being submitted concurrently for any other degree or award (outside of any formal collaboration agreement between the University and a partner organisation).

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This thesis is the result of my own independent work, except where otherwise stated, and the views expressed are my own. Other sources are acknowledged by explicit references. The thesis has not been edited by a third party beyond what is permitted by Cardiff University's Use of Third-Party Editors by Research Degree Students Procedure.

In memory of Deliah Taylor

‘Grandma Dee’

14th September 1949 –

28th March 2023

Acknowledgements

This PhD project could not have been completed without the significant support of a large number of wonderful individuals. Firstly, I would like to extend my heartfelt thanks to my supervisors, Prof Jeremy Hall and Dr Kerrie Thomas. Thank you for taking a chance on me for this project when I had next to no experience and thank you for both your professional and personal support throughout the challenges of the last three years and the times when I thought I wouldn't be able to do it. You have both helped me enormously with my confidence so that I may finish this PhD feeling like 'a proper scientist'.

Numerous others have provided me with support and guidance with this project, to whom I am incredibly grateful. Firstly, the neuroscience power couple that is Dr Nick Clifton and Dr Anna Clifton. Nick, thank you for being an unofficial supervisor and for your patience and clear explanations regarding all things bioinformatics. Anna, thank you for teaching me everything in the lab and for being such a wonderfully friendly presence when I first started. Emma Dalton – the NMHII could not ask for a better lab manager, and we would all be lost without you. You always bring a smile to all our faces, and I cannot thank you enough for your help. Dr Esther Lin, thank you for your generosity of time and patience in teaching me the behavioural work and for finishing off my experiment when I had to take time off, and thank you for being such a lovely desk neighbour for the last three years. Thank you to Dr Peter Richardson for your assistance with some of the dissections and allowing me to bother you with questions at your desk. Thank you to Dr Patricia Gasalla Canto, Prof Dominic Dwyer, Dr Gemma Wilkinson, Dr Jo Haddon, Dr Laura Westacott, Prof Lawrence Wilkinson and Dr Will Davies for your advice and expertise along the way. Thank you to Jo Morgan and the NGS team for your considerable technical help with the RNA-sequencing.

My PhD was funded by a generous legacy donation from David and Mary Mills, and I received a further donation from Robert Rowley (LLB 1970) to support my research in my final year. This is an incredibly selfless thing to do, and I have benefitted enormously from the generosity of people I have never met, so thank you.

This PhD has been difficult and stressful at times, and I am so grateful for the support from those colleagues I am lucky enough to now call friends: Emma Weir, Laura Abram, Olivia Squire, Freya Shepherd, Dr Francesco Bedogni, Cecilia Zuglian, Parinda Prapaiwongs and

Emily Stonelake. Thanks for all the good times at the Woody. Thank you to my friends from ‘the before times’ who have provided me with so much love and support from afar, especially Esmé Curtis, Sophie Boulter, Ben Wind and Jacob Gray. Thank you to my whole family for their continuous encouragement, love and support, particularly my parents and my sister, Lucy. Grandma Dee, to whom this thesis is dedicated, thank you for everything. You made me the person I am today, and I miss you every day.

Finally, I would like to thank my wonderful fiancé, Alex Horne. Thank you for coming with me to Cardiff for this PhD, your unwavering support for my academic and career ambitions and for listening to me talk about nothing but this project for the last three years. I couldn’t have done this without you.

Abbreviations

A2m: Alpha 2-macroglobulin

AA: Active Avoidance

ADHD: Attention Deficit Hyperactivity Disorder

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid

ANOVA: Analysis Of Variance

ARC: Activity-Regulated Cytoskeletal (protein)

ASD: Autism Spectrum Disorder

BDNF: Brain Derived Neurotrophic Factor

BLA: Basolateral Amygdala

CA1/CA2/CA3: Cornu Ammonis 1/2/3

CACNA1C/*Cacna1c*: Calcium voltage-gated channel subunit alpha1-c

CaMKII: Calcium/calmodulin-dependent protein Kinase II

cDNA: complementary Deoxyribonucleic Acid

CeA: Central Amygdala

CER: Conditioned Emotional Response

CFC: Contextual Fear Conditioning

CI: Confidence Interval

CNS: Central Nervous System

CNV: Copy Number Variation

CPM: Counts Per Million

CREB: Cyclic adenosine 3′,5′-monophosphate-responsive element binding protein

CS: Conditioned Stimulus

C_T: Cycle Threshold

CTA: Conditioned Taste Aversion

DEG: Differentially Expressed Gene

DG: Dentate Gyrus

DNA: Deoxyribonucleic Acid

DSM: Diagnostic and Statistical Manual of Mental Disorders

ECM: Extracellular Matrix

ELISA: Enzyme Linked Immunosorbent Assay

eQTL: expression Quantitative Trait Loci

ERK: Extracellular Signal-Related Kinases

E-TC: Excitation-Transcription Coupling

FDR: False Discovery Rate

GABA: γ-aminobutyric acid

GO: Gene Ontology

GR: Glucocorticoid Receptor

GSEA: Gene Set Enrichment Analysis

GWAS: Genome-Wide Association Study

HP: Human Phenotype

HPA: Hypothalamic-Pituitary-Adrenal

ICD: International Classification of Diseases

IEG: Immediate Early Gene

IFN-II: Interferon Type-II

IFN-γ: Interferon Gamma

IL: Infralimbic (medial prefrontal cortex)

LD: Linkage Disequilibrium

LI: Latent Inhibition

LTCC: L-type voltage-gated Calcium Channel

LTP: Long-term potentiation

M6PR: (cation-dependent) Mannose-6-phosphate Receptor

mA: Milli-ampere

MAGMA: Multi-marker Analysis of Genomic Annotation

MAPK: Mitogen Activated Protein Kinase

*μ*l: microlitre

ml: millilitre

mPFC: medial Prefrontal Cortex

MR: Mineralocorticoid Receptor

NAc: Nucleus Accumbens

NAcc: Nucleus Accumbens core

NAcsh: Nucleus Accumbens shell

NDD: Neurodevelopmental Disorder

NGS: Next Generation Sequencing

nm: nanometre

NMDA: N-methyl-D-aspartate

NMDAR: N-methyl-D-aspartate Receptor

PC: Principal Component

PCA: Principal Components Analysis

PE: Pre-Exposure

PFC: Prefrontal Cortex

pg: picogram

PGC: Psychiatric Genetics Consortium

PL: Pre-limbic (medial prefrontal cortex)

PND: Postnatal Day

PTSD: Post-traumatic Stress Disorder

QC: Quality Control

qPCR: quantitative Polymerase Chain Reaction

RBM47: RNA Binding Motif 47

RDoC: Research Domain Criteria

RNA: Ribonucleic Acid

rpm: revolutions per minute

SCHEMA: Schizophrenia Exome-sequencing Meta-Analysis

SNP: Single Nucleotide Polymorphism

US: Unconditioned Stimulus

WGCNA: Weighted Gene Co-expression Network Analysis

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Chapter 1: Introduction

1.1 Genetic risk for psychotic and mood disorders

Neuropsychiatric disorders such as schizophrenia, bipolar disorder and depression are responsible for a considerable proportion of global disease burden, with an estimated 14.3% of global deaths worldwide and around a third of years lived with disability attributable to such disorders (Walker et al., 2015; Vigo et al., 2016). However, despite several decades of research attempting to elucidate the underlying biology of neuropsychiatric disease, minimal progress has been made in the development of novel treatments (Owen, 2023). Psychotic and mood disorders are known to be highly complex and are thought to arise owing to interactions between numerous environmental, genetic, and epigenetic factors. Family and twin studies have estimated the heritability of schizophrenia and bipolar disorder to be ~60-80% (Sullivan et al., 2003; Lichtenstein et al., 2009; Bienvenu et al., 2011; Hilker et al., 2018; Johansson et al., 2019) and slightly lower but nonetheless sizeable heritability estimates have been observed in depression (~30-50%) (Fernandez-Pujals et al., 2015; Kendall et al., 2021). Thus, although they are not simple Mendelian disorders with a single genetic cause, they appear to have a considerable albeit complex genetic component. Consequently, understanding the mechanisms whereby genetic variation leads to the aberrant neurobiology that drives disease will provide improved opportunities for the development of novel treatments.

Early candidate gene studies provided compelling evidence that cases of neuropsychiatric disorders driven by single genes are likely to be decidedly rare, and complex gene-gene interactions between multiple susceptibility loci drive the genetic component of disease risk (O'Donovan and Owen, 1999; Oh et al., 2012; Neale and Sklar, 2015). Early insights regarding the roles of specific genes in disorder aetiology from such candidate gene approaches were limited owing to poor statistical power

(Farrell et al., 2015). Subsequently, the development of whole-genome sequencing technologies has facilitated a new era of psychiatric genomic research in which numerous genome wide association studies (GWAS) have been performed. In GWAS, genome wide SNPs are inferred in a sample of individuals with a specific disorder, as well as a comparable sample of controls. Analysis can then be conducted to identify single nucleotide polymorphism (SNP) variants that are statistically significantly more common in cases compared with controls. Such studies have demonstrated that commonly studied variants in candidate gene approaches were no more associated with schizophrenia than non-candidate genes (Johnson et al., 2017), with similar results also observed in depression (Bosker et al., 2011), highlighting that the candidate-gene approach may not be best poised to elucidate the underlying genetic architecture and downstream implicated biology of neuropsychiatric disease.

Several GWAS have been conducted over the past two decades with the aim of identifying genes that are associated with psychotic and mood disorders. Large consortia of researchers have been formed with the aim of pooling samples to expand power to detect genetic risk variants. These efforts have proven fruitful, and the first three iterations of the Schizophrenia Working Group of the Psychiatric Genomics Consortium (PGC) GWAS have identified far more associations than ever before, with the most recent schizophrenia PGC GWAS having identified a total of 342 linkage-disequilibrium (LD) independent risk variants in 287 genetic loci (Trubetskoy et al., 2022). This most recent PGC schizophrenia GWAS comparing 76,755 schizophrenia patients with 243,649 controls showed that associations were concentrated within genes exclusively expressed in excitatory and inhibitory neurons in the central nervous system (CNS). Using fine-mapping analysis, 120 of the 342 risk SNPs were identified as having a high likelihood of being distinct causal variants, and were implicated in processes related to neuronal functioning such as synaptic organisation, differentiation, and transmission. Furthermore, gene set enrichment analysis (GSEA) identified granule neurons of the dentate gyrus (DG), and pyramidal neurons in cornu Ammonis 1 (CA1) and cornu Ammonis 3 (CA3) regions of the hippocampus as the cell types with the highest level of enrichment of GWAS

associations (Trubetskoy et al., 2022). Thus, the analysis of genetic data in large samples has provided support for the possible role of hippocampal synaptic plasticity processes in the development of schizophrenia.

As with schizophrenia, early studies attempting to identify genetic risk loci for bipolar disorder were limited by poor statistical power and detected few if any associations after correction for multiple comparison (Yosifova et al., 2011; Greenwood et al., 2012, 2013), although early studies did highlight *CACNA1C* as a consistent signal in bipolar disorder (Ferreira et al., 2008; Sklar et al., 2008), a finding which was later replicated in schizophrenia (Nyegaard et al., 2010) and depression (Green et al., 2010). However, the emergence of consortia such as the PGC has facilitated larger sample sizes with greater statistical power. The third and most recent PGC Bipolar Disorder Working Group GWAS compared 41,917 bipolar disorder cases with 371,549 controls, and identified 64 associated genomic loci, 33 of which were novel to the study. Consistent with findings in schizophrenia, gene set association analyses showed enrichment of genes related to synaptic plasticity in bipolar disorder risk variants (Mullins et al., 2021), highlighting the overlap between the biological mechanisms underpinning the two disorders.

As with schizophrenia and bipolar disorder, GWAS of depression using larger sample sizes have been successful in identifying associations. A recent comparison of 135,458 cases and 344,901 controls identified 44 genome-wide significant loci, 30 of which were novel to the study, and many of which were also associated with risk for schizophrenia. Several implicated genomic loci have known roles in the major histocompatibility complex, a multi-gene family playing a key role in both the innate and adaptive immune system (Heijmans et al., 2020), many of which are also known schizophrenia risk loci (Wray et al., 2018). When bipolar disorder risk variants previously identified using GWAS have been assessed in depression cases versus controls, 15 significant associations were observed, indicating some shared genetic risk between depression and bipolar disorder (Ripke et al., 2013). Similarly, genetic risk for a broad depressive phenotype (as opposed to recurrent depression) was found to have a r_g correlation of 0.79 with depression, and 0.24, 0.53, and 0.57 with

recurrent depression, bipolar disorder and schizophrenia respectively (Amare et al., 2020), further providing support for cross-disorder genetic risk. Further analyses of the genetic architectures of schizophrenia and bipolar disorder derived from GWAS data have found that although there remains an element of disorder specificity, a large portion of genetic risk is shared between the two pathologies (Grotzinger, 2021; Hindley et al., 2021; Legge et al., 2021; O'Connell and Coombes, 2021). Similarly, shared genetic architecture has also been identified between schizophrenia and depression (Wray et al., 2018; Howard et al., 2019; McIntosh et al., 2019) and bipolar disorder and depression (Mullins and Huang, 2021).

GWAS have provided highly valuable insights into the underlying genetic architecture of common risk variants in neuropsychiatric disorders. However, a rarer but highly impactful source of genetic variation comes from copy number variation (CNV) in which certain sections of the genome are repeated, with the number of copies of the repeated region varying between individuals (Pös et al., 2021). CNVs that have been implicated in a disorder typically bear greater penetrance, with a greater odds ratio than SNP carriers (exact odds ratios of increased risk differ between specific SNPs and CNVs). Owing to higher disease burden conferred by CNVs compared to SNPs, CNVs are decidedly rarer and affected individuals are less likely to have children than non-carriers. However, CNVs developing *de novo* can maintain mutations within the population despite lower birth rates in carriers (Rees et al., 2011). CNVs are largely studied within the remit of neurodevelopmental disorders (NDD). However, numerous CNVs associated with NDD are also associated with schizophrenia (e.g., 1q21.1 duplication and deletion, 16p11.2 duplication, and 22q11.21 deletion), depression (e.g., 1q21.1 duplication, 15q11.2 duplication, and 16p11.2 duplication), and to a lesser extent, bipolar disorder (e.g., 16p11.2 duplication) (Mollon et al., 2023). Functional GSEA of genes that are disrupted by CNVs associated with schizophrenia has revealed enrichments in genes with functions related to neuronal activity and learning (Raychaudhuri et al., 2010), in line with findings from GWAS studies of common risk SNPs (Pocklington et al., 2015; Hall and Bray, 2022).

Whole-exome sequencing of 24,248 schizophrenia cases compared with 97,322 controls by the Schizophrenia Exome Sequencing Meta-Analysis (SCHEMA) consortium has also implicated ultra-rare variation in ten genes in the development of the disorder: *SETD1A*, *CUL1*, *XPO7*, *TRIO*, *SP4*, *CACNA1G*, *GRIA3*, *GRIN2A*, *RB1CC1*, and *HERC1.* As with the findings in CNVs, there appears to be substantial overlap between genes with ultra-rare variants implicated in schizophrenia and NDD. Specifically, *CACNA1G*, *GRIN2A*, *TRIO* and *SETD1A* have also been identified as loci with ultra-rare variants shown to increase risk for NDD and ASD (Singh et al., 2022). *GRIN2A* and *SP4* were also identified in the most recent PGC schizophrenia GWAS as common risk loci (Trubetskoy et al., 2022) and functional enrichment analysis of both the SCHEMA and PGC studies revealed an enrichment within both the ultra-rare variants and common risk SNPs of genes involved in synaptic transmission, voltage-gated cation channel activity, and regulation of ion transmembrane transport, among other processes (Singh et al., 2022). When comparing 3,987 individuals with bipolar disorder to 5,322 controls, increased burden of pathogenic or likely-pathogenic rare coding variants has been observed in 165 genes, and this has also shown to overlap with bipolar disorder common risk loci. However, despite the known shared common genetic risk burden between the two disorders, the rare coding variants identified in bipolar disorder did not overlap with common risk genes in schizophrenia, suggesting that the loci of rare variation conferring increased risk for bipolar disorder are disorder-specific (Jia et al., 2021). Exome-wide sequencing using the UK Biobank has also revealed ultra-rare variants that increase risk for depression, and the gene set was found to overlap with rare genetic components of schizophrenia and NDD (Tian et al., 2024). Thus, although the rare genetic component of bipolar disorder appears to be somewhat distinct, rare deleterious variations in schizophrenia and depression demonstrate a shared component, and functional enrichment findings in schizophrenia rare variants highlight the importance of voltage-gated ion channels and synaptic transmission in the neurobiology of the disorder.

Together, the sizeable and still growing body of evidence for considerable genetic risk in psychotic and mood disorders suggest that a highly complex genetic

architecture underpins psychotic and mood disorders, with a considerable shared element between pathologies (O'Donovan and Owen, 2016). Common risk variants in particular appear to exert both polygenic and pleiotropic effects in the mechanisms through which they lead to disease susceptibility. SNPs have been shown to explain 17-29% of variance in liability to neuropsychiatric disorders, with a genetic overlap of ~68% between schizophrenia and bipolar disorder, ~43% between schizophrenia and major depressive disorder, and ~47% between bipolar disorder and major depressive disorder (Cross-Disorder Group of the Psychiatric Genomics Consortium, 2013). Shared genetic liability between neuropsychiatric disorders has also been supported by a GWAS across 17 phenotypes that demonstrated strong correlations between common risk variants in schizophrenia, bipolar disorder, major depression and ADHD (Anttila et al., 2018), as well as analyses of polygenic risk scores (Bigdeli et al., 2022). Thus, the current knowledge base raises questions regarding the efficacy of studying biological aetiology of neuropsychiatric disease by investigating discrete diagnostic classifications. Hence, the following section will discuss emerging transdiagnostic frameworks for research and their potential for improved understanding of the underlying biological mechanisms of neuropsychiatric disease and the development of novel precision treatment options.

1.2 Transdiagnostic approaches to psychotic and mood disorders

Psychopathologies have traditionally been considered as discrete diagnostic phenotypes and approaches towards research, diagnosis and treatment in modern medicine have followed this framework. However, conditions such as schizophrenia, bipolar disorder and depression are highly heterogenous in terms of their presentation and responsivity to treatment (Moreno-De-Luca and Martin, 2021). Furthermore, despite several decades of research into the underlying biology of psychotic and mood disorders, no valid biomarkers or biological profiles have been associated with a discrete disorder (Abi-Dargham et al., 2023; Fišar, 2023). Although

numerous associations between biological substrates and psychopathologies, such as neurostructural alterations, aberrant neurotransmitter activity and altered neuroinflammatory mechanisms have been observed, there has been limited clinical utility translated from these findings (Cuthbert and Morris, 2021). Hence, the validity of the current array of neuropsychiatric diagnoses has been questioned, particularly in relation to schizophrenia (Cuthbert and Morris, 2021).

The schizophrenia phenotype is considered to be a combination of positive (e.g., hallucinations and delusions), negative (e.g., depression, anhedonia and social withdrawal) and cognitive symptoms (e.g., deficits in concentration and working memory). The two major frameworks for neuropsychiatric disease classification are the Diagnostic and Statistical Manual of Mental Disorders (DSM), currently in its fifth iteration (American Psychiatric Association, 2013), and the International Classification of Diseases (ICD), currently in its sixth iteration (ICD-11; World Health Organization, 2022). Both current versions of the ICD and DSM are relatively similar in their conceptualisation of schizophrenia, in that, unlike previous versions of the classification systems, the heterogeneity of the symptom profiles is addressed using a spectrum framework rather than previously used subtypes (Valle, 2020). This change has been made in response to considerable criticism of the validity of subtypes in their ability to differentiate outcomes and profiles of patients (Korver-Nieberg et al., 2011). However, schizophrenia is still considered as a distinct diagnosis from schizoaffective disorder, schizotypal disorder, acute and transient psychotic disorder, delusional disorder and other specified schizophrenias or other primary psychotic disorders in the ICD-11, and schizoaffective disorder, delusional disorder, schizophreniform disorder, and brief psychotic disorder in the DSM-V.

In line with schizophrenia, bipolar disorder is classified similarly between the DSM-V and ICD-11 and is characterised by extreme mood swings in which an individual experiences extended periods of both depression and mania. As with schizophrenia, bipolar disorder is also often marked by cognitive symptoms such as deficits in attention and working memory (Lima et al., 2018). Bipolar and related disorders are considered distinct from both schizophrenia spectrum and other psychotic disorders

as well as unipolar depression in the DSM-V, and, as with schizophrenia spectrum and related disorders, comprises a range of diagnoses (bipolar I, bipolar II, cyclothymic disorder and other specified bipolar and related disorders). However, in the ICD-11, both bipolar and related disorders, and depressive disorders, are classified under the umbrella of mood disorders, implying a greater extent of shared conceptualisation of bipolar and unipolar depression than between schizophrenia and bipolar disorder, or schizophrenia and depression. However, bipolar disorder diagnoses can be made both with and without psychotic features. As with the DSM-V, the ICD-11 stratifies bipolar and related disorders into bipolar I, bipolar II, cyclothymic disorder, and other specified bipolar and related disorders, but features the additional diagnosis of bipolar or related disorders, unspecified, which is not found in the DSM-V. The primary distinctions between bipolar disorder categories relate to the extent of mania. Bipolar II is diagnosed when manic symptoms only meet the threshold for hypomania, and if hypomanic episodes are not sufficiently severe or prolonged to meet the threshold for bipolar II, cyclothymic disorder is diagnosed.

On the other hand, depression is not primarily characterised by psychosis or mania, but rather persistent periods of low mood and anhedonia. However, as with schizophrenia and bipolar disorder, individuals with depression also demonstrate impairments in cognition, affecting their working memory, attention, and executive function (Marazziti et al., 2010). In the DSM-V, depressive disorders, often referred to as unipolar depression to demarcate them from bipolar disorders, comprise disrupted mood dysregulation disorder, major depressive disorder, persistent depressive disorder, premenstrual dysphoric disorder, substance/medication induced depressive disorder, depressive disorder due to another medical condition, other specified depressive disorder, and unspecified depressive disorder. Similarly, the ICD-11 delineates depressive disorders into single episode depressive disorder, recurrent depressive disorder, dysthymic disorder, mixed depressive and anxiety disorder, premenstrual depressive disorder, other specified depressive disorders, and depressive disorders, unspecified. Although depressive disorders are demarcated from schizophrenia and bipolar disorders with primary features of low

mood and anhedonia, a diagnosis of a depressive disorder can be made with psychotic features.

Although current classification systems for psychotic and mood disorder diagnosis are largely still based upon discrete diagnoses, there are considerable shared aspects of symptomatology such as cognitive deficits, low mood, and various psychotic features across disorders, a finding in line with the cross-disorder shared genetic architecture previously discussed. The funding frameworks for research into the biological aetiology of neuropsychiatric disease as well as treatment development traditionally require research programmes to be linked to a specific psychopathology. Hence, any limitations in the diagnostic classification of disorders directly influences the ability to effectively research the biological basis of psychopathologies and to translate these findings into much needed novel treatment options. Recent research efforts have been limited in their ability to translate biological findings into meaningful treatment improvements for patients, particularly in the case of schizophrenia (Laughren, 2020). Hence, there has been a need to reconsider the traditionally used research strategies for psychotic and mood disorders.

The Research Domain Criteria (RDoC) framework was developed by the United States National Institute for Mental Health in response to this problem. RDoC aims to research constructs based in empirical neuroscientific observations, rather than the clusters of symptoms that form diagnoses. Specifically, the approach focuses on both upstream and downstream pathways of neural circuitry. Upstream refers to the pathways in which aberrant neural circuitry leads to the development of clinical symptoms, and downstream refers to the underlying genetic, molecular and cellular processes that influence neural circuitry development and maintenance (Insel et al., 2010). The RDoC assume a dimensional structure across six domains: arousal/regulatory, positive valence, negative valence, social processes, sensorimotor and cognitive (Figure 1.1). These domains are transdiagnostic, and the approach aims to take a directly translational approach to targeting the neurobiological basis underpinning each domain, to develop novel treatments and

interventions that target specific biologically meaningful phenomena that may be transdiagnostic, rather than broad heterogenous diagnoses.

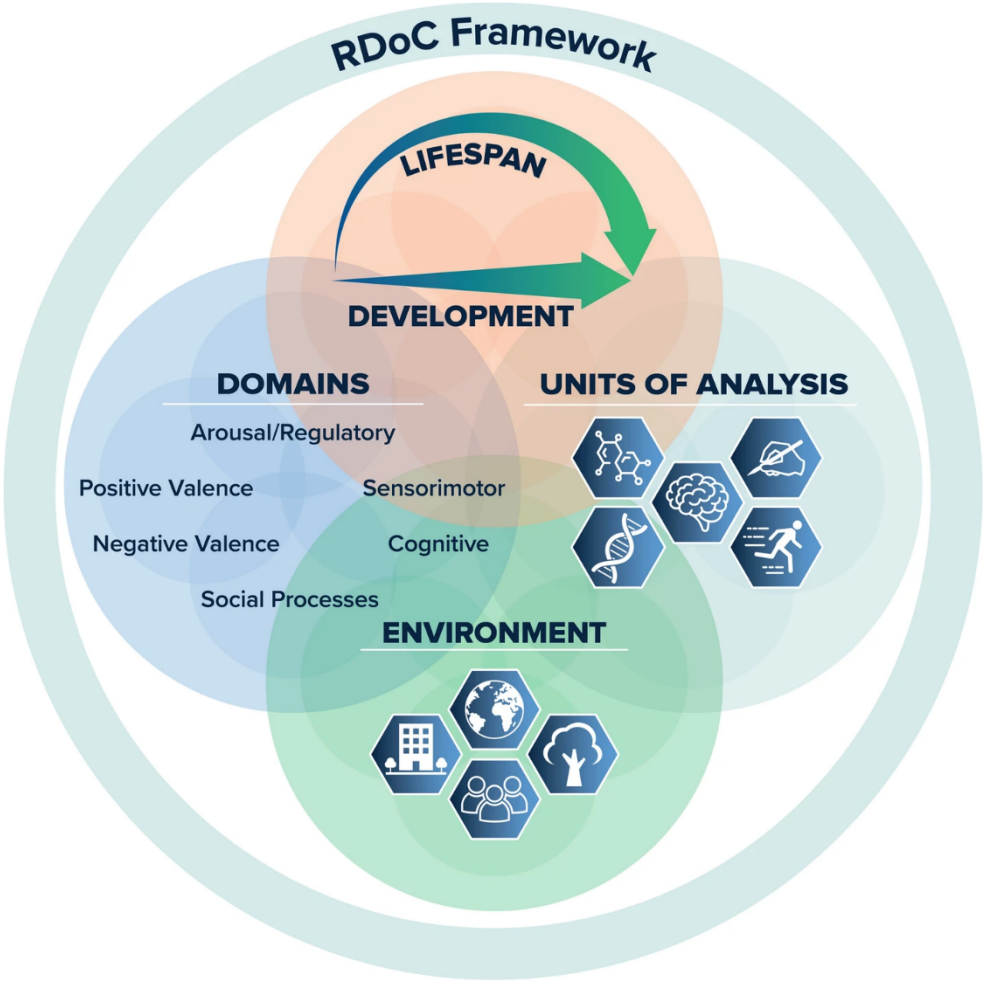

Figure 1.1 The RDoC framework (Morris et al., 2022).

Some have questioned the clinical utility of RDoC, particularly if this were to replace the current diagnostic classification systems (Weinberger et al., 2015; Paris and Kirmayer, 2016). However, retaining the current diagnostic structures within the clinic does not preclude the utilisation of biologically meaningful dimensional approaches in the research of the genetic and neurobiological basis of psychotic and mood disorders. The status quo of neuropsychiatric research has led to an extended period of failed drug trials and no meaningful improvements in patient outcomes, particularly in the case of schizophrenia (Laughren, 2020). Current clinical trials involve comparing the efficacy of a drug against that of a placebo in a clinical group such as schizophrenia patients. However, the biological heterogeneity of schizophrenia

patients may dilute the power for statistically significant efficacy across a whole clinical group, even if a subgroup of patients were to benefit. An alternative strategy would be to target a drug to patients for whom there is a biologically driven hypothesis for the drug's efficacy. This may then inform the development of novel treatment options that are specific to the neurobiology that underpins specific domains such as cognitive functioning, or social processing that may apply to patients across a range of disorders, whilst still maintaining their clinically meaningful diagnosis. Similarly, within disorders, differential domain profiles between patients with the same clinical diagnosis may result in different recommendations for appropriate biologically targeted treatment. The RDoC also particularly help to overcome significant barriers in preclinical animal modelling, in which it is not feasible to model complex psychopathologies such as schizophrenia in a rodent. However, specific transdiagnostic domains driven by specific biological substrates such as cognitive dysfunction can be experimentally modelled and investigated. This approach has more direct translational relevance than previous attempts to model a multifaceted and heterogeneous diagnosis.

1.3 Sex differences in psychotic and mood disorders

A considerable factor influencing biological heterogeneity within clinical groups such as schizophrenia, bipolar disorder or depression patients is sex. Numerous sex differences have been observed in the manifestation of a range of psychopathologies. Schizophrenia is more commonly diagnosed in males compared to females, with a male: female ratio of around 1.4:1 (Abel et al., 2010). The average age of onset in females is also typically older than in males. Females also show an increase in rates of schizophrenia onset during middle age and make up the majority of diagnoses at this time point, which may be attributable to the onset of the menopause (Li et al., 2016). The prevalence of bipolar disorder between sexes is generally thought to be equal; however, as with schizophrenia, an earlier age of onset is observed in males compared to females (Menculini et al., 2022). Type II

bipolar disorder, which is characterised by hypomania as opposed to the mania required for a bipolar type I diagnosis, is also more common in females compared to males (Diflorio and Jones, 2010). These findings from bipolar disorder of a higher prevalence in females of a predominantly depressive phenotype are in line with the prevalence rates of depressive disorders, which are also more commonly diagnosed in women compared to men (Eid et al., 2019).

Symptom profile is also known to differ between sexes in psychotic and mood disorders. Differences have been observed in the manifestation of psychotic phenomena between sexes, with auditory hallucinations as well as sexual and persecutory related delusions reported as more common in females compared to males (Falkenburg and Tracy, 2014). In bipolar disorder, prevalence of manic episodes and cases of unipolar mania appear more common in men compared to women (Diflorio and Jones, 2010), and women typically experience more rapid cycling and longer depressive episodes than men (Menculini et al., 2022). In schizophrenia, pre-morbid functioning is also found to be worse in males compared to females, and females show a greater level of affective symptoms compared to males (Giordano et al., 2021). Further sex differences are seen in bipolar type II, where levels of functioning appear to be worse in females compared to males (Diflorio and Jones, 2010). Sex differences are also observed in the symptom presentation of depression. Females typically show greater symptom severity and are also more likely to show somatic and cognitive-affective symptoms (Eid et al., 2019).

Sex differences are also found in treatment response in schizophrenia, bipolar disorder and depression. All anti-psychotic drugs used to treat schizophrenia exert their effects by the same mechanism of action: dopamine D_2 receptor antagonism. Such drugs appear to be effective at lower doses in women than men, but this advantage in women appears to disappear after the menopause, and differences are also seen in antipsychotic efficacy in women across different stages of the menstrual cycle (Seeman, 2020). However, more nuanced investigations into the underlying mechanisms of the neuropharmacology of antipsychotic treatments have not been

undertaken, as preclinical studies often do not include female animals, and drug trials often have insufficient female sample size (Crawford and DeLisi, 2016). Lithium is the primary drug used to treat mania in bipolar disorder and has been shown to be more commonly prescribed to men (Karanti et al., 2015), likely reflecting the higher rates of manic symptoms in bipolar disorder in men compared to women. However, efficacy rates of lithium appear to be similar between sexes (Viguera et al., 2000). Conversely, women are more likely to be treated with electro-convulsive therapy or antidepressants (Karanti et al., 2015). Regarding antidepressants, tricyclic antidepressants appear to show greater treatment response in males compared to females, with the opposite finding observed in selective serotonin reuptake inhibitors (LeGates et al., 2019).

These differences in onset, clinical presentation, and responsivity to treatment are accompanied by disparities between sexes in biological substrates that are associated with mood and psychotic disorders. Alterations in dopaminergic signalling have been one of the most extensively studied aspects of neurobiology in relation to schizophrenia, and D2/D3 receptor functioning has been implicated in schizophrenia, bipolar disorder and depression (Ashok et al., 2017; Howes et al., 2017; Delva and Stanwood, 2021). It has been long known that there are sex differences in dopamine receptor expression and function; however, very little work has attempted to examine this within clinical populations or in preclinical models to assess whether the mechanisms of dopaminergic dysfunction differ between sexes in psychotic and mood disorders (Williams et al., 2021). Other neurotransmitters such as γ-aminobutyric acid (GABA) and glutamate have also been implicated in neuropsychiatric disease (Glausier and Lewis, 2017; Uno and Coyle, 2019). As with the dopaminergic system, there are numerous known sex differences in the mechanisms of glutamate action in the brain, however scant research has been conducted to assess how this relates to clinical populations (Wickens et al., 2018). Post-mortem analysis of GABA gene expression in the anterior cingulate cortex produced sexually dimorphic results: GABAergic gene expression was found to generally be lower in male schizophrenia cases compared to male controls, whereas

female schizophrenia cases showed increased expression of the same genes compared to female controls (Bristow et al., 2015).

One of the most fundamental biological sex differences is the level and cycling of gonadal hormones, and there is increased risk for depression and psychosis in women during times of significant hormonal change such as the post-natal period and the menopause (Vivian-Taylor and Hickey, 2014; Kirkan et al., 2015; Perry et al., 2021; Culbert et al., 2022; Shitomi-Jones et al., 2024). Sex hormones exert both permanent organizational and transient activational effects, with organizational effects during puberty leading to sexually dimorphic activational responses to sex hormones during adulthood (Schulz and Sisk, 2016). During critical organizational periods, epigenetic effects of sex hormones affect histone protein modifications and DNA methylation that persist throughout life (McCarthy and Nugent, 2013). It has been hypothesised that oestrogen may play a protective role against neuropsychiatric disease, thus explaining the lower rates of schizophrenia diagnosis in females compared to males as well as the delayed onset of symptoms in females. This hypothesis is further supported by findings of an inverse association between age of menarche and age of schizophrenia onset (Kiliçaslan et al., 2014).

Oestrogens, testosterone and progesterone are all known to affect brain function (Fester and Rune, 2021), and sexual dimorphism of aspects of brain functioning such as neuronal gene transcription and brain structure are seen during both early development, puberty and the perinatal and perimenopausal periods (Rehbein et al., 2021; Gegenhuber and Tollkuhn, 2022; Gegenhuber et al., 2022). The asymmetrical inheritance of sex chromosomes is also known to shape sex differences in the brain (Davies and Wilkinson, 2006), in a manner that interacts with the effects of gonadal hormones (Arnold, 2017). Thus, owing to the known sex differences in various aspects of neurobiology associated with psychotic and mood disorders, alongside the known sex differences in onset, clinical presentation and treatment response, it is imperative that pre-clinical studies that aim to investigate the neurobiological mechanisms underpinning genetic risk for neuropsychiatric disease incorporate the impact of sex within their designs.

1.4 The role of *CACNA1C* in psychotic and mood disorders

Although several hundred genes have been associated with psychotic and mood disorders using GWAS, there is variation in how robust these associations are. Among several others, *CACNA1C* is one gene that has consistently been shown to be enriched in neuropsychiatric samples across multiple GWAS, and fine-mapping analysis of the most recent schizophrenia PGC GWAS highlighted *CACNA1C* among genes that are likely to play a causal role in the pathophysiology of schizophrenia (Trubetskoy et al., 2022). *CACNA1C* is located on chromosome 12p13.3 and encodes the $Ca_v1.2$ protein of the α1c subunit of L-type voltage gated calcium channels (LTCCs). It was first identified as a risk gene for bipolar disorder (Ferreira et al., 2008), but has since been associated with schizophrenia (Nyegaard et al., 2010), depression (Green et al., 2010) and autism spectrum disorder (ASD) (Li et al., 2015), and is thus a key candidate in explaining transdiagnostic genetic risk in neuropsychiatric disease. A recent bipolar disorder GWAS also showed a significant enrichment of risk variants in genes encoding targets of calcium channel blockers, providing evidence for the potential of targeting calcium signalling in the development of novel therapeutic agents (Mullins et al., 2021).

The most frequently studied risk allele of *CACNA1C* is rs1006737, located on intron 3 of the gene (Zhu et al., 2019b), and it was the first variant identified in initial GWAS (Ferreira et al., 2008; Nyegaard et al., 2010). Its association with schizophrenia, bipolar disorder and depression has subsequently been replicated numerous times (Sklar et al., 2008; Casamassima et al., 2010; Green et al., 2010, 2013; Nyegaard et al., 2010; Lett et al., 2011; Liu et al., 2011, 2020; Hori et al., 2012; Wray et al., 2012; Gonzalez et al., 2013; Guan et al., 2014; He et al., 2014; Ivorra et al., 2014; Ruderfer et al., 2014; Zheng et al., 2014; Moon et al., 2018). Several other *CACNA1C* risk SNPs have since been identified: rs4765905 (schizophrenia and ASD), rs4765913 (bipolar disorder, schizophrenia and depression), rs1024582 (bipolar disorder, schizophrenia, ASD and attention-deficit hyperactivity disorder (ADHD)), rs2007044 (schizophrenia), rs7297582 (bipolar disorder and depression), rs12898315 (schizophrenia), and rs10744560 (bipolar disorder) (Liu et al., 2011, 2020; Hamshere

et al., 2013; Psychiatric Genetics Cross Disorder Consortium, 2013; Mühleisen et al., 2014; Schizophrenia Working Group of the Psychiatric Genomics Consortium, 2014; Li et al., 2015; Takahashi et al., 2015; Moon et al., 2018; Pardiñas et al., 2018). However, numerous of these risk SNPs are in known LD with one-another (Moon et al., 2018; Wang et al., 2022), indicating they may not all be causal in their effects. All identified *CACNA1C* risk variants are in intronic regions of the gene and hence do not alter the base pair sequence of the transcribed protein, rather they may have a functional impact on the level of gene expression.

Numerous studies have attempted to assess the directionality of the effects of *CACNA1C* risk SNPs on subsequent gene expression and have observed mixed findings. Analysis of post-mortem and foetal tissue, as well as cell lines have suggested *CACNA1C* risk SNPs result in increased expression of the gene (Bigos et al., 2010; Yoshimizu et al., 2015). However, other analyses have found opposing effects, in which rs1006737 has been associated with reduced *CACNA1C* expression in the cerebellum (Gershon et al., 2014), and superior temporal gyrus (Eckart et al., 2016). Eckart et al. (2016) also failed to replicate the findings of Bigos et al. (2010), in that they did not show any changes between rs1006737 genotypes in *CACNA1C* expression in the DLPFC. When comparing schizophrenia patients and controls, *CACNA1C* gene expression has also been shown to be reduced in the temporal cortex, but with no significant changes in the prefrontal cortex, nucleus caudatus and cerebellar vermis (Schmitt et al., 2022). Analysis of expression quantitative trait loci (eQTL) across a range of brain areas has shown increased *CACNA1C* expression observed in the cerebellar hemisphere, cerebellum, putamen and substantia nigra, with concordant decreased expression in the cerebellar cortex (Wang et al., 2022). A recent transcriptome-wide association study of the DG subregion of the hippocampus also showed decreased expression in this region in schizophrenia patients compared with controls (Jaffe et al., 2020). Taken together, the findings regarding the direction of effects of *CACNA1C* risk alleles are complex and show conflicting evidence. However, they do suggest that expression changes may be regionally specific, and that deviations from an optimal level in either direction may confer adverse effects. It is therefore crucial to understand the effects

of altered *CACNA1C* gene expression on downstream neurobiology and associated cognitive processes relevant to neuropsychiatric disease.

1.5 L-type voltage gated calcium channel mediated Ca^{2+} signalling in psychotic and mood disorders

Although the mechanisms mediating the development of psychotic and mood disorders have not been fully elucidated, aberrant calcium signalling and its effects on neuronal circuitry have been implicated in such disorders (Berridge, 2014). Ca_V1 channels are involved in post-synaptic plasticity processes, and long-term potentiation (LTP) models have implicated their involvement. LTP is an electrophysiological model of learning and memory whereby high-frequency afferent stimulation leads to long-term increases in synaptic strength. Upon stimulation of a post-synaptic neuron, glutamate enters the post-synaptic neuron via AMPA receptors. However, with sufficient stimulation, depolarisation of the post-synaptic neuron results in the removal of the Mg^{2+} ion that blocks NMDA receptors (NMDARs) under baseline conditions, thus allowing Ca^{2+} ions to enter the cell via NMDARs. This influx of Ca^{2+} facilitates additional expression of AMPA receptors on the neuronal membrane, thus allowing glutamate to enter the post-synaptic neuron more readily upon subsequent stimulation and increases the sensitivity of the post-synaptic neuron to glutamate (Miyamoto, 2006).

Associative LTP is a highly related process whereby a weak afferent signal, that in itself fails to induce LTP, occurring simultaneously with a strong depolarising input results in the strengthening of the synapse of the weak afferent signal. This process has been most widely studied in the CA1 region of the hippocampus and Ca^{2+} has been shown to enter the post-synaptic neuron through both NMDA and LTCC channels, with different stimulation frequencies differentially activating the two channels. Specifically, when a weak afferent signal is paired with a strong spike-eliciting depolarising input, LTCCs seem to mediate calcium entry, whereas when

depolarisation is prolonged but not spiked, NMDARs facilitate calcium entry. However, serial NMDAR and LTCC activation has also been shown to occur, whereby glutamate-mediated depolarisation induces LTCC-dependent influx of Ca^{2+} (Ma et al., 2023). In turn, this LTCC-dependent calcium influx triggers neuronal excitation-transcription coupling (E-TC), whereby the depolarisation of the neuronal membrane triggers gene transcription, and LTCCs are known to be crucial to this process (Vierra et al., 2021; Ma et al., 2023).

A complex signalling cascade within the cell mediates E-TC. Many hundreds of genes and tens of transcription factors have been shown to be regulated by Ca^{2+} influx into a neuron (Dolmetsch, 2003). Cyclic adenosine 3′,5′-monophosphate-responsive element binding protein (CREB) is one of the most widely studied transcription factors in this process and is known to play a key role in the transcription of gene products that facilitate long-term synaptic plasticity (Benito and Barco, 2010). LTCC-mediated calcium influx is known to activate a complex set of downstream messengers such as calcium/calmodulin-dependent protein kinase II (CaMKII), and mitogen-activated protein kinase (MAPK), which in turn activate CREB within the nucleus, resulting in downstream gene expression which in turn facilitates the protein synthesis required for long-term structural plasticity processes at the synapse (Wang and Peng, 2016). Hence, LTCCs are crucial for many of the associative plasticity processes that are involved in associative learning and memory processing (Moosmang et al., 2005; Qian et al., 2017). Studying the role of *CACNA1C* encoded Cav1.2 in plasticity and long-term memory is of particular promise for treatment as the associative learning and memory processes that would be implicated by altered calcium channel signalling are the very processes which are disrupted in neuropsychiatric disorders (Stone and Hsi, 2011).

1.6 Rodent models of *CACNA1C* risk variants

Numerous rodent models have been constructed with the aim of elucidating the impact of alterations in *Cacna1c* gene dosage and subsequent $Ca_v1.2$ protein levels on various aspects of cognition and neurobiology. Global knockout of *Cacna1c* is embryologically lethal, owing to the involvement of LTCCs in cardiac function (Moon et al., 2018). However, several models of *Cacna1c* knockout specific to regions such as the forebrain, CNS, or to solely neurons have been constructed. Global heterozygote models, with one intact and one deleted copy of *Cacna1c*, are not embryologically lethal and have also been used to investigate the effects of low *Cacna1c* gene dosage. Such models have been comprehensively reviewed by Moon et al. (2018). Briefly, models have shown reduced motor function (Dao et al., 2010; Bader et al., 2011; Kabitzke et al., 2018) and increased anxious and depressive phenotypes (Dao et al., 2010; Bader et al., 2011; Lee et al., 2012; Bavley et al., 2017; Dedic et al., 2018). There has also been evidence to support alterations in cognition and fear conditioning (Moosmang et al., 2005; White et al., 2008; Bader et al., 2011; Temme et al., 2016), however with inconsistent evidence (Langwieser et al., 2010). Several studies also showed evidence for impaired hippocampal neurogenesis in *Cacna1c* rodent models, with reduced rates of cell proliferation and fewer immature neurons observed (Lee et al., 2012; Temme et al., 2016; Moon et al., 2018).

In the time since the review by Moon et al. (2018), numerous studies have provided further detail as to the effects of altered *Cacna1c* gene dosage on cognitive functioning and underlying neurobiological processes. Various cognitive testing paradigms have been implemented with a broad range of results. Subtle deficits in spatial memory have been observed (Braun et al., 2018), with environmental enrichment also having been shown to interact with genotype to affect spatial memory (Braun et al., 2019). Similarly, impairments in reversal learning have been demonstrated (Sykes et al., 2019). Numerous deficits in social cognition and ultrasonic vocalizations have also been observed (Kabitzke et al., 2018; Kisko et al., 2018, 2020; Redecker et al., 2019; Wöhr et al., 2020). In line with earlier findings

detailed in Moon et al. (2018), more recent evidence for hypoactivity in *Cacna1c* heterozygotes has been produced (Kabitzke et al., 2018). However, the opposite finding of increased activity in *Cacna1c* heterozygotes has been found by others (Dedic et al., 2018).

Increased anxiety and depressive-like phenotypes have also been observed in *Cacna1c* rodent models (Dedic et al., 2018; Ehlinger and Commons, 2019), as well as impairments in associative fear learning. Exacerbated contextual fear memory that is also resistant to extinction has been observed in *Cacna1c* heterozygote mice, as well as in male mice (females not assessed) with *Cacna1c* heterozygosity specific to dopamine D1 receptor expressing neurons (Bavley et al., 2021). Moon et al. (2020) showed specifically increased fear responses to irrelevant predictors of an aversive outcome, specifically the conditioning context in delay conditioning and to the cue in trace conditioning. Similar impairments of heightened fear response to irrelevant predictors have been observed in latent inhibition (LI) experiments, in which *Cacna1c*$^{+/-}$ animals do not demonstrate LI of CFC. This deficit was found to be associated with impaired hippocampal synaptic plasticity and network oscillations and was subsequently rescued by administration of an ERK pathway agonist (Tigaret et al., 2021).

Further neurobiological underpinnings of the cognitive deficits found in *Cacna1c* rodent models have been revealed, implicating numerous molecular processes. Synaptic plasticity deficits were also found by Dedic et al. (2018), but impairments were only seen in this study when *Cacna1c* was deleted in the forebrain during embryonic development, not during adulthood, highlighting a critical window for the impact of altered *Cacna1c* gene dosage during early development. Serotonin pathways have also been implicated, as Ehlinger and Commons (2019) found that their depressive phenotype was induced by a *Cacna1c* deletion specific to serotonergic neurons, and the phenotype was rescued by administration of a 5-HT1A receptor agonist. *Cacna1c* deletion during embryonic development targeting neurons destined for the cerebral cortex also induces an anxious phenotype, with concurrent volumetric changes in a wide network of brain regions including the

hippocampus (Smedler et al., 2022). Taken together the body of literature pertaining to modelling the effects of *CACNA1C* risk variants in rodents reveals a range of implicated cognitive and associated cellular and molecular processes. However, a considerable proportion of the evidence is suggestive of deficits in hippocampal-dependent associative memory processes.

1.7 The role of the hippocampus in cognitive processing underlying psychotic and mood disorders

The hippocampus is an allocortical brain area with a critical role in major forms of long-term memory including contextual and spatial memory, as well as relational memories including episodic and declarative types, and it has been extensively implicated in the pathophysiology of schizophrenia, bipolar disorder and depression (Chepenik et al., 2012; Otten and Meeter, 2015; Xu et al., 2020; Tartt et al., 2022; Wegrzyn et al., 2022). Furthermore, as noted earlier, GSEA of SNPs significantly associated with schizophrenia in the most recent PGC GWAS showed an enrichment of risk variants in genes that are highly expressed in pyramidal cells of the CA1 and CA3 regions, and granule cells of the DG region of the hippocampus (Trubetskoy et al., 2022). Many forms of associative memory also rely upon the hippocampus, and such processes allow organisms to use prior experiences to predict future events and direct behaviour in an adaptive manner and is fundamental to our construction of reality (Granger et al., 2019; Biane et al., 2023). Thus, in order to improve our understanding of neuropsychiatric disorders, it is important to elucidate the mediating hippocampal neurobiological pathways whereby risk SNPs such as *CACNA1C* confer deficits in cognition.

One of the fundamental aspects of conscious experience of present and past events is the perception of contexts and events as singular stable constructs. One of the primary functions of the hippocampus is the formation of such cognitive constructs, whereby disparate spatial and temporal signals are integrated (Eichenbaum, 2017).

For past experiences to guide future behaviour, it is important that affective information is integrated into contextual associative memories, and the hippocampus as well as connectivity between the hippocampus and the amygdala and nucleus accumbens has been robustly implicated in the formation of contextual fear memories (Fanselow, 2000; Kim and Cho, 2020).

The hippocampus can be divided into two broad regions: the DG and the 'hippocampus proper' which includes the *cornu Ammonis* areas: CA1, CA2 and CA3, and it can be further broadly divided into dorsal and ventral regions. There are numerous known differences in functioning between hippocampal subregions as well as interconnectivity between each region orchestrating the complex memory processes that depend upon the hippocampus. The CA1 region has been particularly noted for its involvement in associative learning, with dorsal and ventral areas showing differential involvement across the phases of associative learning (Biane et al., 2023). The CA1 is particularly well interlinked with the CA3 and DG to form the 'trisynaptic loop' (Hainmueller and Bartos, 2020). The CA3 is thought to enable the rapid formation of associations between spatial locations, objects and rewards, and also to provide the input that evokes the recall of complete contextual memories (Rolls, 2018). The CA3 is well connected to the DG, which performs a pattern separation function during learning to ensure that separate associations generated by the CA3 are represented by distinct populations of neurons. Subsequently, during recall, the CA3 facilitates pattern completion, whereby discrete cues trigger the retrieval of previously stored activation patterns (Rolls, 2018; Hainmueller and Bartos, 2020).

Neuronal oscillations across the trisynaptic loop of the CA1, CA3 and DG have also been shown to be crucial to associative memory (Trimper et al., 2017). Coordinated firing patterns of both theta and gamma wavelengths allow for the effective encoding of novel associations using LTP (Hasselmo et al., 2002; Bikbaev, 2008). Furthermore, coordination of firing patterns between the hippocampus and entorhinal cortex at 20-40Hz has been shown to develop in unique ensembles of neurons for distinct learning events over the course of the phases of associative learning

(Igarashi et al., 2014). Remapping and realignment of cell ensembles within the hippocampal-entorhinal formation has been shown to guide context dependent associative behaviour (Julian and Doeller, 2021). Such hippocampal associative learning mechanisms have been shown to be directly dependent upon synaptic plasticity processes, which in turn depend upon LTCC-mediated Ca^{2+} influx and downstream cell signalling cascades (Milstein et al., 2021; Yasuda et al., 2022). The dorsal region of the hippocampus is thought to have a predominant function in memory and spatial cognition, whereas the ventral region is thought to be more related to emotional cognition, anxiety and the formation of fear associations, and has greater projections to the amygdala and hypothalamus (Hong and Kaang, 2022). The hippocampus also plays an important negative-feedback role in the homeostatic regulation of the hypothalamic-pituitary-adrenal (HPA) axis, and differences are seen in this functioning along the dorsal-ventral axis. Corticosterone binds to glucocorticoid (GR) and mineralocorticoid receptors (MR) within the hippocampus to trigger the suppression of further downstream corticosterone release, and the ventral hippocampus appears to show a greater involvement in this process (Fanselow and Dong, 2010). Transcriptional and epigenetic changes in response to stress have also been shown to be greater within the ventral compared with dorsal hippocampus (Floriou-Servou et al., 2018). Alterations in HPA-axis functioning have been shown to be involved in numerous psychotic and mood disorders, and early experiences of stress both pre- and postnatally are known to increase risk for neuropsychiatric disorder development (Mikulska et al., 2021). Hence, it is important to elucidate the complex roles of specific hippocampal subregions and the interactions between their functions in associative learning and the HPA-axis in the development of neuropsychiatric disorders.

Associative learning and the mediating synaptic plasticity processes within the hippocampus are also significantly affected by sex. Synaptic plasticity processes such as LTP and synaptic pruning within the hippocampus, as well as learning and memory processes subserved by synaptic plasticity, are known to be influenced by oestradiol and testosterone (Rossetti et al., 2016; Fester and Rune, 2021). Such synaptic plasticity processes that involve structural alterations at the synapse rely

upon RNA transcription, and such gene expression within the hippocampus is known to be modulated by oestradiol (Iqbal et al., 2020). Furthermore, the induction threshold for CA1-LTP is lower in females compared to males prior to puberty, but the opposite effect is true in adulthood (Gall et al., 2024). Sex differences have also been observed in the effects of *Cacna1c* haploinsufficiency on cognition, within the context of animal models of age-related cognitive decline (Zanos et al., 2015). In light of the previously discussed sex differences in the onset, progress and treatment response to psychotic and mood disorders, it is thus crucial that the effect of sex is explored when investigating the effect of aberrant calcium signalling caused by common risk variants in hippocampal-dependent associative learning alongside associated neurobiological substrates.

1.8 Modelling associative learning mechanisms relevant to neuropsychiatric disease

Deficits in associative learning processes have been repeatedly observed in neuropsychiatric disorders. Pavlovian conditioning is the process whereby the pairing of a stimulus that elicits a behavioural response (unconditioned stimulus (US)) with a neutral stimulus that does not elicit a response (conditioned stimulus (CS)) results in the presentation of the CS alone eliciting the response of the US. Acquisition of a conditioned fear response (whereby an aversive US is paired with a neutral CS) has been shown to be impaired in individuals with schizophrenia (Tuominen et al., 2022), as well as deficits in other simple associative learning tasks (Gao et al., 2023). Such CS-US associations may be extinguished when the CS is repeatedly presented without reinforcement of the US, and such extinction learning is an adaptive process that supports behavioural responding to salient stimuli that best predict an outcome. However, the recall of such extinction learning has also been shown to be impaired in schizophrenia patients (Holt et al., 2009), as well as individuals with depression (Tani et al., 2019). Associative learning deficits have also been observed in bipolar disorder (Tournikioti et al., 2017), with performance shown

to be similar to that of individuals with schizophrenia (Brambilla et al., 2011). Furthermore, sex differences in performance on associative learning in schizophrenia patients have been demonstrated, with males showing superior performance compared with females (Charaf et al., 2024). However, in bipolar disorder, when males have outperformed females in a control group, sex differences are attenuated in the patient group (Tournikioti et al., 2018), perhaps indicating a heightened deficit in males.

An associative learning process that has been extensively studied in schizophrenia is latent inhibition (LI) (Myles et al., 2023). LI is the phenomenon whereby pre-exposure to a CS reduces the degree to which it is subsequently entered into an association with a US (Weiner and Feldon, 1997). LI is an adaptive process, as a CS that has previously been presented without co-occurring with a US prior to conditioning is subsequently a poorer predictor of the US compared with a CS that has always co-occurred with the US. However, deficits in this process have been demonstrated in schizophrenia patients (Granger et al., 2019; Myles et al., 2023). Indeed, hemizygous deletion of *Cacna1c* in a rodent model has been shown to result in deficits in LI and accompanying dysfunction in hippocampal synaptic plasticity processes (Tigaret et al., 2021). A similar but distinct process known as blocking occurs when a learning of a CS-US relationship is inhibited by the CS always co-occurring with another CS for which there is an already learned relationship with the US. As with LI, disruptions in this process have also been observed in schizophrenia patients (Jones et al., 1997; Moran et al., 2003; Morris et al., 2013; Granger et al., 2019).

Fear conditioning paradigms have been extensively used in animal model investigations of cognitive processing and associated neurobiological processes related to psychiatric disorders (Namkung et al., 2022), as such processes are highly evolutionarily conserved (Pattwell et al., 2012). Psychotic phenomena also often bear a particularly negative valence and often elicit fear (Gauntlett-Gilbert and Kuipers, 2005). Hence, inferences drawn from such paradigms are likely to be translationally relevant and fear conditioning models allow us to study the acute

threat construct represented under the RDoC negative valence dimension (Sumner et al., 2016), as well as investigating the cognitive dimension (Sanislow et al., 2010). In cued fear conditioning, an aversive US, usually a foot-shock, is associated with and a neutral CS, such as a tone or light, such that the CS then elicits a behavioural and biological fear response owing to its predictive capacity of the US. However, in such an experiment, the animal will also form an association between the training environment, or context, and the US. However, various factors such as pre-exposure to the context and the degree of CS-US contingency will affect the strength of CS-US and context-US associations. A simpler well established paradigm is contextual fear conditioning (CFC), in which the animal is entered into a context, and after a period that has allowed for the formation of a cognitive representation, the US is administered. Upon re-entering the context at a later time, the animal will display fearful behaviour such as freezing, if it has formed an association between the context and the aversive US (Curzon et al., 2009). Owing to the contextual nature of the association, CFC is hippocampally dependent (Kim and Cho, 2020), whereas cued fear conditioning is not (Marschner et al., 2008).

Furthermore, the impact of altered expression of specific risk loci, such as *CACNA1C* can be studied using rodent models, owing to the ability to generate genetically modified lines. Thus, using such models, fine-grained aspects of cellular and molecular mechanisms that may mediate the association between specific genetic risk loci and cognitive deficits observed in patient groups may be better elucidated.

1.9 Aims

There is a known role of common risk SNPs of the *CACNA1C* gene in the genetic risk for psychotic and mood disorders, which bear well established sex differences. Furthermore, the importance of LTCCs in hippocampal-dependent cognitive functioning is also well understood, and aspects of such functioning are also known

to be sexually dimorphic. Hence, the aim of this thesis is to investigate the impact of both sex and *Cacna1c* gene dosage on hippocampal-dependent contextual fear learning and associated gene expression. It is hypothesised that both sex and *Cacna1c* heterozygosity will both independently affect hippocampally dependent associative learning and memory processes, and that sex may interact with *Cacna1c* low gene dosage to elicit differential effects of *Cacna1c* genotype between sexes. It is hoped that the findings from this research may improve understanding of the neurobiological basis of the cross-disorder cognitive dimension that underpins psychotic and mood disorders.

- Aim 1: Further investigate the effect of low *Cacna1c* gene dosage on associative learning performance in both contextual fear memory generalization (Chapter 3) and LI (Chapter 4).

- Aim 2: Further investigate the effect of low *Cacna1c* gene dosage on HPA-axis activation in response to associative contextual fear memory tasks (Chapters 3 and 4).

- Aim 3: Assess the impact of low *Cacna1c* gene dosage on hippocampal gene expression and identify implicated downstream neurobiological pathways (Chapter 7).

- Aim 4: Assess the extent of the current body of knowledge relating to sex differences in LI in both human schizophrenia and schizotypy, and rodent model studies, to ascertain whether *CACNA1C* may be affecting sexually dimorphic cognitive and neurobiological processes (Chapter 5).

- Aim 5: Investigate sex differences in performance of LI of CFC as well as associated immediate early gene (IEG) expression in wild-type animals in a range of brain areas implicated in LI including the hippocampus (Chapter 6).

- Aim 6: Investigate whether the effects of low *Cacna1c* dosage differ between sexes on behavioural performance in associative learning and memory tasks and associated HPA activation (Chapters 3 and 4), as well as on hippocampal gene expression both at baseline and after behaviourally induced hippocampal activation (Chapter 7).

Chapter 2: General Methods

2.1 *Cacna1c*$^{+/-}$ rat model

The *Cacna1c*$^{+/-}$ rat model (strain SD-*Cacna1c*^{tm1Sage}, Sage Research Labs, Pennsylvania, USA) was generated from cryo-preserved embryos which were then maintained and bred at Cardiff University. This line of animals has a Sprague-Dawley background strain and heterozygote (*Cacna1c*$^{+/-}$) animals bear a truncating mutation in exon 6 of the *Cacna1c* gene located on chromosome 4.

The line was maintained by breeding non-sibling wild-type (*Cacna1c*$^{+/+}$) females with *Cacna1c*$^{+/-}$ males which produced roughly even numbers of *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ offspring. This combination of parental genotypes was consistently used to avoid any effects of *Cacna1c* heterozygosity on maternal behaviour, as genetic influences on maternal behaviour are known to influence behaviour in offspring (Tarantino et al., 2011). To avoid effects of inbreeding, the colony was refreshed at least once per year by using wild-type Sprague-Dawley female animals from Charles River UK for breeding rather than *Cacna1c*$^{+/+}$ females from the colony.

Pups were sexed and weaned at 28 days old. Sex was determined by assessment of anogenital distance and rats were housed in same-sex groups of 2-3 (males) or 2-4 (females) per cage. Ear notches were taken for purposes of identification of animals within each cage, and this tissue was also used for genotyping (Transnetyx Inc., USA).

All animals were housed 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 reverse light-dark schedule (light phase 8pm-10am). Cages were lined with wood shavings, a cardboard tube and wooden

stick as basic enrichment. Humidity was maintained at 45-60% and temperature between 19-21°C.

All procedures were conducted in accordance with local Cardiff University Ethical Committee approval and the UK Animals (Scientific Procedures) Act 1986, under UK Home office licenses PPL P0EA855DA and PIL I63097538.

2.2 Behavioural Methods

2.2.1 Pre-experiment handling and weighing

Twenty-four hours prior to the onset of each behavioural experiment, rats were weighed and handled for approximately two minutes or until they were calm to acclimatise the animal to the experimenter. All female animals weighed between 150-300g, and all male animals weighed between 250-600g prior to the onset of each experiment.

2.2.2 Apparatus for Behavioural Testing

Two identical standard modular conditioning chambers were used for each behavioural testing session (internal dimensions: 30.5 cm x 24.1 cm x 21.0 cm) placed inside sound attenuating chambers (55.9 cm x 55.9 cm x 35.6 cm; Med Associates Inc., Vermont, USA). In each experiment, counterbalancing was conducted to ensure an even number of genotypes, behavioural conditions and sexes were assigned to each of the two boxes. The interior of each box had transparent Perspex walls with an infrared camera (JSP Electronics Ltd, China) placed centrally above the box to record the movements of rats. Boxes were cleaned

with 50% ethanol solution before and after each testing session and between each rat. The programmes for each session were controlled through a Med-PC version IV research control and data acquisition system (Med Associates Inc., Vermont, USA). Footage of each behavioural session was recorded using Numeroscope software (Viewpoint, France).

In order to transport animals to the conditioning chamber for each session, rats (singles or pairs) were transported to the minimal light experimental procedure room and placed individually into a conditioning chamber. The experimenter initiated the conditioning session and then left the room. Lights on and off in the conditioning chamber signalled the start and end of the sessions respectively. Rats were immediately placed in the matched transport box and returned to their home cages.

For context discrimination tests, contexts were differentiated by both visual and scent cues: one context featured a black and white checkerboard pattern on the walls as well as a layer of sawdust beneath the grated floor; the other featured a black and white spotted pattern on the walls as well as a lavender scented square of wet tissue paper beneath the grated floor. Each context was assigned to either an AM or PM session in a counterbalanced manner, to aid in distinguishing the two contexts from one another.

Males and females were of the same age range and originated from the same litters for each experiment. The testing of males and females was alternated within each day, to control for any variation between testing days. Conditioning chambers were cleaned between each testing session with 50% ethanol to reduce any influence of the previous animal, which may have been of the opposite sex.

2.2.3 Contextual Fear Conditioning

In each CFC session (Figure 2.1), the Med-PC programme was set so that the lights turned on in the conditioning chamber and after two-minutes (pre-US) a two-second 0.64mA scrambled foot-shock was administered through the metal floor bars. The light remained on for a further minute (post-US), then turned off at the end of the session. When returning animals to their home cage and upon daily inspections, they were monitored for adverse reactions to CFC, and no such adverse reactions were noted in animals included in this thesis.

In some experiments, rats were exposed to the context without conditioning prior to CFC (pre-exposure (PE) sessions). Specific PE durations are detailed in the relevant experimental chapters. In one experiment, rats also underwent extinction training 24 hours after CFC, in which they were entered into the conditioning chamber for 10-minutes, and did not receive a foot-shock. In most experiments, rats underwent a 2-minute recall session either 24 hours after CFC, or 24 hours after extinction training in which they were entered into the conditioning chamber and did not receive a foot shock.

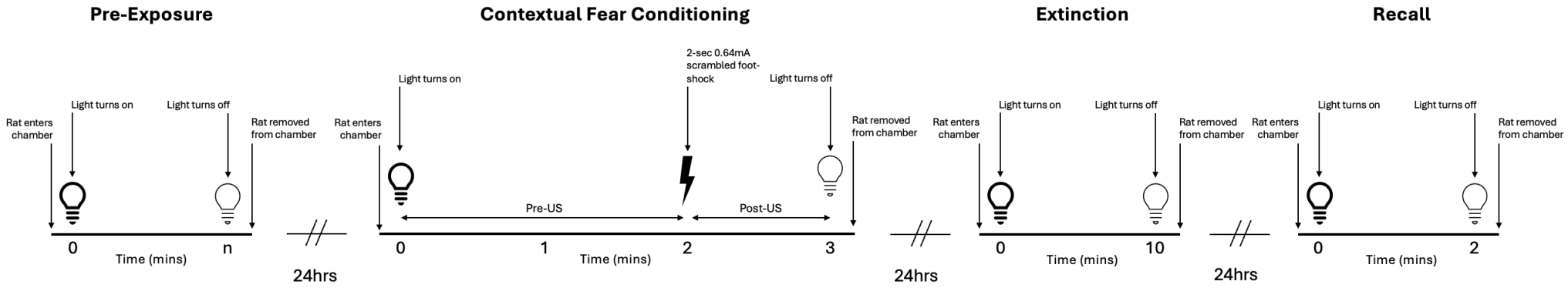

Figure 2.1. The CFC protocol with optional pre-exposure (repeated across two or three consecutive days), and extinction sessions. All CFC experiments were also followed by a 2-minute recall session either 24 hours after CFC, or 24 hours after extinction training. ‘n’ indicates a variable duration of PE session length which is specified in each relevant experimental chapter.

2.2.4 Measurement of Freezing Behaviour

The percentage duration of each pre-US, post-US, extinction, and recall session that animals spent exhibiting freezing behaviour was measured and used as a proxy for fear (Curzon et al., 2009). Pre-US freezing scores were calculated based on the 2-minute period animals spent in the box prior to the onset of the foot-shock during the CFC session and post-US scores were calculated based on the 1-minute animals remained in the box after the foot-shock in the CFC session. A single freezing score was calculated for each 2-minute recall session. The 10-minute extinction sessions were divided into five 2-minute epochs and a score for each epoch was calculated.

The video for each scoring period was split into 5-second intervals and every five seconds it was determined whether the rat was displaying freezing behaviour using a binary response (1 = freezing, 0 = not freezing). Freezing was determined if the rat was stationary and showed no movement other than breathing (Curzon et al., 2009). This was then used to generate a percentage time spent freezing score for each interval by averaging the scores across each interval and multiplying by 100. Scoring was conducted blind to genotype and whether recall session is taking place in the CFC or neutral context. This was achieved by renaming the video files to not include the ID of the rat, and video files were then renamed back to identify the rat after behaviour had been scored. Owing to the size difference between male and female rats, it was not possible to score behaviour blind to sex. All videos were scored by the experimenter on two separate days to assess intra-rater reliability and Pearson's correlation was used to assess the consistency between the two scores and was found to be above 90% for every experiment.

2.2.5 Measurement of Quadrant Crossings

There are known differences in the behavioural expression of fear between sexes, in that freezing is possibly more readily expressed in males compared to females, and females may use alternative behavioural strategies when in a fearful state such as displaying escape or 'darting' behaviours (Gruene et al., 2015; Bauer, 2023). Darting, characterised by a rapid movement across the conditioning chamber (Gruene et al., 2015), was not observed in any animals, in either wild-type Sprague-Dawley animals (Charles River, UK) or animals of either genotype of the *Cacna1c* line bred in house. Hence, darting was not used as an alternative behavioural measure. Changes in movement other than freezing and darting are thought to occur in response to threat and can reflect attempts to escape to a safer environment (de Oca et al., 2007). Thus, quadrant crossings were measured alongside freezing for each session as a proxy for changes in general locomotor activity. A cross with a horizontal and vertical line meeting in the centre of the conditioning chamber was superimposed onto each video using Red Line Tools software (Apple, USA) and the number of crossings made by each rat over the course of each scoring period was counted to generate a score of crossings per minute. A rat was considered to have crossed a quadrant if approximately more than 50% of its body had moved across the line. If a rat moved more than 50% of its body over a line and then moved back to the original quadrant, but part of its body had never left the original quadrant, this was counted as one crossing rather than two. Thus, for each scoring period, each animal had both a time spent freezing (%) and quadrant crossings/minute score. Scoring was conducted blind and intra-rater reliability was assessed using the methods described for freezing behaviour. Intra-rater reliability was found to be above 90% for every experiment.

2.3 Euthanasia

Where blood plasma and/or brain tissue was required for subsequent analysis, euthanasia and brain extraction was carried out using the same method in each experiment. Animals remained in their home cage and were transferred from the home cage room to the CO_2 apparatus in a separate room. A rising concentration of CO_2 was administered over the course of eight minutes, with a slower rate administered during the first four minutes to facilitate unconsciousness, then an increased rate in the latter four minutes to facilitate death. Death was confirmed by dislocation of the cervical spine.

2.4 Tissue Preparation

2.4.1 Tissue Extraction

The maximum available trunk blood was extracted and stored in heparin coated blood collection tubes (Fisher Scientific, UK) which were kept on wet ice for short-term storage. Brains were extracted and flash frozen by submersion for approximately 10 seconds in a bath of isopentane which was surrounded by dry ice. Once fully frozen (colour had completely changed from pink to grey), brains were covered with parafilm and tin foil and transferred to dry ice for short-term storage. Blood samples were immediately processed (see 2.5.1.) and brains were transferred to a -75°C freezer for long-term storage.

2.4.2 Brain Dissection

Isolation of specific brain regions was achieved using micro punches of frozen brain tissue. Brains were taken from -75°C storage and kept on dry ice. A thermal block was set on a bed of dry ice to facilitate dissection at sub-zero temperatures. Glass microscope slides (5cm x 7.6cm; Merck, UK) and a rat coronal brain matrix (Rat 175-300 g, RBMS-300C, 1mm steel, World Precision Instruments, Hertfordshire, UK) were set on the thermal block and were allowed to reduce in temperature. Brains were then placed in the matrix and feathered razor blades were placed into the brain matrix at 2 mm intervals (Figure 2.2).

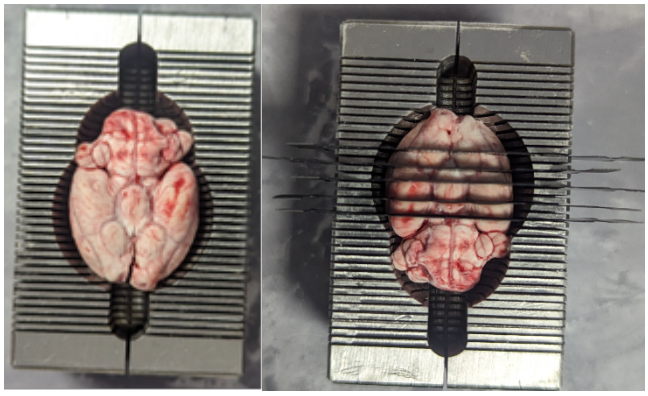

Figure 2.2. Left: Rat brain in Rodent Coronal Brain Matrix (Rat 175-300 g, RBMS-300C, 1mm steel, World Precision Instruments, Hertfordshire, UK). Right: Razor blades placed into the brain matrix to produce 2mm slices.

Coronal slices were then removed from the brain matrix and placed onto the microscope slides. A rat brain atlas (Paxinos and Watson, 2007) was used to identify required brain regions. A precision brain punch 1.5 (±5%) mm Æ (Ted Pella Inc., California, USA) was used to take tissue punches of each region (Figure 2.3). Punches were taken from both left and right hemispheres of each slice. The number of tissue punches taken was dictated by the number of slices in which the required region could be identified and varied between 2-4 tissue punches per region. Tissue punches were then separately submerged for each brain and region in RNAlater (Fisher Scientific, UK) in separate 1.5ml Eppendorf tubes and kept temporarily on dry ice before being moved to -75°C storage.

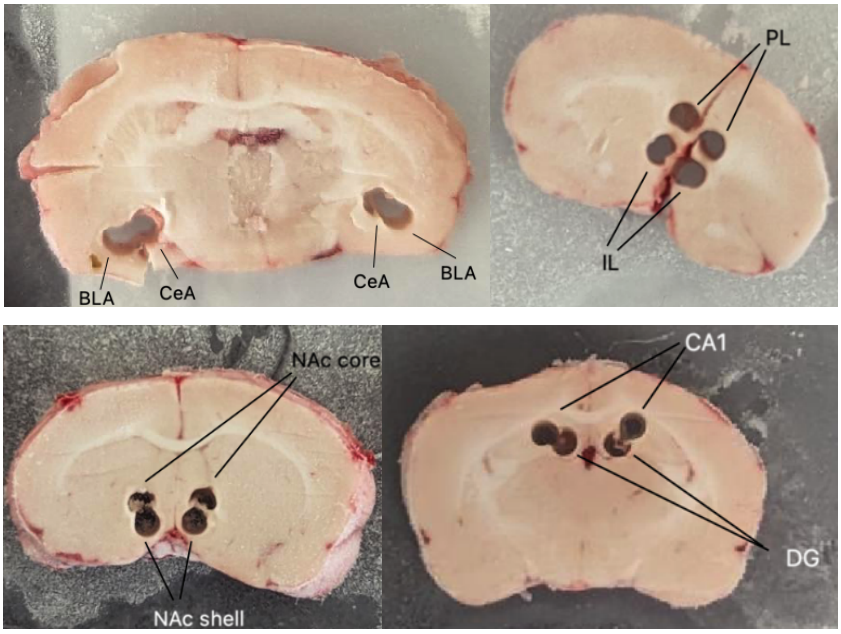

Figure 2.3. Representative neuroanatomical locations of 1.5mm diameter tissue punches. BLA = basolateral amygdala; CeA = central amygdala; PL = prelimbic prefrontal cortex; IL = infralimbic prefrontal cortex; NAc = nucleus accumbens; CA1 = Cornu Ammonis area 1 (CA1) of the hippocampus; DG = dentate gyrus of the hippocampus.

2.5 Plasma Corticosterone Measures

2.5.1 Plasma Extraction

Blood samples were centrifuged at 4000rpm for 10-minutes at 4°C to separate constituent components of blood. Blood plasma was pipetted out from the heparin-coated blood collection tubes and transferred to 1.5ml Eppendorf tubes (Fisher Scientific, UK). The remaining blood components and blood collection tubes were appropriately discarded, and the plasma samples were labelled and transferred to a -75°C freezer for long-term storage.

2.5.2 Quantification of Plasma Corticosterone Concentration

A corticosterone ELISA kit (Enzo Life Sciences, UK) was used to measure corticosterone concentration in blood plasma following the manufacturer's instructions. Milli-Q deionised water (Merck Life Science, UK) was used when water was specified. 1.25ml of Steroid Displacement Reagent (provided in kit) was added to 48.75ml of each plasma sample to free corticosterone from corticosteroid binding globulin. This was then diluted in 450ml of the assay buffer included in the kit to create working solutions of samples. At the end of the assay procedure, the plate was read at 405nm using a microplate reader (CLARIOstar Plus, BMG Labtech, UK). Non-steroid binding wells were used as a negative control and blank wells were used as a positive control to ascertain proper functioning of the ELISA kit. Total activity wells were used to verify proper functioning of the ELISA antibody. ELISA kit functioning was conducted by qualitative analyses of the colour change of the three controls.

Fluorescence values of the corticosterone ELISA plate read from the microplate reader were used to calculate concentration of corticosterone in blood plasma samples. The values of the two blank wells were averaged and subtracted from all other plate readings. Means of the triplicate measures of fluorescence values of standards and samples were calculated. A 4-parameter logistic regression equation was calculated from the mean fluorescence readings of the standards using a four-parameter logistic curve calculator (AAT Bioquest, Inc., USA). This equation was then used to calculate corticosterone concentration values (pg/ml) for each sample from the corresponding mean fluorescence values.

2.6 RNA Quantification

2.6.1 Quantitative polymerase chain reaction (qPCR)

2.6.1.1 RNA extraction

RNA used for qPCR was extracted from tissue samples using the RNeasy Mini Kit (Qiagen, UK) following the manufacturer's instructions. 100*µ*l of β-Mercaptoethanol (β-ME) (Biorad, UK) was added to 10ml of Buffer RLT (supplied in kit), as suggested in the protocol. 70% ethanol was made up with Milli-Q distilled water (Merck, UK) and 100% ethanol (Merck, UK). Samples were disrupted using a Precellys 24 homogeniser (Bertin Technologies, UK) with two five-second blasts. All centrifuge steps that advised >8,000rpm were conducted at 10,000rpm. A RNase-Free DNase set (Qiagen, UK) was used for DNase digestion following the manufacturer's instructions.

2*µ*l of eluted RNA was used to assess RNA concentration and purity using a NanoDrop Microvolume Spectrophotometer (Fisher Scientific, UK). Sample purity was assessed based on 260/280 ratio values and nucleic acid purity was assessed based on 260/230 ratio values. The ideal value for both ratios is approximately 2.0. Almost all 260/280 and 260/230 values were between 1.8 and 2.1.

2.6.1.2 cDNA synthesis

RNA samples were diluted with nuclease free water (Thermo Fisher Scientific, UK) so that for each brain region, all samples were diluted to the concentration of the sample with the lowest concentration as read from the NanoDrop spectrophotometer then kept on ice. A High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, UK) was used to convert RNA to cDNA following the manufacturer's

instructions. cDNA was synthesised using a thermocycler (BioRad, UK). cDNA was then diluted with nuclease free water (Invitrogen, UK) at a 1:15 ratio. cDNA samples were then stored at -75°C.

2.6.1.3 qPCR

qPCR was conducted to assess mRNA expression of the following genes: *Cfos*, *Bdnf-IX*, and *Arc*. The Primer-BLAST tool (National Center for Biotechnology Information, USA) was used to find primer sequences for the three genes of interest and the two house-keeping genes (*Gapdh* and *Hprt1*). The PCR product size was specified as 80-150 and it was specified that the primer must span an exon-exon junction. The optimum melting temperature was set as 60°C with a range of 58-62°C. The top primer result was accepted if it had a G-C content of 45-55%, both strands ended on a GC bond, the primers had a length of 18-22bp and were specific to the gene. The sequences used are displayed in Table 2.1.

Table 2.1. Primer sequences used in qPCR experiments.

Gene	**Strand**	**Primer sequence**
Gapdh	Forward	TCTCTGCTCCTCCCTGTTCT
	Reverse	TACGGCCAAATCCGTTCACA
Hprt1	Forward	TCCTCCTCAGACCGCTTTTC
	Reverse	ATCACTAATCACGACGCTGGG
Arc	Forward	AGTGCTCTGGCGAGTAGTC
	Reverse	CAGTGGCAGTGGCAAGTG
Cfos	Forward	GATACGCTCCAAGCGGAGAC
	Reverse	TCGGTGGGCTGCCAAAATAA
Bdnf-IX	Forward	GGTTATTTCATACTTCGGTTGC
	Reverse	CCCATTCACGCTCTCCAG

Tissue samples for primer efficiency calculations were taken from unused brain tissue in a range of areas from four different brains of both sexes and genotypes during tissue collection for the experiment. Tissue samples from different brain areas were pooled in one sample, RNA was extracted, and cDNA was then synthesised as described above. cDNA for each sample was made up into four dilutions: 20%, 2%, 0.2% and 0.02% of the original concentration. For each gene, a qPCR master mix was made with 79.2*µ*l of SensiMix SYBR Hi-ROX (Meridian Bioscience, USA), 3.16*µ*l of each primer (forward and reverse) and 20.1*µ*l of nuclease free water, and the solution was mixed by pipetting. Primer testing was run in triplicate with 2*µ*l of each cDNA dilution added to 8*µ*l of master mix. The plate was spun down on a plate spinner for approximately ten seconds, before being loaded into a StepOne Plus RT-qPCR machine (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 1 minute and 95°C for 15s.

Mean cycle threshold (C_T) values were calculated for each triplicate run. Outlier C_T were excluded from the mean calculations using Grubbs test if their Z score was equal to or greater than 1.15. The slope of the linear relationship between the log dilution and mean C_T for each primer was determined and percentage efficiency was calculated using the equation: $E = 100(10^{\left(-\frac{1}{m}\right)} - 1)$, where E = primer efficiency, and m = slope. Primers were considered acceptable to use if primer efficiency was between 85-115% (Figure 2.4).

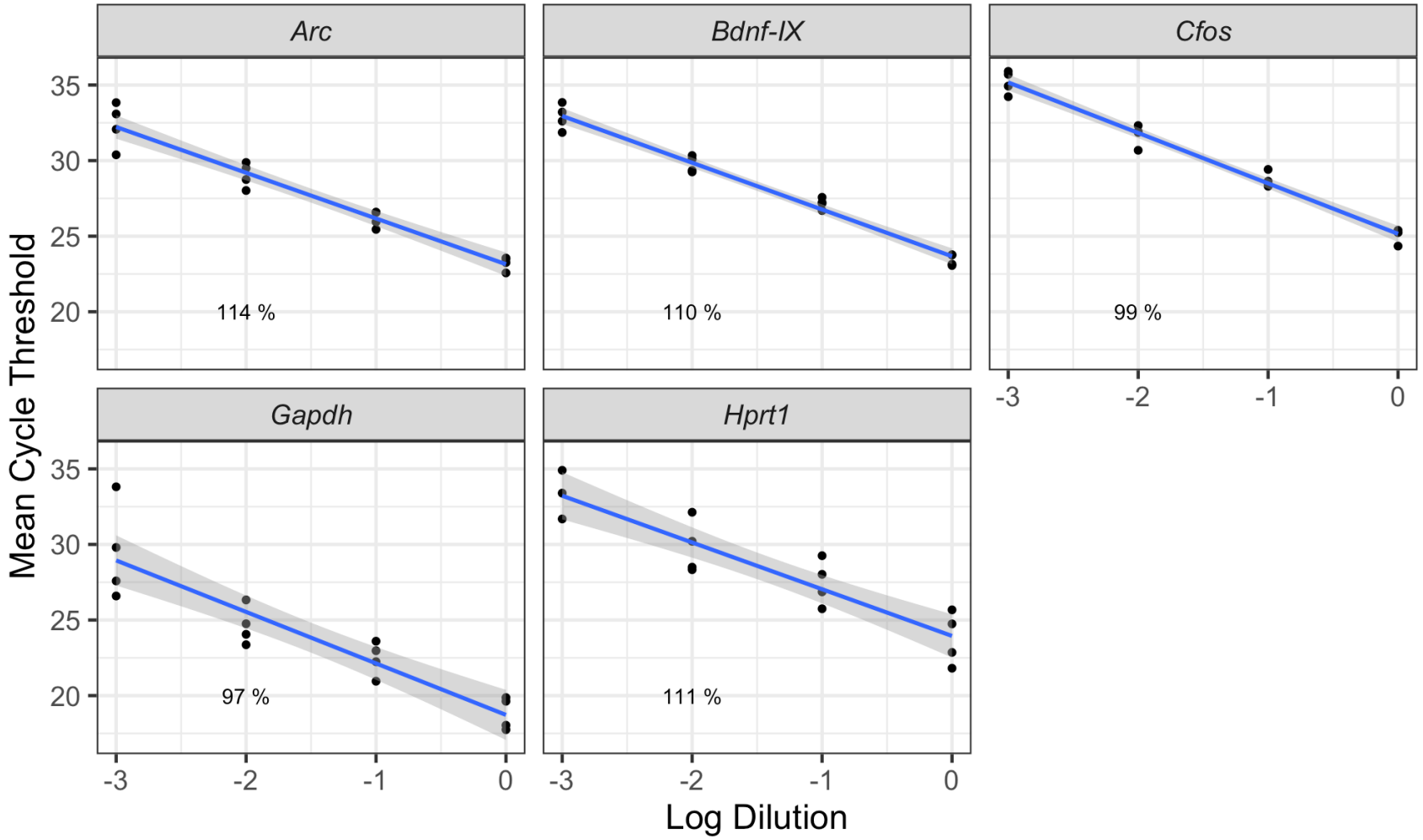

Figure 2.4. Primer efficiency calculations for the primers of the two house-keeping genes (*Gapdh* and *Hprt1*) and the three genes of interest (*Cfos*, *Bdnf-IX*, and *Arc*).

After testing primer efficiency, qPCR was conducted. Each brain region and gene were analysed independently. For each brain region/gene combination, six qPCR plates were conducted, with sex and PE condition evenly divided across plates. The two housekeeping genes, *Hprt1* and *Gapdh* were tested on each plate alongside the gene of interest (*Arc*, *Cfos* or *Bndf-IX*). Master mix for each gene was made up in bulk, using 0.99*µ*l of each primer (forward and reverse), 6.27*µ*l of nuclease free water, and 24.75*µ*l of SensiMix SYBR Hi-Rox per sample to be tested. Each master mix was mixed by gentle pipetting, and 8*µ*l was added to the respective wells of a 96-well plate for each gene. Samples were assessed in triplicate, and after thawing to room temperature and vortexing, 2*µ*l of cDNA for each sample was added to three wells for each gene, of each plate. 8*µ*l of master mix was added to three further wells for each gene, and 2*µ*l of water was added to each of these wells to serve as a non-template control. Plates were spun using a plate spinner for approximately ten seconds, and then loaded onto a RT-qPCR machine 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 1 minute and 95°C for 15s.

2.6.2 RNA sequencing

2.6.2.1 RNA extraction

A RNeasy UCP Micro Kit (Qiagen, UK) for low biomass (less than 45*µ*g total RNA) samples was used to extract RNA from tissue following the manufacturer's instructions. Samples were homogenised for 2x5s bursts using Precellys 24 homogeniser (Bertin Technologies, UK). 100% ethanol (Merck, UK) was used to make the diluted ethanol required in the protocol. An RNAse-Free DNAse Set, (Qiagen, UK) was used following the manufacturer's instructions for DNA digestion.

3μl of each RNA sample was aliquoted separately for quality control (QC). The remaining 11μl was stored at -75°C prior to library preparation. 1.5μl was used to assess RNA purity using a NanoDrop Microvolume Spectrophotometer (Fisher Scientific, UK). Sample purity was assessed based on 260/280 ratio values and nucleic acid purity was assessed based on 260/230 ratio values. The ideal value for both ratios is approximately 2.0. Almost all 260/280 values were between 1.8 and 2.1. However, around one third of 260/230 values were between 0.5 and 1.5. Technical advice was sought from the Cardiff University Next Generation Sequencing (NGS) Core Team (J. Morgan, NGS Technician), and it was determined that library preparation and sequencing should still be successful with low 260/230 values. However, to maximise purity, it was ensured that spin columns were completely dried between each centrifuge step.

The remaining 1.5μl of QC RNA for each sample was diluted with 1.5μl of nuclease free water (ThermoFisher, UK) to allow sufficient volumes for both RNA quality and concentration quantification. A broad range RNA Quantification Kit and Qubit Fluorometer (Invitrogen, UK) were used to assess the concentration of RNA in each sample following the manufacturer's instructions. Each standard was then read by the Qubit Fluorometer to create the standard curve, from which RNA concentration in samples was inferred. Concentration values were then doubled and recorded to account for the previous dilution of QC samples with nuclease free water.

The RNA quality of the remaining 1.5μl of RNA set aside for QC was assessed by the Agilent Fragment Analyzer High Sensitivity (HS) RNA Kit (Agilent Technologies LDA, UK) following the manufacturer's instructions. Samples were analysed in batches of 11 samples, with batches counterbalanced for sex, genotype and condition. Samples were assessed for RNA Quality Number, and values above 7.0 were considered acceptable for sequencing. All samples used in this experiment had quality scores above 9.0.

2.6.2.2 Library preparation

Poly(A) mRNA capture and construction of mRNA sequencing libraries was performed using the KAPA mRNA HyperPrep Kit (KAPA Biosystems, UK) following the manufacturer's instructions. RNA samples were diluted to 50*µ*l using nuclease free water based on Qubit RNA concentration values, so that each sample contained 250ng of total RNA. KAPA Unique Dual-Indexed Adapter Kit (KAPA Biosystems, UK) adapters were used and adapters were diluted to a concentration of 1.5µM. Twelve PCR cycles were used for library amplification. Prepared libraries were stored at -20°C until sequencing took place (within one month).

2.6.2.3 RNA Sequencing

Sequencing of reads of 100 bases in length was conducted at the University Hospital of Wales using an S2 flow cell (20028316, Illumina, UK) on an Illumina NovaSeq 6000 sequencing instrument by NGS Technician, J. Morgan.

2.6.2.4 Data Preparation

The following steps were conducted to generate read counts of each gene for each sample from the raw RNA sequencing files. The command line tool trimmomatic v0.35 (Bolger et al., 2014) was used to cut the Illumina adapter sequence as well as the three low quality leading and trailing base pairs from each read. Reads were then scanned with a four base-wide sliding window to cut reads at a point where average quality dropped below a score of 15. Any reads with a base length less than 36 were then discarded. The command line tool FastQC v0.12.1 (Andrews, 2010) was then used to assess read quality. Reads with every base position scored above 30 were considered to be of good quality. The command line tool STAR v2.7.9a (Dobin and Gingeras, 2015) was used to index the rattus norvegicus genome, using the

mRatBN7.2 genome assembly (National Center for Biotechnology Information, USA). The length of the genomic sequence around the annotated junction to be used in constructing the splice junctions was specified as 99 as the ideal length for the STAR tool is the read length -1. STAR was then used again to align the trimmed reads to the indexed rattus norvegicus genome. The command line tool picard (http://broadinstitute.github.io/picard/) was then used to mark and remove duplicate reads which likely originated from the same strand of RNA within the .bam file of each sample. The command line tool bamtools (Barnett et al., 2011) was then used to assess alignment quality. The command line tool featurecounts (Liao et al., 2014) was then used to provide a read count of each gene for every sample.

2.7 Statistical Analyses

All data analyses were conducted using R versions 4.2.0 – 4.4.0. In all analyses, the threshold for statistical significance was set at $\alpha = 0.05$ unless otherwise stated. For each CFC session, a *t*-test was performed to verify that freezing behaviour was significantly higher post-shock compared to pre-shock and to assess whether there was a change in locomotor activity after the shock was administered.
In analyses where the impact of two or three predictors such as, genotype, sex and/or PE condition on continuous outcomes such as freezing, quadrant crossings, or gene expression, ANOVAs were conducted. Although freezing and quadrant crossings are not strictly continuous variables, they were treated as such here as appropriate transformations of the outcome variable ensured that assumptions of linear models (homoscedasticity and normal distribution of residuals) were adhered to. Models were assessed for normal distribution of residuals and homoscedasticity using visual inspection of QQ plots and histograms of residuals for each model. If assumptions of linear models were transgressed, the 'boxcox' function of the R package MASS was used to determine which transformation would be most appropriate for the data. Any transformations used are specified in the relevant results sections. Models initially included all main effects and interaction terms, and

interaction terms were iteratively dropped using AIC score as a measure of model fit, until the best fitting model was achieved. Test-statistics, degrees of freedom, *p*-values, and confidence intervals (calculated using the pairwise method of the R-package 'emmeans') are reported for each term in each model.

Chapter 3: The effect of *Cacna1c* heterozygosity and sex on contextual fear memory generalisation

3.1 Introduction

One of the key roles of the hippocampus is the integration of disparate sensory features of an environment into a configural contextual representation. Such representations, which can include features such as time points and internal states as well as sensory information from the external environment, are crucial for an organism's ability to generate future expectations, establish contingencies between events, and to create accurate perceptions of ambiguous sensory information (Maren et al., 2013). Deficits in contextual processing have been observed across a range of psychopathologies including schizophrenia and other psychotic disorders (Cohen et al., 1999; Javitt et al., 2000; Barch et al., 2003; Ravizza et al., 2010; Yang et al., 2013; Kang et al., 2019; Brébion et al., 2020; Pokorny et al., 2021) as well as bipolar disorder (Brambilla et al., 2007; Yang et al., 2013; Corrêa et al., 2015; Reilly et al., 2016). Contextual memory processing has been less extensively investigated in patients with depression, however deficits have been observed (Corrêa et al., 2012).

In healthy individuals, associative memories are generally specific to the context in which they were acquired. However, there is evidence of inappropriate memory generalisation in psychotic disorders whereby individuals show a fear response to stimuli similar to but distinct from stimuli with which they have previously formed a fear association (Shohamy et al., 2010; Tuominen et al., 2021). Furthermore, it has been suggested that this occurs in a similar manner between schizophrenia and bipolar disorder, providing further evidence for a shared domain of hippocampal-

dependent cognitive deficits across disorders (Ivleva et al., 2012). Evidence from animal models has shown that the generalisation of contextual fear memories, which occurs to some degree in all individuals, has been shown to be mediated by hippocampal processes, in which the strength of the hippocampal representation of the context degrades over time, reducing the context specificity of the memory (Sauerhöfer et al., 2012). Synaptic plasticity processes within the hippocampus are known to mediate the formation of contextual representations (Zhao et al., 2020), and $Ca_v1.2$ has previously been implicated in the consolidation of contextual fear memories (McKinney et al., 2008), although not unanimously (Tigaret et al., 2021). Hence, aberrant calcium channel functioning caused by low *Cacna1c* gene dosage may affect the stability of contextual representations and result in the inappropriate generalisation of contextual fear memories. Furthermore, corticosterone administration in the DG has been shown to result in increased fear memory generalisation (Lesuis et al., 2021), and recent work has shown aberrant HPA-axis activity in the *Cacna1c* heterozygous deletion model (Moon et al., 2024). Thus, changes in HPA-axis functioning may mediate any impact of *Cacna1c* heterozygosity on contextual fear memory generalisation.

Sex differences have been previously noted in contextual fear memory generalisation as well as HPA-axis functioning. Contextual fear memory generalisation has been shown to occur more readily in female compared to male mice, with differential recruitment of the hippocampus demonstrated between sexes, in that contextual fear memory generalisation was more associated with hippocampal activity in males compared to females (Keiser et al., 2017). However, although the hippocampus is critical to the formation of contextual memories, it functions within a network of other regions including the medial PFC and amygdala, and oestradiol has been shown to affect dendritic spine density of neurons within the CA1 of the hippocampus, amygdala and PFC (Bauer, 2023). Sex differences have also been observed in the expression of corticosteroid receptors in the hippocampus in response to stressors (Heck and Handa, 2019). Hence, it may be the case that the effects of *Cacna1c* heterozygosity on contextual fear memory generalisation differ

between sexes owing to the sexual dimorphism of the mediating neurobiological networks and processes.

Recent studies from our lab have used the *Cacna1c* heterozygous deletion rat model to show that when CFC occurs in a specific context, the fear memory recall is generalised to a distinct neutral context in male *Cacna1c*$^{+/-}$ rats, but not *Cacna1c*$^{+/+}$ (unpublished data). Specifically, all male rats show increased freezing behaviour post-US compared with the pre-US period. However, when undergoing a recall session in both the context in which CFC training was undertaken, and a neutral context, *Cacna1c*$^{+/+}$ animals show increased freezing behaviour in the CFC but not the neutral context, whereas *Cacna1c*$^{+/-}$ animals show freezing at similarly high levels in both contexts. Hence, the aim of this study was to replicate this finding and to assess whether the phenomenon occurs similarly between sexes. Furthermore, analysis was carried out on trunk blood taken 30 minutes after either neutral or CFC context recall, with the aim of assessing changes in blood plasma corticosterone concentration to investigate whether differences in generalisation of contextual fear memories between *Cacna1c* genotypes may be mediated by alterations in HPA-axis functioning.

3.2 Hypotheses

It was hypothesised that…

- *Cacna1c* heterozygosity results in increased generalisation of contextual fear memories.

- This increased contextual fear memory generalization induced by *Cacna1c* heterozygosity is associated with an increased HPA-axis response.

- There are known sex differences in behavioural fear response with males more readily showing freezing behaviour in response to contextual fear memories compared with females (Graham et al., 2009), as well as sex differences in HPA-axis activation, with a more rapid HPA-axis response and greater stress hormone output in females compared with males (Goel et al., 2014). Hence, it was hypothesised that sex would interact with *Cacna1c* heterozygosity to affect fear memory generalisation at both a behavioural and HPA-axis level.

3.3 Methods

3.3.1 Animals

43 female and male Sprague-Dawley rats with either wild-type (*Cacna1c*$^{+/+}$) or *Cacna1c*$^{+/-}$ genotype were used: female *Cacna1c*$^{+/-}$ (n = 13); male *Cacna1c*$^{+/-}$ (n = 13); female *Cacna1c*$^{+/+}$ (n = 12); male *Cacna1c*$^{+/+}$ (n = 12). All testing was conducted during the active (dark) phase of the light cycle. All animals were housed as described in Section 2.1.

3.3.2 Behavioural Paradigm

Aged approximately 110 days, rats underwent a paradigm to test the hypothesis that *Cacna1c*$^{+/-}$ rats would generalise a contextual fear association to a neutral context to a greater extent than *Cacna1c*$^{+/+}$ rats. On the first four days of the experiment, rats were pre-exposed to each context for a single 10-minute PE session in one of the two distinct contexts (Section 2.2.2) each day in an alternating order (Figure 3.1). Each context was assigned to either an AM or PM session in a counterbalanced manner, to aid in distinguishing the two contexts from one another. 24 hours after the

last pre-exposure day, rats underwent a CFC paradigm in one of the two contexts (the assignment of each context to either CFC or neutral was counterbalanced across groups). 24-hours later, they were exposed to the neutral context for a three-minute period to serve as a control for the added exposure to the CFC context during CFC training. 24-hours later, rats underwent a recall session in either their CFC or neutral context. 24 hours after this initial recall session, they underwent a second recall session in the other context. The order of recall sessions was counterbalanced across groups. Animals were euthanised 30-minutes after their second recall session.

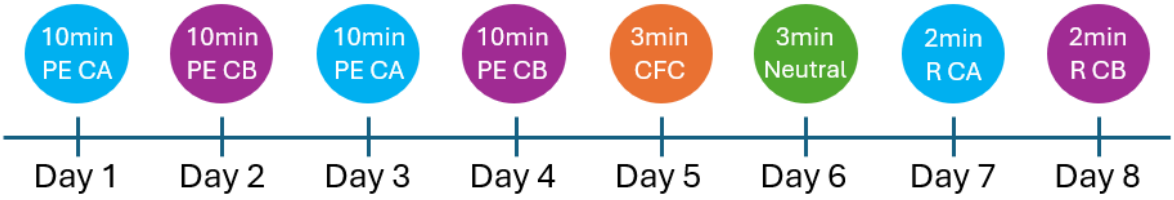

Figure 3.1. Behavioural paradigm of the CFC discrimination experiment. PE = pre-exposure; CA = Context A; CB = Context B; CFC = Contextual Fear Conditioning; Con = Control period; R = Recall. The assignment of Context A or Context B to the CFC or neutral context, as well as the order of pre-exposure (CA on Day 1 and 3 and CB on Day 2 and 4, or vice versa) and recall of each context (either CA on Day 7 and CB on Day 8, or vice versa) was counterbalanced across genotypes and sexes.

3.3.3 Euthanasia

Animals were culled using rising concentration of CO_2 30-minutes after the recall session as described in Section 3.2 and trunk blood samples were extracted as described in Section 2.4.1. For blood plasma isolation and corticosterone concentration ELISA methods, see Section 2.5.

3.3.4 Statistical Analysis

In order to assess whether rats were successfully discriminating between contexts, an ANOVA was performed for each outcome measure: percentage of time spent freezing within each session (Section 2.2.4), and quadrant crossings per minute (Section 2.2.5). All main effect terms (sex, genotype and recall context) and interactions were included in each model. Although all animals underwent a recall session in both their CFC and neutral contexts, the order was counterbalanced across genotypes and sex. Hence, blood was extracted from half the animals 30-minutes after recall in their neutral context, and from the other half of animals 30-minutes after recall in their CFC context. Hence, although context was a within-subject variable in the behavioural analysis, for the blood plasma corticosterone analysis, it is a between-subject variable. Due to logistical constraints induced by stocking density and uneven numbers of genotypes per cage, there is some variation in the number of animals in each group for the corticosterone analysis (Female *Cacna1c*$^{+/-}$ CFC, n = 6; Female *Cacna1c*$^{+/-}$ neutral, n = 7; Female *Cacna1c*$^{+/+}$ CFC, n = 5; Female *Cacna1c*$^{+/+}$ neutral, n = 7; Male *Cacna1c*$^{+/-}$ CFC, n = 6; Male *Cacna1c*$^{+/-}$ neutral, n = 5; Male *Cacna1c*$^{+/+}$ CFC, n = 3; Male *Cacna1c*$^{+/+}$ neutral, n = 9). The same ANOVA analysis method used for the behavioural analysis was used in the corticosterone analysis.

3.4 Results

3.4.1 Behavioural Results

3.4.1.1 Freezing

All rats showed negligible freezing levels prior to administration of the foot-shock, and increased freezing immediately after foot-shock (t_{92} = 28.6, $p < 0.001$, 95%CI =

[69.6, 80.0]) (Figure 3.2). In the immediate post-shock period, freezing behaviour was square transformed. A main effect of genotype was found ($F_{1,43}$ = 7.4, p = 0.009, 95%CI = [464, 3074]), with *Cacna1c*$^{+/-}$ animals showing higher levels of freezing compared with *Cacna1c*$^{+/+}$ animals. There was no main effect of sex on freezing behaviour, however there was a trend towards higher freezing in females compared with males ($F_{1,43}$ = 3.8, p = 0.056, 95%CI = [-2542, 68.3]). The interaction between genotype and sex was not significant ($F_{1,43}$ = 2.7, p = 0.108, 95%CI = [-484, 4737]). However, pairwise post-hoc comparisons showed that greater freezing was found in *Cacna1c*$^{+/-}$ compared to *Cacna1c*$^{+/+}$ rats in females (p = 0.026, 95%CI = [1050, 4615]), a finding which was not observed in males (p = 0.460, 95%CI = [-1201, 2612]). Together, these results suggest that *Cacna1c*$^{+/-}$ rats show greater freezing compared with *Cacna1c*$^{+/+}$ rats immediately following foot-shock, which was likely driven by a female-specific effect.

At recall, there was no main effect of sex, but a trend towards greater freezing in females compared with males was observed ($F_{1,86}$ = 3.9, p = 0.051, 95%CI = [0.52, 20.7]). There was a main effect of context, with greater freezing in the CFC compared to the neutral context ($F_{1,86}$ = 5.5, p = 0.022, 95%CI = [1.46, 21.7]). A main effect of genotype was observed, with greater freezing in *Cacna1c*$^{+/-}$ compared to *Cacna1c*$^{+/+}$ animals ($F_{1,86}$ = 7.5, p = 0.007, 95%CI = [-23.3, -3.05]). The effect of context did not interact with genotype ($F_{1,86}$ = 0.2, p = 0.644, 95%CI = [-24.1, 16.3]). The effect of genotype was found to interact with the effect of sex ($F_{1,86}$ = 5.2, p = 0.025, 95%CI = [2.91, 43.3]). Pairwise post-hoc comparisons revealed that in females, *Cacna1c*$^{+/-}$ animals froze significantly more than *Cacna1c*$^{+/+}$ animals (p < 0.001, 95%CI = [-38.5, -10.9]), whereas there was no significant difference between genotypes in males (p = 0.830, 95%CI = [-16.4, 13.2]), in line with the findings from the post-US period of the CFC session. There was no interaction between sex and context ($F_{1,86}$ = 0.1, p = 0.815, 95%CI = [-17.9, 22.5]) and the three-way interaction was also not significant ($F_{1,86}$ = 1.4, p = 0.248, 95%CI = [16.8, 64.1]).

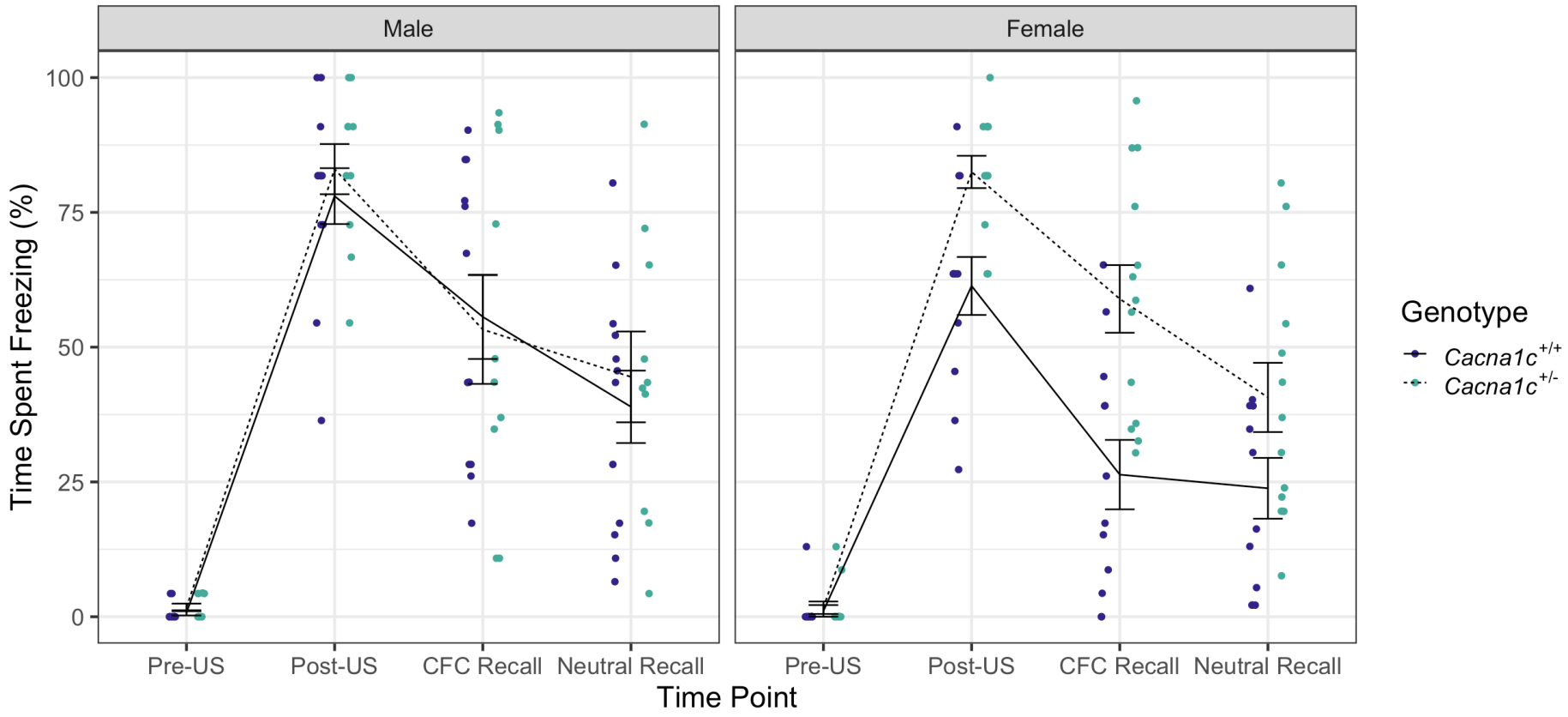

Figure 3.2. Percentage of time spent freezing during pre-US and post-US periods of the CFC session, and the CFC recall and neutral recall sessions.

3.4.1.2 Quadrant Crossings

Rats showed reduced quadrant crossings after the shock was administered (t_{92} = -8.3, $p < 0.001$, 95%CI = [2.8, 4.5]) (Figure 3.3). In the immediate post-shock period, crossings/minute was square root transformed. There was no main effect of either genotype ($F_{1,43} < 0.1$, $p = 0.862$, 95%CI = [-0.5, 0.4]) or sex ($F_{1,43} = 1.5$, $p = 0.233$, 95%CI = [-0.2, 0.7]). There was a trend towards an interaction between genotype and sex ($F_{1,43} = 3.2$, $p = 0.081$, 95%CI = [-1.7, 0.1]). However, pairwise post-hoc comparisons showed there was no difference between *Cacna1c* genotypes in either females ($p = 0.167$, 95%CI = [-1.0, 0.2]) or males ($p = 0.264$, 95%CI = [-0.3, 1.0]).

At recall, crossings/minute was log-transformed. The model showed lower AIC score upon removing the three-way interaction term as well as the two-way interactions between sex and context, and genotype and context. The final model included the main effects of genotype, sex, and context, as well as the two-way interaction between genotype and sex. A main effect of sex was found ($F_{1,89} = 7.7$, $p = 0.007$, 95%CI = [-0.6, -0.1]), with females showing more frequent quadrant crossings compared to males. A main effect of context was also observed ($F_{1,89} = 4.9$, $p = 0.030$, 95%CI = [-0.5, 0.0]), with animals making more frequent quadrant crossings in the neutral compared to CFC context. There was also a main effect of genotype ($F_{1,89} = 6.8$, $p = 0.011$, 95%CI = [0.1, 0.6]), with *Cacna1c*$^{+/-}$ animals showing fewer quadrant crossings compared with *Cacna1c*$^{+/+}$ animals. There was no interaction genotype and sex ($F_{1,89} = 2.1$, $p = 0.149$, 95%CI = [-0.9, 0.1]).

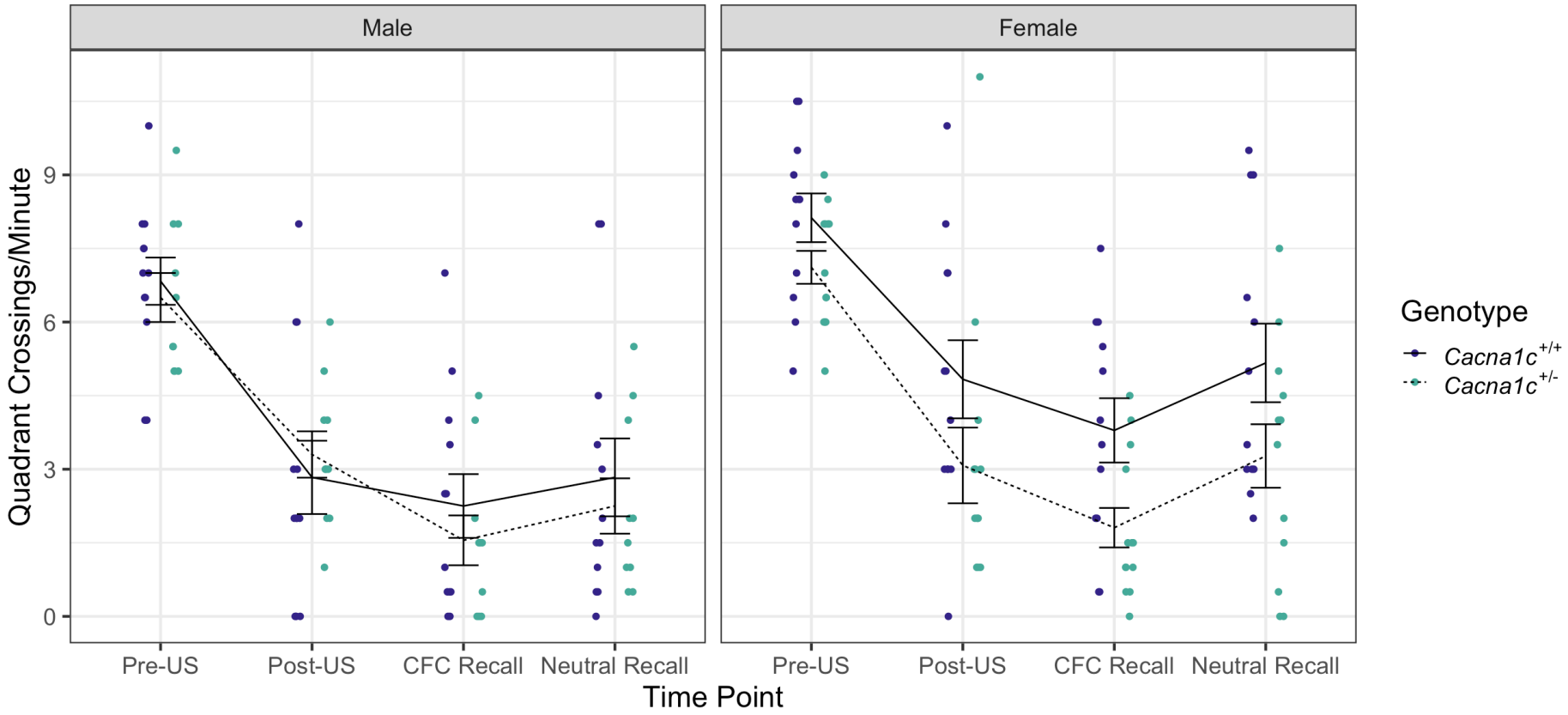

Figure 3.3. Quadrant crossings per minute during the pre-US and post-US periods of the CFC session, and the CFC recall and neutral recall sessions. .

3.4.2 Blood plasma corticosterone concentration

Corticosterone concentration was log-transformed. The model showed lower AIC score upon removing the three-way interaction term as well as the two-way interaction between sex and genotype. The final model included main effects of sex, genotype, and context, as well as the interactions between sex and context, and genotype and context. There was no main effect of genotype ($F_{1,41} < 0.1$, $p = 0.899$, 95%CI = [-0.6, 0.5]) or context ($F_{1,41} = 0.5$, $p = 0.505$, 95%CI = [-0.3, 0.9]). There was a main effect of sex, in that females had higher blood plasma corticosterone concentration than males ($F_{1,41} = 6.1$, $p = 0.018$, 95%CI = [-1.4, -0.3]). However, this appeared to be driven by a significant interaction between context and sex ($F_{1,41} = 14.5$, $p < 0.001$; 95%CI = [1.0, 3.2]) (Figure 3.4A). Pairwise post-hoc comparisons revealed that females showed significantly higher blood plasma corticosterone concentrations in the CFC compared with neutral context ($p = 0.045$, 95%CI = [-1.5, -0.0]) However, males show significantly lower blood plasma corticosterone concentration in the CFC compared with neutral context ($p = 0.012$, 95%CI = [0.5, 2.2]). The interaction between genotype and context was not found to be significant ($F_{1,41} = 1.2$, $p = 0.276$, 95%CI = [-1.7, 0.5]) (Figure 3.4B).

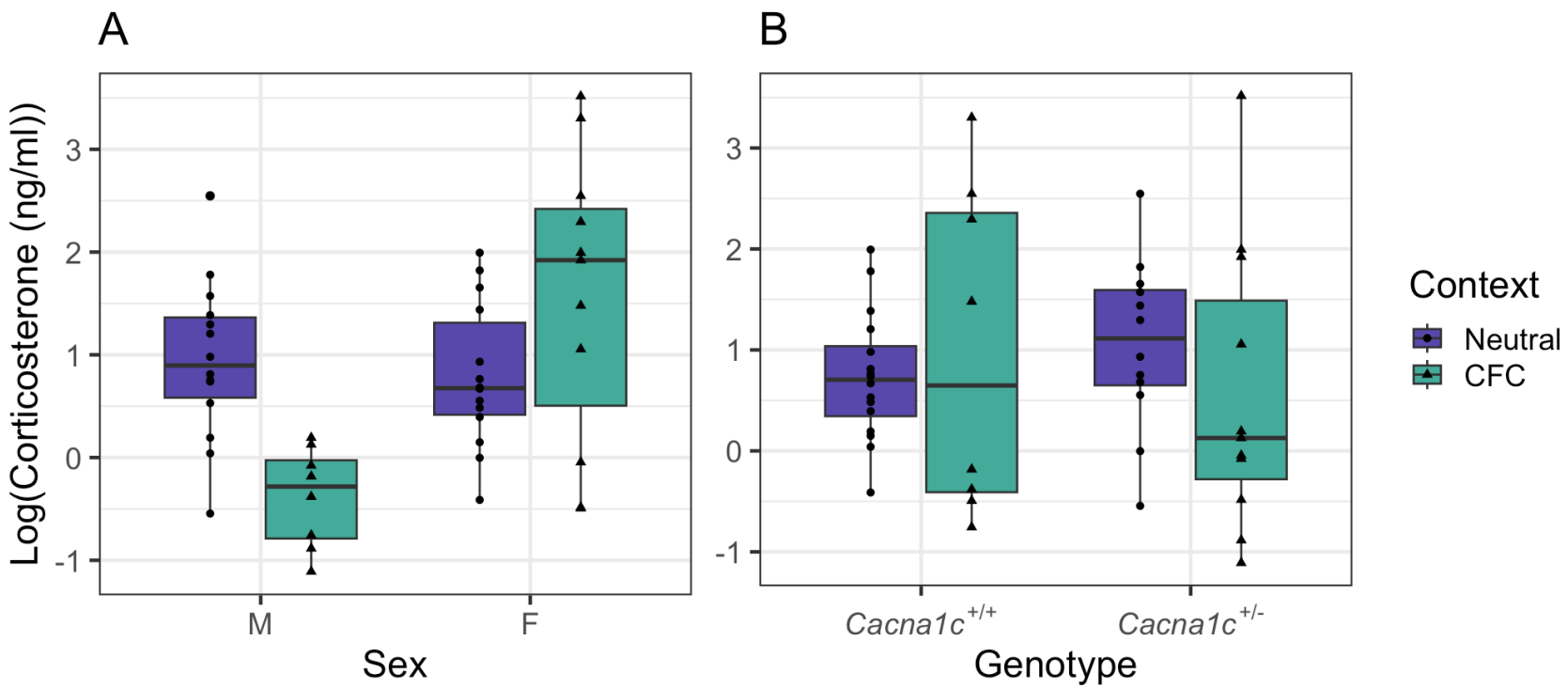

Figure 3.4. Blood plasma corticosterone concentration (ng/ml) between sexes and recall contexts (A) and *Cacna1c* genotypes and recall contexts (B). Y-axis is log transformed to aid visualisation.

3.4.3 Summary of Results

The main findings of the experiment are outlined in Table 3.1. At recall, animals in the CFC group froze more and made fewer quadrant crossings compared with animals in the neutral context. This did not interact with genotype, indicating that *Cacna1c*$^{+/-}$ do not show increased contextual fear memory generalisation compared with *Cacna1c*$^{+/+}$ animals. There was also no interaction between genotype and context in corticosterone concentration. *Cacna1c*$^{+/-}$ animals showed greater freezing and fewer quadrant crossings compared with *Cacna1c*$^{+/+}$ animals. These freezing findings appear to be driven by an effect specific to females. However, sex did not interact with genotype to affect quadrant crossings. At recall, males froze more and made fewer quadrant crossings compared with females. Females had higher corticosterone concentration compared to males. There was also an interaction between sex and context, with higher corticosterone concentration in the CFC compared to the neutral context in females, but the opposite finding of higher corticosterone concentration in the neutral compared with CFC context in males.

Table 3.1 Summary of the effect of sex, genotype, and context on freezing, quadrant crossings and plasma corticosterone concentration. G = genotype; S = sex; C = context; N = neutral; CFC = contextual fear conditioning; M = male; F = female.

Effect	Dependent Variable				
	Freezing		Quadrant Crossings		Plasma Corticosterone
	Post-US	Recall	Post-US	Recall	
Genotype	$^{+/-} > ^{+/+}$	$^{+/-} > ^{+/+}$	-	$^{+/-} < ^{+/+}$	-
Sex	-	M > F	-	M < F	F > M
Context		CFC > N		CFC < N	-
G x S	F:$^{+/-}$ > F:$^{+/+}$	F:$^{+/-}$ > F:$^{+/+}$	-	-	-
G x C		-		-	-
S x C		-		-	F: CFC > N M: CFC < N
G x S x C		-		-	-

3.5 Discussion

This experiment aimed to replicate the findings observed in a previous study by our group of increased fear memory generalisation in male *Cacna1c*$^{+/-}$ compared to male *Cacna1c*$^{+/+}$ rats. Furthermore, this experiment aimed to explore the impact of sex, and to determine whether sex would interact with genotype to affect fear memory generalisation. Contrary to expectations, the fear memory generalisation observed in *Cacna1c*$^{+/-}$ animals in the previous experiment was not replicated in this study in either males or females. It was found that freezing behaviour was higher, and quadrant crossings were lower in the CFC context at recall compared to the neutral context across all animals, in a manner that did not interact with genotype and/or sex, indicating no genotype differences in fear memory generalisation.

In order to clarify why the earlier findings of the lab group were not replicated in this experiment, the raw data of the original experiment were reanalysed. Upon reanalysis of the original data of this previous experiment, an order effect was found to be driving the genotype-by-context interaction seen in freezing at recall (Figure 3.5). As part of the design of this original experiment, the order of the CFC and neutral context recall sessions was counterbalanced between genotypes. Upon conducting an ANOVA, a trend towards a three-way interaction was observed between genotype, context, and recall order ($F_{1,36}$ = 3.5, p = 0.070, 95%CI = [-153.0, 6.2]). Irrespective of whether the recall session of CFC or neutral context was first or second, *Cacna1c*$^{+/+}$ animals froze more during the CFC recall session than the neutral recall session, indicating they were not generalizing from the CFC to the neutral context. In *Cacna1c*$^{+/-}$ animals, if the neutral context recall session took place prior to the CFC context recall session, they also showed discrimination, with lower freezing during neutral recall compared to CFC recall. However, if the CFC context recall session took place prior to the neutral context recall session, similar levels of freezing were observed in both sessions in *Cacna1c*$^{+/-}$ animals. Hence, the overall effect observed of a lack of discrimination between contexts in *Cacna1c*$^{+/-}$ animals compared to *Cacna1c*$^{+/+}$ in the original experiment appears to have been driven by the half of animals that underwent CFC recall prior to neutral recall.

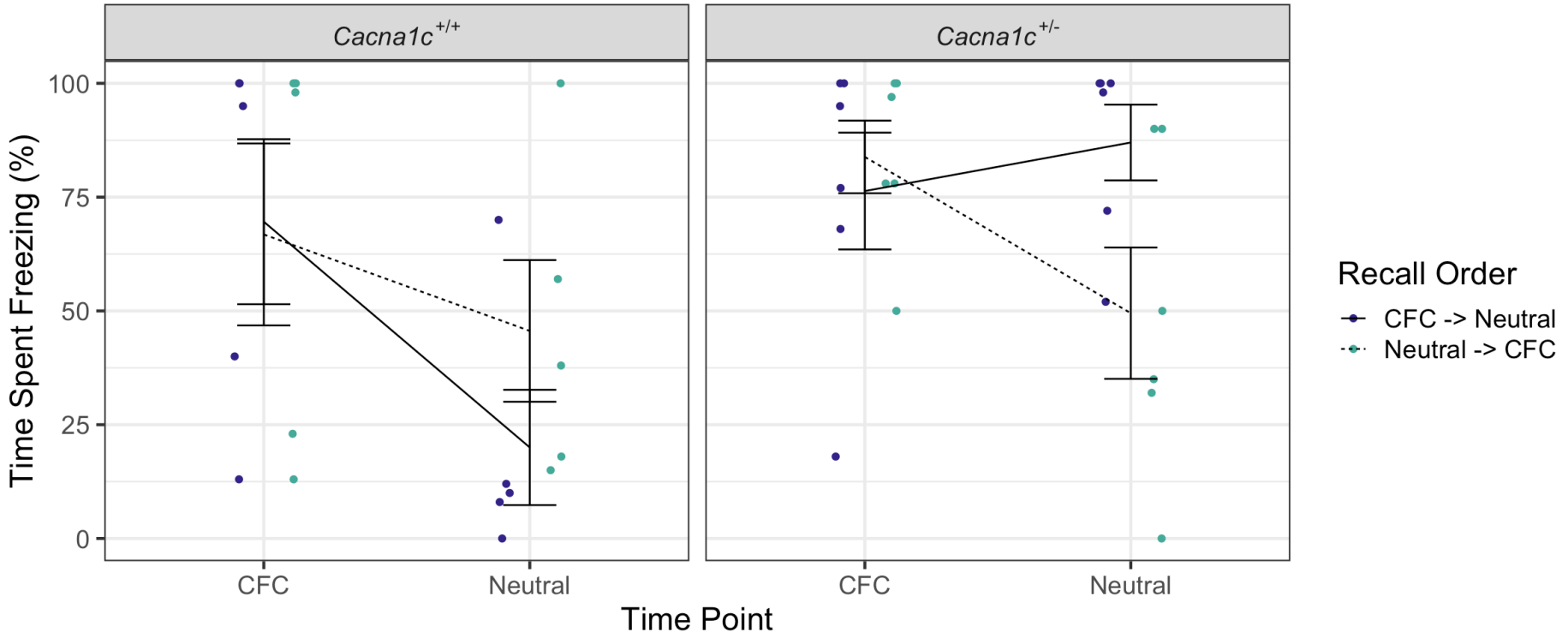

Figure 3.5. Re-analysis of data from the original experiment conducted by the research group. Duration of each recall session spent freezing, split by order of context recall and genotype.

In addition, the earlier behavioural experiment differed from the data reported here in that both recall sessions took place on the same day. In contrast, in this experiment, recall sessions took place 24-hours apart. Hence, the discrepancy between the two data sets may be explained by an order effect taking place in the original experiment, whereby in *Cacna1c*$^{+/-}$ animals, having an experience of fear memory recall in the CFC context only a few hours earlier resulted in increased freezing when re-entering the neutral context, whereas this order effect did not occur in *Cacna1c*$^{+/+}$ animals. In the later experiment, this order effect did not take place, as neutral context recall sessions took place either 24 hours before or 24 hours after the CFC recall session. It may be that in *Cacna1c*$^{+/-}$ animals, a proactive interference effect is caused by the recent CFC memory reactivation, affecting responses to the later neutral context recall, that does not occur in *Cacna1c*$^{+/+}$ animals. Further experiments are required to specifically test this hypothesis, particularly as females were not included in the initial experiment.

An interesting sex-by-genotype interaction was also observed in this study, in that in males, there were no differences between genotypes in the overall levels of freezing, however in females, a higher level of freezing was observed in *Cacna1c*$^{+/-}$ compared to *Cacna1c*$^{+/+}$ animals across both recall sessions. This was not expected, as previous experiments using this model have not assessed possible sex differences in the effects of *Cacna1c* heterozygosity on fear memory learning processes. Hence, further experiments are required to verify this finding and further investigate possible causal explanations. Several possibilities may explain this finding. For instance, *Cacna1c* heterozygosity may lead to a general heightening of behavioural fear responsivity in females. Increased freezing in *Cacna1c*$^{+/-}$ females compared to *Cacna1c*$^{+/+}$ was not just observed in the two recall sessions, but also in the immediate post-shock period. Hence, the effect may not be specific to long-term memory recall, but also extend to immediate fear responses and short-term fear memory. During the pre-shock period, both *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ females showed negligible levels of freezing, indicating that this finding is not occurring as a result of spontaneous, non-fear-related freezing.

Behavioural experiments rely on outcome measures that are thought to be representative of an internal state. Hence, when analysing freezing behaviour as a proxy for fear, it is important to consider what freezing, or a lack thereof, represents. Freezing behaviour has been robustly associated with states of fear in rodents; however, an absence of fear behaviour does not necessarily mean animals are not fearful, as other behavioural strategies may be employed such as avoidance and escape, that may be mistaken for exploratory behaviour when simple measures of locomotion are taken. There are known sex differences in the behavioural strategies employed during a state of fear, with freezing more readily displayed by males compared with females (Bauer, 2023), and an overall higher level of freezing across recall sessions was found in males compared with females. This main effect of sex was also reflected in quadrant crossings, with more frequent crossings made by females compared with males. However, there was no interaction between sex and genotype in quadrant crossings. Hence, it may be that *Cacna1c* heterozygosity does not increase fear in females as such, but alters the behavioural strategies employed towards a greater propensity for freezing behaviour. As the finding of an interaction between genotype and sex in freezing behaviour has not previously been observed in this model, future work should aim to replicate the phenomenon and to assess the underlying causal mechanisms such as whether this effect is driven by *Cacna1c* heterozygosity interacting with oestrous state or organisational sex differences in HPA-axis functioning. However, if this finding were driven by oestrous state, variance of freezing in females would be expected to be higher than that of males, which was not found in this study.

Several studies have demonstrated increased fear memory generalisation in response to corticosterone administration (Bahtiyar et al., 2020; dos Santos Corrêa et al., 2021), with effects mediated by increased engram cell populations in the DG of the hippocampus (Krugers et al., 2020; Lesuis et al., 2021). Alterations in the HPA-axis have been implicated in the pathophysiology of psychotic and mood disorders (Mikulska et al., 2021), and experience of prenatal or early life stress are two of the most robust risk factors for schizophrenia, bipolar disorder and depression (Carr et al., 2013; Saleh et al., 2017; Garcia-Rizo and Bitanihirwe, 2020; LeMoult et

al., 2020; Quidé et al., 2020; Rodriguez et al., 2021; Mawson and Morris, 2023; Shintani et al., 2023). Indeed, the hippocampus plays an important role in the negative feedback suppression of HPA activity upon exposure to a stressor (Heck and Handa, 2019). Hence, it is important to explore whether genetic risk factors such as *CACNA1C* risk variants may confer their effects via aberrant HPA-axis activity that increases susceptibility to environmental risk factors such as early life stress, and whether a subsequently altered stress axis may disrupt memory generalisation. Indeed, recent data from the *Cacna1c*$^{+/-}$ animal model used in this thesis has shown increased circulating blood plasma corticosterone levels at baseline in *Cacna1c*$^{+/-}$ animals compared with *Cacna1c*$^{+/+}$ (Moon et al., 2024), indicating that HPA-axis alterations may mediate the effects of *Cacna1c* heterozygosity on fear memory generalisation.

An unexpected interaction between sex and context was observed in blood plasma corticosterone concentration, with males showing higher corticosterone levels 30-minutes after recall in the neutral context compared to males who had undergone recall in their CFC context. Conversely, females showed the opposite pattern of higher plasma corticosterone concentration in the CFC context recall group compared with the neutral context recall group. The direction of this effect in males was particularly surprising, as it would be anticipated that re-exposure to the CFC context would elicit a greater stress response compared to the neutral context (dos Santos Corrêa et al., 2019), not the other way around. This could perhaps be explained because of the time-point of 30-minutes post recall chosen for tissue extraction. This was chosen as plasma corticosterone concentration after a stressor peaks around this period (Koolhaas et al., 1997). It may be the case that an earlier significant release of corticosterone in the CFC recall group resulted in a negative feedback mechanism to subsequently reduce corticosterone expression in this group at the time that blood was taken.

In the corticosterone analysis, context was a between-subject independent variable as blood was taken 30-minutes after their CFC or neutral context recall session. Conversely, as animals underwent recall sessions in both contexts and behaviour

was recorded in both sessions, sample size for each group was reduced in the corticosterone analysis compared with the behavioural analysis, which reduces the statistical power to detect possible interactions. All animals in each cage were in the same context recall order, so that social behaviour between animals who had different behavioural experiences (CFC or neutral context recall) did not influence one another's stress response. As genotypes were not evenly balanced across cages, this resulted in uneven group sizes across each combination of sex, genotype and context (range 3-9). Thus, statistical power to make comparisons was further constrained in the corticosterone compared with behavioural analysis.

The initial hypothesis was that *Cacna1c*$^{+/-}$ animals would show increased contextual fear memory generalisation compared with *Cacna1c*$^{+/+}$ animals. Although this was not substantiated, these data are nonetheless informative in demonstrating that *Cacna1c* heterozygosity does not result in a broad increase in fear memory generalisation, but that this specifically occurs in response to interference from a recently recalled fear memory, although this was only tested in males. The majority of the pre-clinical fear memory generalisation literature pertains to post-traumatic stress disorder (PTSD), as increased fear memory generalisation has been demonstrated in numerous PTSD human samples (Morey et al., 2015; Lopresto et al., 2016; Lis et al., 2020). GWAS for PTSD have not revealed associations between *CACNA1C* SNPs and the disorder (Gelernter et al., 2019; Nievergelt et al., 2019; Stein et al., 2021). However, a recent study investigating risk variants in a sample of traumatised police officers showed an association between the *CACNA1C* risk variant rs1990322 and PTSD, a finding which was subsequently replicated in a sample of traumatised children (Krzyzewska et al., 2018). Hence, these findings of fear memory generalisation in response to a recent fear memory recall may play a role in the aberrant cognition that underpins the association between *CACNA1C* genetic variation and neuropsychiatric disorders including PTSD.

Chapter 4: The effect of *Cacna1c* heterozygosity and sex on latent inhibition

4.1 Introduction

LI is the phenomenon whereby pre-exposure to a CS or context prior to conditioning impairs the formation of a CS/US or context/US association, compared with an association in which the individual has not been pre-exposed to the CS or context. The aberrant salience hypothesis of schizophrenia argues that directed attentional processes are dysfunctional in schizophrenia and alterations in dopaminergic signalling lead to misdirection of attention to irrelevant stimuli, resulting in hallucinations and delusions (Kapur, 2003; Lubow, 2005). LI studies in both humans and rodents can be used to model aberrant salience, and indeed, deficits in LI tasks have been observed in patients with schizophrenia as well as highly psychosis-prone individuals (Lubow et al., 1992; De la Casa et al., 1993; Allan et al., 1995; Gray et al., 1995; Williams et al., 1998; Vaitl et al., 2002; Tsakanikos et al., 2003; Cohen et al., 2004; Yogev et al., 2004; Kraus et al., 2016; Myles et al., 2023) (although see exceptions: Lubow et al. (1987); Swerdlow et al. (1996)).

A recent study from the lab group using the *Cacna1c*$^{+/-}$ rat model observed a deficit in LI in male *Cacna1c*$^{+/-}$ relative to male *Cacna1c*$^{+/+}$ rats (Tigaret et al., 2021), and that this was associated with altered synaptic plasticity processes in the hippocampus. Hence, this suggests that *CACNA1C* risk variants may play a causal role in affecting the learning of contextual information or inappropriate contextual memory engram formation and/or retrieval (Vasudevan et al., 2024). However, female animals were not included in this experiment. Hence, it is not known whether LI deficits conferred by *Cacna1c* heterozygosity are sexually dimorphic. The aim of this study was to determine whether *Cacna1c*$^{+/-}$ females show impaired LI and to

replicate the finding in males. Furthermore, as in the previous chapter, associated changes in peripheral plasma corticosterone concentrations were also investigated.

4.2 Hypotheses

It was hypothesised that…

- *Cacna1c* heterozygosity results in impaired LI of contextual fear conditioning.

- As alterations in HPA-axis function have previously been observed in *Cacna1c*$^{+/-}$ animals (Moon et al., 2024), it was hypothesised that this impairment in LI induced by *Cacna1c* heterozygosity is associated with increased HPA-axis response in the group of animals pre-exposed to the context.

- Owing to the known sex differences in behavioural fear responses such as freezing and darting (Graham et al., 2009) as well as HPA-axis activation (Goel et al., 2014), sex would interact with *Cacna1c* heterozygosity to affect LI of contextual fear memory at both a behavioural and HPA-axis level.

4.3 Experiment 1

Tigaret et al. (2021) showed that four hours of pre-exposure (PE) to a novel context resulted in an LI effect in male *Cacna1c*$^{+/+}$ animals, whereby they showed reduced freezing 24-hours after CFC compared to *Cacna1c*$^{+/+}$ animals who had not been pre-exposed to the context prior to CFC. However, in male *Cacna1c*$^{+/-}$ animals, there was no difference in the level of conditioning between PE conditions, indicating a deficit in LI. However, no female animals were used in the experiment. Hence, the aim of this experiment was to assess whether the LI deficit was also present in

female *Cacna1c*$^{+/-}$ rats. It was also decided that a shorter PE duration spread across multiple sessions should be used to ascertain how *Cacna1c* heterozygosity may affect LI when contextual learning has taken place over multiple separate instances, as more frequent instances of PE has been shown to enhance LI (Miller et al., 2022), and more frequent shorter PE sessions was more practically feasible than a single multiple-hour PE session. However, the total PE duration was shortened so that the LI effect would not be so strong as to overshadow any possible subtle effect of *Cacna1c* heterozygosity as no *a priori* hypotheses regarding sex differences in the degree of deficit had been made. Furthermore, as there are known sex differences in contextual memory processing (Subramaniapillai et al., 2019; Colon and Poulos, 2020), it was important to first conduct an experiment to ascertain the minimal PE duration required to achieve LI. Thus, this initial experiment was conducted in *Cacna1c*$^{+/+}$ animals to test whether two 10-minute sessions of PE over two days would induce LI in both males and females.

4.3.1 Methods

4.3.1.1 Animals

Twenty-three *Cacna1c*$^{+/+}$ rats from the *Cacna1c* heterozygote deletion line were used in this study (10 males and 13 females). All testing was conducted during the active (dark) phase of the light cycle. All animals were housed as described in Section 2.1.

4.3.1.2 Behavioural Paradigm

On the first two days of the experiment, rats in the PE group (N males = 7; N females = 9) received two 10-minute context pre-exposure sessions over two consecutive days whilst rats in the no-PE group remained in their home cage (N males = 3; N

females = 4) (Figure 4.1). A greater number of animals were assigned to the PE compared with the no-PE condition as a limited number of animals were available and it was deemed more important to minimise variation in the PE group relative to the reference group when the aim of this experiment was to find an ideal threshold for PE duration in both sexes in *Cacna1c*$^{+/+}$ animals. On the third day of the experiment all rats underwent a 3-minute CFC session, and on the fourth day, returned to the conditioning chamber for a 2-minute recall session.

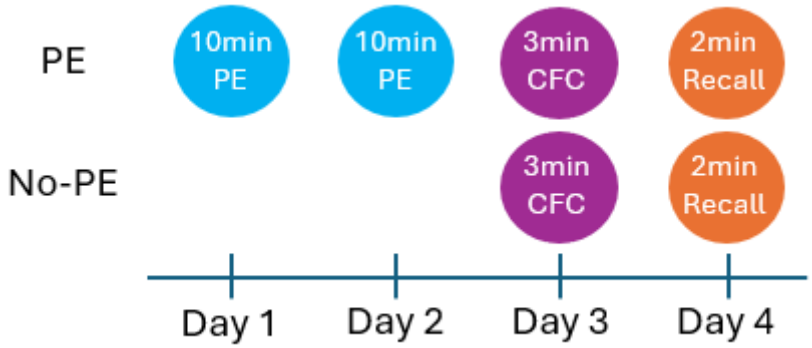

Figure 4.1. Behavioural paradigm of the first LI experiment. PE = pre-exposure; CFC = Contextual Fear Conditioning.

4.3.1.3 Behavioural and Statistical Analysis

For the pre-US and post-US periods of the CFC session, and the recall session, percentage of time spent displaying freezing behaviour was measured (as described in Section 2.2.4). Statistical analysis was performed as described in Section 2.7. ANOVA models were used to assess whether sex, pre-exposure group, or the interaction between the two variables affected freezing behaviour during the post-US period, and at recall.

4.3.2 Results

All animals showed negligible freezing prior to administration of the foot-shock, and increased freezing immediately after foot-shock (t_{44} = 12.3, $p < 0.001$, 95%CI = [59.0, 82.2]) (Figure 4.2). During the post-US period of the CFC session, there was a main effect of PE condition with higher freezing in the no-PE compared with PE group was found ($F_{1,19}$ = 7.1, p = 0.015, 95%CI = [1.73, 42.2]). At this time point there was no main effect of sex. However, there was a trend towards greater freezing in males compared with females ($F_{1,19}$ = 3.9, p = 0.064, 95%CI = [-12.9, 27.5]). An interaction was also observed between sex and PE condition ($F_{1,19}$ = 7.4, p = 0.014, 95%CI = [-93.0, -12.1]). Pairwise post hoc analysis showed greater freezing in the no-PE compared with PE group in females (p = 0.001, 95%CI = [21.7, 74.8]), but no difference was found between PE conditions in males (p = 0.769, 95%CI = [-34.8, 26.2]).

During the recall period 24 hours after the CFC session, there was no main effect of sex ($F_{1,19}$ = 1.2, p = 0.293, 95%CI = [-21.4, 29.0]) or PE condition ($F_{1,19}$ = 2.9, p = 0.104, 95%CI = [-7.78, 42.7]) on freezing. There was a trend towards an interaction between the two variables ($F_{1,19}$ = 3.1, p = 0.095, 95%CI = [-93, -12.1]). Pairwise post hoc analysis showed that in females, there was greater freezing in animals in the no-PE compared with PE group (p = 0.001, 95%CI = [21.7, 74.8]), whereas there was no difference in males (p = 0.769, 95%CI = [-34.8, 26.2]).

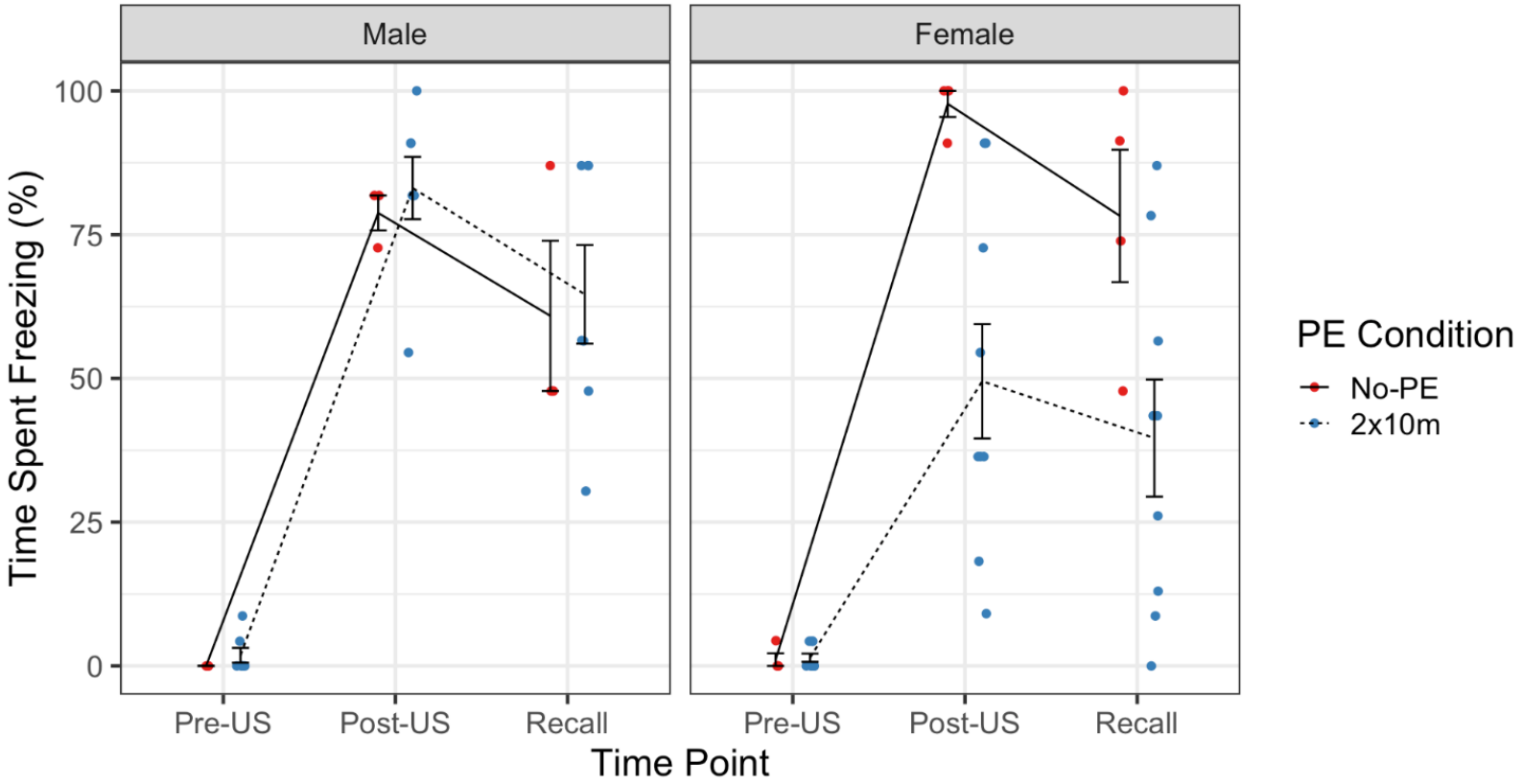

Figure 4.2. Percentage of time *Cacna1c*$^{+/+}$ animals spent freezing during the pre-US and post-US periods of the CFC session, and the recall session, split by sex and PE condition.

4.3.3 Discussion

The results of Experiment 1 showed that two 10-minute sessions of context PE over two days was sufficient to induce LI in females but not in males. Although a single four-hour PE session would likely induce LI in both sexes, the lower threshold of PE required to induce LI in females observed in this experiment would possibly mean that a four-hour PE session could overshadow an effect of *Cacna1c* heterozygosity. As deficits in LI have already been observed in male *Cacna1c*$^{+/-}$ animals, the primary aim of this study was to assess whether this also occurred in females. However, a secondary aim of the study was to replicate the effect in males. Hence, for Experiment 2, it was decided that three sessions of 20-minutes PE over three-days would be used in animals in the PE group. It was hoped that this would be sufficient to induce a small LI effect in males, but not be so strong as to overshadow any effect of *Cacna1c* heterozygosity in females.

4.4 Experiment 2

4.4.1 Methods

4.4.1.1 Animals

50 female and male Sprague-Dawley rats aged between five and eight months with either *Cacna1c*$^{+/+}$ or *Cacna1c*$^{+/-}$ genotype were used: female *Cacna1c*$^{+/-}$ (n = 17); male *Cacna1c*$^{+/-}$ (n = 8); female *Cacna1c*$^{+/+}$ (n = 16); male *Cacna1c*$^{+/+}$ (n = 9). All testing was conducted during the active (dark) phase of the light cycle. All animals were housed as described in Section 2.1. The number of males used in the experiment was considerably lower than the number of females owing to a higher number of females produced from the breeding cohort used (*Cacna1c*$^{+/-}$ Female PE group, n = 8; *Cacna1c*$^{+/-}$ Female no-PE group, n = 9; *Cacna1c*$^{+/+}$ Female PE group,

n = 8; *Cacna1c*$^{+/+}$ Female no-PE group, n = 8; *Cacna1c*$^{+/-}$ Male PE group, n = 4; *Cacna1c*$^{+/-}$ Male no-PE group, n = 5; *Cacna1c*$^{+/+}$ Male PE group, n = 5; *Cacna1c*$^{+/+}$ Male no-PE group, n = 3).

4.4.1.2 Behavioural paradigm

All behavioural sessions for each animal took place in one of the two conditioning chambers described in Section 2.2.2. The assignment of either context to each animal was counterbalanced across groups. On days 1-3 of the experiment, half of the rats in each genotype/sex group were pre-exposed to the conditioning context for a single 20-minute PE session each day (PE group), and the other half of the animals in each group remained in their home cage during the pre-exposure days (no-PE group) (Figure 4.3). Twenty-four hours after the last PE day, rats in both PE and no-PE groups underwent a CFC paradigm, and then a 2-minute recall session a further 24 hours later.

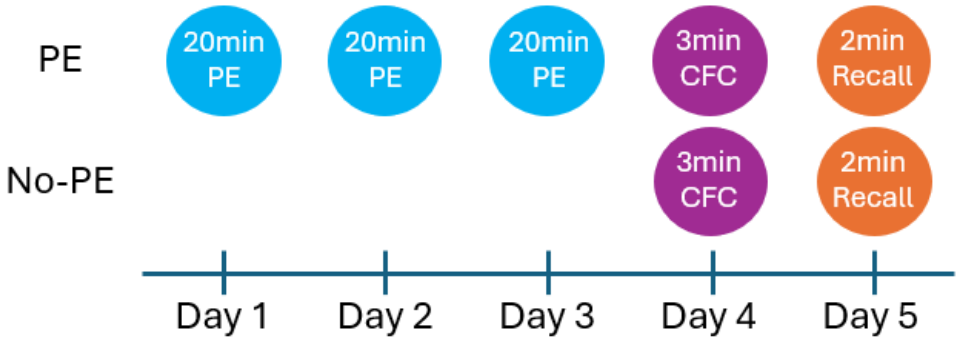

Figure 4.3. Behavioural paradigm of the LI experiment. PE = pre-exposure; CFC = Contextual Fear Conditioning.

4.4.1.3 Euthanasia and Plasma Corticosterone Concentration Quantification

Animals were culled using rising concentration of CO_2 30-minutes after the recall session as described in Section 3.2 and trunk blood samples were extracted as described in Section 2.4.1. For blood plasma isolation and corticosterone concentration ELISA methods, see Section 2.5.

4.4.1.4 Behavioural and Statistical analysis

For the pre-US and post-US periods of the CFC session, and the recall session, percentage of time spent displaying freezing behaviour as well as number of quadrant crossings per minute were measured (as described in Section 2.2.4 and Section 2.2.5 respectively). Statistical analysis was performed as described in Section 2.7. ANOVA models were used to assess whether genotype, sex, pre-exposure group, or any of the interactions between the three variables affected freezing behaviour during the post-US period, and at recall. The same analyses were carried out to assess the dependent variable of quadrant crossings, with an additional model during the pre-US period. Freezing behaviour was not assessed during this time point as this behaviour is not typically displayed prior to US onset. A single ANOVA was performed to assess the impact of *Cacna1c* genotype, sex and PE condition, as well as the interactions between the three variables on plasma corticosterone concentration 30-minutes after the recall session.

4.4.2 Results

4.4.2.1 Freezing

All animals showed negligible freezing prior to administration of the foot-shock, and increased freezing immediately after foot-shock ($t_{97} = 27.4$, $p < 0.001$, 95%CI = [-

75.3, -65.1]) (Figure 4.4). An ANOVA was performed to assess the effect of sex, genotype and PE condition on freezing during the post-US period of the CFC session. Percentage of time spent freezing was square transformed. It was found that there was a main effect of genotype ($F_{1,41}$ = 6.1, p = 0.017, 95%CI = [-3014, -496]), with *Cacna1c*$^{+/-}$ animals freezing more than *Cacna1c*$^{+/+}$. There was a main effect of PE condition ($F_{1,41}$ = 6.4, p = 0.016, 95%CI = [85.8, 2604]), with animals in the no-PE group freezing more than those in the PE group, and a main effect of sex ($F_{1,41}$ = 6.1, p = 0.017 , 95%CI = [280, 2798]), with males freezing more than females. There was no interaction between genotype and sex ($F_{1,41}$ = 1.9, p = 0.170, 95%CI = [-4357, 680]), genotype and PE condition ($F_{1,41}$ = 1.1, p = 0.300, 95%CI = [-2054, 2983]) or sex and PE condition ($F_{1,41}$ = 0.2, p = 0.693, 95%CI = [-3080, 1957]). The three-way interaction was also not significant ($F_{1,41}$ = 2.8, p = 0.100, 95%CI = [-9234, 840]).

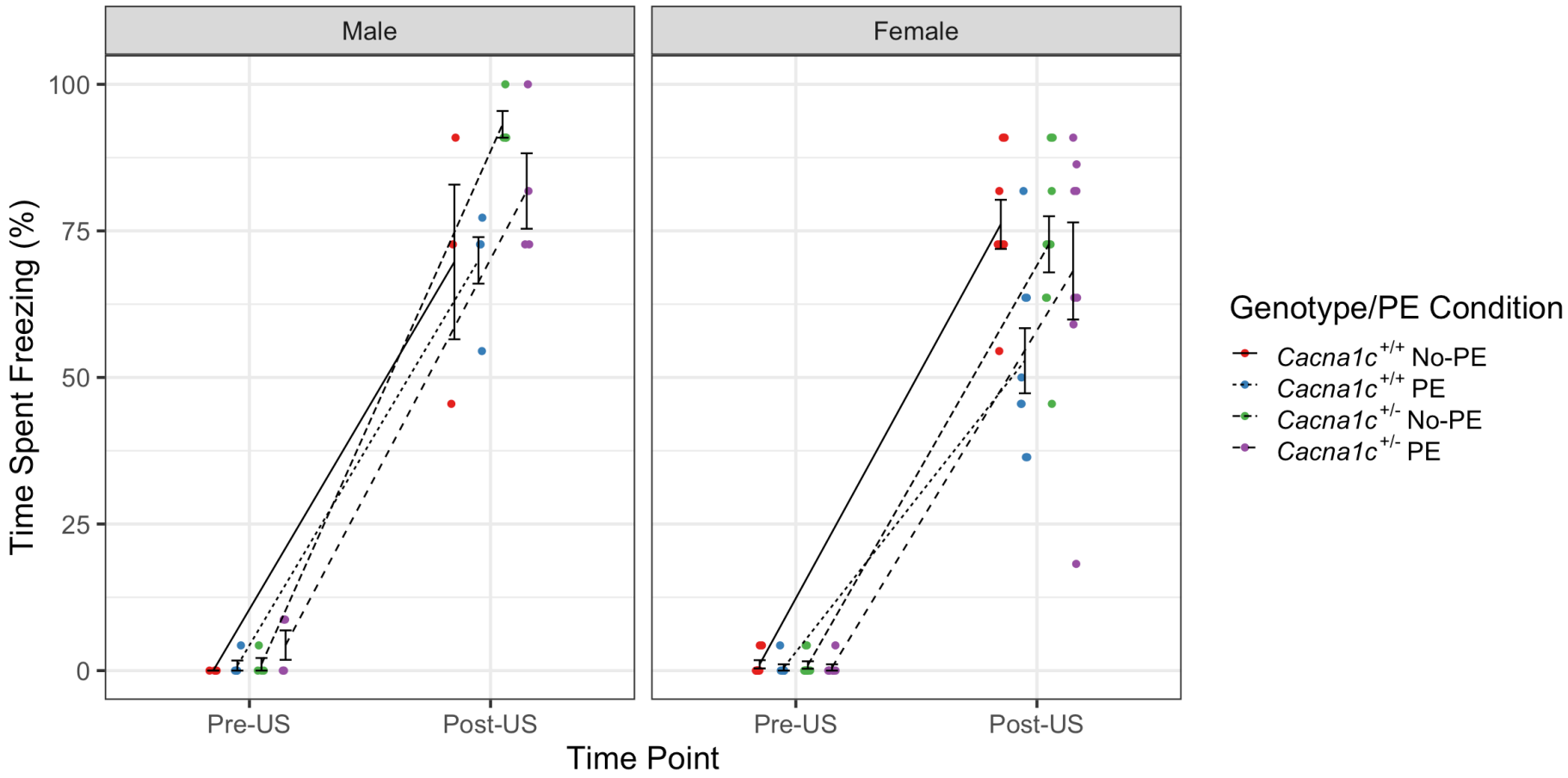

Figure 4.4. Percentage of time spent freezing for each sex, genotype and PE condition during the pre-US and post-US periods of the CFC session.

For the 2-minute recall period, the three-way interaction and the interaction between sex and PE condition were dropped from the model as this improved model fit according to AIC score. The final model included the main effects of genotype, sex, PE condition, and the interactions between genotype and sex, and genotype and PE condition. There was no effect of genotype ($F_{1,44} = 0.4$, $p = 0.555$, 95%CI = [-21.6, 7.79]) (Figure 4.5A), or PE condition ($F_{1,44} = 2.2$, $p = 0.153$, 95%CI = [-3.94, 23.9]) (Figure 4.5C). However, there was an effect of sex ($F_{1,44} = 18.3$, $p < 0.001$, 95%CI = [17.2, 46.6]), with males freezing more than females (Figure 4.5B).

There was no interaction between genotype and sex ($F_{1,44} = 0.8$, $p = 0.382$, 95%CI = [-41.4, 17.4]) (Figure 4.6A), or genotype and PE condition ($F_{1,44} = 2.2$, $p = 0.145$, 95%CI = [-48.3, 7.4]) (Figure 4.6B).

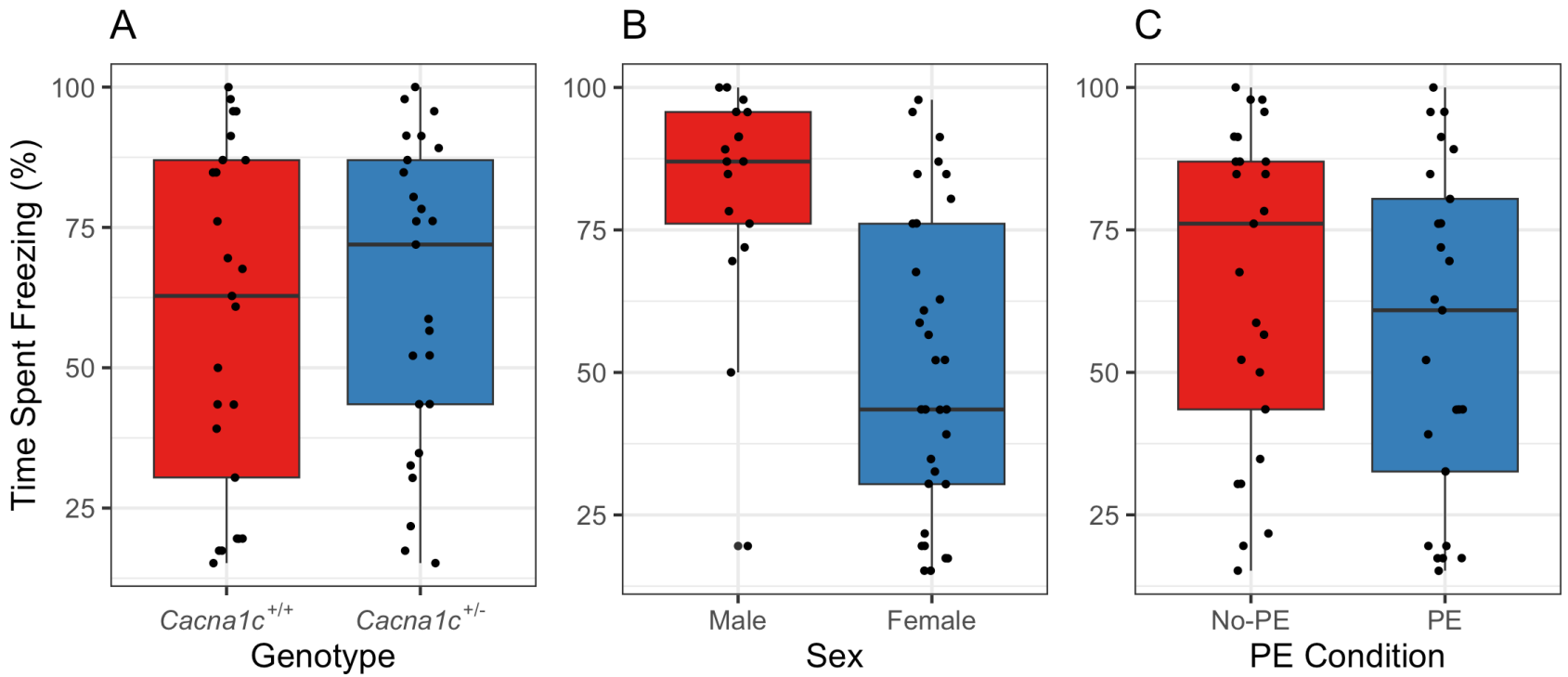

Figure 4.5. Percentage of time spent freezing for each genotype, sex and PE condition during the recall session.

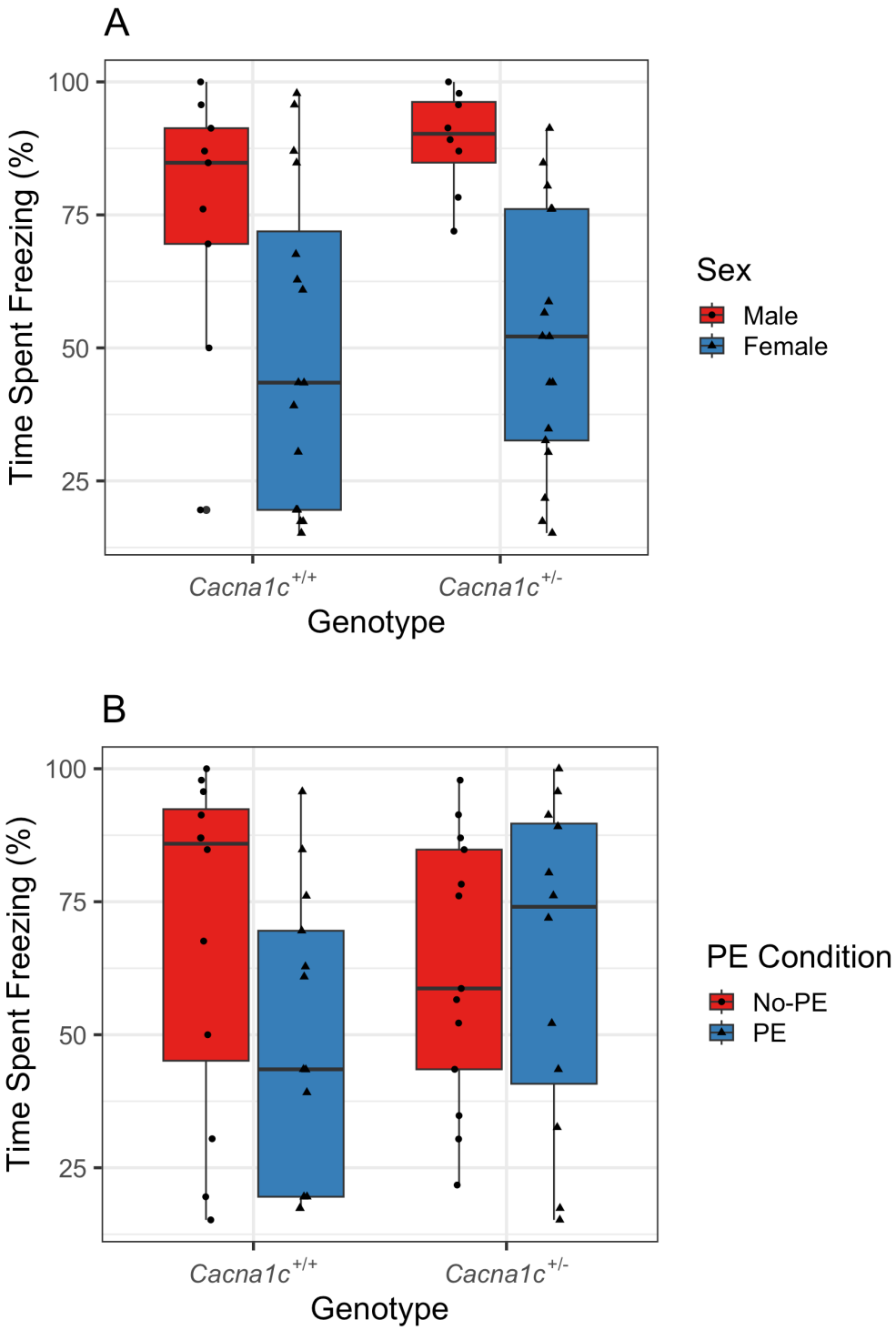

Figure 4.6. Percentage of time spent freezing for each genotype and sex (A) and each genotype and PE condition (B) during the recall session.

In order to increase statistical power to assess the effect of genotype and sex differences on LI, an ANOVA was performed with the freezing data from the post-US and recall periods pooled. Hence, this pooled data contained two data points per animal, from both the immediate post-US period, and the recall session 24 hours later. The dependent variable, percentage of time spent freezing, was square transformed. There was no effect of genotype, however there was a trend towards higher freezing in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ rats ($F_{1,91} = 3.2$, $p = 0.079$, 95%CI = [-2278, -170]) (Figure 4.7A). There was a main effect of sex ($F_{1,91} = 27.4$, $p < 0.001$, 95%CI = [1689, 3797]), with males freezing more than females (Figure 4.7B), and a main effect of PE condition ($F_{1,91} = 5.7$, $p = 0.019$, 95%CI = [190, 2298]), with animals in the no-PE group freezing more than those in the PE group (Figure 4.7C).

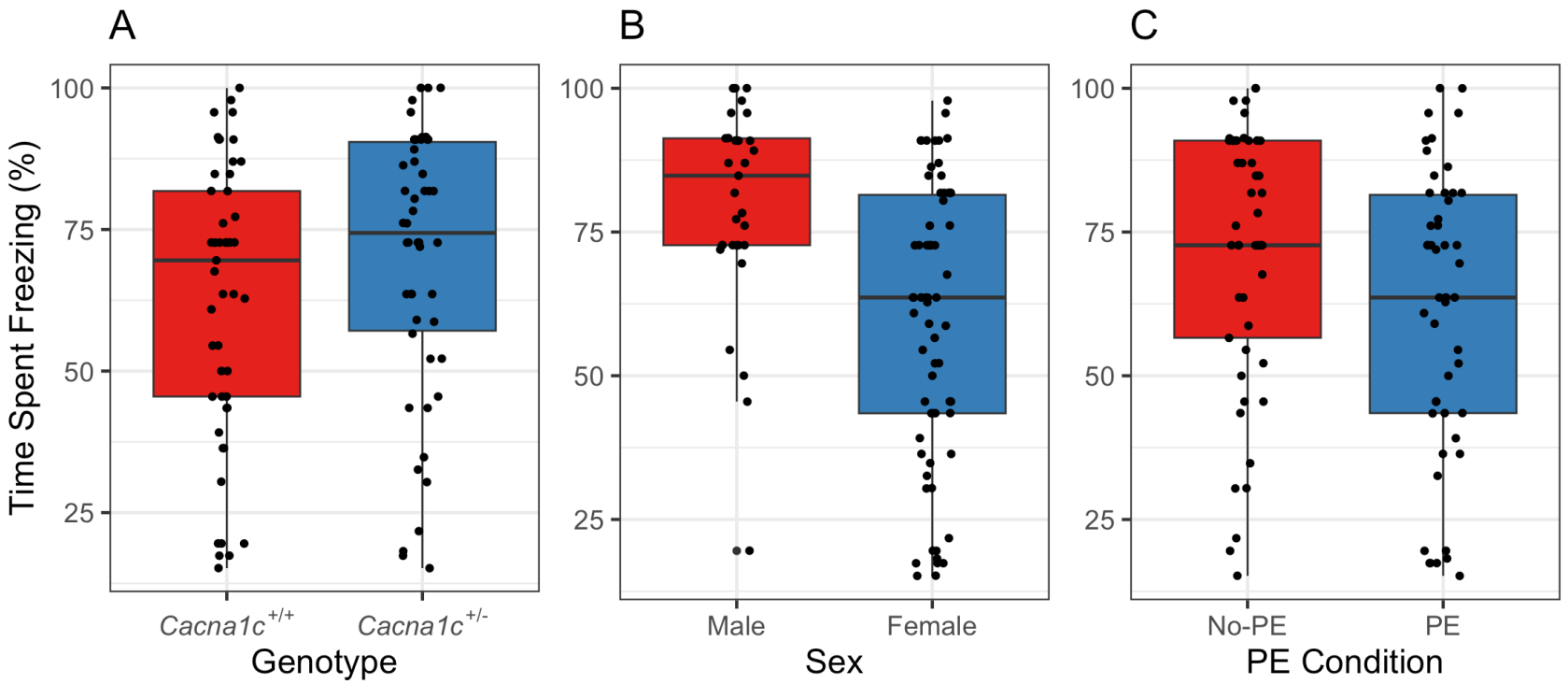

Figure 4.7. Percentage of time spent freezing for each genotype (A), sex (B), and PE condition (C) with data pooled between the post-US period of the CFC session and the recall session. Thus, two data points for each animal are represented within each plot.

In this pooled model, there was also an interaction between genotype and PE condition, indicating that *Cacna1c*$^{+/-}$, but not *Cacna1c*$^{+/+}$, animals showed a deficit in LI ($F_{1,91}$ = 4.9, p = 0.029, 95%CI = [-465, 3750]) (Figure 4.8). Pairwise post hoc analysis showed no significant difference in freezing between the no-PE and PE groups in the *Cacna1c*$^{+/-}$ animals (p = 0.574, 95%CI = [-1067, 1913]), whereas in the *Cacna1c*$^{+/+}$ animals, there was higher freezing in the no-PE compared with PE group (p = 0.007, 95%CI = [575, 3555]), indicating an LI impairment in *Cacna1c*$^{+/-}$ animals relative to *Cacna1c*$^{+/+}$. Crucially, this interaction between genotype and PE condition was not found to interact with sex in a three-way manner ($F_{1,91}$ = 1.9, p = 0.182, 95%CI = [-7068, 1362]), indicating that the effect of *Cacna1c* genotype on LI occurs similarly between sexes. The other two-way interactions were not found to be significant (genotype-by-sex: $F_{1,91}$ = 2.5, p = 0.115, 95%CI = [-3899, 316]; PE condition-by-sex: $F_{1,91}$ = 0.3, p = 0.601, 95%CI = [-2664, 1551]).

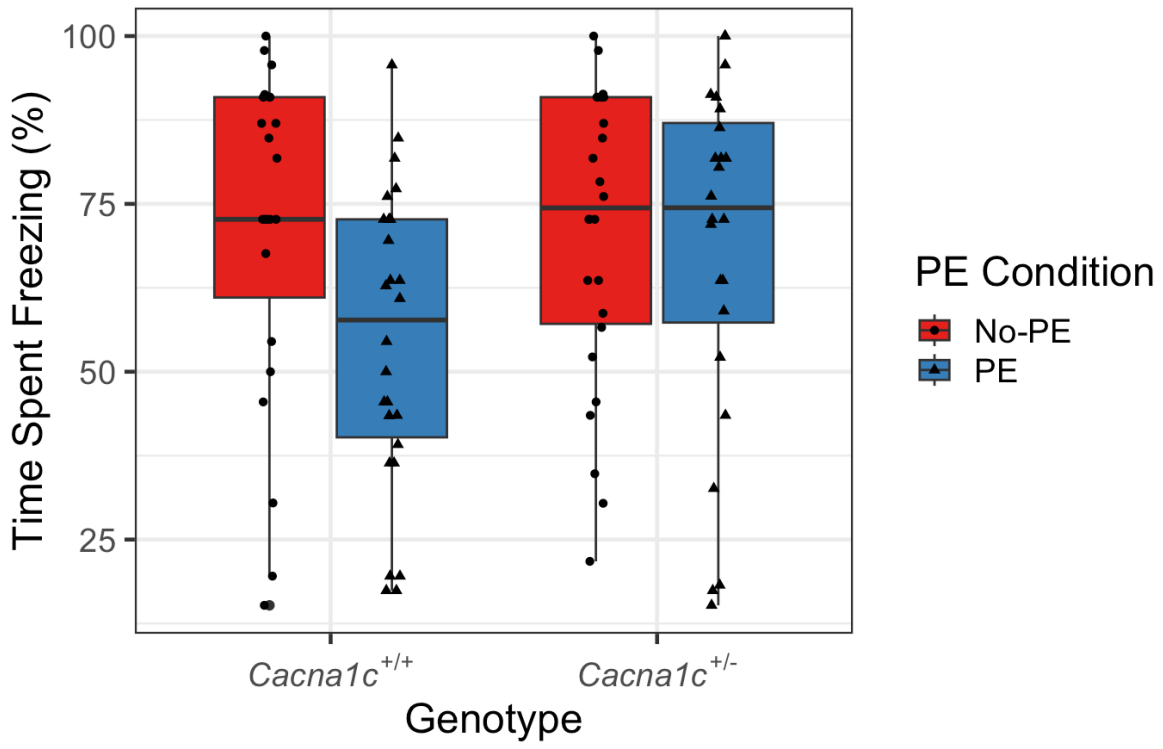

Figure 4.8. Percentage of time spent freezing for each genotype and PE condition with data pooled from the post-US period of the CFC session and the recall session. Thus, two data points for each animal are represented within each plot.

Although the overall interaction term did not reach statistical significance at $\alpha = 0.05$, pairwise post-hoc analysis of the interaction between genotype and sex showed that in females, there was no difference in freezing between *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ animals ($p = 0.592$, 95%CI = [-1539, 883], whereas in males there was greater freezing in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals ($p = 0.027$, 95%CI = [-3845, -395]) (Figure 4.9).

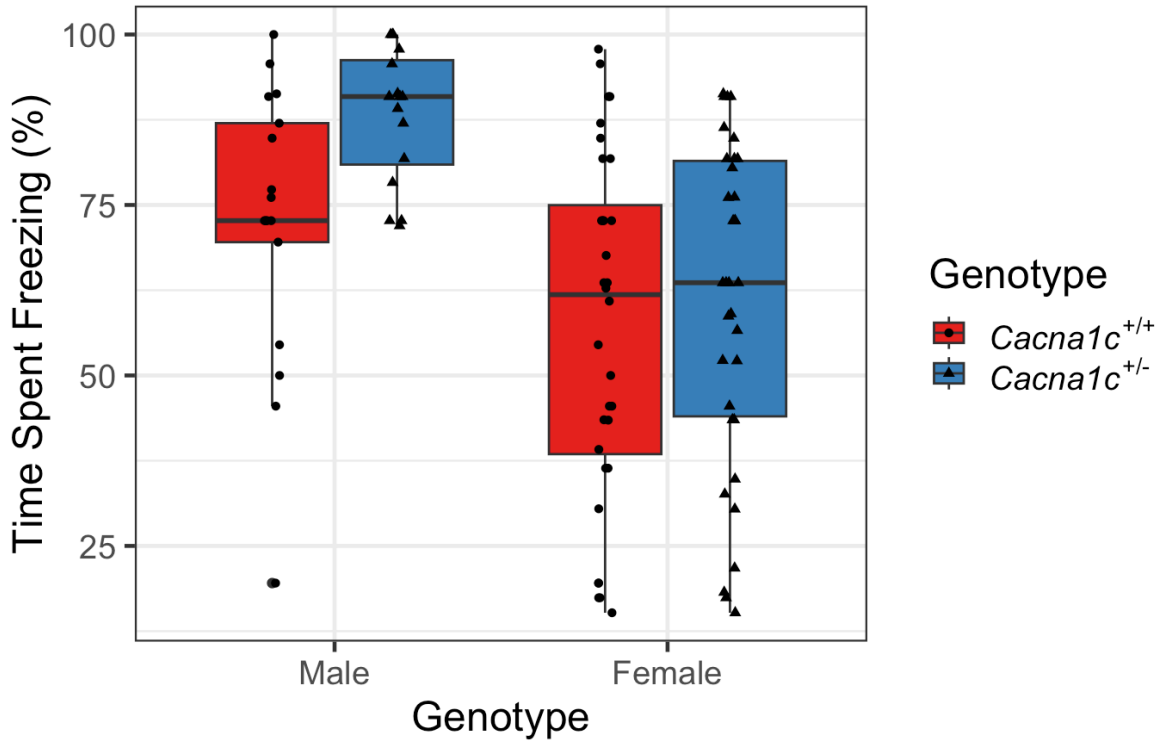

Figure 4.9. Percentage of time spent freezing for each sex and genotype with data pooled from the post-US period of the CFC session and the recall session. Thus, two data points for each animal are represented within each plot.

4.4.2.2 Quadrant crossings

An ANOVA was carried out to assess whether there were any significant effects of sex, genotype, PE condition, or the interactions between these variables on the number of quadrant crossings made during the pre-US period of the CFC session (Figure 4.10). The three-way interaction was removed from the model as this

improved AIC score. This was also the case for the two-way interactions between genotype and PE condition, and sex and PE condition. The final model included the main effects of genotype, sex, and PE condition as well as the two-way interaction between genotype and sex. There was no main effect of genotype ($F_{1,45}$ = 0.3, p = 0.609, 95%CI = [-1.05, 1.43]). However, there was a main effect of sex ($F_{1,45}$ = 5.2, p = 0.028, 95%CI = [-2.5, 0.0]), with females making a greater number of quadrant crossings compared to males, and there was a main effect of PE condition ($F_{1,45}$ = 15.0, $p < 0.001$, 95%CI = [1.08, 3.43]), with more crossings made by rats in the no-PE group compared with those in the PE group. The interaction between genotype and sex was not significant ($F_{1,45}$ = 1.6, p = 0.208, 95%CI = [-4.1, 0.9]).

There was an overall decrease in quadrant crossings between the pre-US and post-US periods (t_{97} = -12.7, $p < 0.001$, 95%CI = [-6.6, -4.8]). An ANOVA was performed to assess the effect of sex, genotype and PE condition on quadrant crossings during the post-US period of the CFC session. There was no main effect of genotype ($F_{1,41}$ = 2.2, p = 0.145, 95%CI = [-0.5, 1.7]). However, there was a main effect of sex ($F_{1,41}$ = 4.6, p = 0.039, 95%CI = [-2.3, -0.1]), with females making a greater number of crossings compared to males, and a main effect of PE condition ($F_{1,41}$ = 10.9, p = 0.002, 95%CI = [-2.3, -0.1]), with a greater number of crossings made by rats in the PE compared to the no-PE group. This appeared to be driven by an interaction between sex and PE condition ($F_{1,41}$ = 5.6, p = 0.023, 95%CI = [0.4, 4.7]). Pairwise post-hoc analysis revealed that in males, there was no difference in the number of quadrant crossings made between PE groups (p = 0.963, 95%CI = [-1.8, 1.8]). However, in females, a greater number of quadrant crossings were made by the PE compared with no-PE group ($p < 0.001$, 95%CI = [-3.8, -1.3]), indicating an LI effect in females, but not in males. There was no interaction between genotype and sex ($F_{1,41}$ = 0.4, p = 0.496, 95%CI = [-2.8, 1.6]) or genotype and PE condition ($F_{1,41}$ = 0.4, p = 0.545, 95%CI = [-3.0, 1.4]). The three-way interaction was also not significant ($F_{1,41}$ = 0.3, p = 0.590, 95%CI = [-5.6, 3.2]).

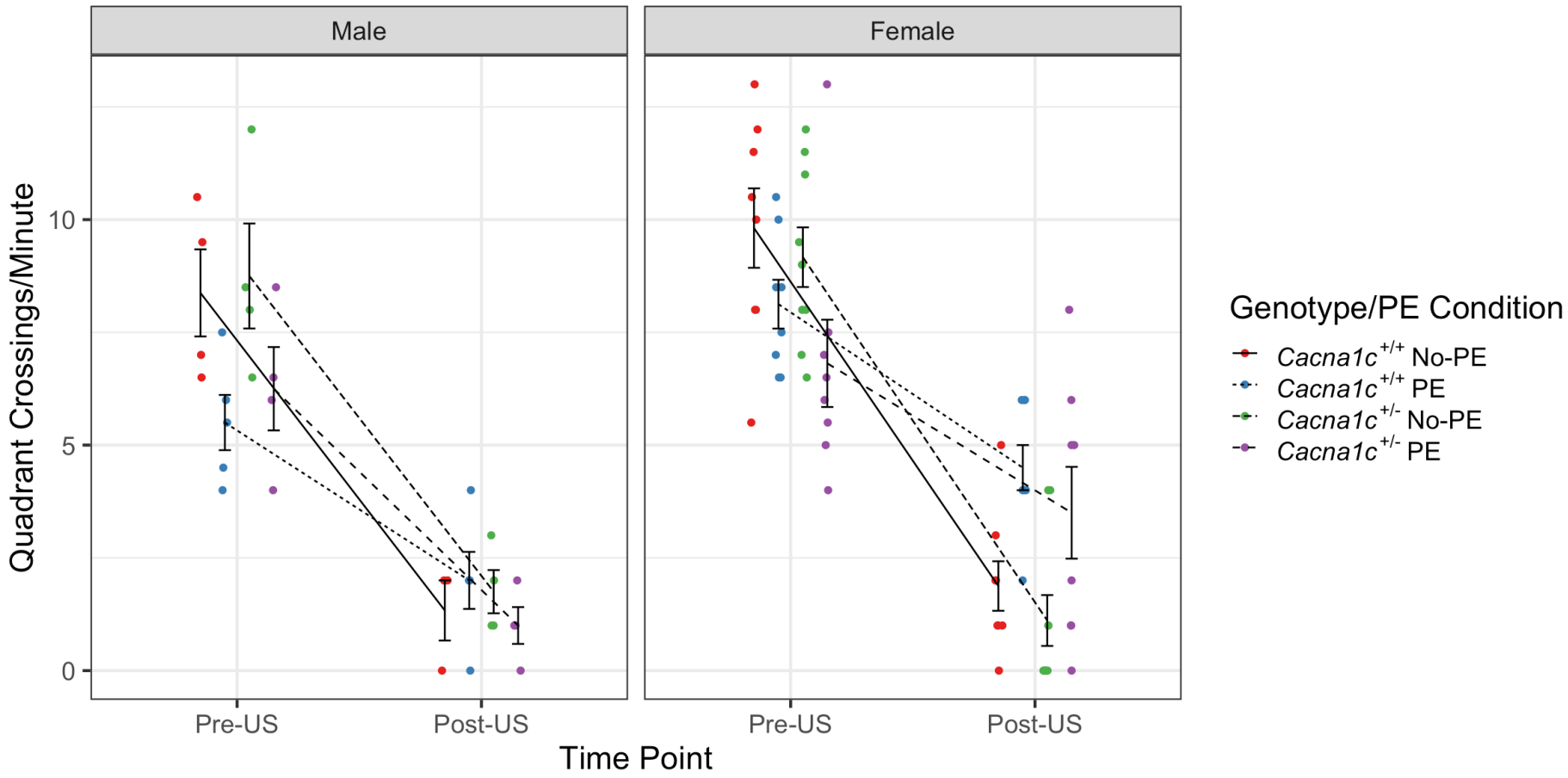

Figure 4.10. Number of quadrant crossings per minute for each sex, genotype and PE condition during the pre-US and post-US periods of the CFC session.

An ANOVA was performed to assess the effect of sex, genotype and PE condition on quadrant crossings during the recall session. Quadrant crossings per minute was inverse square root transformed. There was found to be no main effect of genotype ($F_{1,42}$ = 2.0, p = 0.164, 95%CI = [-0.2, 0.0]) (Figure 4.11A) or PE condition ($F_{1,42}$ = 0.6, p = 0.426, 95%CI = [-0.1, 0.2]) (Figure 4.11C). However, there was a main effect of sex ($F_{1,42}$ = 19.5, $p < 0.001$, 95%CI = [0.1, 0.4]), with females making a greater number of quadrant crossings compared with males (Figure 4.11B). There was no interaction between genotype and sex ($F_{1,42}$ = 0.1, p = 0.762, 95%CI = [-0.3, 0.2]), genotype and PE condition ($F_{1,42}$ = 1.1, p = 0.301, 95%CI = [-0.1, 0.3]) or sex and PE condition ($F_{1,42}$ = 0.1, p = 0.720, 95%CI = [-0.3, 0.2]). The three-way interaction was also not significant ($F_{1,42}$ = 0.8, p = 0.391, 95%CI = [-0.7, 0.3]).

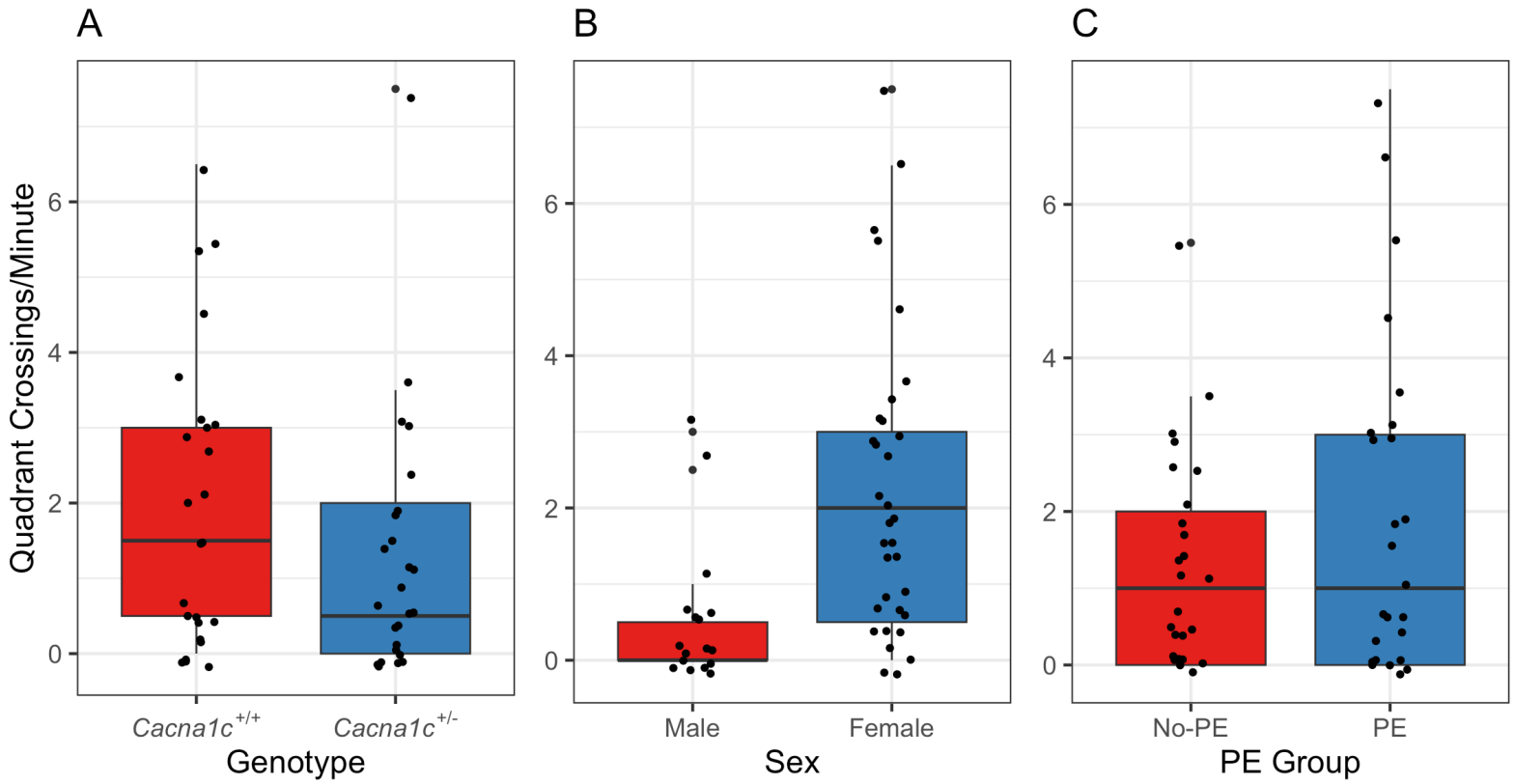

Figure 4.11. Number of quadrant crossings per minute for each genotype (A), sex (B) and PE-group (C) during the recall session.

In order to increase statistical power to assess the effects of genotype and sex on LI, an ANOVA was performed with the quadrant crossings data from the post-US and recall periods pooled. Percentage of time spent freezing was log transformed. The three-way interaction and the interaction between genotype and sex were removed from the model as this improved AIC score. The final model included the main effects of genotype, sex and PE condition, as well as the interactions between genotype and PE condition, and sex and PE condition. There was a main effect of genotype ($F_{1,93} = 5.5$, $p = 0.021$, 95%CI = [0.0, 0.5]), with more frequent quadrant crossings in *Cacna1c*$^{+/-}$ compared to *Cacna1c*$^{+/+}$ animals (Figure 4.12A), a main effect of sex ($F_{1,93} = 16.3$, $p < 0.001$, 95%CI = [-0.7, -0.2]), with more frequent quadrant crossings in females compared to males (Figure 4.12B), and a main effect of PE condition ($F_{1,93} = 6.6$, $p = 0.012$, 95%CI = [-0.5, 0.0]), with more frequent quadrant crossings in the PE compared with no-PE group (Figure 4.12C).

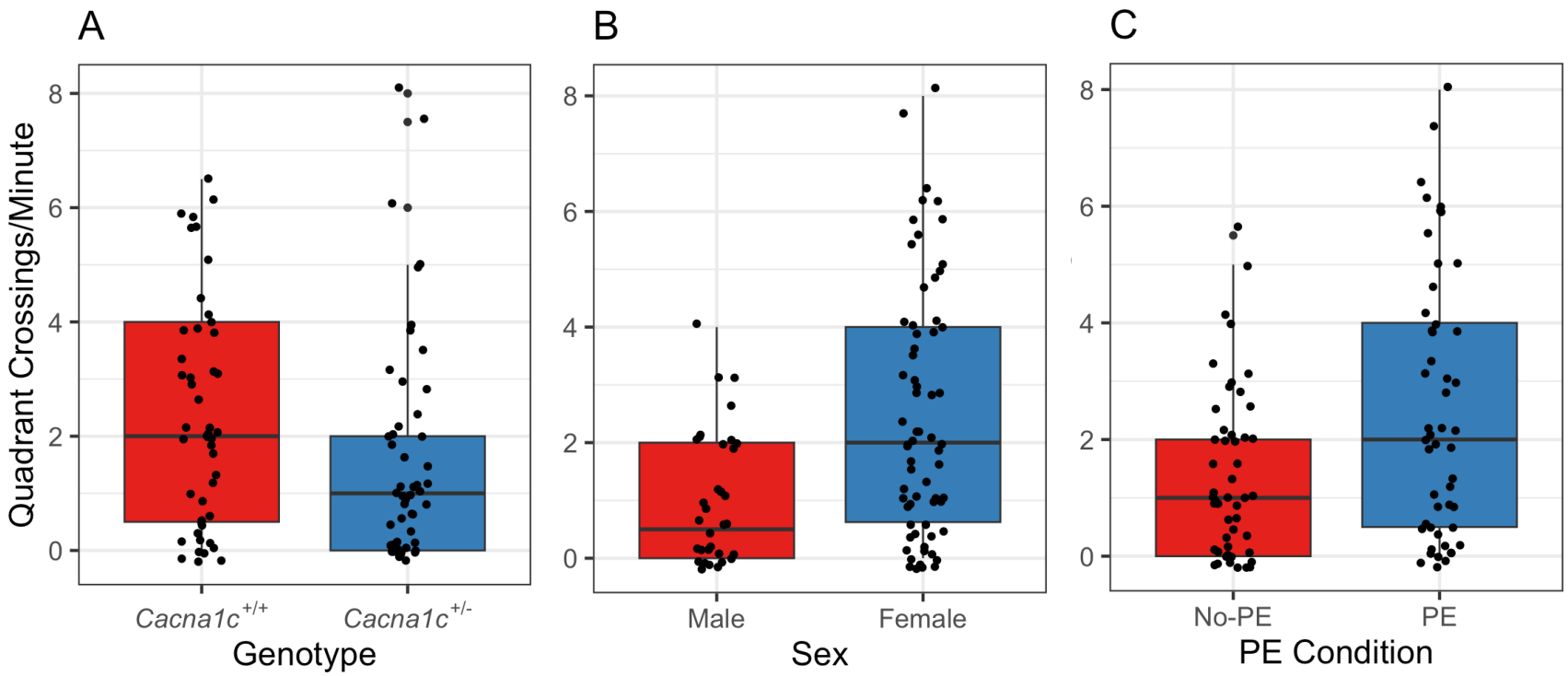

Figure 4.12. Quadrant crossings per minute for each genotype (A), sex (B), and PE condition (C) with data pooled between the post-US period of the CFC session and the recall session.

The interaction between genotype and PE condition was not significant ($F_{1,93}$ = 0.9, p = 0.354, 95%CI = [-0.7, 0.2]). There was a trend towards an interaction between sex and PE condition ($F_{1,93}$ = 3.8, p = 0.05, 95%CI = [-0.0, 1.0]) (Figure 4.13). Pairwise post-hoc analysis revealed that in males, there was no difference in quadrant crossings between animals in the PE and no-PE group (p = 0.993, 95%CI = [-0.4, 0.4]), whereas in females, animals in the PE group made more quadrant crossings than those in the no-PE group (p = 0.005, 95%CI = [-0.8, -0.2]).

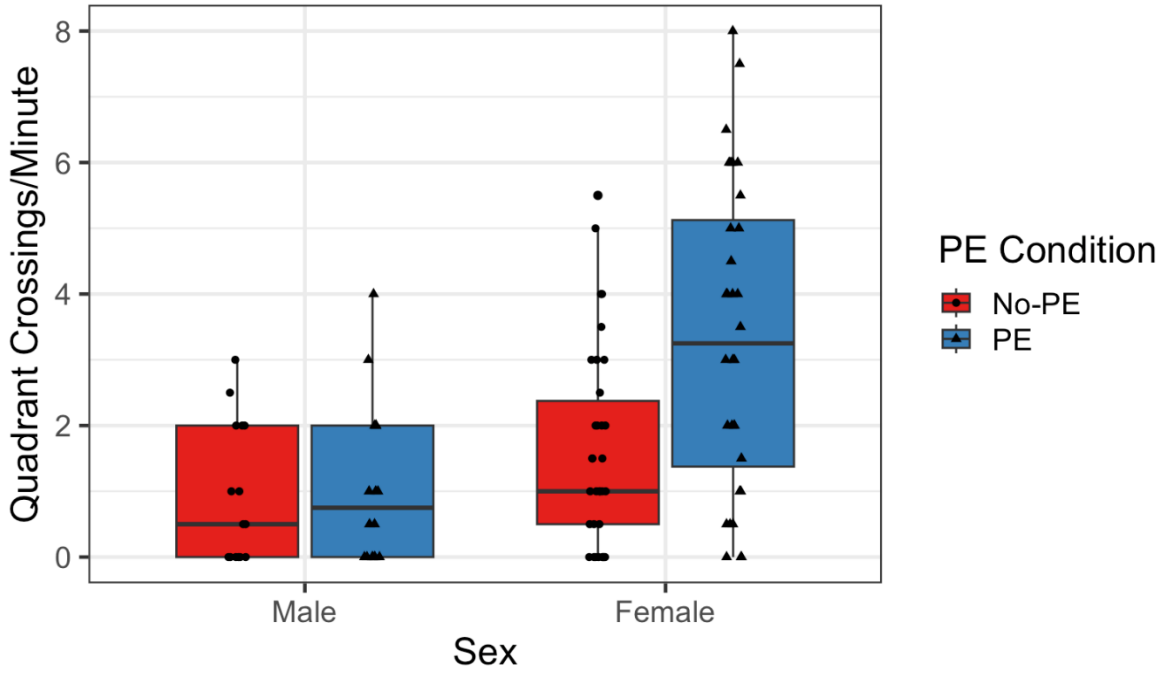

Figure 4.13. Quadrant crossings per minute for each sex and PE condition with data pooled from the post-US period of the CFC session and the recall session.

4.4.2.3 Plasma corticosterone concentration

Plasma corticosterone concentration was square root transformed. There was no main effect of genotype ($F_{1,42}$ = 1.0, p = 0.327, 95%CI = [-0.3, 3.1]), or PE condition ($F_{1,42}$ = 1.2, p = 0.270, 95%CI = [-0.5, 2.9]). However, there was a main effect of sex ($F_{1,42}$ = 19.6, p < 0.001 95%CI = [-5.5, -2.1]), with greater blood plasma corticosterone concentration in males compared to females. There was no

interaction between genotype and sex ($F_{1,42}$ = 2.4, p = 0.130, 95%CI = [-6.0, 0.9]), genotype and PE condition ($F_{1,42}$ = 1.2, p = 0.275, 95%CI = [-4.0, 2.9]), or sex and PE condition ($F_{1,42}$ = 0.8, p = 0.378, 95%CI = [-5.1, 1.8]). However, there was a three-way interaction ($F_{1,42}$ = 4.4, p = 0.042, 95%CI = [-14.0, -0.3]) (Figure 4.14). Pairwise post hoc analysis of the three-way interaction revealed that in female *Cacna1c*$^{+/-}$ rats, there was no difference in blood plasma corticosterone concentration between no-PE and PE groups (p = 0.234, 95%CI = [-4.5, 1.1]). There was no effect of PE group in female *Cacna1c*$^{+/+}$ rats. However, there was a trend towards higher corticosterone concentration in the no-PE group compared with the PE group (p = 0.089, 95%CI = [-0.4, 5.4]). In males, the opposite finding was observed, with no difference between PE and no-PE groups in the *Cacna1c*$^{+/+}$ animals (p = 0.789, 95%CI = [-3.3, 4.4]), but a trend towards higher corticosterone concentration in the no-PE group compared with the PE group in *Cacna1c*$^{+/-}$ animals (p = 0.088, 95%CI = [-0.5, 7.6]).

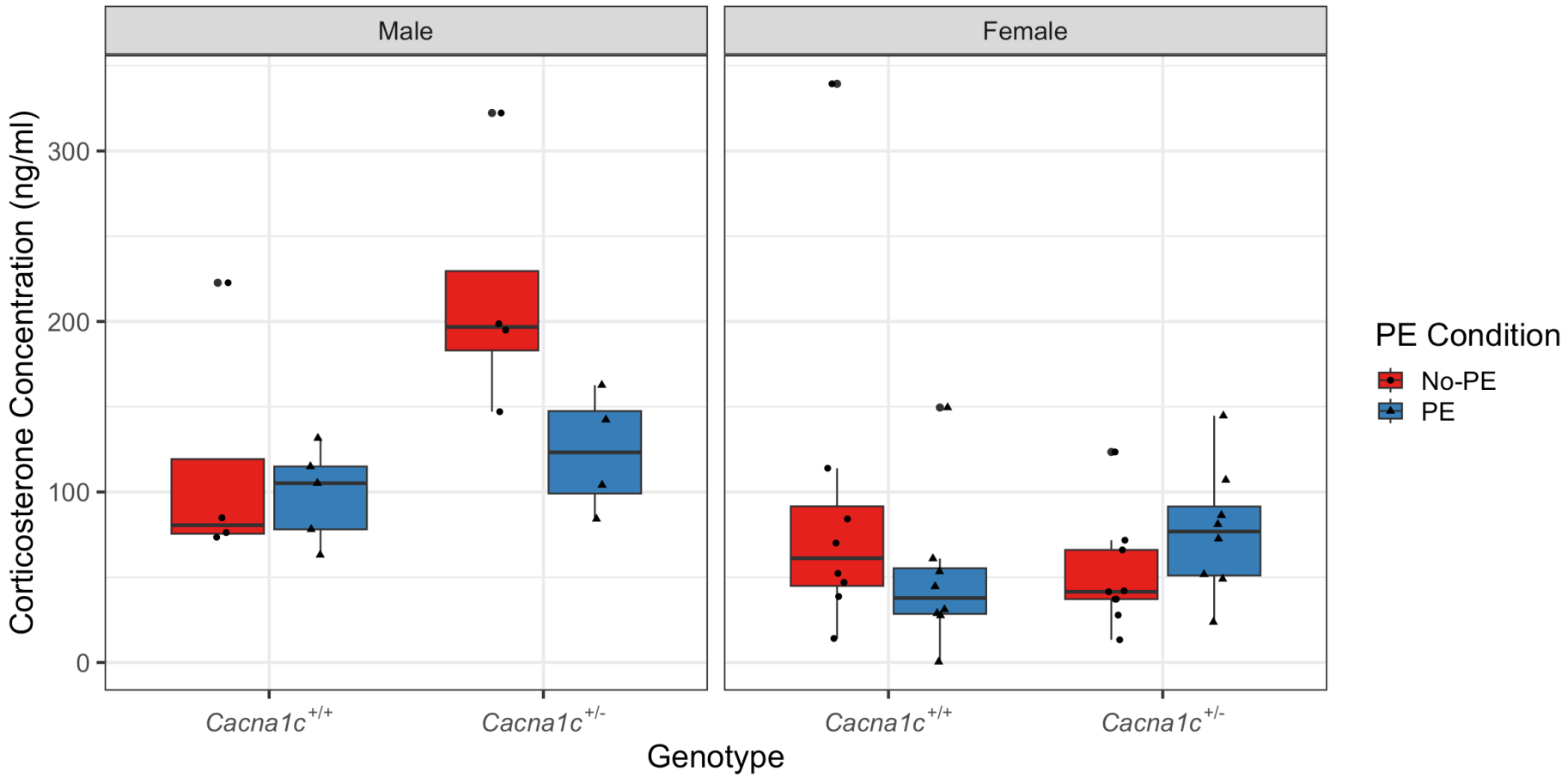

Figure 4.14. Blood plasma corticosterone concentration (ng/ml) 30 minutes after the recall session across sex, genotype and PE condition.

4.4.2.4 Summary of Results

All results are summarised in Table 4.1. Using freezing behaviour as an outcome measure, there was greater freezing in the no-PE compared with PE groups in both the post-US period and when the post-US and recall periods were pooled, indicating an LI effect. There was no interaction between *Cacna1c* genotype and PE condition in either the post-US or recall sessions. However, when data from the post-US and recall periods were pooled, *Cacna1c*$^{+/+}$ animals showed an LI effect of greater freezing in the no-PE compared with PE groups. However, this difference was not observed in *Cacna1c*$^{+/-}$ animals, indicating a deficit in LI in these animals. This interaction between *Cacna1c* genotype and PE group did not interact with sex, indicating the LI deficit in *Cacna1c*$^{+/-}$ animals was present in both males and females. Across all time points, males displayed greater freezing behaviour than females. In the pooled data, *Cacna1c*$^{+/-}$ males showed greater freezing than *Cacna1c*$^{+/+}$ males, a finding which was not observed in females.

There were more frequent quadrant crossings in the no-PE compared to PE group during the pre-US period, but the opposite finding of more frequent crossings in the PE compared with no-PE group was observed during the post-US and pooled post-US and recall data. This was found to be specific to females, indicating that LI is reflected in locomotor activity specifically in females. However, using this measure, a deficit in LI was not observed in *Cacna1c*$^{+/-}$ animals. Females showed more frequent quadrant crossings compared with males at all time points. No overall genotype differences were observed at any individual time point; however, more frequent crossings were observed in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ rats when data from the post-US and recall periods were pooled.

Males showed higher plasma corticosterone concentration compared to females. Female *Cacna1c*$^{+/+}$ showed higher plasma corticosterone concentration in the no-PE compared with PE group, but no difference in *Cacna1c*$^{+/-}$ animals. However, in males, there was no difference in *Cacna1c*$^{+/+}$ animals, but higher plasma corticosterone concentration in the no-PE compared with PE *Cacna1c*$^{+/-}$ rats.

Table 4.1 Summary of the effect of sex, genotype, and PE condition on freezing, quadrant crossings and plasma corticosterone concentration. * indicates this did not reach statistical significance at $\alpha = 0.05$ but was statistically significant at $\alpha = 0.10$. † indicates a non-statistically significant interaction term but a post-hoc finding is significant at $\alpha = 0.05$. ‡ indicates a statistically significant interaction term, but a post-hoc finding only significant at $\alpha = 0.10$.

		Dependent Variable							
		Freezing			Quadrant Crossings				Corticosterone Concentration
		Post-US	Recall	Post-US/Recall Pooled	Pre-US	Post-US	Recall	Post-US/Recall Pooled	
Effect	Genotype	$^{+/-} > ^{+/+}$	-	$^{+/-} > ^{+/+}$ *	-		-	$^{+/-} > ^{+/+}$	-
	PE Condition	No-PE > PE	-	No-PE > PE	No-PE > PE	PE > No-PE	-	PE > No-PE	-
	Sex	M > F	M > F	M > F	F > M	F > M	F > M	F > M	M > F
	G x PE	-	-	$^{+/+}$: No-PE > PE	-	-	-	-	-
	G x S	-	-	M: $^{+/-} > ^{+/+}$ †	-	-	-	-	-
	PE x S	-	-	-	-	F: PE > No-PE	-	F: PE > No-PE*†	-
	G x PE x S	-	-	-	-	-	-	-	F $^{+/+}$: No-PE > PE ‡ M $^{+/-}$: No-PE > PE ‡

4.5 Discussion

Processes such as LI allow us to selectively attend to environmental stimuli which best predict salient outcomes, thus facilitating adaptive behaviour. LI deficits have been repeatedly observed in schizophrenia patients and high-schizotypal individuals (Myles et al., 2023). Hence, the underlying aberrant cognitive processes and neurobiological substrates that lead to such deficits may explain the development of altered perceptions that occur in individuals experiencing psychosis, as well as the co-occurring cognitive impairments. Previous work by the lab group (Tigaret et al., 2021) demonstrated LI deficits in *Cacna1c*$^{+/-}$ male rats with co-occurring aberrant synaptic plasticity in the hippocampus, indicating that the schizophrenia risk gene *CACNA1C* may play a role in the development of LI deficits in patients by affecting hippocampal synaptic plasticity processes. The aim of this thesis chapter was to assess whether this deficit was also seen in *Cacna1c*$^{+/-}$ females, with a secondary aim of replicating this effect in males with a shorter PE duration split across multiple days.

As a single four-hour PE session was used in the experiment by Tigaret et al. (2021), Experiment 1 was carried out to determine whether two sessions of 10-minutes of context PE split over 48 hours would be sufficient to induce LI in both male and female *Cacna1c*$^{+/+}$ animals. It was found that this initial duration of context PE was largely sufficient to induce LI in females, but not in males. This led to the decision to use a longer duration of three sessions of 20-minutes of context PE over three days during the second experiment. However, this was still a considerably lower amount of PE than was used in the original experiment by Tigaret et al. (2021). This finding of a sex difference in the threshold of PE at which LI would be exhibited was unanticipated, and to the author's knowledge, has not been previously reported in the literature, and thus requires further investigation to verify. If the relationship between context PE and subsequent CFC is sexually dimorphic, this may mean that the impact of neuropsychiatric risk variants in genes such as *CACNA1C* may interact

with such differences to produce sex-specific effects, possibly contributing to the sex differences observed in psychotic and mood disorders.

In Experiment 2, it was found that the LI deficit previously observed in males *Cacna1c*$^{+/-}$ rats was also found in females. However, pooling the freezing data from both the post-US period of the CFC session and the recall session 24 hours later was required to detect this at a statistically significant level. The female sample size in this experiment was similar to that of males used in Tigaret et al. (2021). Hence, it may be that the shorter pre-exposure duration used in this experiment resulted in less LI. This was not found to interact with sex, indicating that the effect of *Cacna1c* heterozygosity on latent inhibition occurs in a similar manner between sexes. However, owing to constraints of available male animals, it is entirely possible that this experiment was not sufficiently powered to detect a sex difference. Hence, although the data appears to show that *Cacna1c* heterozygosity results in LI deficits in both sexes, there may be slight differences in the extent to and circumstances in which this occurs between males and females that cannot be inferred from this experiment. Despite these constraints, the fact that pooling the data from both time points resulted in a statistically significant finding indicates that the effect of *Cacna1c* heterozygosity on LI is relatively broad in affecting both the immediate fear response to the US, as well as later recall of the fear memory, despite the fact that CFC acquisition and short-term memory, and long-term memory retrieval are cognitively distinct processes with differing contributions of hippocampus subregions subserving these processes (Lee and Kesner, 2004; Rudy et al., 2004).

An overall effect of more frequent quadrant crossings and less time spent freezing was observed in females compared to males at every time point. This is in line with the data from Chapter 3, as well as literature relating to sex differences in fear associated behaviour, with females showing generally lower rates of freezing compared with males in response to fearful stimuli (Graham et al., 2009), and generally being more active (Simpson and Kelly, 2012). During the pre-US period, there was also a main effect of PE condition on quadrant crossings, with animals in the no-PE group showing more locomotor activity relative to those in the PE group.

Exposure to a novel environment is known to increase locomotor activity through exploratory behaviour, which is thought to be an adaptive behaviour to enhance the security of the animal (Whishaw et al., 2006). Thus, this finding is likely as a result of the novelty of the context to the animals who had not undergone PE as they had never previously entered the context, or any other conditioning chamber, whereas animals in the PE group had been in the conditioning chamber during the three previous days.

Locomotor activity and freezing are inherently antagonistic measures, as when an animal freezes there is an absence of movement. However, locomotor activity was chosen as an alternative measure of fear behaviour, as it may encapsulate behavioural changes such as more cautious movement that would not be captured by measuring only freezing that may reflect increased anxiety (Sestakova et al., 2013). An overall LI effect was observed in females when analysing quadrant crossings, with fewer crossings made by female animals in the no-PE compared with PE group. However, this did not interact with genotype, even when data from the post-US and recall sessions were pooled. This finding indicates that the overall effect of LI in females is relatively strong and is reflected in both measures of fear, but that the LI deficit induced by *Cacna1c* heterozygosity is specific to freezing behaviour in females. As males showed such a high level of freezing, they showed very little locomotor activity. Hence, this floor effect diminishes the ability to investigate whether a LI effect and a concurrent deficit of this effect in *Cacna1c*$^{+/-}$ animals would be reflected in changes to locomotor activity in males.

When measuring freezing behaviour pooled between the post-US and recall periods, an interaction between sex and genotype was observed, with more freezing in male *Cacna1c*$^{+/-}$ animals compared to *Cacna1c*$^{+/+}$ animals, but no differences in freezing between genotypes in females, albeit with this interaction not reaching statistical significance. This finding was surprising, as in the contextual fear memory generalisation experiment of the previous chapter, an interaction was observed between sex and genotype, but in a different direction. In the generalisation experiment, *Cacna1c*$^{+/-}$ females froze more than *Cacna1c*$^{+/+}$ females, with no

differences in males. As the sample size of males in this experiment was small, it may be that the genotype difference observed here was spurious. However, the lack of genotype effect in females here, despite a previously observed difference, may indicate that the sex-specific effects of *Cacna1c* heterozygosity on the behavioural response to fear are complex, and may be sensitive to specific experimental parameters such as pre-exposure duration, and pre-exposure to one or multiple contexts.

Blood plasma corticosterone concentration was measured to assess whether effects of genotype on LI would be associated with changes in the HPA-axis response. A three-way interaction was observed between the effects of *Cacna1c* genotype, sex and PE condition. Pairwise post-hoc analyses of this interaction showed opposing effects of *Cacna1c* heterozygosity on LI between sexes. In females, higher blood plasma corticosterone concentration was seen in the no-PE compared with PE group in *Cacna1c*$^{+/+}$ animals (albeit not at the level of statistical significance), with no difference in *Cacna1c*$^{+/-}$ animals. This is in line with the hypothesis of *Cacna1c* heterozygosity leading to a deficit in LI, whereby no difference in HPA-axis response between PE groups would be anticipated in *Cacna1c*$^{+/-}$ rats. However, in males, the direction of effect was surprising, with no difference between PE conditions in *Cacna1c*$^{+/+}$ animals, but greater blood plasma corticosterone concentration in the no-PE compared with PE group in *Cacna1c*$^{+/-}$ animals (albeit not at the level of statistical significance). This is contrary to the expectation of a greater stress response in animals showing higher behavioural fear expression. Owing to the small sample size of males, these results must be interpreted with caution. Altered HPA-axis activity has been observed in this model (Moon et al., 2024) and there are also known sex differences in the stress response (Heck and Handa, 2019), which is known to impact hippocampal fear learning processes (Lesuis et al., 2021). Thus, the neurobiological mechanisms involving the HPA-axis underpinning the effects of *Cacna1c* heterozygosity on LI may be sexually dimorphic.

A main effect of sex was found, with greater blood plasma corticosterone concentration in males compared with females. This was contrary to the prediction

that females would have higher corticosterone concentration than males, which would be in line with the literature, which suggests that at baseline, females have greater blood plasma corticosterone concentrations compared to males (Goel et al., 2014). Similarly, results of the fear memory generalisation experiment of Chapter 3 showed a main effect of sex, in which females had higher corticosterone concentration compared to males, an observation which was found to be specific to recall in the CFC context. Hence, it would be anticipated that 30-minutes after a recall session in a context in which they previously had experienced CFC, females would show a greater stress response compared to males. However, in the presence of the three-way interaction, this main effect must be interpreted with caution and may reflect the differential impact of genotype and PE condition between males and females.

Taken together, these results suggest that *Cacna1c* heterozygosity broadly affects both sexes in a similar manner of leading to deficits in LI in *Cacna1c*$^{+/-}$ relative to *Cacna1c*$^{+/+}$ animals. However, genotype may lead to sex-specific changes to the associated stress response, but further experiments are required to verify this finding. Sex differences in *Cacna1c*$^{+/+}$ animals in the relationship between context PE and fear memory learning and recall were also observed in Experiment 1. This may mean that psychotic and mood disorder risk variants that affect such processes may lead to sex-specific impacts of genotype. Thus, it is important to fully characterise these differences in a healthy model to better enable predictions of the impacts of genetic risk variants as well as possibly sexually dimorphic responses to treatment. Hence, the following two chapters will investigate this finding further in *Cacna1c*$^{+/+}$ animals. Owing to the results of these first two experimental chapters that demonstrate impacts of *Cacna1c* heterozygosity on aspects of hippocampal dependent associative contextual learning processes, the final set of experiments (Chapter 7) will investigate the impact of *Cacna1c* genotype, sex, and exposure to a novel context on gene expression in both the DG and CA1 regions of the hippocampus.

Chapter 5: A systematic literature review of sex differences in latent inhibition

5.1 Introduction

Experiment 1 of Chapter 4 indicated that there may be differences between sexes in the threshold of context PE that induces LI of contextual fear conditioning. Specifically, two 10-minute sessions of context PE over two days was sufficient to induce LI in females, but not in males. This suggests that there are differences between sexes in aspects of contextual associative fear learning, which may pertain to contextual encoding, the formation of context-US associations, or the integration of previously learned information regarding context-no US contingencies when the context is subsequently associated with a US. Owing to the known sex differences in the course of illness and outcomes of individuals with schizophrenia, it is important to ascertain whether the healthy processing of LI differs between sexes, in order to understand how genetic risk variants such as *CACNA1C* may differentially affect males and females.

Over the past three decades, LI has been extensively studied in individuals with schizophrenia, as well as studies investigating the association between LI performance and measures of schizotypy in healthy samples (Lubow et al., 1987; Swerdlow et al., 1996; Myles et al., 2023). Schizotypy refers to a dimensional construct that can be measured in all individuals, ranging from mild instances of positive, negative and cognitive factors that underlie schizophrenia, such as ‘odd beliefs’, ‘mild withdrawal’ and ‘disorganised thinking’ to pathological symptoms of schizophrenia such as ‘hallucinations’, ‘anhedonia’ and ‘thought disorder’. Studying this construct in healthy populations is thought to provide insight into the aetiology and developmental pathways of schizophrenia (Kwapil and Barrantes-Vidal, 2015). The misattribution of salience to irrelevant stimuli, such as those that are poor

predictors of an outcome, has been hypothesised to play a role in the cognitive dysfunction that underpins psychosis (Kapur, 2003; Lubow, 2005). Thus, ascertaining the extent of sex differences observed in LI studies of both schizophrenia patient samples and schizotypy in healthy samples will help to elucidate the role aberrant associative learning processes play in explaining the sex differences observed in psychosis.

Similarly, extensive literature exists pertaining to studies of LI in rodents, which facilitate a more extensive investigation of the neurobiological underpinnings of LI, as well as the effects of environmental stressors and administration of substances such as dopaminergic agonists and antagonists. Thus, sex differences observed in both behavioural performance on LI tasks as well as neurobiological substrates can elucidate pathways that may be differentially implicated in neuropsychiatric disorders between sexes. In light of the importance of furthering our understanding of sex differences in associative learning processes implicated in neuropsychiatric disease, the aim of this chapter is to examine the existent literature pertaining to sex differences in LI in both human (schizophrenia patient and schizotypy) and rodent studies.

5.2 Hypotheses

- Owing to the known sex differences in neuropsychiatric disorders, and the fact that LI deficits have been observed in schizophrenia, it was hypothesised that sex differences in LI would also have been observed in the literature in both human and rodent studies.

5.3 Methods

The study selection protocol is outlined in Figure 5.1.

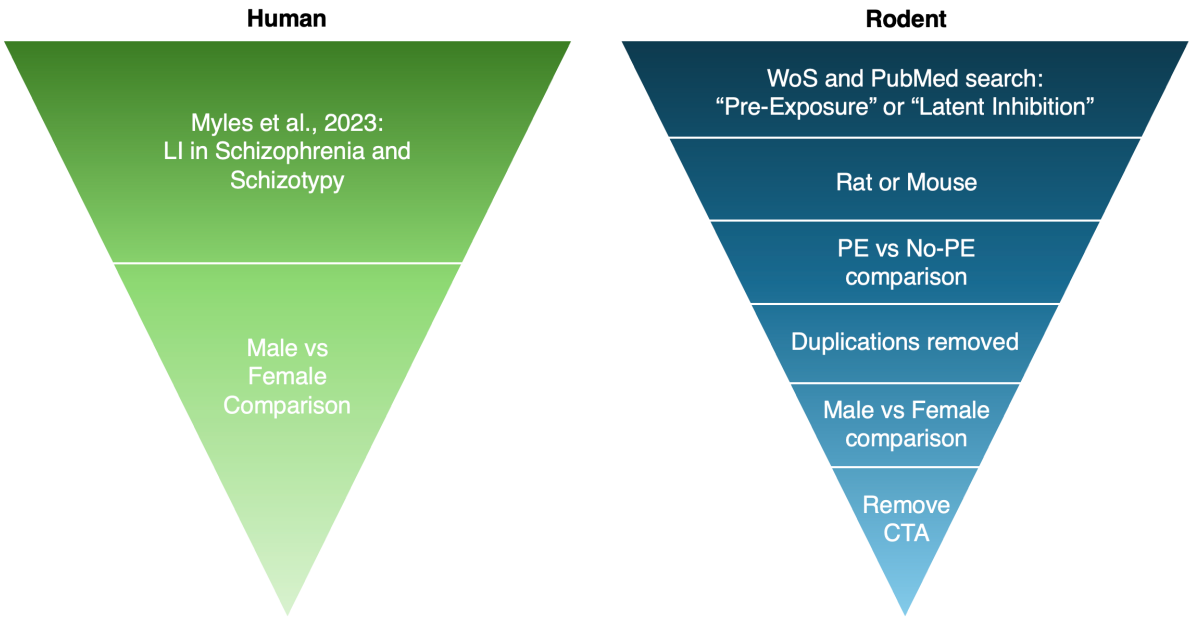

Figure 5.1. Study selection protocol for human and rodent studies included in the systematic literature review of sex differences in LI.

PubMed and Web of Science were searched using the terms “pre-exposure” and “latent inhibition” to identify potential studies. Any study in English or with English translation was accepted with no limitations on year of publication. Abstracts (and methods sections where necessary) were reviewed to include studies that were conducted in either mouse or rat and included a comparison between a group which had been pre-exposed to the CS or context, and a group which had experienced no PE. Duplications between PubMed and Web of Science were removed, and of those that remained, methods sections were examined to determine whether a comparison of the effects of CS or context PE had been made between sexes. Studies that included both sexes but did not present an analysis in each sex independently or examine the interaction between sex and CS or context PE were excluded from the review. Studies using conditioned taste aversion (CTA) paradigms were also excluded as in such designs, there is a considerable latency between CS and US

onset and CTA is thought to be underpinned by a distinct neurobiological network that differs from that of classical conditioning (Chambers, 1990).

A recent review (Myles et al., 2023) had comprehensively summarised the literature pertaining to latent inhibition in schizophrenia and schizotypy in humans. Hence, in order to assess sex differences within the schizophrenia clinical population and related studies examining schizotypy in healthy individuals, the studies included in this review were examined to identify those that included an analysis of both sexes independently or examined the interaction between sex and CS or context PE. As with the rodent literature, studies that included both sexes but did not report sex specific analyses were not included in the review.

5.4 Results

5.4.1 Schizophrenia and Schizotypy

A review by Myles et al. (2023) detailed 48 studies which either assessed LI in schizophrenia patients compared with healthy controls or assessed the effect of schizotypy as a personality dimension on LI within samples of the healthy population. These 48 studies were examined for the purposes of assessing the current understanding of sex differences within the clinical schizophrenia and schizotypy literature. Although most of the studies included in the review included both male and female participants, 29 did not stratify their analysis by sex (Baruch et al., 1988; Lipp and Vaitl, 1992; Lubow et al., 1992; De la Casa et al., 1993; De la Casa and Lubow, 1994; Lipp et al., 1994; Allan et al., 1995; Gray et al., 1995, 2002; Guterman et al., 1996; Williams et al., 1996, 1997; Braunstein-Bercovitz and Lubow, 1998; Kathmann et al., 2000; Rascle et al., 2001; Vaitl et al., 2002; Braunstein-Bercovitz, 2003; Tsakanikos et al., 2003; Cohen et al., 2004; Tsakanikos, 2004; Tsakanikos and Reed, 2004; Yogev et al., 2004; Orosz et al., 2008, 2011; Gal et al., 2009; Schmidt-Hansen et al., 2009; Le Pelley et al., 2010; Granger et al., 2016; Chun et al., 2019).

Only one study used solely female participants (Gibbons and Rammsayer, 1999), and a further two studies included solely male participants (McCartan et al., 2001; Young et al., 2005).

The remaining sixteen studies in the review included both male and female participants and stratified their analysis by sex, revealing mixed findings. Thirteen studies did not observe any effect of sex on LI (Schizotypy: Della Casa et al., 1999; Höfer et al., 1999; Burch et al., 2004; Evans et al., 2007; Shrira and Kaplan, 2009; Shrira and Tsakanikos, 2009; Granger et al., 2012; Dawes et al., 2022. Schizophrenia: Swerdlow et al., 1996, 2005; Serra et al., 2001; Leumann et al., 2002; Kraus et al., 2016). Conversely, three other studies (all from the same research group) did observe sex differences in the effect of schizophrenia or schizotypy in LI, whereby schizophrenia and high schizotypy and seems to confer a LI deficit in females, but not in males, whereas controls of both sexes showed intact LI (Lubow et al., 2000, 2001; Lubow and De la Casa, 2002). Hence, these studies provide limited evidence for sex differences in latent inhibition deficits in psychosis, and the inconsistent nature of these three studies compared with the wider literature may be attributable to the specific paradigm used by this research group. Thus, possible sex differences in LI deficits seen in schizophrenia and schizotypy appear to be subtle.

5.4.2 Rodent literature

A total of 16 studies published between 1985 and 2017 conducted in mice or rats were identified in which a comparison was made between animals who were not pre-exposed to a CS or context and a group which had undergone CS or context PE prior to conditioning. Crucially, all studies identified were conducted in both sexes, and analysis was either stratified by sex, or a specific assessment of the interaction between sex and PE group was conducted (Table 5.1). Most studies (14/16) were conducted in rat (Weiner et al., 1985, 1987; Shalev et al., 1998; Lehmann et al., 2000b, 2000a; Maes, 2002; Jongen-Rêlo et al., 2004; Arad and Weiner, 2008,

2010a; Piontkewitz et al., 2011; Wang et al., 2012; Vorhees et al., 2015; Marks et al., 2016; Huang et al., 2017) and the remaining two were carried out in mice (Caldarone et al., 2000; Mizuno et al., 2006).

Most studies (14/16) also used a paradigm in which the animals in the PE group were pre-exposed to a CS which was subsequently paired with a US (Weiner et al., 1985, 1987; Shalev et al., 1998; Caldarone et al., 2000; Lehmann et al., 2000b, 2000a; Maes, 2002; Jongen-Rêlo et al., 2004; Mizuno et al., 2006; Arad and Weiner, 2008, 2010; Piontkewitz et al., 2011; Vorhees et al., 2015; Marks et al., 2016). Only two studies used a paradigm with no CS, in which animals in the PE group were pre-exposed to the conditioning context, which was later entered into association with a US (Wang et al., 2012; Huang et al., 2017). In all studies, a foot-shock was used as the aversive US. Three studies used a light as the discrete CS (Lehmann et al., 2000b, 2000a; Jongen-Rêlo et al., 2004), however in most cases a tone was used (Weiner et al., 1985, 1987; Shalev et al., 1998; Caldarone et al., 2000; Lehmann et al., 2000b, 2000a; Maes, 2002; Mizuno et al., 2006; Arad and Weiner, 2008, 2010b; Piontkewitz et al., 2011; Vorhees et al., 2015; Marks et al., 2016). Both studies by the Lehmann et al. detailed two experiments, one in which a tone was used, and another in which a light was used as the CS.

Several different response measures were used for the fourteen studies in which a CS-US relationship was assessed. Four studies used active avoidance paradigms in which animals had to make an active locomotor response to the tone in order to move into a separate compartment of the conditioning chamber to avoid receiving a shock (Weiner et al., 1985; Lehmann et al., 2000b, 2000a; Jongen-Rêlo et al., 2004). Seven studies used conditioned emotional response paradigms, and in every case the conditioned emotional response was the suppression of licking behaviour. In such studies, water-deprived animals are presented with the CS whilst consuming water, and the reduction in water consumption in response to the CS is assessed as a measure of fear (Weiner et al., 1987; Shalev et al., 1998; Lehmann et al., 2000a, 2000b; Arad and Weiner, 2008, 2010b; Piontkewitz et al., 2011). In five studies, freezing behaviour was assessed as a measure of fear in response to the CS

(Caldarone et al., 2000; Maes, 2002; Mizuno et al., 2006; Vorhees et al., 2015; Marks et al., 2016). The two studies that assessed context-US relationships both used passive avoidance paradigms, in which the animal suppresses their innate preference for the dark compartment which has previously been associated with the US and moves to the light compartment in order to avoid the US (Wang et al., 2012; Huang et al., 2017).

Of the studies assessing LI of a CS-US relationship, six of the fourteen found no sex differences in LI, with similar differences between CS pre-exposed and non-pre-exposed animals observed between males and females (Caldarone et al., 2000; Lehmann et al., 2000b; Maes, 2002; Jongen-Rêlo et al., 2004; Mizuno et al., 2006; Vorhees et al., 2015). Lehmann et al. (2000a) did not observe sex differences in their CER paradigm. However, in their active avoidance experiment, there was a significant interaction between the effect of sex and the effect of CS pre-exposure, in that there were no sex differences in the CS pre-exposed animals in escape latencies, whereas in the non-pre-exposed group, females had decreased escape latencies compared to males, indicating a reduced LI effect in females compared with males. However, the authors note that there was a tendency towards an interaction with sex in the CER paradigm, and propose that both effects may be explained by increased fear in females compared with males.

Arad and Weiner (2008) examined sex differences in LI during various phases of the female oestrous cycle and found no sex differences during the oestrus-metestrus phase of the cycle. However, during the metestrus-dioestrus, dioestrus-proestrus and proestrus-oestrus phases of the cycle, LI was not observed in females. In a later study, the authors also observed sex-dependent effects of LI in relation to oestrogen (Arad and Weiner, 2010). No sex differences were seen between control males and females. However, LI was diminished by amphetamine administration in both males and females, and this effect was rescued by 17β-oestradiol in both sexes as well as ovariectomised females. However, when LI was disrupted by administration of the NMDA antagonist MK-801, this was rescued by 17β-oestradiol in males and ovariectomised females, but not in intact females. Thus, taken together, these results

suggest that circulating gonadal hormones elicit sex differences in LI. However, the fact that 17β-oestradiol did not rescue the effects of MK-801 of LI in intact females, but did in ovariectomised females, suggests the impact of gonadal hormones on LI is wider than oestrogen alone. No sex differences in LI were seen between control males and females.

Piontkewitz et al. (2011) assessed how stimulation of the immune system interacted with sex to affect LI. No sex differences were observed in control animals. However, in animals that were administered with the immunostimulant polyinosinic:polycytidylic (polyI:C) during gestational day 15, LI was disrupted in males at postnatal day (PND) 70 but was not disrupted in females. However, at PND 90, LI was disrupted in both sexes in the polyI:C group. Heightened inflammation resulting from maternal infection during gestational development has been robustly implicated in the development of psychotic disorders (Haddad et al., 2020). This study provides evidence for maternal immune activation differentially impacting LI between male and female offspring during early post-natal life.

Three studies from the same group investigated the effects of juvenile handling on LI and were consistent with one another in that no sex differences were observed in LI in animals that had been handled during the juvenile period (Weiner et al., 1985, 1987; Shalev et al., 1998). Sex differences in LI were seen in the non-handled groups in all three studies, with a consistent direction of effect across all three studies of LI in non-handled females, but not non-handled males (Weiner et al., 1985, 1987; Shalev et al., 1998). However, Shalev et al. (1998) found that by 16 months of age, a time point corresponding with middle age, LI was not observed in either sex of the non-handled group. Thus, these results suggest that early environmental experiences of enrichment can modulate the effects of sex on LI, which such effects appearing to vary across post-natal development.

In the two studies that were found which assessed the LI of a context-US relationship, there were no sex differences observed in control animals (Wang et al., 2012; Huang et al., 2017). However, Wang et al. (2012) found that amphetamine

administration decreased LI in males but increased LI in females, whereas ascorbic acid decreased LI in males but did not affect LI in females. However, the $D_{1/2}$ receptor agonist apomorphine enhanced LI in both sexes. A significant interaction between sex, PE condition and drug (NMDA, MK-108, NaCl) was observed in Huang et al. (2017), indicating that the effects of each drug on LI differ between sexes. However, specific pairwise effects were not reported. Both studies assessed foreground contextual fear conditioning, in which no CS is presented and the primary association formed is between the context and the US. However, Vorhees et al. (2015) assessed background contextual fear conditioning as well as discrete cued CS-US conditioning. Context can be still relevant to CS-US paradigms as all conditioning occurs within the context of the conditioning chamber. Hence, although in cued conditioning experiments, the primary association is between the CS and the US, this CS-US relationship is typically context specific, and the US may also enter into association with the context as well as the US, a phenomenon known as background contextual fear conditioning (Phillips and LeDoux, 1994). Thus, behavioural fear response to the context alone can be assessed in CS-US experiments. Using this design, Vorhees et al. (2015) found no sex differences in either the CS-US relationship or the context-US relationship.

Table 5.1. Summary of the 16 rodent studies which have compared male and female performance in latent inhibition. CS: Conditioned stimulus. AA: Active avoidance. CER: Conditioned emotional response. Grey denotes findings of no sex differences. Blue denotes findings of greater LI in females compared with males. Orange denotes findings of greater LI in males compared with females.

Study	Species	CS/Context	Paradigm	Findings
Weiner et al., 1985	Rat	CS (tone)	AA (foot shock)	No sex differences in LI in juvenile handled animals. LI observed in juvenile non-handled females, but not juvenile non-handled males.
Jongen-Rêlo et al., 2004	Rat	CS (light)	AA (foot shock)	No sex differences in LI.
Weiner et al., 1987	Rat	CS (tone)	CER: Conditioned suppression of licking (foot shock).	No sex differences in LI in juvenile handled animals. LI observed in juvenile non-handled females, but not juvenile non-handled males.
Shalev et al., 1998	Rat	CS (tone)	CER: Conditioned suppression of licking (foot shock).	No sex differences in LI in juvenile handled rats at age 3 months. LI observed in juvenile non-handled females, but not

				juvenile non-handled males at 3 months. At 16 months old, neither non-handled sex demonstrated LI.
Lehmann et al., 2000a	Rat	CER: CS (light) AA: CS (tone)	CER: Conditioned suppression of licking (foot shock); AA (foot shock)	CER: No sex differences in LI. AA: no sex differences in escape latency in pre-exposed animals but decreased escape latency in non-pre-exposed females compared to non-pre-exposed males.
Lehmann et al., 2000b	Rat	CER: CS (light). AA: CS (tone)	CER: Conditioned suppression of licking (foot shock); AA (foot shock).	No sex differences in LI in either CER or AA.
Maes, 2002	Rat	CS (tone)	Freezing behaviour (foot shock).	No sex differences in LI.
Caldarone et al., 2000	Mouse	CS (tone)	Freezing behaviour (foot shock).	No sex differences in LI.
Mizuno et al., 2006	Mouse	CS (tone)	Freezing behaviour (foot shock).	No sex differences in LI.

Vorhees et al., 2015	Rat	CS (tone)	Freezing behaviour (foot shock)	No sex differences in LI to either context (without CS) or CS.
Marks et al., 2016	Rat	CS (tone)	Freezing behaviour (foot shock)	LI observed in males but not in females.
Arad and Weiner, 2008	Rat	CS (tone)	CER: Conditioned suppression of licking (foot shock).	Similar LI observed in males and females when females are in oestrus-metestrus phase of cycle. No LI observed in females in metestrus-dioestrus, dioestrus-proestrus or proestrus-oestrus phases.
Arad and Weiner, 2010	Rat	CS (tone)	CER: Conditioned suppression of licking (foot shock).	No sex differences in LI in control animals. 17β-oestradiol exerted similar effects to antipsychotics in rescuing LI disrupting effects of amphetamine in males, intact females and ovariectomised females. However, 17β-oestradiol reversed MK-801 potentiation of LI in males, ovariectomised females, but not intact females.
Piontkewitz et al., 2011	Rat	CS (tone)	CER: Conditioned suppression of licking (foot shock).	No sex differences in LI in control animals. Prenatal poly-I:C disrupted LI in males but not females at PND 70. At PND 90 LI was absent in both sexes of the prenatal poly-I:C condition.

Wang et al., 2012	Rat	Context	Passive avoidance (foot shock)	No sex differences in LI in control animals. Methamphetamine administration decreased LI in males but enhanced LI in females. Ascorbic acid administration decreased LI in males but did not affect LI in females. $D_{1/2}$ receptor agonist apomorphine enhanced LI in both sexes.
Huang et al., 2017	Rat	Context	Passive avoidance (foot shock)	No sex differences in LI in control animals. A significant ($p <$ 0.05) three-way interaction between sex, drug (MK08, NMDA, NaCl) and condition (pre-exposure vs no pre-exposure) was reported. However, post hoc testing to reveal the specific effects of drugs on LI between sexes was not reported.

5.5 Discussion

Experiments in both humans and rodents have been used to model attentional and associative learning deficits in schizophrenia, a disorder with known sex differences. The first experiment of Chapter 4 showed sex differences in the LI of CFC, with the same context pre-exposure duration eliciting greater LI in females compared with males. Hence, it was hypothesised that both the human and rodent literature would show evidence supporting sex differences in LI. However, the majority of studies (13/16) that assessed the effect of sex on LI in humans did not show sex differences. The three studies that did observe sex differences in the effects of schizophrenia (one study) or high schizotypy (two studies) all showed impairments in females, but not in males. However, although the review of the rodent literature was conducted systematically, the review of the human literature was not, as papers used in a recent review of LI in schizophrenia and schizotypy (Myles et al., 2023) were assessed, rather than the entirety of the literature. Hence, important findings may have been missed.

Considerations of the paradigms used in such studies are required when interpreting these findings. Although the majority of human studies used both males and females, the majority did not perform or include stratified analysis by sex, despite the availability of the data. Almost all studies in humans use similar paradigms in which a discrete CS and US are associated with one another, with two PE participant groups. In the PE group, the CS has been presented to the participant prior to being associated with the US and in the non-PE group, the CS is not presented to the participant prior to association with the US. Owing to important ethical considerations required in human studies and particularly those including patients, the stimuli used generally bear a neutral valence (e.g., letters on a screen or a flashing light). The use of similar tasks between studies allows for replicability and may have been adapted for use in scanners, facilitating assessment of the neurobiological underpinnings of LI. However, the psychotic phenomena that occur in psychosis often bear a negative valence and are fearful to the individual (Gauntlett-Gilbert and

Kuipers, 2005). Fear conditioning and the formation of fear associations is known to have a distinct neuronal circuitry heavily involving connectivity between the amygdala, hippocampus and PFC (Delgado et al., 2008; Maren et al., 2013; Moustafa et al., 2013), and such networks are known to be sexually dimorphic (Bauer, 2023). Hence, if these networks are not activated by the current array of LI tasks commonly used in humans, important sex differences in LI relevant to psychosis may be missed.

The majority of the evidence from rodent studies relating to sex differences in LI pertains to cued rather than contextual fear conditioning experiments. While most of this evidence suggests that in control animals, there are very few sex differences in the LI of cued conditioning, there are several factors such as juvenile handling and oestrous cycle stage which were found to influence LI in a sexually dimorphic manner. Piontkewitz et al. (2011) showed that, although there were no differences in LI between sexes in control animals, the administration of the immunostimulant poly-I:C during prenatal development disrupted LI in males but not females at PND 70, and the effect was restored at PND 90. Thus, sexes may be differentially vulnerable to aberrant immune activation during foetal development. Other studies observed that neurotransmitter modulators such as methamphetamine (Wang et al., 2012) and MK801 (Huang et al., 2017) disrupted LI of cued conditioning in males but not females. Thus, the underlying neurobiology mediating LI processes may differ between sexes, even if behavioural expression is similar between males and females in control animals. It is worthy of note that most studies did not investigate underlying neurobiological processes alongside behavioural expression of LI, which may mean that sex differences in mediating processes that may lead to differential susceptibility to psychopathology between sexes may be missed, even when behavioural performance is comparable between sexes (Shansky and Murphy, 2021; Gall et al., 2024).

Experiment 1 of Chapter 4 showed that 2x10-minute context pre-exposure sessions (24 hours apart) induced LI in females but not in males. Hence, it was hypothesised that other studies assessing LI of CFC would show sex differences. In the three

studies found that examined sex differences in the LI of either foreground or background contextual fear conditioning, sex differences were not observed. However, both foreground conditioning LI studies used the same paradigm of passive avoidance. The inconsistency of the evidence from cued conditioning studies indicates that there may be several factors, relating to both biology and study design, that may influence whether sex differences are observed.

Upon initial appraisal the existing literature does not appear to concur with the findings of Chapter 4 Experiment 1 of sex differences in LI of CFC. However, it cannot be said that this question has been exhaustively examined and a study using a paradigm similar to that of the experiment in Chapter 4 was not found in the literature. Thus, a wider range of studies in both humans and rodents that assess factors such as oestrous phase, age, prior environmental experiences, assessing both behavioural LI performance and associated neurobiological underpinnings ought to be conducted. Furthermore, CFC is known to be a highly hippocampally dependent process and numerous aspects of sexual dimorphism have been observed in this brain structure (Rossetti et al., 2016; Iqbal et al., 2020; Fester and Rune, 2021; Gall et al., 2024). Hence, owing to the relevance of latent inhibition to psychopathologies with known sex differences, such as schizophrenia, it is of clinical relevance for sex differences in the LI of CFC to be explored in more detail.

Chapter 6: Sex differences in the effect of context pre-exposure duration on contextual fear conditioning

6.1 Introduction

The findings of the systematic review in Chapter 5 showed few prior studies that have investigated whether sex differences exist in the relationship between context PE and subsequent CFC and contextual fear memory recall. Furthermore, even fewer studies examined the biological underpinnings of LI processing, despite plausible differences between sexes in the biological substrates of the same behavioural processes. The hippocampus is particularly critical to the encoding of environmental contexts, whereby the disparate aspects of the environment are integrated into a configural cognitive representation (Eichenbaum, 2017). Hence, the LI of CFC rather than cued conditioning is likely to be hippocampally dependent to a greater degree. Owing to the known aspects of sexual dimorphism within the hippocampus that have been previously discussed, it is plausible that sex differences may arise in LI that are specific to contextual associations. Hence, this chapter aims to investigate sex differences in the relationship between context PE duration and CFC, as well as IEG expression associated with fear memory recall.

Neuronal response to afferent stimulation takes place over a time course ranging from an early response of milliseconds to minutes and a later response of hours to days, and it is possible to study neurobiological processes associated with a cognitive process across this time range. IEGs are expressed as part of the early response to neuronal stimulation and in turn activate the downstream expression of numerous other genes involved in a wide array of cellular processes (Pérez-Cadahía et al., 2011). Thus, the expression of different IEGs may indicate differing neuronal

responses and downstream processes associated with a particular experimental stimulus. *Cfos* encodes the activity-dependent transcription factor c-Fos protein and *Arc* encodes the activity-regulated cytoskeletal associated protein (ARC). Both c-Fos and ARC have been linked to learning and memory retrieval in behavioural training paradigms, and ARC has a known role in synaptic plasticity mechanisms such as long-term potentiation and depression (Gallo et al., 2018). Hence, the differing levels of expression of both *Cfos* and *Arc* between context PE duration groups in each sex can provide an indication of whether a specific brain region is both active and undergoing memory consolidation processes during post-extinction recall. *Bdnf* transcribes the brain derived neurotrophic factor (BDNF) protein and is crucial to activity-dependent synaptic plasticity, whereby it mediates ion channel and neurotransmitter receptor expression on the neuronal membrane, as well as triggering downstream gene expression via the Erk signalling pathway and via binding with TrkB (Bekinschtein et al., 2014). BDNF is also known to mediate *Arc* expression, however, *Arc* may also be expressed via a BDNF-independent pathway (Zheng et al., 2009). Hence, analysing both *Arc* and *Bdnf* expression can provide insights into related but distinct synaptic plasticity processes underpinning memory recall and consolidation that may be taking place. Multiple transcripts of the *Bdnf* gene exist, and *Bdnf-IX* was chosen as this contains the exon necessary for BDNF protein translation (Bach et al., 2024).

Several brain regions with known involvement in LI were chosen for analysis in order to develop a comprehensive picture of the regions that may be implicated in the sex differences observed behaviourally, and to ascertain whether this would include or be specific to the hippocampus. The CA1 and DG regions of the hippocampus were chosen as both have known involvement in contextual fear memory processing (Hernández-Rabaza et al., 2008; Bernier et al., 2017; Jimenez et al., 2020; Ratigan et al., 2023). Both the infralimbic (IL) and prelimbic (PL) regions of the mPFC have been implicated in LI processing, with pharmacological blockade of the mPFC having been shown to disrupt LI (Lingawi et al., 2016), but substructure heterogeneity within the mPFC on the impact of LI has been demonstrated (George et al., 2010; Nelson et al., 2010). Numerous investigations have also demonstrated the role of the

basolateral amygdala (BLA) in the LI of fear conditioning (Schauz and Koch, 2000; Coutureau et al., 2001. Although, see exception: Stevenson and Gratton, 2004), and Schiller and Weiner (2004) found that the BLA may play a specific role in the suppression of previously learned LI when the CS-US relationship is repeatedly reinforced. The central amygdala (CeA) is also known to be involved in LI, particularly in the cognitive flexibility that is required to respond to stimuli that were previously irrelevant but are now significant predictors of an aversive outcome (Ashby et al., 2021). Pharmacological and optogenetic disruption of the core and shell subregions of the nucleus accumbens (NAc. Core: NAcc. Shell: NAcsh) has been shown to disrupt LI (Gray et al., 1997; Weiner and Feldon, 1997; Kutlu et al., 2022). Thus, *Cfos*, *Arc* and *Bdnf-IX* expression in these regions was assessed in this experiment.

In summary, owing to the limitations of the current body of literature pertaining to sex differences in LI, the following experiment aims to expand on the results of Chapter 4 Experiment 1 which suggested a sex difference in the threshold of PE duration required to induce LI. In this experiment, the effect of three separate durations of context PE were compared in both males and females to assess whether the relationship between context PE duration and fear conditioning is sexually dimorphic. Furthermore, assessment of the expression of the IEGs, *Cfos*, *Arc* and *Bdnf-IX*, in various brain regions including the hippocampus was conducted to gain insight into the underlying neurobiology of possible sex differences in LI.

6.2 Hypotheses

It was hypothesised that…

- In both sexes, longer context PE durations result in greater LI, reflected by reduced behavioural fear response compared with shorter durations of PE exposure.

- A sex difference exists in the threshold for LI between sexes, with LI more readily occurring in females compared with males, reflected by females showing a reduced fear response compared with males at shorter durations of context PE, but not at longer durations of PE.

- Behavioural differences are reflected in IEG expression in the hippocampus as well as a network of brain regions known to be involved in LI (mPFC, amygdala, and nucleus accumbens). Predictions were not made regarding specific effects of sex and PE durations on gene expression owing to limited existing evidence.

6.3 Methods

6.3.1 Animals

Forty-eight Sprague-Dawley wild-type rats (24 males and 24 females) aged 60-80 days were ordered from Charles River Laboratories (UK). They remained in their home cage room for 14 days from the point of arrival in the animal unit to recover from any transportation stress and to habituate to their new environment. All testing was conducted during the active (dark) phase of the light cycle. All animals were housed as described in Section 2.1. with the exception that all animals in this experiment were housed in pairs.

6.3.2 Behavioural paradigm

Animals were split into three context PE duration conditions: 3x5-minutes, 3x20-minutes, and 3x60-minutes each across three days. An equal number of males and

females were assigned to each group, so each sex/PE duration group had an n of 8. Over the first three days, animals underwent PE sessions of their assigned duration. On day 4, animals underwent a CFC session, followed by a 10-minute extinction session on day 6, and a 2-minute recall session on day 8 (Figure 6.1).
30-minutes after the recall session, rats were euthanised. All further details of the experimental design of the behavioural experiment are described in Section 2.2.

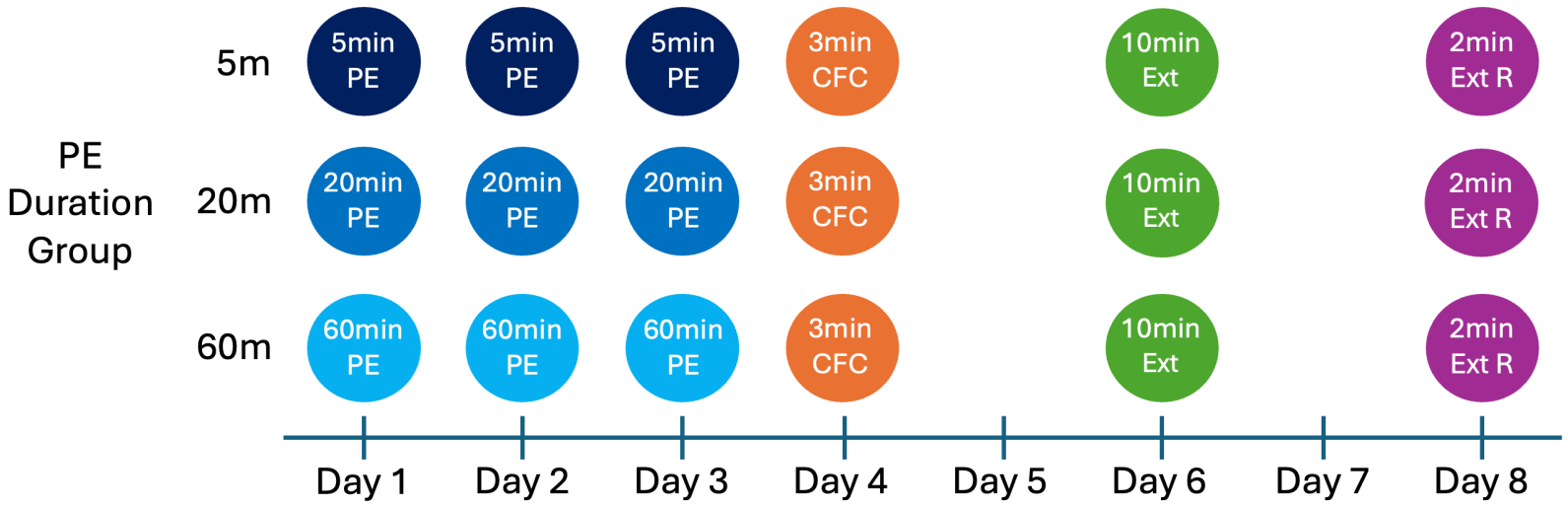

Figure 6.1. Behavioural paradigm of the experiment. PE = pre-exposure; CFC = contextual fear conditioning; Ext = extinction; Ext R = extinction recall.

6.3.3 Euthanasia and Tissue Extraction

Animals were culled using rising concentration of CO_2 30-minutes after the extinction recall session as described in Section 2.3. Brains were extracted and dissected as described in Section 2.4.

6.3.4 Quantification of RNA

RNA extraction, cDNA synthesis and qPCR was carried out as described in Chapter 2. Pre-limbic (PL) and infralimbic (IL) regions of the mPFC, core (NAcc) and shell (NAcsh) regions of the NAc, central (CeA) and basolateral (BLA) amygdala, and DG and CA1 regions of the hippocampus were extracted from each brain. Owing to a laboratory error in regulating the temperature of the thermal block, it was unfortunately not possible to carry out dissections of two of the brains (male 3x60m of PE and male 3x20m of PE).

6.3.5 Statistical analysis

The time points used for ANOVAs were the post-US period of the CFC session, the extinction session, and the extinction recall session. Time was also included as a variable within the extinction session ANOVA, to assess whether freezing or locomotor activity differed between each 2-minute measurement period, and to determine whether within-session extinction had occurred. An additional model to assess between-session extinction was carried out between the first 2-minute epoch of the extinction session, and the 2-minute extinction recall session, and to determine whether this differed between sexes and PE durations. For quadrant crossings, a further model was carried out to determine whether differences between

sexes and PE durations occurred during the pre-US period of the CFC session. This was not conducted for freezing as pre-US freezing levels were expected to be negligible.

qPCR data was analysed using the $2^{\Delta\Delta CT}$ method. Outlier C_T values were excluded using Grubbs' test and a mean value for each sample/gene combination was taken. For each sample, an overall housekeeping gene C_T value was derived from the mean of the *Gapdh* and *Hprt1* values. The C_T values for each gene of interest was the normalised to the housekeeping gene C_T value by subtracting the housekeeping gene C_T value from the gene of interest C_T value. A calibrator value was calculated from the mean of normalised C_T values of males in the 5m PE group. $\Delta\Delta C_T$ values were generated by subtracting the calibrator from each C_T value. Final fold change values were generated by calculating 2 raised to the power of each sample's $\Delta\Delta C_T$ value. An ANOVA was then performed to assess the impact of sex and PE group, as well as the interaction between the two variables on fold change values for each gene in each brain region, in the same manner as the behavioural data.

6.4 Results

6.4.1 Freezing

Increased freezing was found between the pre-US and post-US periods of the CFC session ($t_{94} = 11.33$, $p < 0.001$, 95%CI = [31.2, 44.5]) (Figure 6.3). Freezing scores of the post-US period were square root transformed. There was no main effect of sex ($F_{1,42} < 0.1$, $p = 0.869$, 95%CI = [-1.0, 1.2]). There was also no main effect of PE duration; however, there was a trend towards greater freezing in the 5m and 20m groups compared with the 60m group ($F_{2,42} = 2.7$, $p = 0.078$, 5m vs 20m 95%CI = [-1.3, 1.9], 5m vs 60m 95%CI = [-0.1, 3.1], 20m vs 60m 95%CI = [-0.5, 2.8]). The interaction between sex and PE condition was not significant ($F_{2,42} = 1.6$, $p = 0.213$.

Males: 5m vs 20m 95%CI = [-1.1, 2.7], 5m vs 60m 95%CI = [-1.1, 2.7], 20m vs 60m 95%CI = [-1.9, 1.9]. Females: 5m vs 20m 95%CI = [-2.1, 1.7], 5m vs 60m 95%CI = [0.3, 4.0], 20m vs 60m 95%CI = [0.4, 4.2]). However, confidence intervals indicate that there may be some between PE duration group differences in females that are not present in males, as the 5m vs 60m and 20m vs 60m 95% CIs in females did not straddle zero.

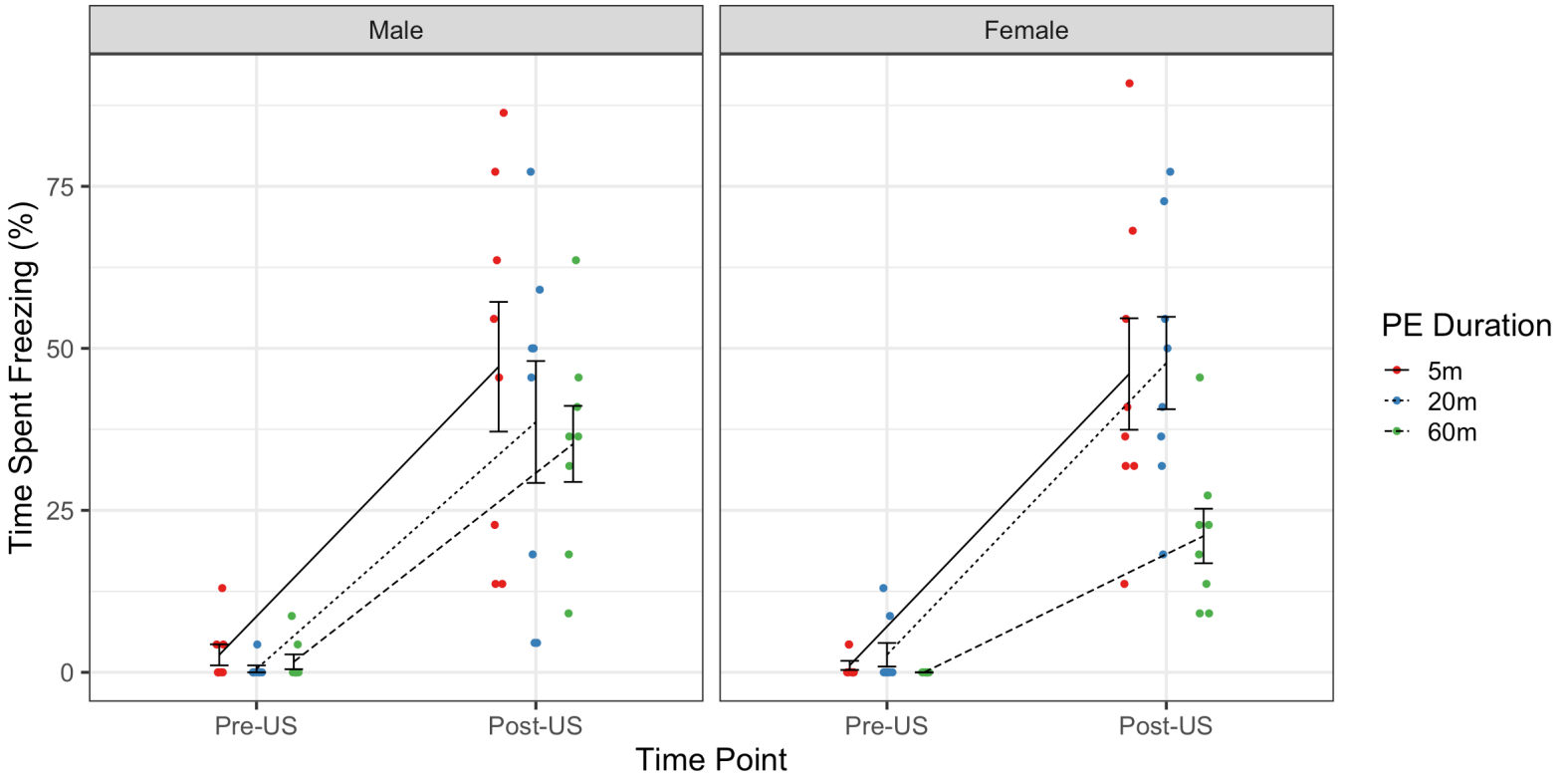

Figure 6.3. Percentage of time spent freezing for each sex and PE duration during the pre-US and post-US periods of the CFC session.

Freezing behaviour during the 10-minute extinction session was square root transformed. Model fit improved by removing the three-way interaction, the two-way interactions between PE duration and time, and sex and time, and the main effect of time. The removal of the main effect of time and interactions between sex and PE duration and time suggests that there was no within-session extinction across the 10-minute extinction period (Figure 6.4). The final model included the main effects of sex, PE duration and the interaction between these two variables. There was no main effect of sex ($F_{1,232}$ = 0.2, p = 0.660, 95%CI = [-0.4, 0.7]). There was a main effect of PE duration with greater freezing in the 5m and 20m groups compared with the 60m group ($F_{2,232}$ = 9.8, $p < 0.001$, 5m vs 20m 95%CI = [-0.8, 0.8], 5m vs 60m 95%CI = [0.5, 2.1], 20m vs 60m 95%CI = [0.5, 2.1]). There was also an interaction between sex and PE duration with greater freezing in the 5m and 20m groups compared with the 60m group in males, but with reduced freezing in the 5m and 60m groups compared with the 20m group in females ($F_{2,232}$ = 3.5, p = 0.032. Males: 5m vs 20m 95%CI = [-0.1, 1.8], 5m vs 60m 95%CI = [0.9, 2.8], 20m vs 60m 95%CI = [0.1, 2.0]. Females: 5m vs 20m 95%CI = [-1.9, 0.0], 5m vs 60m 95%CI = [-0.2, 0.16], 20m vs 60m 95%CI = [0.7, 2.6]).

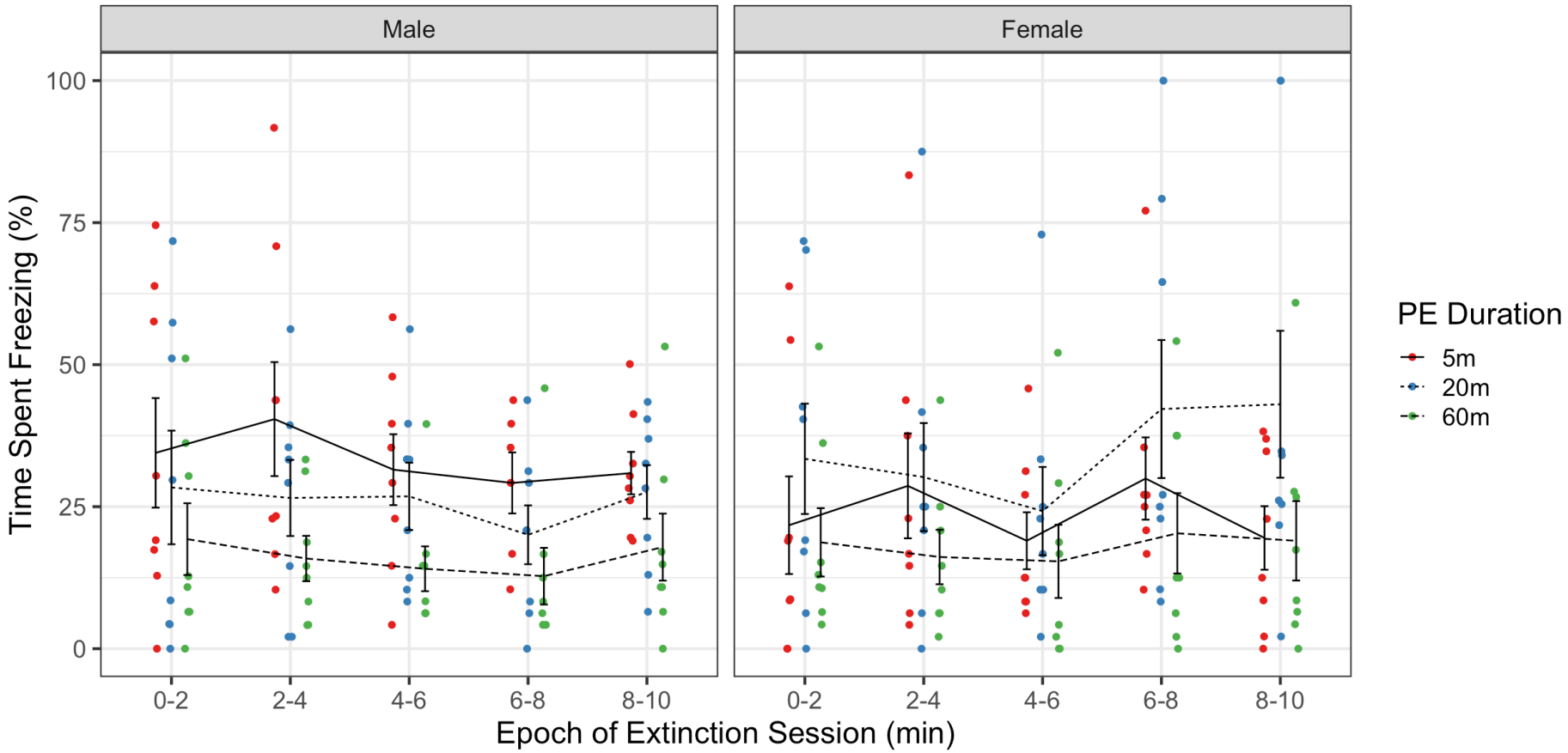

Figure 6.4. Percentage of time spent freezing for each sex and PE condition during each 2-minute epoch of the extinction session.

When comparing the first 2-minutes of the extinction session and the 2-minute extinction recall session to assess between session extinction, freezing was square root transformed. The three-way interaction term, as well as the two-way interaction terms between time and PE duration and time and sex were removed from the model as this improved model fit. There was no main effect of time ($F_{1,87}$ = 2.7, p = 0.106, 95%CI = [-0.2, 1.7]) (Figure 6.5), sex ($F_{1,87}$ < 0.1, p = 0.939, 95%CI = [-1.0, 0.9]), or PE duration ($F_{2,87}$ = 2.1, p = 0.134, 5m vs 20m 95%CI = [-1.4, 1.4], 5m vs 60m 95%CI = [-0.4, 2.4], 20m vs 60m 95%CI = [-0.4, 2.5]). There was no interaction between sex and PE duration. However, there was a trend towards males in the 5m group showing greater freezing relative to those in the 60m group, which was not found in females ($F_{2,87}$ = 2.8, p = 0.064. Males: 5m vs 20m 95%CI = [-0.4, 3.0], 5m vs 60m 95%CI = [0.5, 3.8], 20m vs 60m 95%CI = [-0.9, 2.5]. Females: 5m vs 20m 95%CI = [-3.0, 0.3], 5m vs 60m 95%CI = [-1.8, 1.5], 20m vs 60m 95%CI = [-0.4, 2.9]). Together, these results do not provide evidence of between-session extinction.

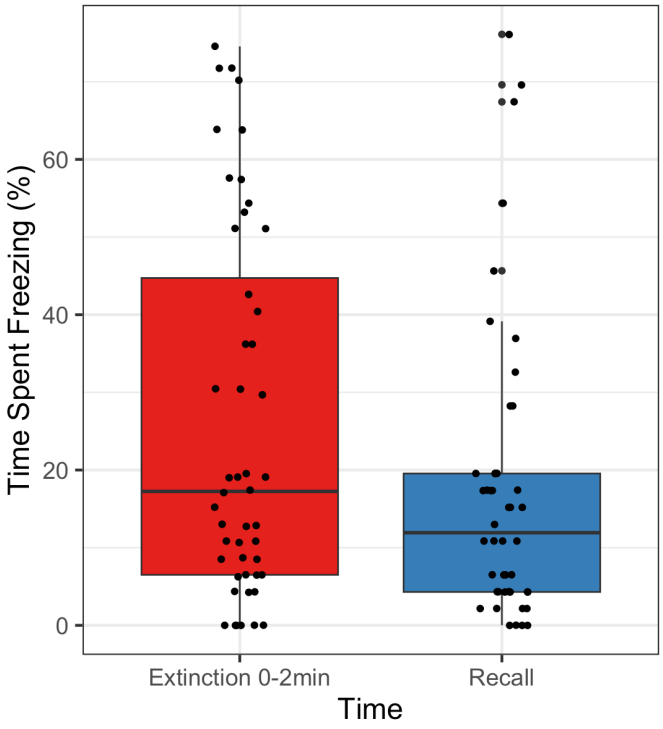

Figure 6.5. Percentage of time spent freezing during the first two-minute epoch of the extinction session compared with the extinction recall session.

Freezing behaviour in the 2-minute extinction session was square root transformed. No main effects of sex ($F_{1,40}$ = 0.3, p = 0.606, 95%CI = [-1.6, 0.0]), or PE duration ($F_{2,40}$ = 2.3, p = 0.116, 5m vs 20m 95%CI = [-1.6, 2.1], 5m vs 60m 95%CI = [-0.3, 3.2], 20m vs 60m 95%CI = [-0.7, 3.0]) on freezing behaviour were observed. There was no interaction between sex and PE duration (Figure 6.6). However, there was a trend towards males showing greater freezing in the 5m compared with the 60m group, which was not observed in females ($F_{2,40}$ = 2.8, p = 0.074. Males: 5m vs 20m 95%CI = [-0.4, 4.1], 5m vs 60m 95%CI = [0.8, 4.9], 20m vs 60m 95%CI = [-1.3, 3.2]. Females: 5m vs 20m 95%CI = [-3.4, 0.8], 5m vs 60m 95%CI = [-2.0, 2.1], 20m vs 60m 95%CI = [-0.7, 3.4]).

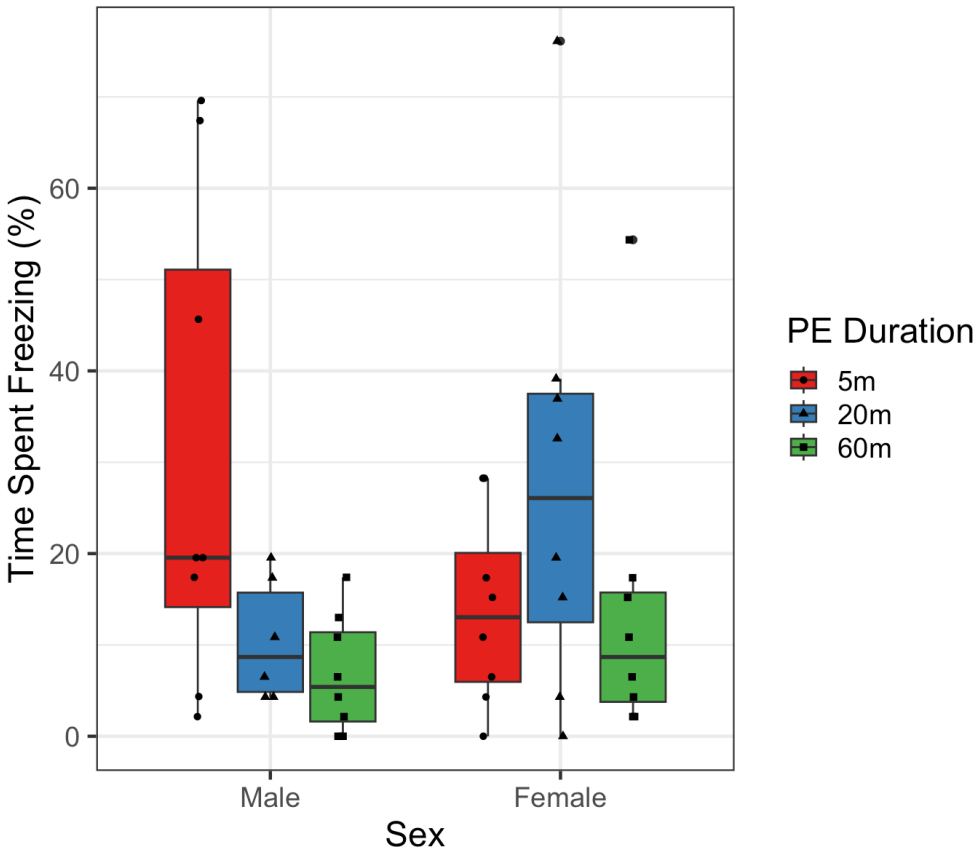

Figure 6.6. Percentage of time spent freezing for each sex and PE condition during the extinction recall session.

6.4.2 Quadrant Crossings

In the pre-US period of the CFC session, model fit was improved by removing the two-way interaction between sex and PE duration. There was no main effect of sex ($F_{1,44}$ = 1.3, p = 0.254, 95%CI = [-2.0, 0.5]) or PE duration ($F_{2,44}$ = 1.1, p = 0.349, 5m vs 20m 95%CI = [-1.0, 2.8], 5m vs 60m 95%CI = [-0.8, 2.9], 20m vs 60m 95%CI = [-1.7, 2.1]) on quadrant crossings per minute (Figure 6.7). Thus, prior to the onset of the foot shock, there do not appear to be any differences in locomotor activity between sexes and PE durations.

No change in quadrant crossings was observed between the pre-US and post-US periods of the CFC session (t_{94} = -0.3, p = 0.737, 95%CI = [-1.4, 1.0]). A main effect of sex on quadrant crossings in the post-US period was observed, with more frequent crossings in females compared with males ($F_{1,42}$ = 9.6, p = 0.004, 95%CI = [-4.4, -1.0]). There was no main effect of PE duration. However, there was a trend towards greater freezing in the 5m compared with the 60m group ($F_{2,42}$ = 2.4, p = 0.099, 5m vs 20m 95%CI = [-4.0, 1.1], 5m vs 60m 95%CI = [-4.9, 0.3], 20m vs 60m 95%CI = [-3.4, 1.7]). There was no interaction between sex and PE duration. However, there was a trend towards females in the 20m and 60m groups showing more frequent crossings compared with the 5m group, whilst no differences were observed in males ($F_{2,42}$ = 2.7, p = 0.076. Males: 5m vs 20m 95%CI = [-2.0, 4.0], 5m vs 60m 95%CI = [-3.8, 2.3], 20m vs 60m 95%CI = [-4.8, 1.3]. Females: 5m vs 20m 95%CI = [-6.9, -0.9], 5m vs 60m 95%CI = [-6.9, -0.9], 20m vs 60m 95%CI = [-3.0, 3.0]).

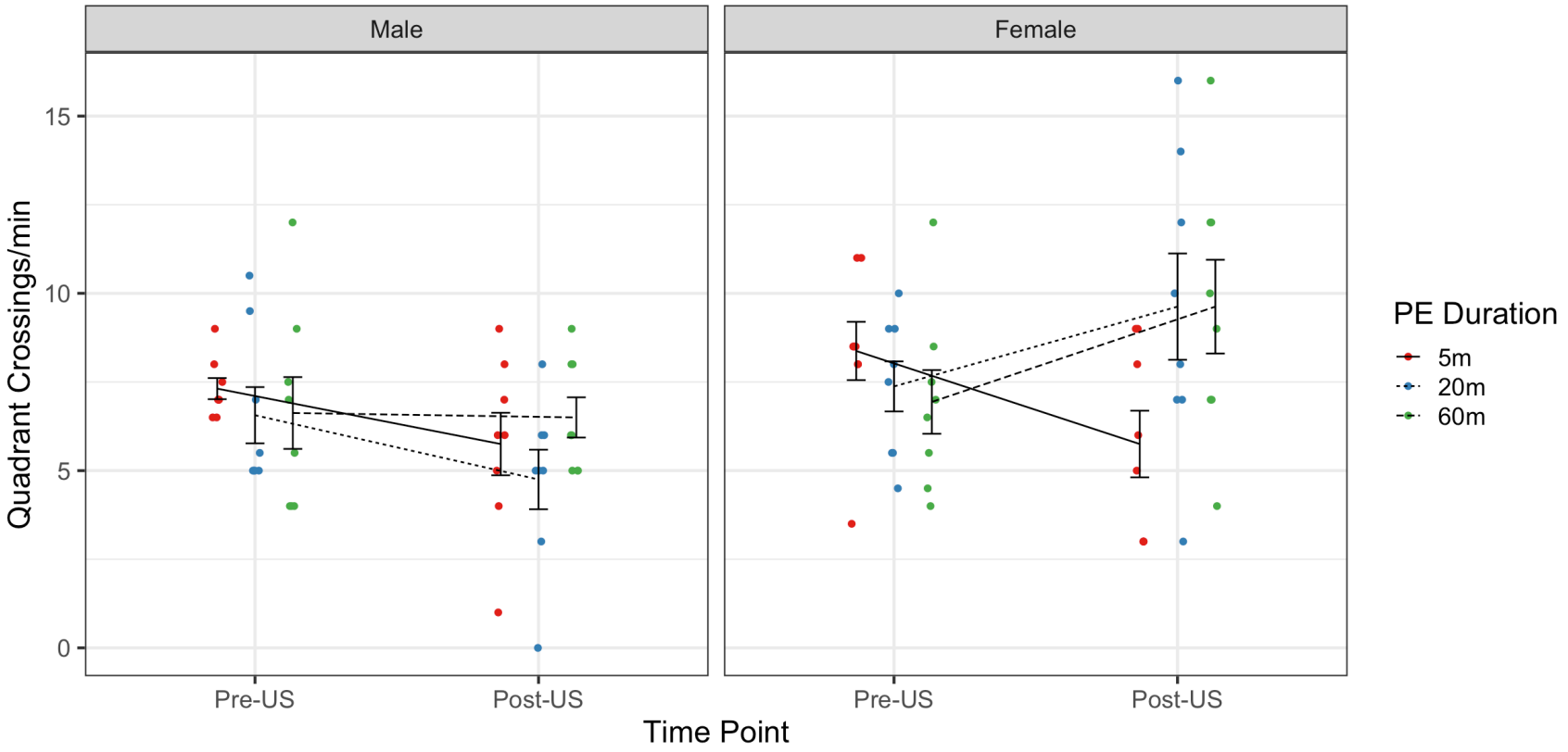

Figure 6.7. Quadrant crossings per minute for each sex and PE duration during the pre-US and post-US periods of the CFC session.

During the extinction session, model fit was improved by removing the three-way interaction term, as well as the two-way interaction terms between PE duration and time, and sex and time. The final model included the main effects of sex, PE duration and time, as well as the interaction term between sex and PE duration. There was no main effect of sex ($F_{1,228}$, $p = 0.129$, 95%CI = [-1.0, 0.1]), but there was a main effect of PE condition, with more frequent quadrant crossings in the 5m compared with 20m and 60m PE duration groups ($F_{2,228} = 10.9$, $p < 0.001$, 5m vs 20m 95%CI = [0.7, 2.3], 5m vs 60m 95%CI = [0.4, 2.1], 20m vs 60m 95%CI = [-1.1, 0.6]) (Figure 6.8). There was also a main effect of time with more frequent crossings in the first 2-minutes compared with the last 2-minutes ($F_{4,228} = 3.5$, $p = 0.009$, 0-2m vs 2-4m 95%CI = [-0.5, 2.0], 0-2m vs 4-6m 95%CI = [-0.5, 2.0], 0-2m vs 6-8m 95%CI = [-0.2, 2.5], 0-2m vs 8-10m 95%CI = [0.4, 2.9], 2-4m vs 4-6m 95%CI = [-1.3, 1.2], 2-4m vs 6-8m 95%CI = [-0.9, 1.7], 2-4m vs 8-10m 95%CI = [-0.4, 2.1], 4-6m vs 6-8m 95%CI = [-0.8, 1.7], 4-6m vs 8-10m 95%CI = [-0.4, 2.1], 6-8m vs 8-10m 95%CI = [-0.8, 1.7]). There was an interaction between sex and PE duration, with more frequent crossings in females in the 5m compared with 20m and 60m groups, but fewer crossings in the 20m compared with 5m and 60m groups in males ($F_{2,228} = 8.4$, $p <$ 0.001. Males: 5m vs 20m 95%CI = [-0.2, 1.8], 5m vs 60m 95%CI = [-1.2, -0.8], 20m vs 60m 95%CI = [-2.0, -0.0]. Females: 5m vs 20m 95%CI = [1.2, 3.2], 5m vs 60m 95%CI = [1.8, 3.7], 20m vs 60m 95%CI = [-0.5, 1.5]).

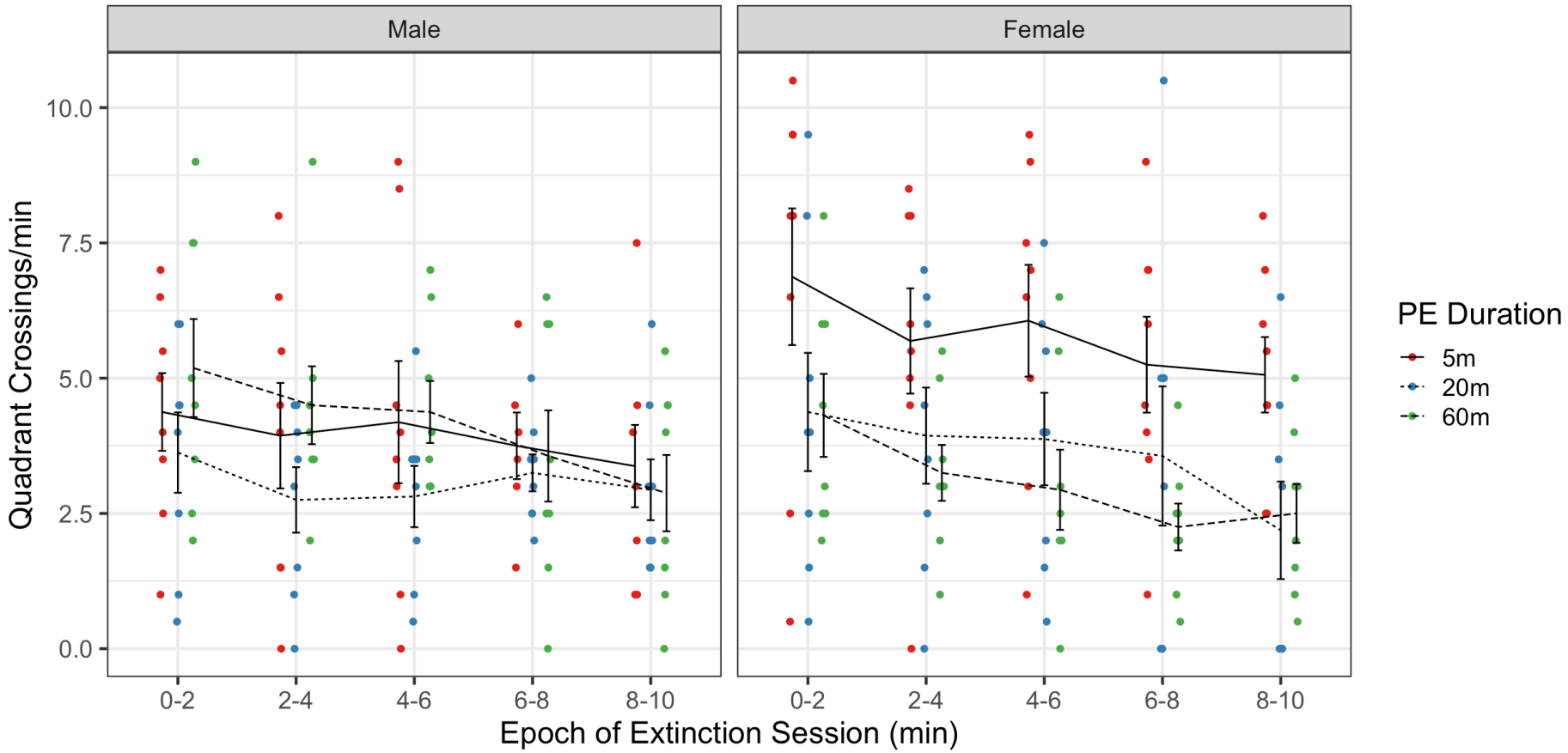

Figure 6.8. Quadrant crossings per minute for each sex and PE condition during each two-minute epoch of the extinction session.

When comparing the first 2-minutes of the extinction session with the 2-minute extinction recall session to assess between-session extinction, model fit improved by removing the three-way interaction term as well as the two-way interaction terms between time and PE duration, and time and sex. The final model included the main effects of time, sex, PE duration and the interaction between sex and PE duration. There was no main effect of time. However, there was a trend towards increased locomotor activity in the extinction recall session compared with the first 2-minute period of the extinction session ($F_{1,87}$ = 3.1, p = 0.082, 95%CI = [-2.0, 0.2]) (Figure 6.9). There was no main effect of sex ($F_{1,87}$ = 0.5, p = 0.466, 95%CI = [-1.5, 0.6]) or PE duration ($F_{2,87}$ = 1.9, p = 0.155, 5m vs 20m 95%CI = [-0.3, 2.9], 5m vs 60m 95%CI = [-1.0, 2.1], 20m vs 60m 95%CI = [-2.3, 0.8]). There was no interaction between sex and PE duration. However, there was a trend towards females in the 20m and 60m groups showing more frequent quadrant crossings compared with the 5m group, with no differences between PE groups found in males ($F_{2,87}$ = 2.8, p = 0.066. Males: 5m vs 20m 95%CI = [-1.4, 2.4], 5m vs 60m 95%CI = [-2.8, 0.8], 20m vs 60m 95%CI = [-3.4, 0.4]. Females: 5m vs 20m 95%CI = [0.2, 3.9], 5m vs 60m 95%CI = [0.3, 3.9], 20m vs 60m 95%CI = [-1.8, 1.9]).

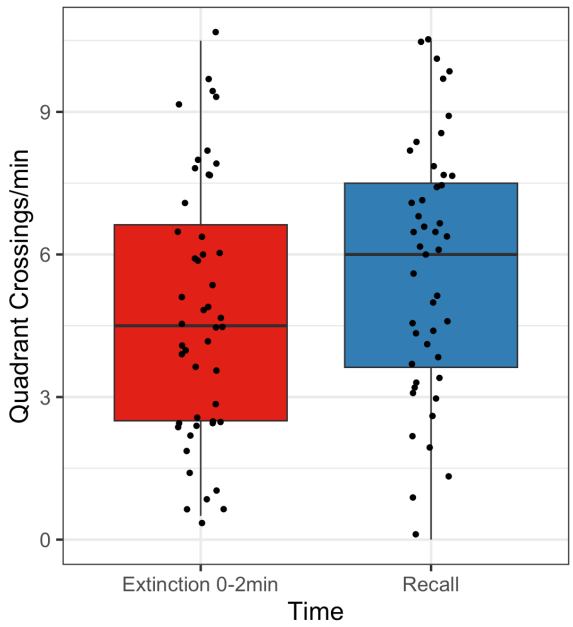

Figure 6.9. Quadrant crossings per minute during the first two-minute epoch of the extinction session compared with the extinction recall session 48 hours later.

In the 2-minute extinction recall session, no main effects or interactions were observed (Figure 6.10): sex ($F_{1,40}$ = < 0.1, p = 0.979, 95%CI = [-1.7, 1.6]); PE duration ($F_{2,40}$ = 0.5, p = 0.635, 5m vs 20m 95%CI = [-1.5, 3.3], 5m vs 60m 95%CI = [-2.1, 2.6], 20m vs 60m 95%CI = [-3.1, 1.7]); sex X PE duration ($F_{2,40}$ = 1.01, p = 0.349. Males: 5m vs 20m 95%CI = [-2.7, 3.2], 5m vs 60m 95%CI = [-3.9, 1.5], 20m vs 60m 95%CI = [-4.4, 1.6]. Females: 5m vs 20m 95%CI = [-1.1, 4.4], 5m vs 60m 95%CI = [-1.1, 4.4], 20m vs 60m 95%CI = [-2.7, 2.7]). Thus, neither sex, PE duration, nor the interaction between the two variables appear to significantly affect locomotor activity during the extinction recall session.

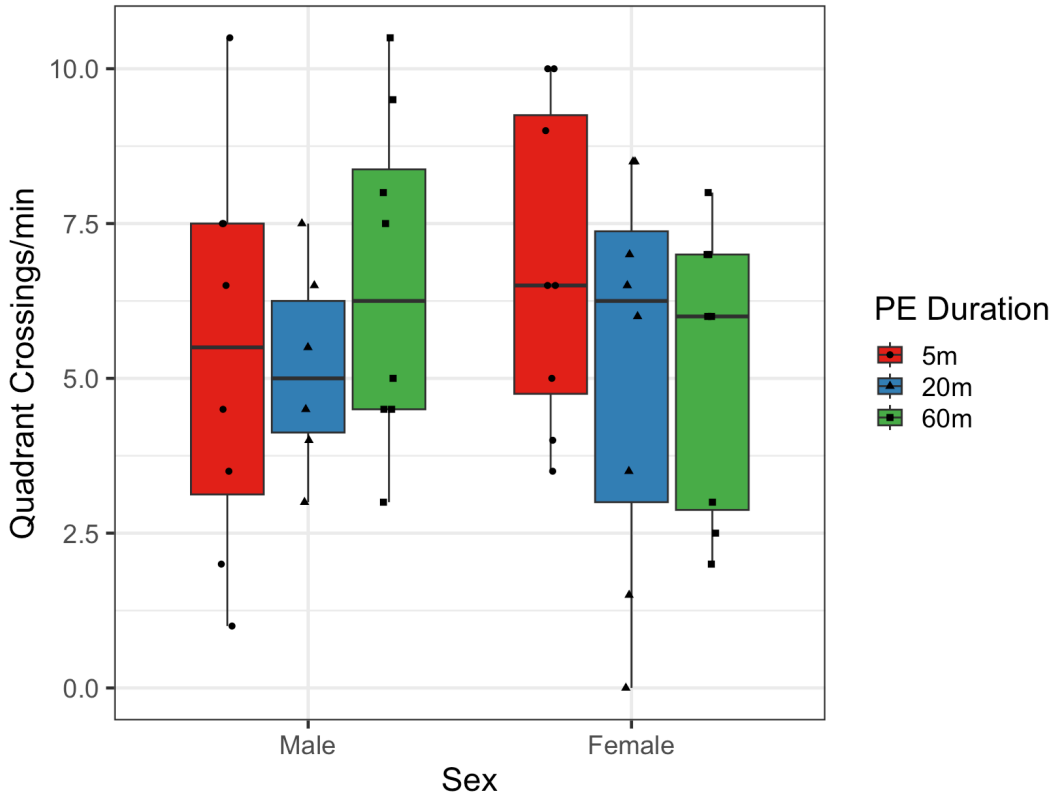

Figure 6.10. Quadrant crossings per minute for each sex and PE condition during the extinction recall session.

6.4.3 qPCR

6.4.3.1 CA1

All models were log transformed.

There was a main effect of sex on *Cfos* expression levels, with greater expression in females compared to males ($F_{1,40}$ = 10.6, p = 0.002, 95%CI = [-0.5, -0.1]) (Figure 6.11). There was no main effect of sex on *Arc* expression; however, there was a trend towards greater expression in females compared to males ($F_{1,40}$ = 2.7, p = 0.10, 95%CI = [-0.6, 0.0]). No main effect of sex was observed on *Bdnf-IX* levels ($F_{1,40}$ = 0.4, p = 0.558, 95%CI = [-0.2, 0.3]).

There was no significant main effect of PE duration for any of the three genes. *Arc*: ($F_{2,40}$ = 0.9, p = 0.413, 5m vs 20m 95%CI = [-0.5, 0.4], 5m vs 60m 95%CI = [-0.3, 0.7], 20m vs 60m 95%CI = [-0.2, 0.8]). *Cfos*: ($F_{2,40}$ = 1.7, p = 0.193, 5m vs 20m 95%CI = [-0.3, 0.2], 5m vs 60m 95%CI [-0.1, 0.4], 20m vs 60m 95%CI = [-0.1, 0.4]). *Bdnf-IX*: ($F_{2,40}$ = 0.6, p = 0.547, 5m vs 20m 95%CI = [-0.3, 0.4], 5m vs 60m 95%CI = [-0.4, 0.3], 20m vs 60m 95%CI = [-0.5, 0.2]).

In both *Cfos* and *Bdnf-IX* expression, there was an interaction between sex and PE duration. In both cases, no differences were found in males. However, in females, the 20m group showed higher *Cfos* expression, but lower *Bdnf-IX* expression, than the 5m and or 60m groups. No interaction between sex and PE duration was observed in *Arc* expression. *Cfos*: ($F_{2,40}$ = 5.7, p = 0.007. Males: 5m vs 20m 95%CI = [-0.0, 0.6], 5m vs 60m 95%CI = [-0.1, 0.5], 20m vs 60m 95%CI = [-0.4, 0.2]. Females: 5m vs 20m 95%CI = [-0.7, -0.1], 5m vs 60m 95%CI = [-0.2, 0.4], 20m vs 60m 95%CI = [0.2, 0.8]). *Bdnf-IX*: ($F_{2,40}$ = 4.0, p = 0.025. Males: 5m vs 20m 95%CI = [-0.7, 0.1], 5m vs 60m 95%CI = [-0.7, 0.1], 20m vs 60m 95%CI = [-0.4, 0.5]. Females: 5m vs 20m 95%CI = [0.1, 0.8], 5m vs 60m 95%CI = [-0.3, 0.5], 20m vs 60m 95%CI = [-0.7, 0.1]). *Arc*: ($F_{2,40}$ = 0.3, p = 0.722. Males: 5m vs 20m 95%CI = [-

0.5, 0.7], 5m vs 60m 95%CI = [-0.3, 0.9], 20m vs 60m 95%CI = [-0.4, 0.8]. Females: 5m vs 20m 95%CI = [-0.7, 0.4], 5m vs 60m 95%CI = [-0.5, 0.7], 20m vs 60m 95%CI = [-0.3, 0.9]).

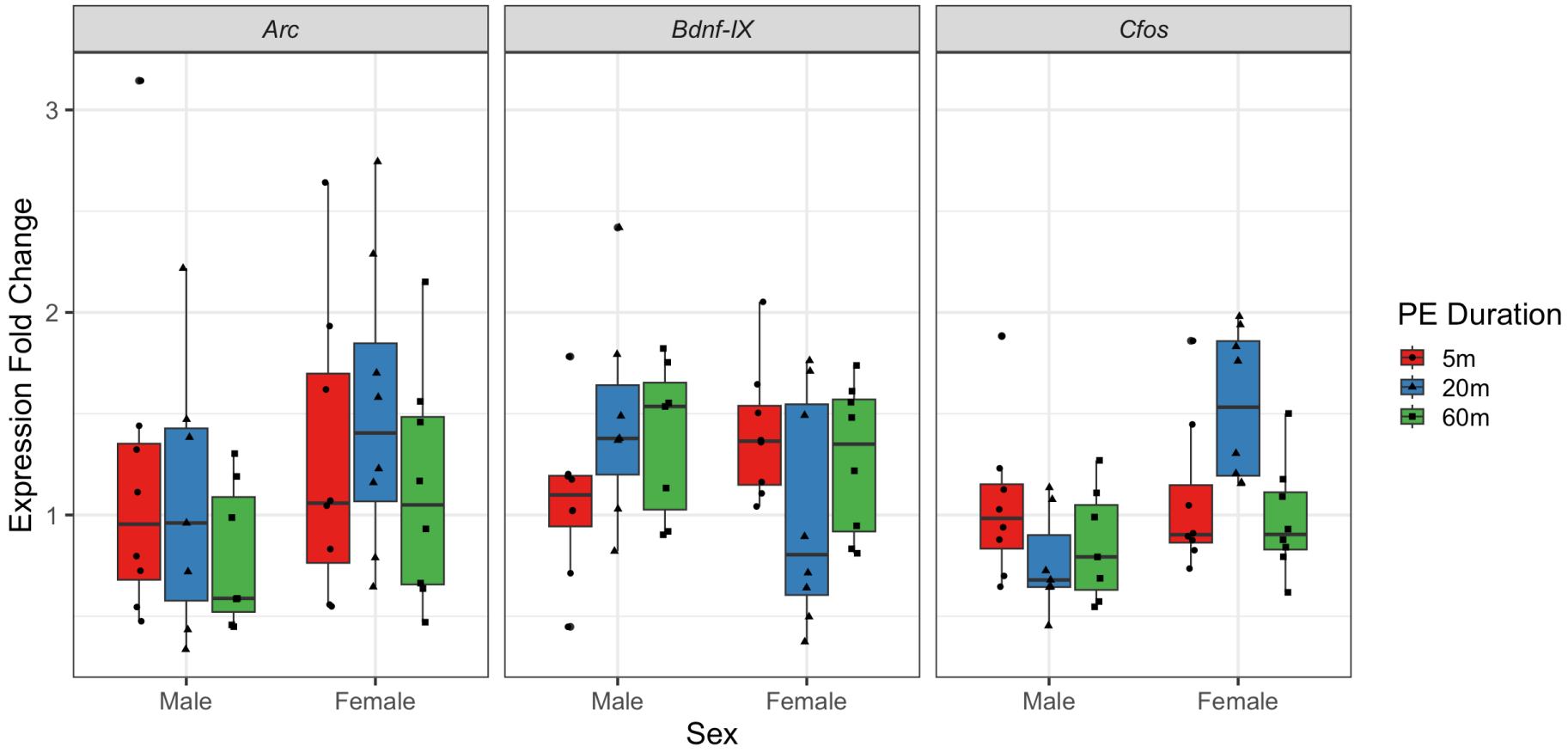

Figure 6.11. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the CA1 region of the hippocampus.

6.4.3.2 Dentate gyrus

Arc and *Cfos* fold change data was inverse square root transformed. No transformation was required for *Bdnf-IX* fold change data.

There was a main effect of sex for both *Arc* and *Cfos* expression, with higher expression in females compared with males in both genes (Figure 6.12). *Arc*: ($F_{1,40}$ = 5.6, p = 0.023, 95%CI = [0.0, 0.3]). *Cfos*: ($F_{1,40}$ = 4.5, p = 0.040, 95%CI = [0.0, 0.2]). There was no main effect of sex on *Bdnf-IX* expression ($F_{1,40}$ = 0.4, p = 0.540, 95%CI = [-0.1, 0.2]).

There was no main effect of PE duration in any IEG. *Arc*: ($F_{2,40}$ = 0.5, p = 0.596, 5m vs 20m 95%CI = [-0.2, 0.2], 5m vs 60m 95%CI = [-0.3, 0.1], 20m vs 60m 95%CI = [-0.3, 0.1]). *Cfos*: ($F_{2,40}$ = 2.3, p = 0.115, 5m vs 20m 95%CI = [-0.2, 0.2], 5m vs 60m 95%CI = [-0.3, 0.0], 20m vs 60m 95%CI = [-0.3, 0.0]). *Bdnf-IX*: ($F_{2,40}$ = 0.8, p = 0.440, 5m vs 20m 95%CI = [-0.2, 0.4], 5m vs 60m 95%CI = [-0.3, 0.2], 20m vs 60m 95%CI = [-0.4, 0.1]).

There was no interaction between sex and PE duration on *Cfos* expression. However, there was a trend towards females in the 20m group showing higher *Cfos* expression compared with females in the 5m or 60m PE duration groups, whereas in males, there was greater expression in the 5m compared with 60m group ($F_{2,40}$ = 4.8, p = 0.072. Males: 5m vs 20m 95%CI = [-0.4, 0.1], 5m vs 60m 95%CI = [-0.4, -0.0], 20m vs 60m 95%CI = [-0.3, 0.2]. Females: 5m vs 20m 95%CI = [-0.0, 0.4], 5m vs 60m 95%CI = [-0.2, 0.2], 20m vs 60m 95%CI = [-0.4, -0.0]). No interaction between sex and PE duration was observed in *Arc* expression ($F_{2,40}$ = 1.0, p = 0.362. Males: 5m vs 20m 95%CI = [-0.4, 0.1], 5m vs 60m 95%CI = [-0.4, 0.1], 20m vs 60m 95%CI = [-0.3, 0.2]. Females: 5m vs 20m 95%CI = [-0.1, 0.3], 5m vs 60m 95%CI = [-0.2, 0.2], 20m vs 60m 95%CI = [-0.3, 0.1]), or *Bdnf-IX* expression ($F_{2,40}$ = 1.1, p = 0.337. Males: 5m vs 20m 95%CI = [-0.4, 0.3], 5m vs 60m 95%CI = [-0.5, 0.1], 20m vs 60m 95%CI = [-0.5, 0.2]. Females: 5m vs 20m 95%CI = [-0.1, 0.5], 5m vs 60m 95%CI = [-0.2, 0.4], 20m vs 60m 95%CI = [-0.4, 0.2]).

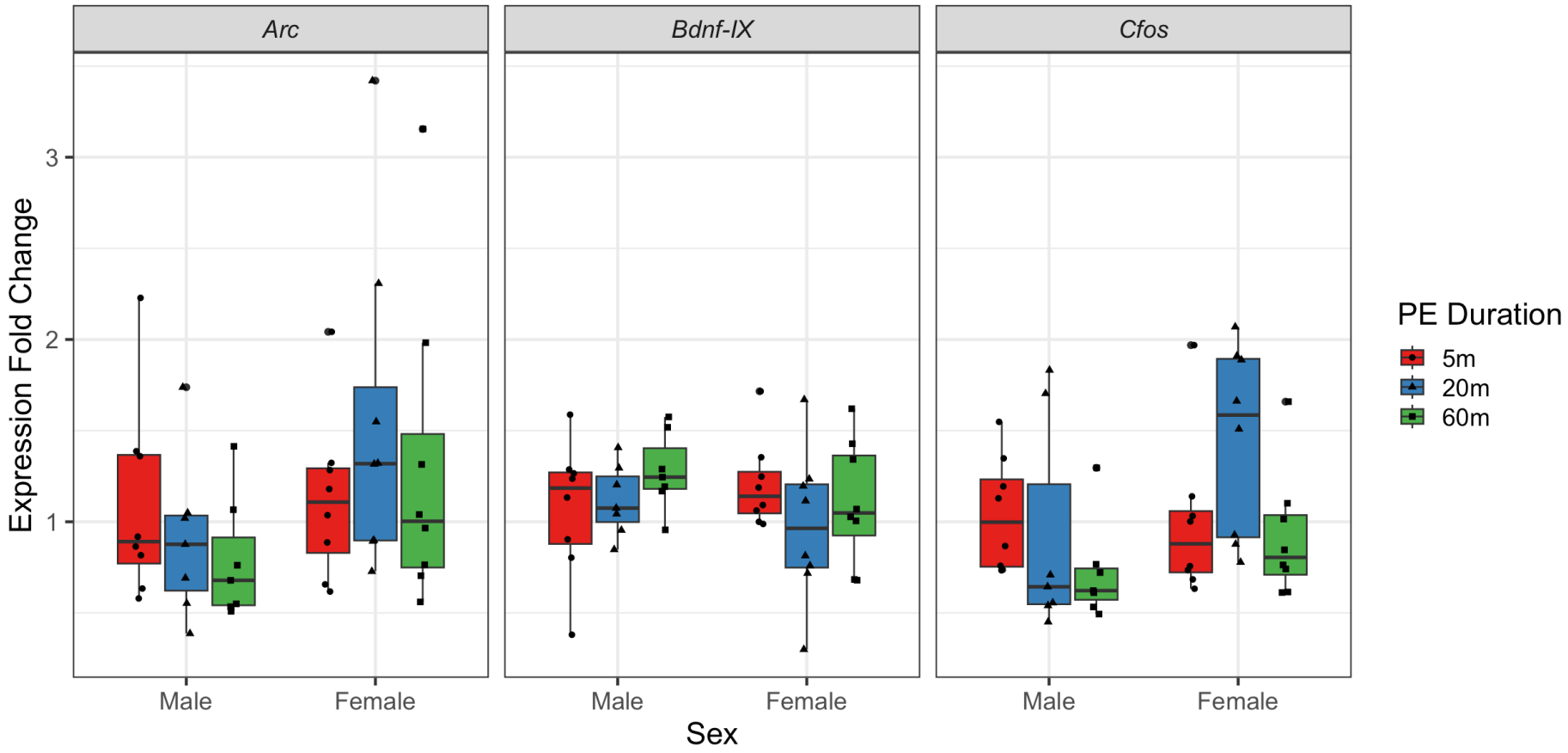

Figure 6.12. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the DG region of the hippocampus.

6.4.3.3 Infralimbic mPFC

Arc fold change data was square root transformed. No transformation was required for *Bdnf-IX* or *Cfos* fold change data. For both *Cfos* and *Bdnf-IX* expression, model fit improved by removing the interaction term between sex and PE duration.

There was no main effect of sex on expression of any of the IEGs (Figure 6.13). *Arc*: ($F_{1,40}$ = 0.2, p = 0.629, 95%CI = [-0.1, 0.1]). *Cfos*: ($F_{1,42}$ = 0.7, p = 0.405, 95%CI = [-0.2, 0.1]). *Bdnf-IX*: ($F_{1,42}$ = 2.7, p = 0.108, 95%CI = [-0.3, 0.0]).

There was no main effect of sex on *Cfos* expression. However, there was a trend towards higher expression in the 20m compared with 60m group ($F_{2,42}$ = 3.1, p = 0.053, 5m vs 20m 95%CI = [-0.3, 0.1], 5m vs 60m 95%CI = [-0.1, 0.4], 20m vs 60m 95%CI = [0.0, 0.5]). There was no main effect of PE group on expression of either *Arc* ($F_{2,40}$ = 0.8, p = 0.478, 5m vs 20m 95%CI = [-0.2, 0.1], 5m vs 60m 95%CI = [-0.1, 0.2], 20m vs 60m 95%CI = [-0.1, 0.2]) or *Bdnf-IX* ($F_{2,42}$ = 0.2, p = 0.781, 5m vs 20m 95%CI = [-0.3, 0.2], 5m vs 60m 95%CI = [-0.2, 0.2], 20m vs 60m 95%CI = [-0.2, 0.3]).

Although it was retained within the model, there was no interaction between sex and PE duration on *Arc* expression ($F_{2,40}$ = 0.5, p = 0.597. Males: 5m vs 20m 95%CI = [-0.2, 0.2], 5m vs 60m 95%CI = [-0.2, 0.2], 20m vs 60m 95%CI = [-0.2, 0.2]. Females: 5m vs 20m 95%CI = [-0.3, 0.1], 5m vs 60m 95%CI = [-0.1, 0.3], 20m vs 60m 95%CI = [-0.0, 0.3]).

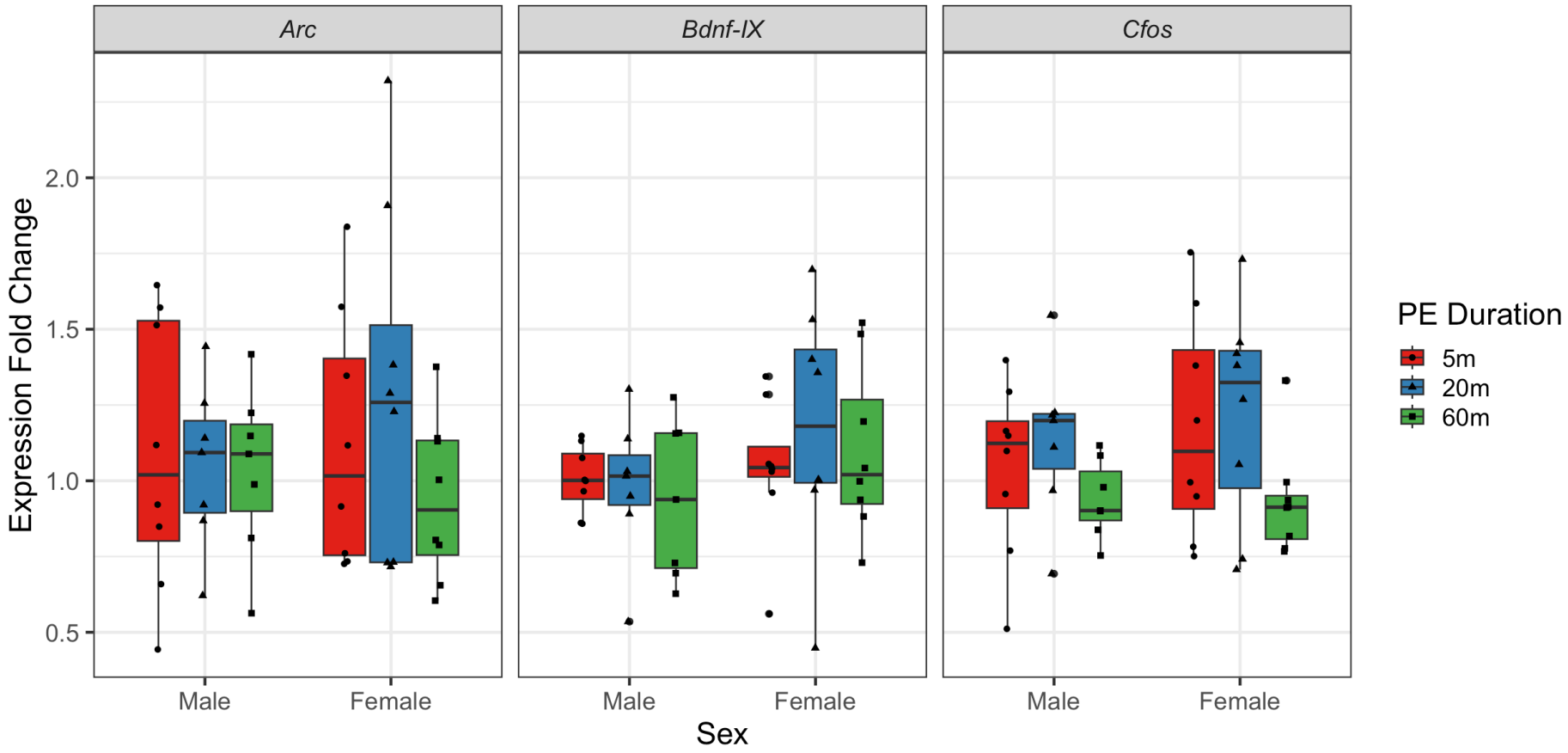

Figure 6.13. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the IL region of the mPFC.

6.4.3.4 Prelimbic mPFC

Cfos fold change data was log transformed and *Bdnf-IX* data was inverse square root transformed. No transformation was required for *Arc* fold change data. Model fit of *Bdnf-IX* expression improved by removing the interaction term between sex and PE duration.

There was no main effect of sex on *Arc* expression. However, there was a trend towards higher expression in females compared with males ($F_{1,40}$ = 2.8, p = 0.099, 95%CI = [-0.3, 0.0]) (Figure 6.14). There was no main effect of sex on the expression of either *Cfos* ($F_{1,40}$ = 1.3, p = 0.263, 95%CI = [-0.2, 0.1]) or *Bdnf-IX* ($F_{1,42}$ = 0.2, p = 0.661, 95%CI = [-0.1, 0.1]).

There was no main effect PE duration on expression of any of the IEGs. *Arc*: ($F_{2,40}$ = 1.0, p = 0.389, 5m vs 20m 95%CI = [-0.4, 0.2], 5m vs 60m 95%CI = [-0.2, 0.3], 20m vs 60m 95%CI = [-0.1, 0.4]). *Cfos*: ($F_{2,40}$ = 1.1, p = 0.359, 5m vs 20m 95%CI = [-0.3, 0.1], 5m vs 60m 95%CI = [-0.2, 0.3], 20m vs 60m 95%CI = [-0.1, 0.4]). *Bdnf-IX*: ($F_{2,42}$ = 1.4, p = 0.266, 5m vs 20m 95%CI = [-0.1, 0.2], 5m vs 60m 95%CI = [-0.2, 0.1], 20m vs 60m 95%CI = [-0.2, 0.0]).

There was no interaction between sex and PE duration for either *Cfos* or *Arc*. However, for both genes, there was a trend towards higher expression in females in the 20m compared with the 5m or 60m groups, with no differences seen in males. *Arc*: ($F_{2,40}$ = 3.1, p = 0.055. Males: 5m vs 20m 95%CI = [-0.2, 0.5], 5m vs 60m 95%CI = [-0.2, 0.4], 20m vs 60m 95%CI = [-0.4, 0.3]. Females: 5m vs 20m 95%CI = [-0.7, -0.1], 5m vs 60m 95%CI = [-0.4, 0.2], 20m vs 60m 95%CI = [0.0, 0.6]). *Cfos*: ($F_{2,40}$ = 2.6, p = 0.083. Males: 5m vs 20m 95%CI = [-0.2, 0.4], 5m vs 60m 95%CI = [-0.3, 0.3], 20m vs 60m 95%CI = [-0.4, 0.2]. Females: 5m vs 20m 95%CI = [-0.5, -0.0], 5m vs 60m 95%CI = [-0.2, 0.3], 20m vs 60m 95%CI = [0.0, 0.6]).

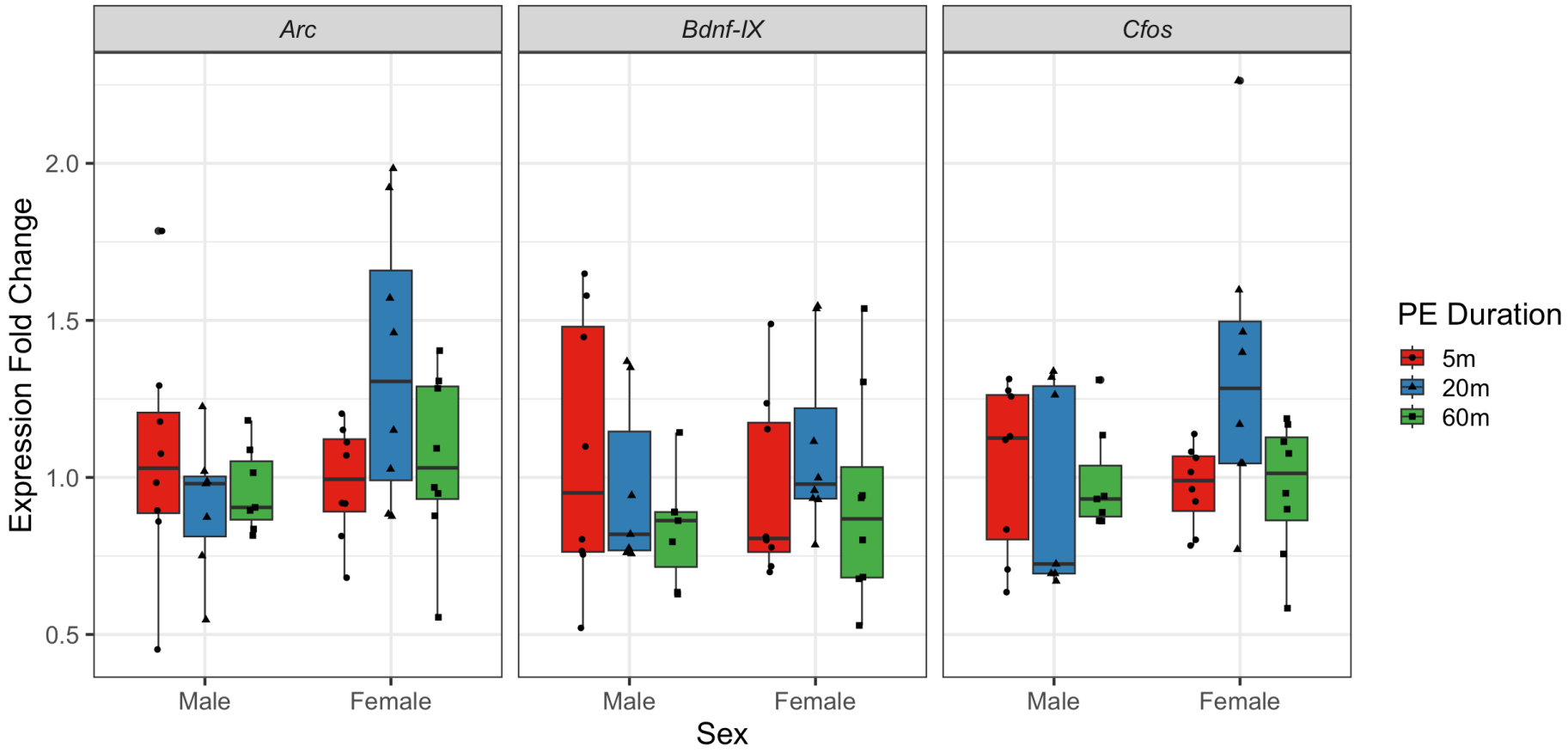

Figure 6.14. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the PL region of the mPFC.

6.4.3.5 Central amygdala

Arc fold change data was square root transformed, and *Cfos* and *Bdnf-IX* data were log transformed. Model fit of all three IEGs improved by removing the interaction term between sex and PE duration.

There was no main effect of sex on expression of any of the three IEGs (Figure 6.15). *Arc*: ($F_{1,42}$ = 0.1, p = 0.764, 95%CI = [-0.2, 0.1]). *Cfos*: ($F_{1,40}$ = 0.1, p = 0.767, 95%CI = [-0.4, 0.3]). *Bdnf-IX*: ($F_{1,41}$ < 0.1, p = 0.996, 95%CI = [-1.0, 0.9]).

There was also no main effect of PE duration on expression of any of the three IEGs. *Arc*: ($F_{2,41}$ = 0.5, p = 0.600, 5m vs 20m 95%CI = [-0.2, 0.3], 5m vs 60m 95%CI = [-0.1, 0.3], 20m vs 60m 95%CI = [-0.2, 0.3]). *Cfos*: ($F_{2,40}$ = 1.8, p = 0.173, 5m vs 20m 95%CI = [-0.2, 0.8], 5m vs 60m 95%CI = [-0.2, 0.8], 20m vs 60m 95%CI = [-0.5, 0.5]). *Bdnf-IX*: ($F_{2,41}$ = 1.6, p = 0.225, 5m vs 20m 95%CI = [-0.4, 2.4], 5m vs 60m 95%CI = [-0.8, 2.0], 20m vs 60m 95%CI = [-1.8, 1.0]).

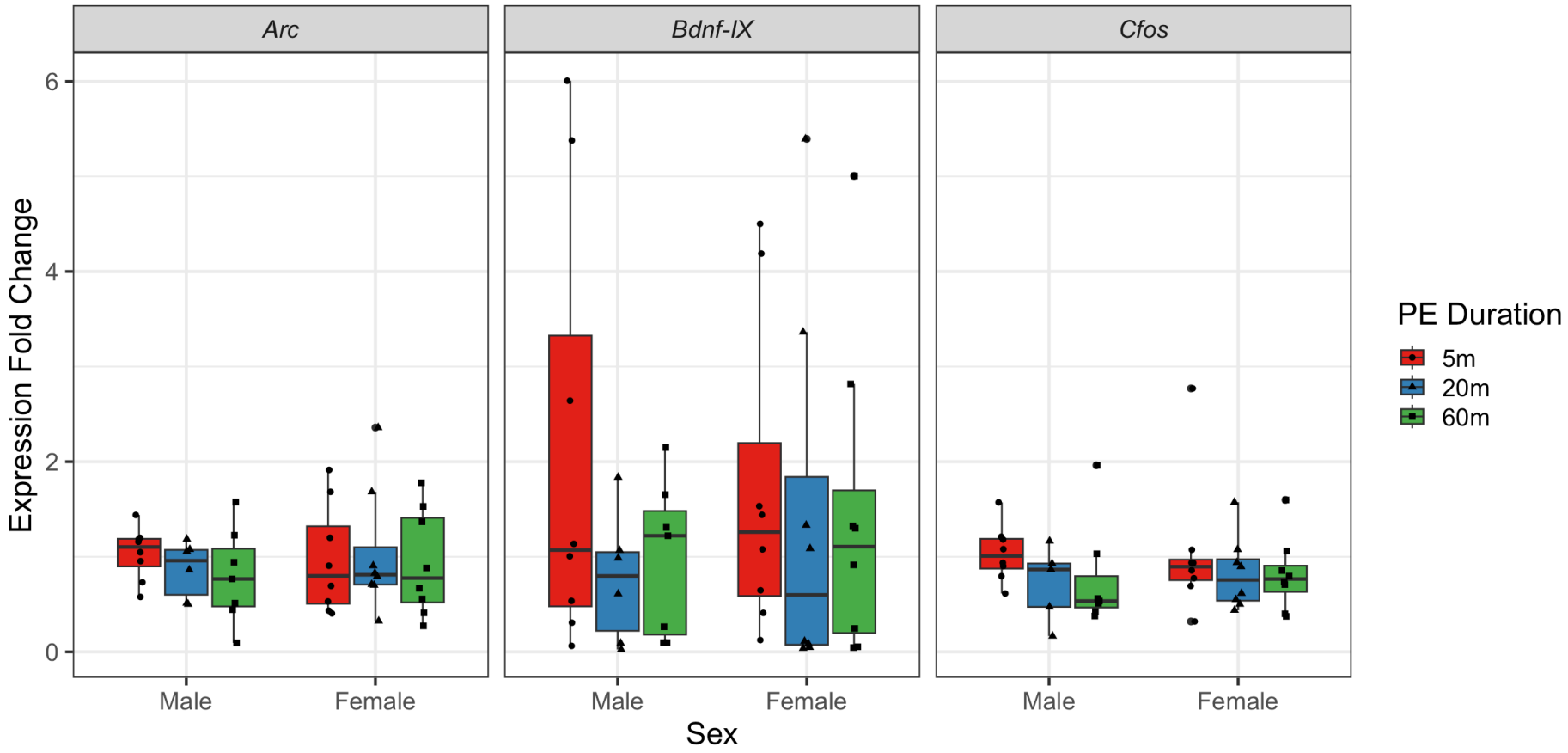

Figure 6.15. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the CeA.

6.4.3.6 Basolateral amygdala

Fold change data for all three genes was log transformed. Model fit of all three IEGs improved by removing the interaction term between sex and PE duration.

There was no main effect of sex on expression of any of the three IEGs (Figure 6.16). *Arc*: ($F_{1,41}$ = 0.6, p = 0.453, 95%CI = [-0.3, 0.2]). *Cfos*: ($F_{1,41}$ = 0.1, p = 0.725, 95%CI = [-0.2, 0.2]). *Bdnf-IX*: ($F_{1,41}$ < 0.1, p = 0.996, 95%CI = [-1.0, 0.9]).

There was also no main effect of PE duration on expression of any of the three IEGs. *Arc*: ($F_{2,41}$ = 0.4, p = 0.681, 5m vs 20m 95%CI = [-0.5, 0.3], 5m vs 60m 95%CI = [-0.4, 0.4], 20m vs 60m 95%CI = [-0.3, 0.5]). *Cfos*: ($F_{2,41}$ = 0.3, p = 0.712, 5m vs 20m 95%CI = [-0.4, 0.3], 5m vs 60m 95%CI = [-0.3, 0.4], 20m vs 60m 95%CI = [-0.2, 0.4]). *Bdnf-IX*: ($F_{2,41}$ = 1.6, p = 0.225, 5m vs 20m 95%CI = [-0.4, 2.4], 5m vs 60m 95%CI = [-0.8, 2.0], 20m vs 60m 95%CI = [-1.8, 1.0]).

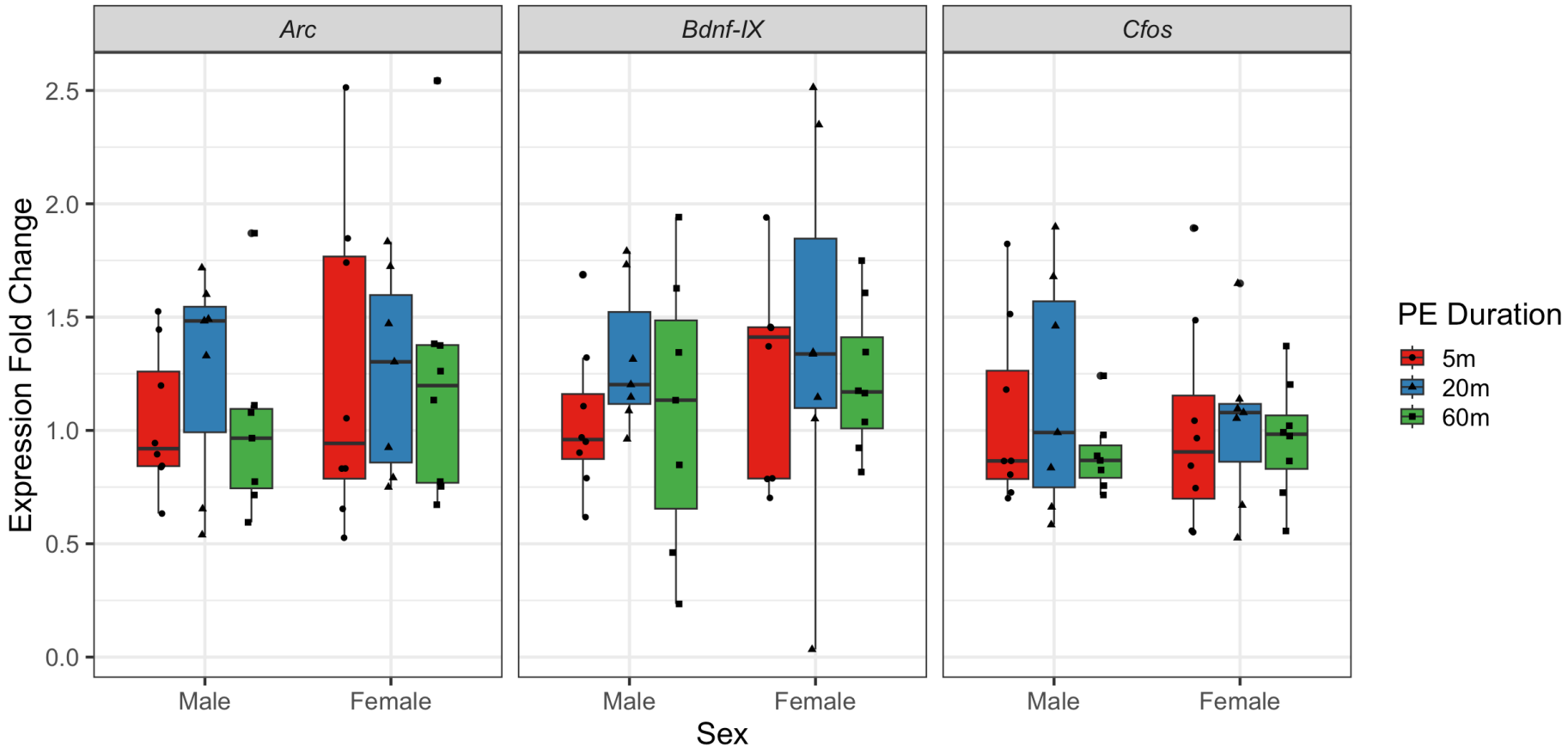

Figure 6.16. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the BLA.

6.4.3.7 Nucleus accumbens core

Cfos fold change data was inverse square root transformed, and *Arc* and *Bdnf-IX* data were log transformed. Model fit of *Arc* and *Bdnf-IX* expression improved by removing the interaction term between sex and PE duration.

There was no main effect of sex on expression of any of the three IEGs (Figure 6.17). *Arc*: ($F_{1,42} < 0.1$, $p = 0.863$, 95%CI = [-0.2, 0.3]). *Cfos*: ($F_{1,40} = 0.1$, $p = 0.823$, 95%CI = [-0.1, 0.1]). *Bdnf-IX*: ($F_{1,42} = 0.1$, $p = 0.789$, 95%CI = [-1.2, 0.9]).

There was also no main effect of PE duration on expression of any of the three IEGs. *Arc*: ($F_{2,42} = 0.9$, $p = 0.416$, 5m vs 20m 95%CI = [-0.4, 0.4], 5m vs 60m 95%CI = [-0.2, 0.6], 20m vs 60m 95%CI = [-0.2, 0.6]). *Cfos*: ($F_{2,40} = 2.0$, $p = 0.155$, 5m vs 20m 95%CI = [-0.2, 0.1], 5m vs 60m 95%CI = [0.3, 0.0], 20m vs 60m 95%CI = [-0.3, 0.1]). *Bdnf-IX*: ($F_{2,42} = 0.6$, $p = 0.551$, 5m vs 20m 95%CI = [-1.1, 1.9], 5m vs 60m 95%CI = [-0.8, 2.2], 20m vs 60m 95%CI = [-1.2, 1.8]).

Although it was retained within the model, there was no interaction between sex and PE duration on *Cfos* expression ($F_{2,40} = 1.5$, $p = 0.228$. Males: 5m vs 20m 95%CI = [-0.2, 0.2], 5m vs 60m 95%CI = [-0.4, 0.0], 20m vs 60m 95%CI = [-0.4, -0.0]. Females: 5m vs 20m 95% CI = [-0.3, 0.1], 5m vs 60m 95%CI = [-0.2, 0.1], 20m vs 60m 95%CI = [-0.2, 0.2]).

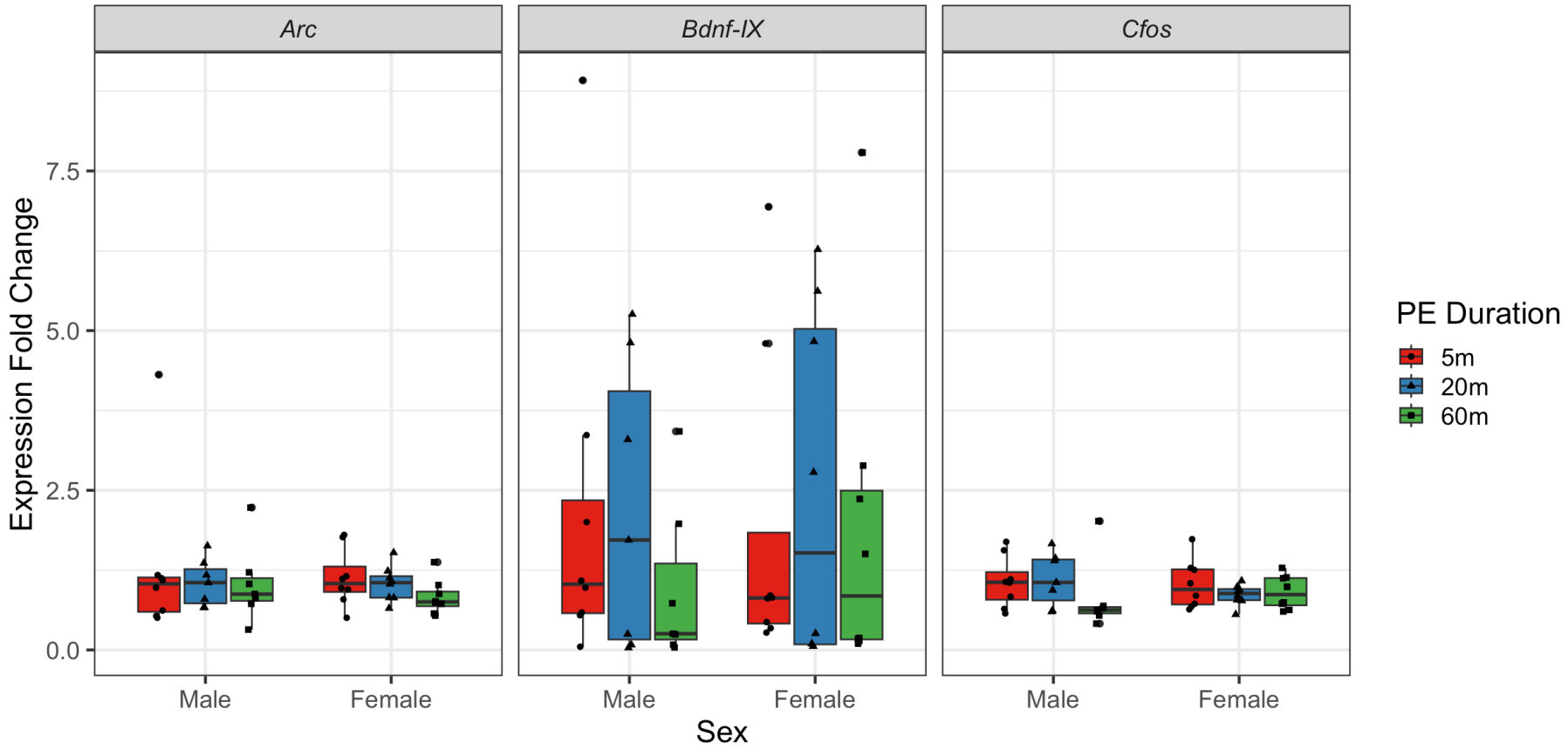

Figure 6.17. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the NAcc.

6.4.3.8 Nucleus accumbens shell

Fold change data for all three genes was log transformed. Model fit of all three IEGs improved by removing the interaction term between sex and PE duration.

There was no main effect of sex on expression of any of the three IEGs (Figure 6.18). *Arc*: ($F_{1,42}$ = 0.7, p = 0.404, 95%CI = [-0.2, 0.6]). *Cfos*: ($F_{1,42}$ = 0.6, p = 0.434, 95%CI = [-0.1, 0.3]). *Bdnf-IX*: ($F_{1,42}$ = 1.0, p = 0.325, 95%CI = [-0.8, 0.3]).

There was also no main effect of PE duration on expression of any of the three IEGs. *Arc*: ($F_{2,42}$ = 1.4, p = 0.258, 5m vs 20m 95%CI = [-0.8, 0.4], 5m vs 60m 95%CI = [-0.4, 0.8], 20m vs 60m 95%CI = [-0.2, 1.1]). *Cfos*: ($F_{2,42}$ = 0.6, p = 0.553, 5m vs 20m 95%CI = [-0.4, 0.3], 5m vs 60m 95%CI = [-0.3, 0.4], 20m vs 60m 95%CI = [-0.2, 0.5]). *Bdnf-IX*: ($F_{2,42}$ = 2.3, p = 0.108, 5m vs 20m 95%CI = [-0.2, 1.4], 5m vs 60m 95%CI = [-0.8, 0.8], 20m vs 60m 95%CI = [-1.4, 0.2]).

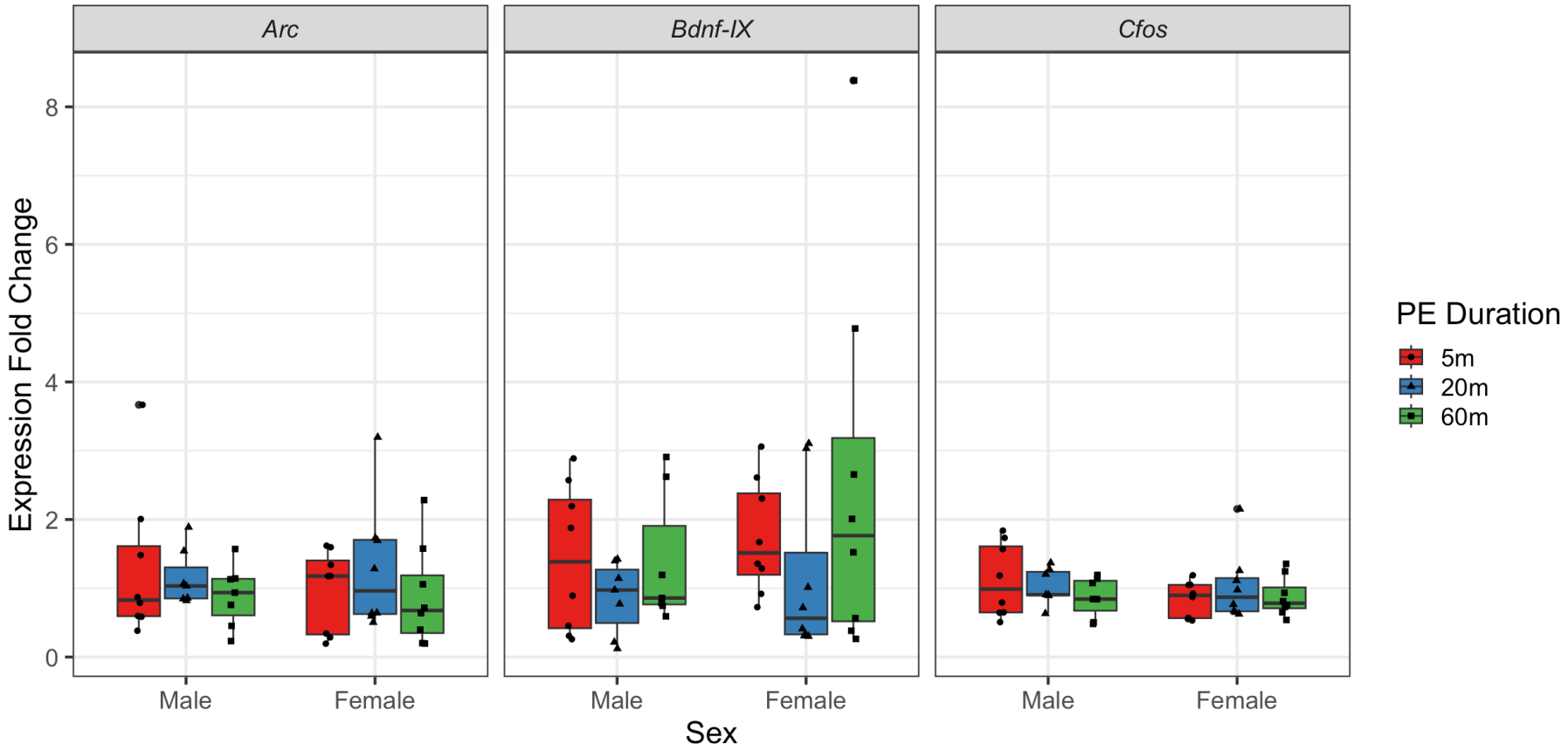

Figure 6.18. Gene expression fold change relative to the mean expression value of males in the 5m PE duration group for the genes *Arc*, *Bdnf-IX* and *Cfos* in the NAcsh.

6.4.4 Summary of Results

All behavioural results are summarised in Table 6.1. There were no overall sex differences in the level of freezing. During the extinction session, there was an overall effect of greater freezing in the 5m and 20m PE groups compared with the 60m PE group. However, this effect interacted with sex, with females showing the highest level of freezing in the 20m PE group compared to the 5m and 60m groups, whereas in males there was higher freezing in the 5m and 20m groups compared with the 60m groups. There was also a trend towards this finding in females during the post-US period of the CFC session, and a trend towards the effect in males during the extinction recall session. Although these findings were not statistically significant, they showed a consistent direction of effect. Thus, these results suggest that there are sex differences in the relationship between context PE duration and subsequent contextual fear memory processing.

Females made more frequent quadrant crossings compared with males in the post-US period of the CFC session, with no other effects seen during the CFC session. During the extinction session, animals in the 5m PE group made more frequent quadrant crossings compared with those in the 20m and 60m groups. However, this effect interacted with sex, with males making more frequent quadrant crossings in the 60m and 5m PE groups compared with the 20m PE group, whereas females made more frequent crossings in the 5m compared with 20m and 60m groups. Thus, these findings also indicate sex differences in the relationship between PE duration and subsequent contextual fear memory processing. However, this is reflected differently between the two behavioural measures.

All qPCR results are summarised in Table 6.2. Females showed higher *Cfos* expression compared with males in both hippocampal subregions, but no other areas. Higher *Arc* expression was observed in females compared with males in the DG, CA1 and PL (although not reaching statistical significance at α= 0.05 in CA1 and PL. No overall sex differences were seen in *Bdnf-IX* expression in any region. There was a trend towards higher *Cfos* expression in the 20m compared with 60m

PE group in the IL, but no other overall effects of PE group were seen in IEG expression in any region.

The majority of interactions between sex and PE group involved differences between PE groups observed in females that were not observed in males. In the CA1 region, *Bdnf-IX* and *Cfos* showed a mirrored pattern in females with higher *Cfos* expression in the 5m compared with 20m PE group, but higher *Bdnf-IX* expression in the 5m compared with 20m PE group. There was also a trend towards higher *Cfos* expression in the 20m compared with 5m and 60m groups that was specific to females in both the DG and PL. This was also found to be the case for *Arc* expression in the PL. A trend towards higher *Cfos* expression in males in the 5m group compared with males in the 60m group was seen in the DG. No effects of either sex or PE duration or interactions between the two variables were seen in the CeA, BLA, NAcc or NAcsh. Thus, these results suggest that there are sex differences in the effects of context PE duration on IEG expression after CFC, extinction and recall in the DG, CA1 and PL, but not IL, CeA, BLA, NAcc or NAcsh.

Table 6.1. Summary of effects of sex and PE duration (3x5m, 3x20m or 3x60m) on behavioural expression of fear (freezing and quadrant crossings) during CFC, extinction and extinction recall sessions. * indicates this did not reach statistical significance at α = 0.05 but was statistically significant at α = 0.10. † indicates a non-statistically significant interaction term but a post-hoc finding that is significant at α = 0.05.

		Dependent Variable						
		Freezing			Quadrant Crossings			
		Post-US	Extinction	Extinction Recall	Pre-US	Post-US	Extinction	Extinction Recall
Effect	Sex	-	-	-	-	F > M	-	-
	PE Duration	-	5m > 60m 20m > 60m	-	-	-	5m > 20m 5m > 60m	-
	Time		-				0-2m > 8-10m	
	Sex * PE	F: 5m > 60m† F: 20m > 60m†	M: 5m > 60m M: 20m > 60m F: 20m > 5m F: 20m > 60m	M: 5m > 60m†	-	-	M: 5m > 20m M: 60m > 20m F: 5m > 20m F: 5m > 60m	-
	Time * Sex		-				-	
	Time * PE		-				-	

Table 6.2. Summary of effects of sex and PE duration (3x5m, 3x20m or 3x60m) on gene expression of IEGs *Arc*, *Cfos*, and *Bdnf-IX* in regions with known involvement in LI. * indicates this did not reach statistical significance at $\alpha = 0.05$ but was statistically significant at $\alpha = 0.10$. † indicates a non-statistically significant interaction term but a post-hoc finding that is significant at $\alpha = 0.05$.

		Effect		
Region	Gene	Sex	PE Duration	Sex * PE Duration
CA1	*Arc*	F > M*	-	-
	Cfos	F > M	-	F: 20m > 5m; 20m > 60m
	Bdnf-IX	-	-	F: 5m > 20m; 60m > 20m
DG	*Arc*	F > M	-	-
	Cfos	F > M	-	F: 20m > 5m; 20m > 60m† M: 5m > 60m†
	Bdnf-IX	-	-	-
IL	*Arc*	-	-	-
	Cfos	-	20m > 60m†	-
	Bdnf-IX	-	-	-
PL	*Arc*	F > M*	-	F: 20m > 5m; 20m > 60m†
	Cfos	-	-	F: 20m > 5m; 20m > 60m†
	Bdnf-IX	-	-	-
CeA	*Arc*	-	-	-
	Cfos	-	-	-
	Bdnf-IX	-	-	-
BLA	*Arc*	-	-	-
	Cfos	-	-	-
	Bdnf-IX	-	-	-
NAcc	*Arc*	-	-	-
	Cfos	-	-	-
	Bdnf-IX	-	-	-
NAcsh	*Arc*	-	-	-
	Cfos	-	-	-
	Bdnf-IX	-	-	-

6.5 Discussion

The aim of this experiment was to assess whether the relationship between PE duration and subsequent CFC differed between sexes. Furthermore, after extinction learning and recall, expression of IEGs (*Arc*, *Cfos* and *Bdnf-IX*) in the hippocampus, mPFC, amygdala and NAc was measured. Taken together, the behavioural findings support the hypothesis of sex differences in the relationship between PE duration and contextual fear memory recall and extinction. Few overall sex differences were observed in the behavioural measures of freezing and quadrant crossings. However, several sex-specific differences between PE durations were observed. During the immediate post-US period, females in the shorter PE duration groups froze more than those in the longest PE duration group, a finding which is in line with the expectation of longer PE durations producing a stronger LI effect. However, no differences were observed between PE durations in males in the post-US period. Unexpectedly, during extinction learning, the intermediate PE duration group showed greater freezing compared to the high and low groups in females. Conversely, males showed greater freezing in the shorter PE duration groups compared with the longest PE duration group, which was the same finding observed in females in the post-US period. At extinction recall, there was greater freezing in males in the low compared with high PE duration groups, with no differences seen in females. Thus, it appears that the predicted relationship of increased behavioural fear expression with longer PE durations that was seen in males is not displayed in females other than in the post-US period. Conversely, during extinction training, the intermediate PE duration elicited the highest behavioural fear response in females. This pattern was also observed during extinction recall, albeit not at a statistically significant level.

Quadrant crossings were measured as a proxy for locomotor behaviour as a secondary behavioural measure of fear expression and sex differences in the effect of PE duration were found that differed to the findings from freezing behaviour. During the immediate post-US period, no differences between PE durations were observed in males. However, in females, those in the low PE duration group showed less locomotor activity compared to those in the higher PE duration groups. This

female-specific finding may indicate that females in this group are showing a heightened fear response that is expressed by more tentative movement. Thus, although no main effect of sex was seen in freezing behaviour in the post-US period, these results may mean that sex differences are only reflected in changes in locomotor activity. Conversely, during extinction training, females in the low PE duration group showed higher locomotor activity compared with intermediate and high PE duration groups, in contrast to the freezing findings. This may reflect the fact that animals in the low PE duration group had limited prior experience in the conditioning context. Hence, increased locomotor activity in this group may indicate increased exploratory behaviour, and thus this may reflect a sex difference in the effect of PE duration on the behavioural response to novelty rather than CFC. Males did not show this difference but did show a slight decrease in locomotor activity in the intermediate PE duration group compared to the high PE duration group. However, this finding does not appear clear cut across all epochs of the extinction session. The results from Chapter 4 indicated that the threshold for eliciting LI would differ between males and females, but it was still thought that the relationship between PE duration and fear response would be generally linear, with greater context PE durations resulting in a lower fear response. The results from this experiment suggest otherwise, as the highest fear response in females was elicited by the intermediate PE duration group.

RT-qPCR was conducted on numerous brain regions from tissue taken 30 minutes after the extinction recall session to assess group differences in the expression of the IEGs, *Cfos*, *Arc* and *Bdnf-IX*. In the CA1 region, interactions between the effects of sex and PE duration were seen in both *Cfos* and *Bdnf-IX*, but not in *Arc* expression. An opposing pattern was seen in the two genes, with the intermediate PE duration in females resulting in increased *Cfos* but decreased *Bdnf-IX* expression relative to the other two PE durations. In males, there was marginal evidence for increased *Cfos* expression in the intermediate compared with low PE duration group. However, this was not as clear as the findings in females. The pattern seen in *Cfos* in the CA1 in females was also found in the DG, and in males there was evidence for increased *Cfos* expression in the high compared to low PE duration in this region.

However, the interaction between sex and PE duration in *Bdnf-IX* seen in the CA1 was not found in the DG. Together, this suggests that both regions have some involvement in contextual fear memory recall and that the differing PE duration differences seen between sexes is reflected in differential activation in these regions. However, synaptic plasticity processes associated with sex differences in LI after extinction recall may be specific to the CA1 region. Although this method cannot comprehensively examine the intricacies of plasticity mechanisms taking place, it is worthy of note that the sex difference in the effect of PE duration was reflected in *Bdnf-IX*, but not *Arc* expression, indicating that the underlying plasticity processes responsive to PE duration in females may be specific to BDNF.

No effects of sex on gene expression in the IL region of the mPFC were observed. However, PE duration appeared to impact *Cfos* expression, with lower expression in the highest PE duration group relative to the two other durations, and this effect did not differ according to sex. In the PL region, interactions between the effects of sex and PE duration on both *Cfos* and *Arc* approached significance but not in *Bdnf-IX* expression. No differences were seen in males between PE group durations. However, in females, expression of both *Cfos* and *Arc* was higher in the intermediate PE duration group compared with the low and high duration groups, in a manner that mirrors the results of freezing behaviour and *Cfos* expression in the CA1 and DG regions of the hippocampus. Thus, the mPFC may preferentially be used in females in conjunction with the hippocampus during the recall of contextual fear memories when compared with males. This is in line with previous evidence to suggest that the PFC may be required in females for fear memory extinction, but not in males (Baran et al., 2010), and that this may be specific to the PL region of the mPFC (Fenton et al., 2016). Thus, this importance of the mPFC in females may extend to degree of learned irrelevance of context. However, it is interesting that the interaction between sex and PE duration was reflected in *Arc* but not *Bdnf-IX* expression in the PL area of the mPFC, in contrast with the CA1 region of the hippocampus, in which the interaction was reflected in *Bdnf-IX* but not *Arc* expression. Thus, plasticity mechanisms underpinning memory processing may be distinct between these two areas in females. Despite previously discussed evidence for the involvement of the

amygdala and NAc in LI, no effects of either sex or PE duration, or interaction between the two variables was observed in expression of any of the three genes in the CeA, BLA, NAcc or NAcsh. Thus, although the amygdala and NAc may be involved in LI, the functioning of these regions may not be modulated by the degree of learned irrelevance.

Taken together, these results suggest that the interaction between sex and PE duration observed in the behavioural results is reflected in changes in IEG expression in the CA1 and DG regions of the hippocampus and the PL area of the mPFC, but not the IL region of the mPFC, amygdala or NAc. The results of this experiment are suggestive of sex differences in the LI of CFC, a process which has been considerably understudied relative to the LI of cued fear conditioning, despite its relevance to neuropsychiatric disease. Thus, it is important to consider whether risk SNPs for neuropsychiatric conditions such as *CACNA1C* may interact with this sexual dimorphism in the hippocampus and mPFC to subsequently differentially affect males and females. In order to investigate this further, the following chapter will discuss RNA-sequencing experiments which have sought to assess the effect of *Cacna1c* genotype on downstream gene expression in the CA1 and DG regions of the hippocampus, and whether this interacts with sex and/or the encoding of a novel context.

Chapter 7: The effect of *Cacna1c* heterozygosity and sex on hippocampal gene expression

7.1 Introduction

As previously discussed, the hippocampus is a site of known importance for cognitive functions implicated in neuropsychiatric disorders such as schizophrenia, bipolar disorder and depression. *CACNA1C* risk SNPs have been implicated in all three disorders, and recent fine-mapping analysis of GWAS data has shown variation in this gene is a likely causal candidate for contributing to risk for the development of schizophrenia. Furthermore, the same study showed that granule neurons of the DG and pyramidal neurons of the CA1 and CA3 were highly enriched for expression of genes implicated by the GWAS analysis (Trubetskoy et al., 2022). The functional role of *CACNA1C* in calcium signalling is crucial to healthy functioning of the hippocampus in mechanisms underpinning learning and memory, and $Ca_v1.2$ mediated Ca^{2+} entry into the cell is a pivotal component of numerous downstream synaptic plasticity processes (Langwieser et al., 2010; Sridharan et al., 2020; Navakkode et al., 2022; Ma et al., 2023).

Both NMDA and LTCC mediated calcium entry into the cell are required for E-TC to take place, a process whereby neuronal activity results in downstream gene transcription which is known to be vital to synaptic plasticity processes underpinning learning and memory (Ma et al., 2023). Hence, disruptions to these processes caused by alterations in $Ca_v1.2$ channel expression may have a variety of deleterious consequences affecting learning and memory processes, which may then result in the development of neuropsychiatric symptoms. $Ca_v1.2$ channels have been highlighted as a possible highly druggable target and thus a site of interest for

the development of novel pharmacological therapeutics (Cipriani et al., 2016; Harrison et al., 2022). However, LTCCs play a crucial role in cardiac function (Shaw and Colecraft, 2013). Hence, a considerable challenge for the use of drugs targeting LTCCs is avoiding unintended off-target cardiac effects. Thus, understanding the downstream consequences of altered *CACNA1C* expression that are specific to sites such as the hippocampus, which plays a specific role in the development of psychiatric symptoms, may facilitate the development of pharmacological targets that have a precise mechanism of action with limited adverse effects.

Chapters 3 and 4 have detailed experiments which demonstrate the deleterious impact of *Cacna1c* heterozygosity on specific hippocampal dependent fear learning and memory processes. The experiment in Chapter 6 was conducted in wild-type animals and demonstrated that aspects of hippocampal dependent fear learning and associated molecular mechanisms may be sexually dimorphic. However, these findings are limited in their ability to provide insights into underlying mechanisms, as in Chapters 3 and 4, only a single peripheral measure of HPA-axis activity were taken, and in Chapter 6, only three genes were investigated. Hence, the aim of the two experiments described below is to use RNA-sequencing and subsequent gene-set enrichment analysis to explore the impact of *Cacna1c* heterozygosity on downstream gene expression in both DG and CA1 regions of the hippocampus. The first experiment will analyse differential gene expression in an existing RNA-sequencing dataset of both *Cacna1c*$^{+/+}$ and *Cacna1c*$^{+/-}$ males. The second experiment will conduct an expanded RNA-sequencing analysis of hippocampal gene expression in the two *Cacna1c* genotypes in both males and females, as well as assessing the impact of hippocampal activation induced by exposure to a novel environmental context.

7.2 Experiment 1

In order to assess the effects of *Cacna1c* heterozygosity on hippocampal gene expression, existing DG and CA1 RNA-sequencing data in male *Cacna1c*$^{+/-}$ and *Cacna1c*$^{+/+}$ animals at baseline was analysed. Owing to the low sample size of this data set (six animals per genotype), Weighted Gene Correlation Network Analysis (WGCNA) was used (Langfelder and Horvath, 2008). Traditional methods of assessing differential gene expression have a high burden of correction for multiple comparison owing to the analysis of differential expression of many thousands of genes at an individual level. WGCNA is an alternative method with a smaller burden of correction for multiple comparison. In this approach, a network is constructed in which genes are clustered into modules based on degree of co-expression. Hence, differences in gene expression between genotypes can be compared on a module rather than individual gene basis.

7.2.1 Hypotheses

It was hypothesised that…

- *Cacna1c* heterozygosity alters gene expression in the hippocampus.

- Differentially expressed genes overlap between the DG and CA1, however a degree of regional specificity remains.

- Differentially expressed genes are associated with biological processes involved in synaptic plasticity, learning and memory.

7.2.2 Methods

7.2.2.1 Animals and RNA Expression Data

Tissue collection, sequencing and data preparation was conducted by Dr Nicholas Clifton and Dr Simon Trent with assistance from NGS technician Joanne Morgan prior to the start of this PhD project. 12 male rats of the *Cacna1c*$^{+/-}$ line were used in this experiment (6 *Cacna1c*$^{+/-}$ and 6 *Cacna1c*$^{+/+}$). Animals were 60-80 days old at the time of tissue extraction, and brain tissue was extracted as described in section 2.3. and DG and CA1 hippocampal subregions were dissected as outlined in section 2.4.3. Owing to a technical issue with sample preparation, one *Cacna1c*$^{+/+}$ animal was missing from the DG analysis. For the analysis in this experiment, data was provided for 16,305 genes for each animal as a ranged summarized experiment (RSE) object with $\log_2$ transformation of counts per million (CPM) values.

7.2.2.2 Dimensionality Reduction

The goodSamplesGenes function from the 'WGCNA' package was used to filter out samples and genes with too many missing entries. Hierarchical clustering and principal components analysis (PCA) were then conducted to inspect the relationship between samples and identify those that may be considered to be outliers. Hierarchical clustering was conducted using the hclust function and PCA was conducted using the prcomp function. The hierarchical clustering dendrogram was then visually inspected to identify any samples in which the gene expression profile was particularly distinct from the other samples. Similarly, PCA cluster plots of the first principal components (PCs) that cumulatively explained more than 50% of the variance in gene expression were plotted against one another and visually inspected to identify outliers requiring removal.

7.2.2.3 Weighted Gene Co-expression Network Analysis (WGCNA)

In the gene co-expression model, initially all genes are connected to one another, and a thresholding value for the expression correlation between genes must be set, below which edges between genes will be removed. Pearson's correlation is used to assess the relatedness between genes, and this is then raised to a soft thresholding power to enhance the connections between highly related genes and diminish the connections between poorly related genes. Thus, for each network (CA1 and DG), the scale independence and mean connectivity of a range of soft thresholding powers between 1 and 20 was modelled. For each model, the lowest soft thresholding power was selected at which the scale free topology model fit (R^2) was above 0.8, and the mean connectivity was approaching 0, indicating that the network exhibited scale free topology in which significant gene-gene relationships are distinguished from noise, and the network consists of a small number of highly connected genes and a larger number of genes with relatively few connections (Langfelder and Horvath, 2008).

The network was then constructed with the chosen soft thresholding power using the blockwiseModules function. WGCNA networks may be either signed, unsigned or hybrid, which determines whether the direction of the correlation between is considered. A signed network, in which the directionality of the association is retained, was chosen as this approach conserved biologically meaningful information relating to the relationships between genes. A biweight midcorrelation was set as the correlation type to minimise the impact of outliers or non-normal associations on network construction. The minimum module size was set to 50 genes and merge cut height set to 0.25.

Module eigengenes were extracted from the network, which reflect the first principal component of each module. *T*-tests were then conducted to test whether eigengenes for any modules significantly differed between *Cacna1c* genotypes, to assess the differential expression of each of the modules between genotypes. A false discovery

rate (FDR) correction for multiple comparison was then applied to account for the genotype comparison being conducted for each module.

7.2.2.4 Gene Set Enrichment Analysis

For any module that was significantly differentially expressed between *Cacna1c* genotypes, gene set enrichment analysis (GSEA) was conducted using the R package 'gprofiler2'. Owing to a bug in the rat version of the package affecting the term sizes of each gene ontology (GO) term, the human analogues of the rat genes were used. GSEA was conducted to assess whether any GO or human phenotype (HP) terms were enriched within the genes of the module of interest. The human analogues of all genes expressed within the tissue were used as the background gene list. FDR correction for multiple comparison was also applied.

7.2.3 Results

7.2.3.1 Dimensionality Reduction

After filtering genes for low expression and missing entries, 16,299 genes were found to be expressed within the DG tissue and 16,300 genes in CA1 tissue. The dendrogram of the hierarchical clustering analysis did not suggest that any samples ought to be designated as outliers and removed (Supplementary Figure 1A). PCA revealed that the majority of the variance in gene expression was explained by the first three PCs in the DG (Supplementary Figure 1B). Scatter plots of the first three PCs indicated that, in the DG data, sample 1 (*Cacna1c*$^{+/+}$) had an extreme score of -90.2 on PC1, sample 15 (*Cacna1c*$^{+/+}$) had an extreme score of 74.0 on PC2 and sample 18 (*Cacna1c*$^{+/-}$) had an extreme score of -62.5 on PC 3. However, removal of these samples would reduce the sample size below that which would be required for the analysis, and all deviating scores were within three standard deviations of the

mean for each principal component, so were retained for analysis (Supplementary Figure 1C).

In the CA1, the dendrogram suggested that sample 1 may be an outlier (Supplementary Figure 2A). PCA revealed that the majority of the variance in gene expression was explained by the first four PCs in the CA1 (Supplementary Figure 2B). Scatter plots of the first four PCs in the CA1 showed sample 1 had an extreme score of -92.6 on PC1 and thus concurred with the findings of the hierarchical clustering analysis indicating that this sample ought to be designated as an outlier and subsequently removed (Supplementary Figure 2C).

Subsequently, sample 1 was removed and hierarchical cluster analysis and PCA were reconducted (Supplementary Figure 3A). PCA then revealed that the majority of the variance in gene expression in the CA1 was explained by the first four principal components (Supplementary Figure 3B). Scatter plots of the first four principal components after the removal of the outlier sample are shown in Supplementary Figure 3C.

T-tests were then used to assess whether scores of any of the first ten principal components differed between *Cacna1c* genotypes, and this was not found to be the case for any PCs in the DG (PC1: $t_9 = 1.1$, $p = 0.318$. PC2: $t_9 = 0.7$, $p = 0.474$. PC3: $t_9 = 0.9$, $p = 0.383$. PC4: $t_9 = 1.0$, $p = 0.334$. PC5: $t_9 = 1.0$, $p = 0.331$. PC6: $t_9 = -1.3$, $p = 0.217$. PC7: $t_9 = 1.2$, $p = 0.277$. PC8: $t_9 = -0.6$, $p = 0.554$. PC9: $t_9 = -0.8$, $p = 0.429$. PC10: $t_9 = -1.2$, $p = 0.269$). In the CA1, there was a trend towards scores of PC10 differing between *Cacna1c* genotypes ($t_9 = 2.1$, $p = 0.060$), with this PC explaining 6.26% of variation in gene expression. However, there were no differences between genotypes in any other PC scores (PC1: $t_9 = 0.2$, $p = 0.844$. PC2: $t_9 = -0.7$, $p = 0.511$. PC3: $t_9 = 0.8$, $p = 0.426$. PC4: $t_9 = -0.1$, $p = 0.909$. PC5: $t_9 = 1.8$, $p = 0.105$. PC6: $t_9 = 0.4$, $p = 0.713$. PC7: $t_9 = 1.5$, $p = 0.171$. PC8: $t_9 = -0.6$, $p = 0.589$. PC9: $t_9 = -0.5$, $p = 0.661$). Thus, very little variation in gene expression in either the DG or CA1 tissue appears to be attributable to *Cacna1c* genotype.

7.2.3.2 WGCNA

Scale independence and mean connectivity of soft threshold powers between 1 and 20 were modelled, and a soft thresholding power of 12 for the DG model and 10 for the CA1 model was found to be the point at which scale-free topology was achieved (Figure 7.1).

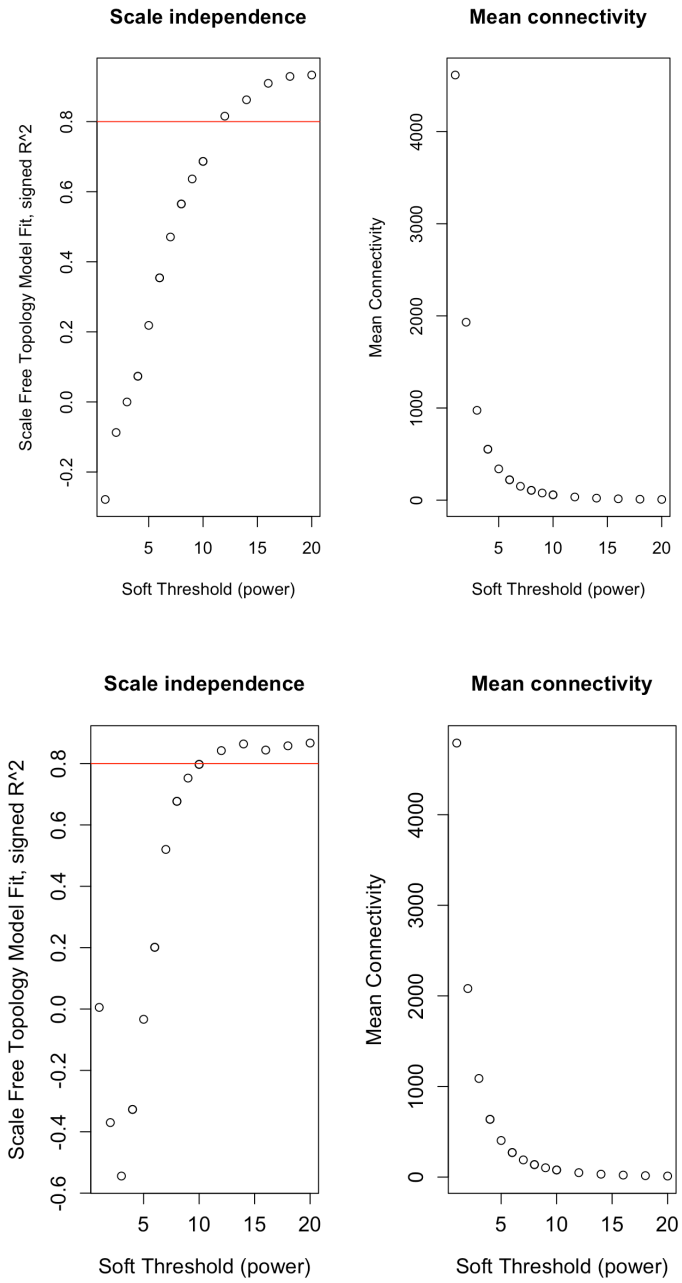

Figure 7.1. Scale free topology model fit and mean connectivity for each modelled soft threshold power. Top: DG. Bottom: CA1.

For each region, a signed network was then created and clustered into modules of co-expression. This generated networks with 96 modules of genes for the DG and 58 modules of genes for the CA1. For the purposes of visualisation and identification, a colour was assigned to each module (Figure 7.2).

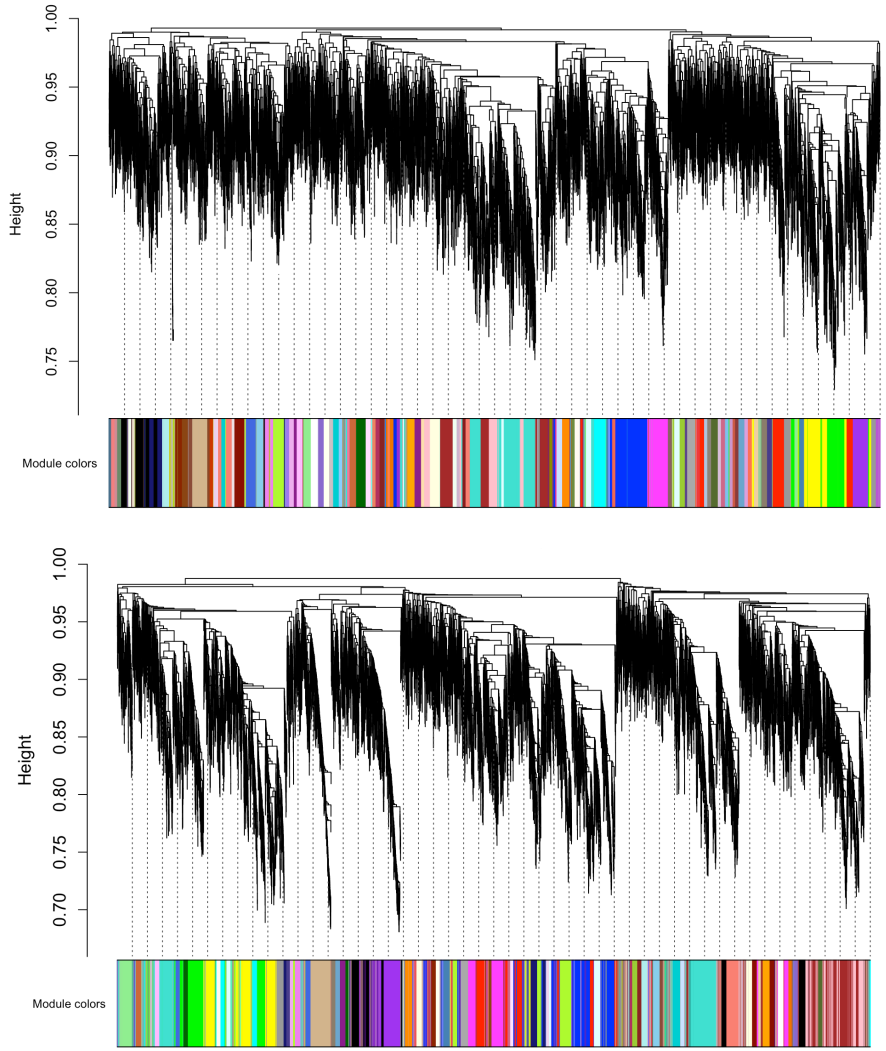

Figure 7.2. Cluster dendrogram of gene associations and module memberships for the signed WGCNA network generated for gene expression in DG (top) and CA1 (bottom) tissue. Each module of co-expressed genes is represented by a colour.

In the DG network, six module eigengenes were found to significantly differ between genotypes at $\alpha = 0.05$, with Module 60 surviving FDR correction for multiple comparison (Supplementary Table 1). In the CA1, no modules eigengenes were found to be significantly related to genotype (Supplementary Table 2).

Degree of module membership to DG module 60 was calculated by correlating gene expression with the eigengene value of this module. Within the 88 genes assigned to this module, individual *p*-values for the association between genotype and gene expression was calculated for each gene. The *p*-value for differential expression between *Cacna1c* genotypes of genes within this module was found to be highly significantly negatively associated with degree of module membership ($r^2 = -0.56$, $p = 1.4 \times 10^{-9}$). This indicates that within genes assigned to module 60, those that are highly differentially expressed between *Cacna1c* genotypes (i.e., small *p* values) have a higher degree of module membership of module 60, compared with genes that are less differentially expressed between *Cacna1c* genotypes (Figure 7.3). The genes with significant module membership to module 60 are displayed in Supplementary Table 3. Genes assigned to the module do not necessarily have a significant degree of module membership, as assignment to each module is based on the WGCNA network co-expression structure, rather than module eigengene scores.

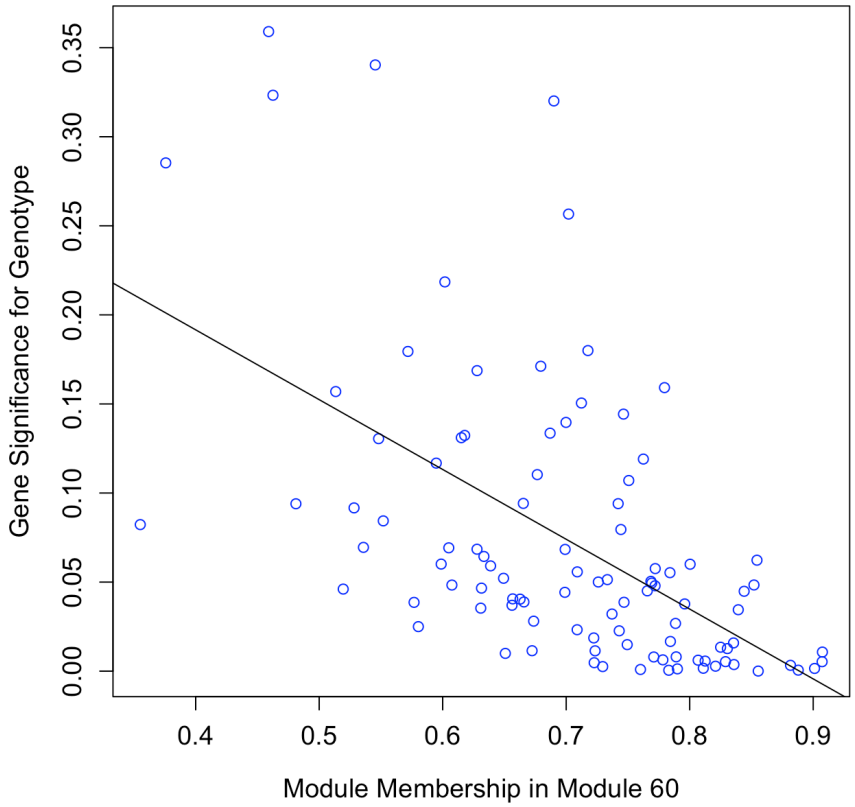

Figure 7.3. The relationship between degree of module membership (Pearson r^2 correlation between module 60 eigengene and gene expression) and *p*-value of differential expression between genotypes.

The GSEA of module 60 featured 86 human analogues of the genes with significant module membership, and 14,503 human analogues in the background gene list corresponding to all genes expressed within the tissue. However, no GO or HP terms were found to be enriched within the gene set.

7.2.4 Discussion

It was initially hypothesised that *Cacna1c* heterozygosity alters gene expression in the hippocampus. However, WGCNA did not observe any modules with eigengene scores that differed according to *Cacna1c* genotype in CA1. One module of genes in which eigengene score significantly differed according to *Cacna1c* genotype after FDR correction for multiple comparison was found in the DG, with this module containing 88 genes. Thus, this indicates that there is a degree of regional specificity in the effects of *Cacna1c* heterozygosity on gene expression within the hippocampus. It was further hypothesised that any differentially expressed genes would be associated with biological processes involved in synaptic plasticity, learning and memory. However, GSEA did not reveal any GO or HP terms that were enriched among the gene list, indicating that the gene list may not be concentrated around shared underlying biological functions.

Although one module of genes was found to be differentially expressed, scatter plots of the first three principal components in the DG indicated that three samples did not cluster well with the others. The minimum number of samples recommended for use in WGCNA is fifteen and only eleven were used in these analyses, which after outlier removal would have been reduced to eight. Re-analysis of the data after the removal of the two samples with extreme scores on either of the first two principal components did not show any differentially expressed modules of genes between *Cacna1c* genotypes (data not reported here). Owing to the low sample size, it is not possible to determine whether the module of differentially expressed genes observed in the initial DG analysis was driven by outlier samples, or if this finding was not observed after outlier removal because of low sample size. Thus, although WGCNA may serve as a viable alternative approach to differential expression analysis at an individual gene level, it is not possible to ascertain whether the null results observed in this this initial experiment were genuine, or if the experiment was not sufficiently powered to highly effectively use this tool.

7.3 Experiment 2

7.3.1 Introduction

As the initial experiment did not reveal conclusive results, and there was evidence to suggest that there was insufficient statistical power, the following experiment was conducted with a larger sample size. As the statistical power was considerably increased in this second experiment, the R package DESeq2 (Love et al., 2014) was used for analysis of differential expression instead of WGCNA, as this provides more fine-grained detail gene expression differences by testing genes on an individual level. Furthermore, the design was expanded to include animals of both sexes, as well as a comparison between a baseline group, and a group in which animals had recently been exposed to a novel environmental context. Thus, it may then be possible to assess whether the effect of *Cacna1c* genotype on CA1 and DG gene expression interacted with either sex or novel context exposure.

7.3.2 Hypotheses

- Owing to the known role of LTCCs in hippocampal encoding of context and CFC, and specific underlying processes such as synaptic plasticity and E-TC, *Cacna1c* heterozygosity results in altered gene expression in the hippocampus (both DG and CA1 regions).

- There is known sexual dimorphism in the structure and function of the hippocampus, as well as its involvement in contextual processing. Furthermore, the results of Chapter 6 indicate that males and females differ in their relationship between context PE and subsequent extent of fear conditioning, reflected in both behavioural differences and differences in hippocampal gene expression. Thus, it was hypothesised that gene

expression differs between sexes, and context exposure groups, and that sex and context exposure interact with *Cacna1c* genotype to affect gene expression.

- Experiment 1 was likely underpowered to detect differential gene expression between *Cacna1c* genotypes. Hence, increasing sample size will increase power to detect differential gene expression between genotypes, sexes and context exposure groups.

- Furthermore, GSEA will show differentially expressed genes between *Cacna1c* genotypes to be enriched for involvement in processes such as synaptic plasticity, learning and memory.

- MAGMA gene set association analysis was also used to assess whether degree of differential gene expression between *Cacna1c* genotypes would be associated with the gene's degree of association with schizophrenia, as derived from GWAS summary statistics. As this was an exploratory assessment, no specific directional hypotheses were made *a priori*.

7.3.3 Methods

7.3.3.1 Animals

64 rats from the *Cacna1c*$^{+/-}$ line aged between 80-106 days were used in this experiment, with 16 animals of each sex/genotype combination (*Cacna1c*$^{+/+}$ male; *Cacna1c*$^{+/+}$ female: *Cacna1c*$^{+/-}$ male; *Cacna1c*$^{+/-}$ female). All animals were behaviourally naïve and had not been used in previous experiments.

7.3.3.2 Behavioural Paradigm

Half of the animals of each sex and genotype (8 *Cacna1c*$^{+/+}$ males; 8 *Cacna1c*$^{+/+}$ females; 8 *Cacna1c*$^{+/-}$ males; 8 *Cacna1c*$^{+/-}$ females) underwent a behavioural paradigm whereby they were exposed to a novel context (fear conditioning chamber) for 20 minutes. Rats in the novel context condition were euthanised 30-minutes after returning to their home cage.

7.3.3.3 Brain dissection and RNA-Sequencing

Brain tissue was extracted and DG and CA1 hippocampal subregions were dissected as outlined in Section 2.4.2. RNA extraction, library preparation and RNA-sequencing were performed as described in Section 2.6.2. Owing to a technical issue in the RNA extraction step, RNA was not available for eight animals (1x female *Cacna1c*$^{+/-}$ baseline, 1x female *Cacna1c*$^{+/+}$ baseline, 4x female *Cacna1c*$^{+/+}$ context exposed, 1x male *Cacna1c*$^{+/+}$ baseline, 1x male *Cacna1c*$^{+/+}$ context exposed).

7.3.3.4 Assessment of Differential Gene Expression

Sequencing data was pre-processed as described in Chapter 2. The R package DESeq2 (Love et al., 2014) and associated analysis pipeline was used to assess differential gene expression between genotypes. Read count files for each sample were concatenated into a single data frame. A dds object was generated from the read count data and sample information (genotype, sex and context exposure group) using the DESeqDataSetFromMatrix function. The dds object was then pre-filtered to remove genes with low expression within the tissue. The DESeq2 pipeline dictates that genes with a read count of lower than 10 for at least the number of samples in the smallest group size should be removed. In this experiment, eight groups were used (male *Cacna1c*$^{+/-}$ baseline; male *Cacna1c*$^{+/-}$ context exposure; female

Cacna1c$^{+/-}$ baseline; female *Cacna1c*$^{+/-}$ context exposure; male *Cacna1c*$^{+/+}$ baseline; male *Cacna1c*$^{+/+}$ context exposure; female *Cacna1c*$^{+/+}$ baseline; female *Cacna1c*$^{+/+}$ context exposure), each with eight animals. Hence, in DG tissue, genes with a read count of less than 10 in at least eight animals were removed. Unfortunately, due to a technical fault, RNA samples for eight CA1 tissue samples were unusable for sequencing. This affected four of the samples in one group (female *Cacna1c*$^{+/+}$ context exposure). Hence, for the CA1 pre-filtering of read counts, genes with a read count of less than 10 in at least four animals were removed.

The nature of RNA-sequencing data is such that the variance increases with mean read count, which can complicate downstream statistical analyses such as principal components analysis (PCA). Hence, a variance stabilizing transformation was applied to dds object to create a vsd object. PCA using the prcomp R function and hierarchical clustering using the hclust function was then conducted to identify possible outlier samples and to examine the major drivers of variance between samples, with the first ten PCs calculated. *T*-tests were conducted to assess whether PC scores significantly differed according to genotype, sex and pre-exposure condition.

Likelihood ratio tests were then conducted using the DESeq function to compare each possible two-way interaction between the effects of genotype, sex and context condition with the model with only main effects, to determine whether any genes were significantly differentially expressed as a result of any of the interaction terms. If no genes were differentially expressed as a result of the interaction term, it was removed from the model. Comparisons of $\log_2$ fold changes between genotype, sex and context exposure group were then calculated using the lfcShrink function from the R package apeglm (Zhu et al., 2019), and results were inspected to identify any genes with FDR adjusted *p*-values of less than 0.05, indicating significant differential expression between groups.

7.3.3.5 Gene Set Enrichment Analysis

Gene set enrichment analysis (GSEA) was then conducted using the gost function of the R package gprofiler2 (Kolberg et al., 2020). Unfortunately, a bug was present in the rat version of the package, affecting the term sizes. Hence, differentially expressed genes were converted to their human analogues using the getLDS function of the biomaRt R package (Smedley et al., 2009). GSEA was then conducted for each gene list of DEGs using the human analogues of all expressed genes as the background gene list. Enrichment terms were sourced from GO and HP databases.

7.3.3.6 Gene Set Analysis

Gene set analysis to assess whether there was an association between differential gene expression between *Cacna1c* genotypes and degree of association of the gene with schizophrenia as derived from GWAS summary statistics (Trubetskoy et al., 2022) was conducted using the command line tool Multi-marker Analysis of GenoMic Annotation (MAGMA). MAGMA requires three steps: an annotation step, a gene analysis step, and a gene-level analysis step (de Leeuw et al., 2015).

In the annotation step, SNP locations are mapped to the human genome based on genomic location to provide an output required for subsequent gene analysis. The gene locations file for homo sapiens build 37 and SNP locations for European ancestry from Phase 3 of the 1,000 Genomes project were used in the analysis (available at: cncr.nl/research/magma/). The European ancestry data was used as 74.3% of the samples from the PGC third wave Schizophrenia GWAS were of European ancestry (Trubetskoy et al., 2022). An annotation window of 35kb upstream and 10kb downstream was added to allow for the inclusion of possible regulatory regions (Maston et al., 2006; Trubetskoy et al., 2022).

The gene analysis step is used to compute the *Z*-scores and *p*-values of association with schizophrenia for each gene as well as neighbouring genes. The PGC3 schizophrenia GWAS summary statistics contained 7659767 SNPs. After filtering to retain only SNPs with imputation quality INFO scores of > 0.8 and minor allele frequencies of > 0.05 in both cases and controls (Trubetskoy et al., 2022), 7035714 SNPs remained. In the gene analysis step, the *Z*-scores and *p*-values for each gene as well as neighbouring genes are computed using the gene annotation output file, the GWAS summary statistic *p*-values for each SNP, and the European ancestry Phase 3 data of the 1,000 Genomes project were used as a reference for linkage disequilibrium. The GWAS summary statistic *p*-value file contained an additional column which specified the total sample size for each SNP.

For the gene-level analysis step, continuous gene property analysis was used to assess whether there was any association between the *Z*-score representing the association between each gene and schizophrenia calculated in the gene analysis step from the GWAS summary statistics, and the $-\log_{10}$ *p*-value of the differential expression of the human analogues of each gene expressed within either the CA1 or DG. $-\log_{10}$ *p*-values were used as this enhances the prominence of significant DEGs and reduces noise generated by a large number of high *p*-values.

7.3.4 Results

7.3.4.1 Dimensionality Reduction

A total of 30,562 genes were sequenced. After filtering genes for low expression, 19,508 genes were found to be expressed within the DG tissue, and 19,950 genes were found to be expressed within CA1 tissue. The dendrogram of the hierarchical clustering analysis did not indicate that any samples should be designated as outliers in DG (Supplementary Figure 4). However, in CA1, four possible outliers

were found (1757, 1704, 1765, 1785). PCA was subsequently conducted to investigate this further.

In the DG, scatter plots of the first four principal components, which explained the majority of variance in gene expression (Table 7.1), were not suggestive of any outlier samples (Supplementary Figure 5). However, in the CA1, the scatter plots concurred with the findings of the hierarchical clustering analysis indicating that four samples ought to be removed as outliers (Supplementary Figure 5), with sample 1704 showing an extreme score of -130.7 on PC1, sample 1757 showing an extreme score of 175.0 on PC3, and sample 1765 showing extreme scores of 100.0 on PC2 and -139.8 on PC4. Subsequently, samples 1757 (male, *Cacna1c*$^{+/-}$, context exposure), 1704 (male, *Cacna1c*$^{+/+}$, context exposure), and 1765 (female, *Cacna1c*$^{+/-}$, baseline) were removed. Sample 1785 (male, *Cacna1c*$^{+/-}$, context exposure) did not show extreme scores on the first four PCs, which explained the majority of the variation in gene expression. However, it did have an extreme score of 121.0 on PC5 (remaining range -12.9 – 33.3) and its position on the dendrogram also supported its outlier status, hence it was removed. Hierarchical clustering and PCA revealed that after the removal of the four outlier samples, the majority of the variance in gene expression in the CA1 was explained by the first three principal components (Table 7.1; Supplementary Figure 6).

T-tests were then used to assess whether scores of any of the first ten principal components significantly differed between either sex, genotype or context exposure (summarised in Table 7.1). In the DG, scores of PCs 1, 5 and 8 were both found to significantly differ between genotype (PC1: $t_{62} = 2.3$, $p = 0.025$. PC5: $t_{62} = 2.5$, $p = 0.016$. PC8: $t_{62} = 2.2$, $p = 0.035$). No other PCs were found to differ according to genotype (PC2: $t_{62} = 0.4$, $p = 0.663$. PC3: $t_{62} = 0.8$, $p = 0.413$. PC4: $t_{62} = -0.3$, $p = 0.746$. PC6: $t_{62} = 0.3$, $p = 0.733$. PC7: $t_{62} = -0.0$, $p = 0.973$. PC9: $t_{62} = -0.2$, $p = 0.843$. PC10: $t_{62} = -1.5$, $p = 0.141$). In the CA1, no PCs were found to differ according to genotype (PC1: $t_{50} = 1.5$, $p = 0.129$. PC2: $t_{50} = -1.3$, $p = 0.204$. PC3: $t_{50} = -0.7$, $p = 0.488$. PC4: $t_{50} = -0.7$, $p = 0.458$. PC5: $t_{50} = 0.0$, $p = 0.978$. PC6: $t_{50} = -1.5$, $p = 0.140$.

PC7: t_{50} = -1.6, p = 0.124. PC8: t_{50} = -1.7, p = 0.095, PC9: t_{50} = -1.1, p = 0.260. PC10: t_{50} = 0.8, p = 0.438).

In the DG, PC6 was found to highly significantly differ between sexes (t_{62} = -13.0, $p < 2x10^{-16}$). No other PCs were found to differ between sexes (PC1: t_{62} = -0.1, p = 0.954. PC2: t_{62} = -1.7, p = 0.099. PC3: t_{62} = 1.96, p = 0.054. PC4: t_{62} = -1.7, p = 0.098. PC5: t_{62} = 1.5, p = 0.147. PC7: t_{62} = -0.3, p = 0.786. PC8: t_{62} = -1.5, p = 0.140. PC9: t_{62} = 0.9, p = 0.379. PC10: t_{62} = -0.7, p = 0.514). In the CA1, scores of PCs 3, 5, 6 and 9 were found to significantly differ between sexes (PC3: t_{50} = -2.0, p = 0.049. PC5: t_{50} = 7.9, $p < 0.001$. PC6: t_{50} = 3.5, p = 0.001. PC9: t_{50} = -2.1, p = 0.045). No other PCs were found to differ between sexes (PC1: t_{50} = 0.3, p = 0.755. PC2: t_{50} = 0.3, p = 0.735. PC4: t_{50} = -0.7, p = 0.508. PC7: t_{50} = 0.1, p = 0.904. PC8: t_{50} = 0.5, p = 0.643. PC10: t_{50} = -0.1, p = 0.913).

No scores of the first ten principal components were found to significantly differ between context exposure groups in the DG (PC1: t_{62} = -1.1, p = 0.295. PC2: t_{62} = 0.7, p = 0.476. PC3: t_{62} = 0.7, p = 0.499. PC4: t_{62} = -0.6, p = 0.557. PC5: t_{62} = -0.0, p = 0.975. PC6: t_{62} = 0.1, p = 0.887. PC7: t_{62} = 0.5, p = 0.592. PC8: t_{62} = -0.7, p = 0.458. PC9: t_{62} = 0.2, p = 0.850. PC10: t_{62} = 1.1, p = 0.277). In the CA1, score of PC8 was found to significantly differ according to context exposure group (t_{50} = -2.7, p = 0.010). However, no other PCs were found to differ according to context exposure condition (PC1: t_{50} = -0.3, p = 0.760. PC2: t_{50} = 1.4, p = 0.166. PC3: t_{50} = 0.4, p = 0.671. PC4: t_{50} = -0.5, p = 0.622. PC5: t_{50} = 0.4, p = 0.679. PC6: t_{50} = -0.6, p = 0.575. PC7: t_{50} = 0.6, p = 0.562. PC9: t_{50} = 0.3, p = 0.734. PC10: t_{50} = -0.9, p = 0.388).

Table 7.1. Percentage of the variance in gene expression explained by each principal component in the DG and CA1 after outlier removal. Significantly differing PC values between *Cacna1c* genotypes are highlighted in green, between sexes are highlighted in blue, and between context exposure groups are highlighted in orange.

		Region	
		DG	CA1
PC	1	18.64	29.91
	2	15.76	17.55
	3	11.31	7.56
	4	9.63	5.46
	5	6.55	3.92
	6	3.56	3.41
	7	3.21	2.79
	8	2.67	2.69
	9	2.12	2.05
	10	2.05	1.56

7.3.4.2 Differential Gene Expression

In both the DG and CA1 tissues, likelihood ratio tests showed that the model fit did not improve by including any of the two-way interaction terms between *Cacna1c* genotype, sex, and context exposure for any genes. Hence, the final model contained only the main effects of *Cacna1c* genotype, sex and context exposure condition.

Eighty genes in the DG and 10 genes in the CA1 were significantly differentially expressed between *Cacna1c* genotypes after FDR correction for multiple comparison (Figure 7.4 and 7.5). Only 7 of the 80 DG DEGs and 2 of the 10 CA1 DEGs showed reduced expression in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ rats, indicating that low *Cacna1c* gene dosage primarily leads to increased expression of affected downstream genes. DEGs between *Cacna1c* genotypes are displayed in Tables 7.2 and 7.3 for the DG and CA1 respectively. Four DEGs were common to both DG and CA1, with all showing increased expression in *Cacna1c*$^{+/-}$ compared to *Cacna1c*$^{+/+}$ rats in both regions.

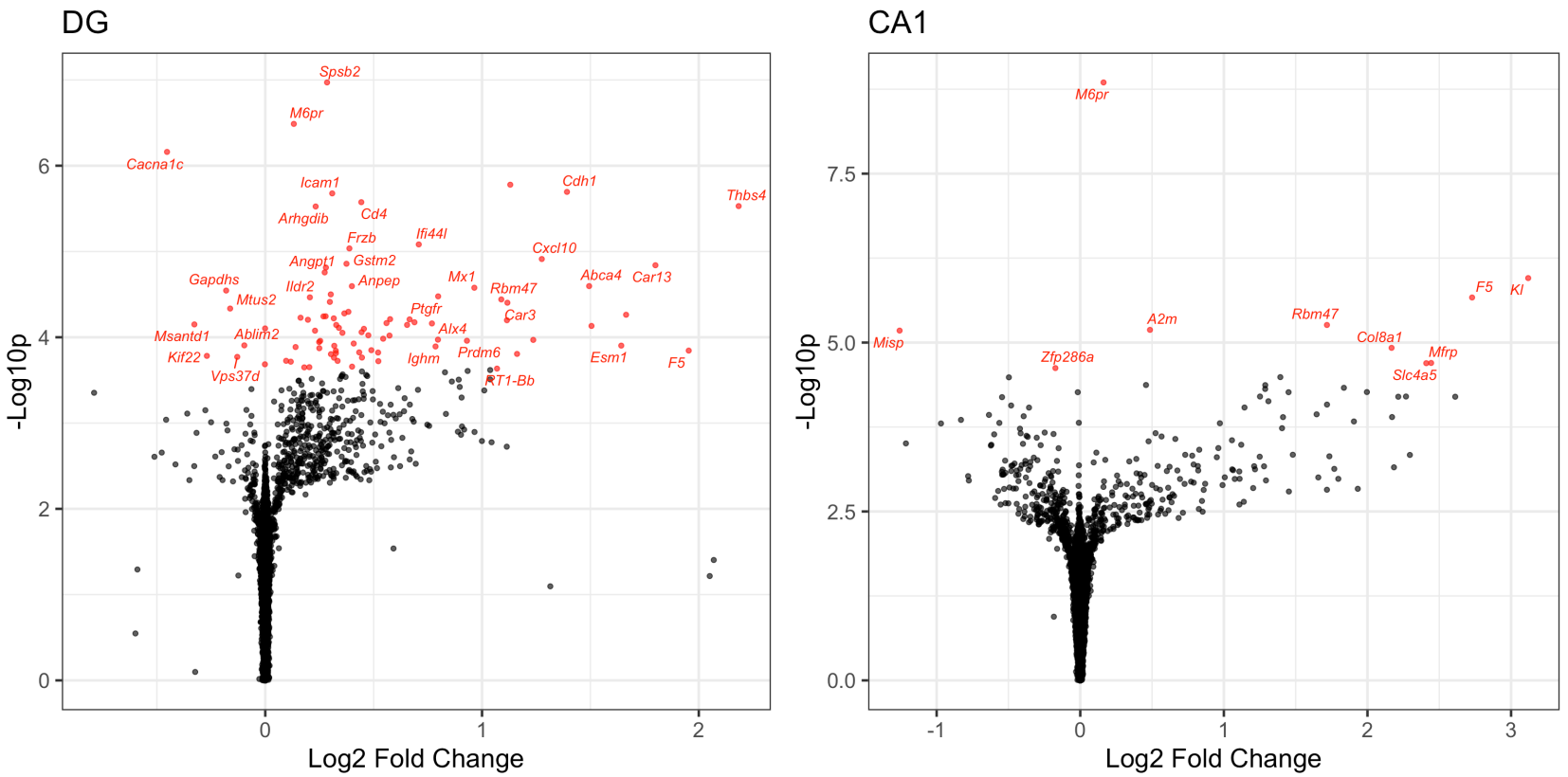

Figure 7.4. Differential gene expression in the DG (left) and CA1 (right) in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. Red points indicate significant DEGs at an FDR corrected alpha threshold of $\alpha = 0.05$.

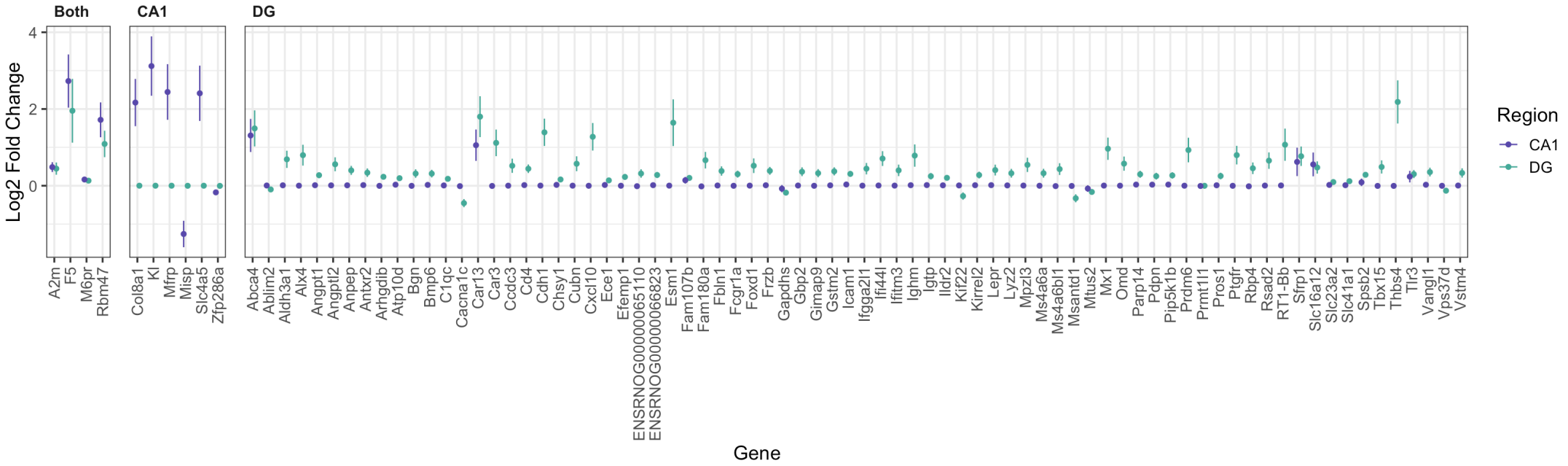

Figure 7.5. Log_2 fold change (and standard error) in *Cacna1c*$^{+/-}$ relative to *Cacna1c*$^{+/+}$ rats in CA1 and DG at FDR adjusted $\alpha = 0.05$.

Table 7.2. Differentially expressed genes in DG between *Cacna1c* genotypes. Positive Log_2 Fold Change values reflect higher expression in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. NA values correspond to Ensembl IDs of novel genes with no existing gene symbols. Genes in bold were also found to be DEGs in the CA1.

Ensembl ID	Gene Symbol	Base Mean	Log_2 Fold Change	L_2FC SE	*p*	FDR *p*
ENSRNOG00000015125	*Spsb2*	368.48	0.29	0.06	1.00E-07	2.00E-03
ENSRNOG00000014992	***M6pr***	**6027.3**	**0.13**	**0.03**	**3.00E-07**	**3.00E-03**
ENSRNOG00000007090	*Cacna1c*	4916.63	-0.45	0.11	7.00E-07	4.00E-03
ENSRNOG00000020151	*Cdh1*	69.42	1.39	0.36	2.00E-06	6.00E-03
ENSRNOG00000020679	*Icam1*	294.05	0.31	0.08	2.10E-06	6.00E-03
ENSRNOG00000071219	*Cd4*	560.68	0.44	0.11	2.70E-06	6.00E-03
ENSRNOG00000012471	*Thbs4*	21.47	2.18	0.56	3.00E-06	6.00E-03
ENSRNOG00000005809	*Arhgdib*	528.3	0.23	0.06	3.00E-06	6.00E-03
ENSRNOG00000049994	*Ifi44l*	87.23	0.71	0.19	8.30E-06	1.60E-02
ENSRNOG00000007765	*Frzb*	1862.52	0.39	0.1	9.20E-06	1.60E-02
ENSRNOG00000022256	*Cxcl10*	25.15	1.28	0.36	1.22E-05	1.90E-02
ENSRNOG00000060939	*Gstm2*	736.56	0.37	0.11	1.39E-05	1.90E-02
ENSRNOG00000021323	*Car13*	99.38	1.8	0.53	1.45E-05	1.90E-02
ENSRNOG00000066823	*N/A*	783.25	0.28	0.08	1.54E-05	1.90E-02
ENSRNOG00000005854	*Angpt1*	621.36	0.27	0.08	1.75E-05	2.00E-02
ENSRNOG00000012892	*Abca4*	223.52	1.49	0.47	2.53E-05	2.50E-02
ENSRNOG00000014610	*Anpep*	237.49	0.4	0.12	2.54E-05	2.50E-02
ENSRNOG00000001959	*Mx1*	96.95	0.96	0.29	2.65E-05	2.50E-02
ENSRNOG00000021009	*Gapdhs*	200.38	-0.18	0.05	2.85E-05	2.50E-02
ENSRNOG00000021199	*Fcgr1a*	89.58	0.3	0.09	3.16E-05	2.60E-02
ENSRNOG00000046468	*Ptgfr*	86.33	0.8	0.24	3.34E-05	2.60E-02
ENSRNOG00000025151	*Ildr2*	5004.98	0.21	0.06	3.42E-05	2.60E-02
ENSRNOG00000002408	***Rbm47***	**105.86**	**1.09**	**0.34**	**3.62E-05**	**2.70E-02**
ENSRNOG00000023334	*Parp14*	536.4	0.3	0.09	3.87E-05	2.70E-02
ENSRNOG00000010079	*Car3*	145.11	1.12	0.35	3.96E-05	2.70E-02
ENSRNOG00000032410	*Mtus2*	3441.66	-0.16	0.05	4.63E-05	3.00E-02
ENSRNOG00000014137	*Fbln1*	2239.13	0.38	0.12	5.05E-05	3.00E-02
ENSRNOG00000031743	*Gbp2*	197.47	0.36	0.12	5.27E-05	3.00E-02
ENSRNOG00000020864	*Kirrel2*	210.71	0.28	0.09	5.69E-05	3.00E-02
ENSRNOG00000015232	*Pip5k1b*	3135.04	0.27	0.08	5.71E-05	3.00E-02

ENSRNOG00000012698	*Chsy1*	1833.85	0.16	0.05	5.92E-05	3.00E-02
ENSRNOG00000013717	*Bmp6*	1847.11	0.32	0.1	6.02E-05	3.00E-02
ENSRNOG00000039560	*Omd*	336.4	0.58	0.19	6.15E-05	3.00E-02
ENSRNOG00000011750	*Fam180a*	66.91	0.67	0.22	6.19E-05	3.00E-02
ENSRNOG00000002312	*Atp10d*	350.24	0.2	0.06	6.25E-05	3.00E-02
ENSRNOG00000002331	*Aldh3a1*	36.87	0.69	0.22	6.69E-05	3.00E-02
ENSRNOG00000016678	*Angptl2*	330.3	0.56	0.18	6.83E-05	3.00E-02
ENSRNOG00000017783	*Sfrp1*	1084.48	0.77	0.25	6.90E-05	3.00E-02
ENSRNOG00000064777	*Msantd1*	54.52	-0.33	0.1	7.08E-05	3.00E-02
ENSRNOG00000007539	*Rsad2*	106.83	0.65	0.21	7.18E-05	3.00E-02
ENSRNOG00000024569	*Gimap9*	51.04	0.33	0.11	7.19E-05	3.00E-02
ENSRNOG00000000081	*Antxr2*	520.22	0.34	0.11	7.77E-05	3.10E-02
ENSRNOG00000015518	*Rbp4*	933.81	0.46	0.15	7.97E-05	3.10E-02
ENSRNOG00000003553	*Efemp1*	653	0.23	0.08	8.38E-05	3.20E-02
ENSRNOG00000019542	*Ifgga2l1*	421.21	0.44	0.15	8.72E-05	3.30E-02
ENSRNOG00000016477	*Vangl1*	831.11	0.36	0.11	8.87E-05	3.30E-02
ENSRNOG00000021916	*Slc16a12*	224.63	0.48	0.16	9.48E-05	3.40E-02
ENSRNOG00000029047	*Cubn*	130.16	0.57	0.19	9.56E-05	3.40E-02
ENSRNOG00000068913	*Mpzl3*	290.96	0.54	0.19	1.04E-04	3.60E-02
ENSRNOG00000000008	*Alx4*	25.07	0.8	0.27	1.07E-04	3.60E-02
ENSRNOG00000030486	*Prdm6*	61.4	0.93	0.32	1.10E-04	3.60E-02
ENSRNOG00000048723	*Pros1*	692.01	0.25	0.09	1.10E-04	3.60E-02
ENSRNOG00000027008	*Igtp*	410.36	0.25	0.08	1.14E-04	3.60E-02
ENSRNOG00000023664	*Lepr*	525.96	0.41	0.14	1.18E-04	3.70E-02
ENSRNOG00000007882	*Ablim2*	3853.54	-0.1	0.03	1.24E-04	3.70E-02
ENSRNOG00000010797	*Esm1*	90.46	1.64	0.61	1.25E-04	3.70E-02
ENSRNOG00000065110	*N/A*	65.74	0.32	0.11	1.26E-04	3.70E-02
ENSRNOG00000034190	*Ighm*	57.79	0.79	0.29	1.28E-04	3.70E-02
ENSRNOG00000014241	*Ece1*	3809.38	0.14	0.05	1.30E-04	3.70E-02
ENSRNOG00000014961	*Pdpn*	1449.51	0.25	0.09	1.34E-04	3.80E-02
ENSRNOG00000019565	*Tbx15*	103.72	0.49	0.17	1.42E-04	3.80E-02
ENSRNOG00000057855	***F5***	**1542.07**	**1.95**	**0.83**	**1.43E-04**	**3.80E-02**
ENSRNOG00000005825	*Lyz2*	1390.23	0.33	0.11	1.44E-04	3.80E-02
ENSRNOG00000050395	*Ms4a6bl1*	95.25	0.43	0.15	1.50E-04	3.90E-02
ENSRNOG00000017933	*Ccdc3*	412.67	0.52	0.18	1.51E-04	3.90E-02
ENSRNOG00000020991	*Ms4a6a*	69.75	0.33	0.12	1.53E-04	3.90E-02
ENSRNOG00000021726	*Tlr3*	333.86	0.3	0.11	1.57E-04	4.00E-02
ENSRNOG00000020281	*Kif22*	107.24	-0.27	0.1	1.65E-04	4.10E-02

ENSRNOG00000048699	*Vps37d*	594.27	-0.13	0.05	1.69E-04	4.10E-02
ENSRNOG00000028896	***A2m***	**2635.81**	**0.45**	**0.16**	**1.73E-04**	**4.10E-02**
ENSRNOG00000055962	*Bgn*	2157.15	0.32	0.11	1.73E-04	4.10E-02
ENSRNOG00000021262	*Slc23a2*	9668.26	0.1	0.03	1.88E-04	4.30E-02
ENSRNOG00000020078	*Vstm4*	207.77	0.33	0.12	1.89E-04	4.30E-02
ENSRNOG00000043332	*Foxd1*	83.6	0.52	0.19	1.90E-04	4.30E-02
ENSRNOG00000042320	*Slc41a1*	7328.53	0.12	0.04	1.93E-04	4.30E-02
ENSRNOG00000004090	*Prmt1l1*	96.23	0	0.01	2.07E-04	4.60E-02
ENSRNOG00000015078	*Ifitm3*	314.32	0.4	0.15	2.20E-04	4.80E-02
ENSRNOG00000014886	*Fam107b*	1084.34	0.2	0.07	2.23E-04	4.80E-02
ENSRNOG00000012804	*C1qc*	1003.65	0.18	0.07	2.24E-04	4.80E-02
ENSRNOG00000032708	*RT1-Bb*	116.89	1.07	0.42	2.32E-04	4.90E-02

Table 7.3. Differentially expressed genes in CA1 between *Cacna1c* genotypes. Positive Log_2 Fold Change values reflect higher expression in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. Genes in bold were also found to be DEGs in the DG.

Ensembl ID	Gene Symbol	Base Mean	Log_2 Fold Change	L_2FC SE	*p*	FDR *p*
ENSRNOG00000014992	***M6pr***	**8706.32**	**0.16**	**0.03**	**1.41E-09**	**2.81E-05**
ENSRNOG00000001092	*Kl*	1932.01	3.12	0.77	1.11E-06	1.11E-02
ENSRNOG00000057855	***F5***	**827.67**	**2.73**	**0.69**	**2.15E-06**	**1.43E-02**
ENSRNOG00000002408	***Rbm47***	**114.45**	**1.72**	**0.45**	**5.49E-06**	**2.21E-02**
ENSRNOG00000028896	***A2m***	**2502.83**	**0.49**	**0.13**	**6.49E-06**	**2.21E-02**
ENSRNOG00000010423	*Misp*	14.12	-1.26	0.34	6.65E-06	2.21E-02
ENSRNOG00000039668	*Col8a1*	186.01	2.17	0.62	1.20E-05	3.40E-02
ENSRNOG00000039107	*Mfrp*	2146.49	2.44	0.73	2.00E-05	4.47E-02
ENSRNOG00000010378	*Slc4a5*	1013.72	2.41	0.72	2.02E-05	4.47E-02
ENSRNOG00000003218	*Zfp286a*	727.22	-0.17	0.05	2.38E-05	4.75E-02

It is worthy of note that, in the DG, *Cacna1c* was the third most significantly differentially expressed gene. However, *Cacna1c* was not differentially expressed between *Cacna1c* genotypes in the CA1 after FDR correction for multiple comparison. The difference in *Cacna1c* expression in the CA1 between *Cacna1c* genotypes is significant prior to correction for multiple comparison ($p = 0.043$). However, Log_2FC is -0.010 in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals in CA1, whereas it is -0.453 in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals in DG (Figure 7.6).

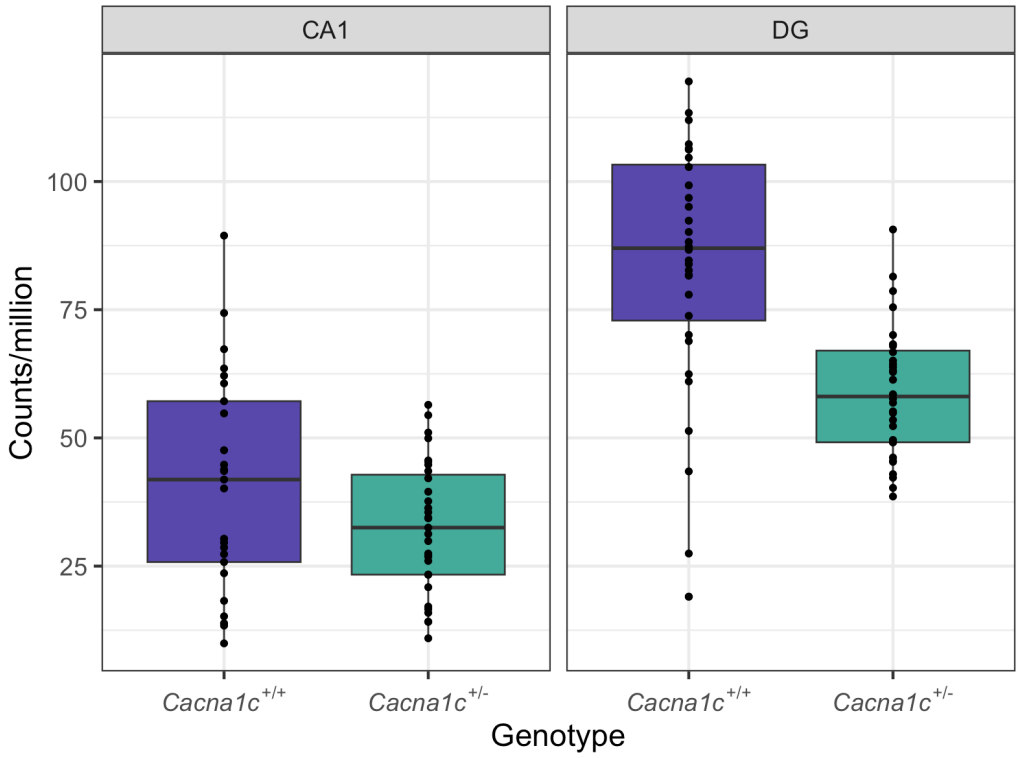

Figure 7.6. Differential expression of *Cacan1c* genotypes in DG and CA1 between *Cacna1c* genotypes.

Twenty-seven genes in the DG and 46 genes in CA1 were found to be significantly differentially expressed between sexes after FDR correction for multiple comparison (Figure 7.7 and 7.8). Fifteen of the 27 DG DEGs and 27 of the 46 CA1 DEGs showed reduced expression in females compared with males. DEGs are displayed in Table 7.4 for DG and Table 7.5 for CA1. Thirteen DEGs were common to both DG and CA1, with all showing consistent directions of effects between regions. The majority of the thirteen DEGs common to DG and CA1 were the most significantly differentially expressed genes between sexes.

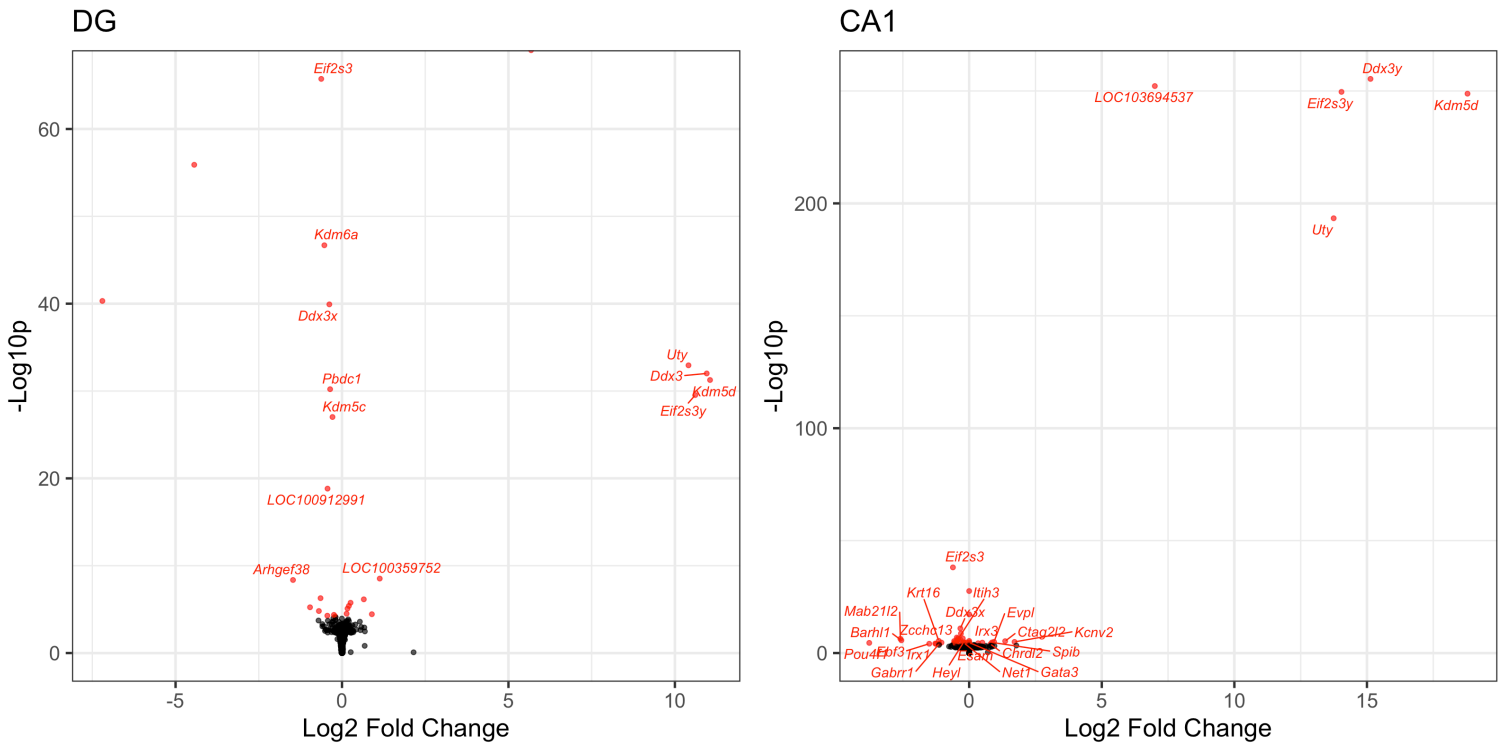

Figure 7.7. Differential gene expression in the DG (left) and CA1 (right) in female compared with male animals. Red points indicate significant DEGs at an FDR corrected alpha threshold of $\alpha = 0.05$.

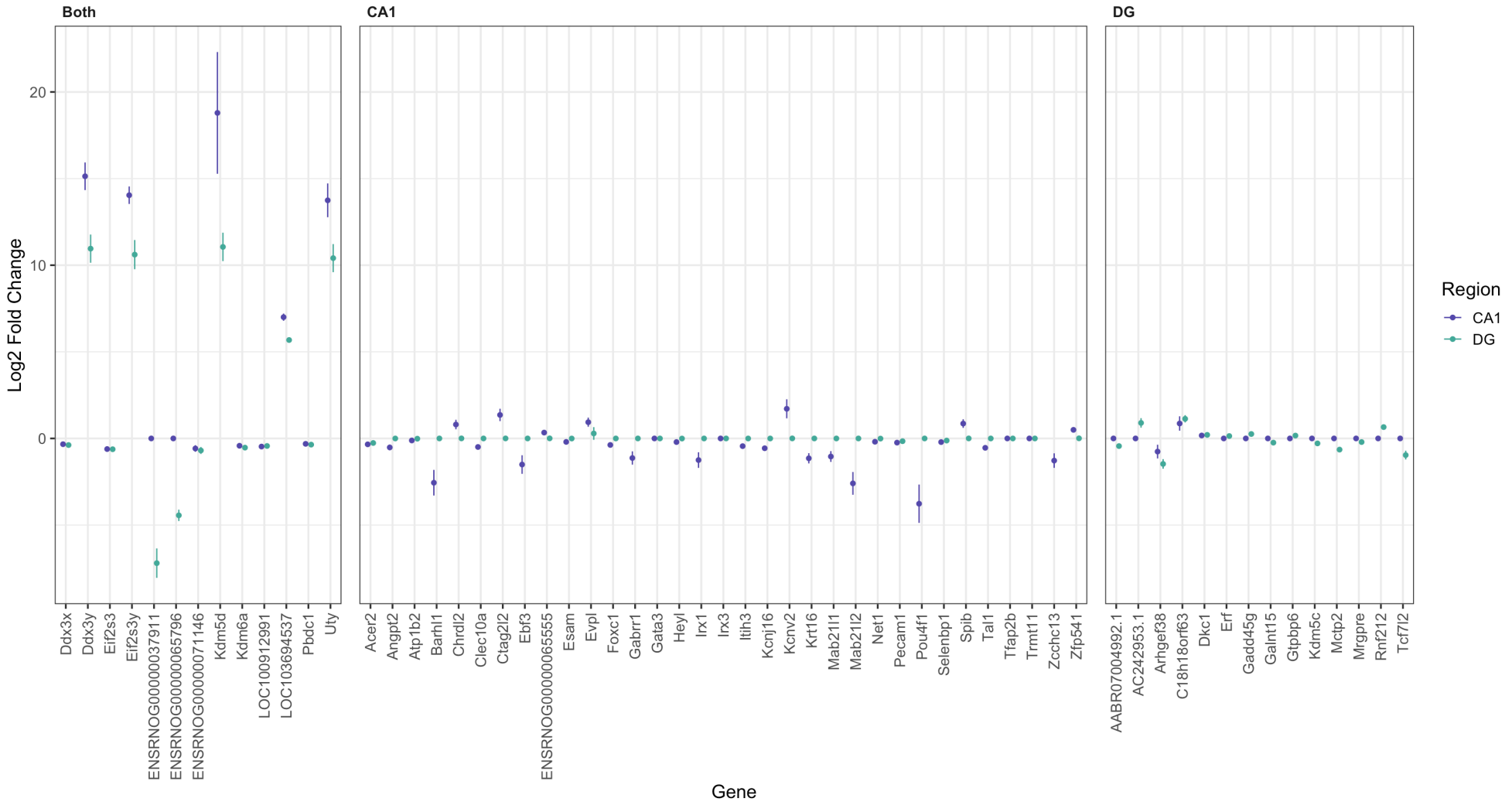

Figure 7.8. Log_2 fold change (and standard error) in female relative to male rats in the 13 genes that were DEGs in both CA1 and DG (left), the 33 CA1 specific DEGs (middle) and the 14 DG specific DEGs (right) at FDR adjusted $\alpha = 0.05$. *Zcchc13* was removed from the DG data during initial filtering owing to low expression.

Table 7.4. Differentially expressed genes in DG between sexes. Positive Log_2 Fold Change values reflect higher expression in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. NA values correspond to Ensembl IDs of novel genes with no existing gene symbols. Genes in bold were also found to be DEGs in the CA1.

Ensembl ID	Gene Symbol	Base Mean	Log_2 Fold Change	L_2FC SE	*p*	FDR *p*
ENSRNOG00000060437	***LOC103694537***	**409.14**	**5.68**	**0.08**	**0.00E+00**	**0.00E+00**
ENSRNOG00000060793	***Eif2s3***	**8569.14**	**-0.62**	**0.04**	**1.84E-66**	**1.79E-62**
ENSRNOG00000065796	***N/A***	**2768.80**	**-4.44**	**0.33**	**1.26E-56**	**8.17E-53**
ENSRNOG00000052721	***Kdm6a***	**2299.75**	**-0.53**	**0.04**	**2.06E-47**	**1.00E-43**
ENSRNOG00000037911	***N/A***	**960.08**	**-7.20**	**0.85**	**4.88E-41**	**1.90E-37**
ENSRNOG00000023383	***Ddx3x***	**20699.21**	**-0.39**	**0.03**	**1.20E-40**	**3.91E-37**
ENSRNOG00000060617	***Uty***	**633.06**	**10.41**	**0.81**	**1.14E-33**	**3.18E-30**
ENSRNOG00000057231	***Ddx3y***	**2424.80**	**10.96**	**0.81**	**9.71E-33**	**2.37E-29**
ENSRNOG00000060496	***Kdm5d***	**1549.07**	**11.06**	**0.82**	**5.48E-32**	**1.19E-28**
ENSRNOG00000002662	***Pbdc1***	**2533.28**	**-0.36**	**0.03**	**6.19E-31**	**1.21E-27**
ENSRNOG00000060048	***Eif2s3y***	**2011.04**	**10.61**	**0.84**	**2.91E-30**	**5.16E-27**
ENSRNOG00000057706	*Kdm5c*	4860.04	-0.29	0.03	9.47E-28	1.54E-24
ENSRNOG00000068101	***LOC100912991***	**306.13**	**-0.43**	**0.05**	**1.50E-19**	**2.25E-16**
ENSRNOG00000038241	*LOC100359752*	32.29	1.13	0.21	2.97E-09	4.14E-06
ENSRNOG00000023965	*Arhgef38*	40.08	-1.47	0.28	4.31E-09	5.60E-06
ENSRNOG00000009932	*Mctp2*	181.72	-0.65	0.15	5.20E-07	6.34E-04
ENSRNOG00000059010	*Rnf212*	33.02	0.66	0.15	7.07E-07	8.11E-04
ENSRNOG00000013090	*Gadd45g*	344.35	0.26	0.06	1.74E-06	1.89E-03
ENSRNOG00000055562	*Dkc1*	2886.46	0.21	0.05	4.00E-06	4.11E-03
ENSRNOG00000049232	*Tcf7l2*	2172.71	-0.96	0.25	5.75E-06	5.61E-03
ENSRNOG00000062521	*Gtpbp6*	934.02	0.16	0.04	8.13E-06	7.55E-03
ENSRNOG00000071146	***N/A***	**33.50**	**-0.69**	**0.20**	**1.55E-05**	**1.37E-02**
ENSRNOG00000020426	*Erf*	1888.77	0.14	0.04	3.13E-05	2.66E-02
ENSRNOG00000062115	*AC242953.1*	6.30	0.90	0.27	3.57E-05	2.90E-02
ENSRNOG00000019718	*Galnt15*	316.38	-0.24	0.07	4.22E-05	3.29E-02
ENSRNOG00000052236	*AABR07004992.1*	30.62	-0.44	0.14	5.27E-05	3.95E-02
ENSRNOG00000065330	*Mrgpre*	1184.13	-0.21	0.07	6.19E-05	4.47E-02

Table 7.5. Differentially expressed genes in CA1 between sexes. Positive Log_2 Fold Change values reflect higher expression in females compared with males. NA values correspond to Ensembl IDs of novel genes with no existing gene symbols. Genes in bold were also found to be DEGs in the DG.

Ensembl ID	Gene Symbol	Base Mean	Log_2 Fold Change	L_2FC SE	*p*	FDR *p*
ENSRNOG00000057231	***Ddx3y***	**2208.66**	**15.13**	**0.80**	**4.27E-256**	**8.51E-252**
ENSRNOG00000060437	***LOC103694537***	**430.03**	**7.00**	**0.21**	**6.49E-253**	**6.46E-249**
ENSRNOG00000060048	***Eif2s3y***	**2586.99**	**14.04**	**0.50**	**2.55E-250**	**1.69E-246**
ENSRNOG00000060496	***Kdm5d***	**1477.78**	**18.79**	**3.51**	**1.60E-249**	**7.97E-246**
ENSRNOG00000060617	***Uty***	**563.63**	**13.74**	**0.98**	**4.10E-194**	**1.63E-190**
ENSRNOG00000060793	***Eif2s3***	**10739.00**	**-0.61**	**0.05**	**8.05E-39**	**2.67E-35**
ENSRNOG00000065796	***N/A***	**1319.99**	**-3.81**	**0.37**	**2.63E-28**	**7.48E-25**
ENSRNOG00000037911	***N/A***	**574.42**	**0.00**	**0.00**	**6.31E-18**	**1.57E-14**
ENSRNOG00000023383	***Ddx3x***	**20982.42**	**-0.33**	**0.05**	**1.09E-11**	**2.41E-08**
ENSRNOG00000002662	***Pbdc1***	**3161.01**	**-0.31**	**0.06**	**2.05E-09**	**4.07E-06**
ENSRNOG00000068101	***LOC100912991***	**429.46**	**-0.47**	**0.10**	**1.25E-07**	**2.26E-04**
ENSRNOG00000052721	***Kdm6a***	**2049.86**	**-0.42**	**0.09**	**3.05E-07**	**4.81E-04**
ENSRNOG00000066008	*Pecam1*	1592.21	-0.24	0.05	3.14E-07	4.81E-04
ENSRNOG00000031398	*Mab21l2*	20.00	-2.59	0.66	4.41E-07	6.27E-04
ENSRNOG00000007637	*Acer2*	1488.77	-0.34	0.08	8.41E-07	1.12E-03
ENSRNOG00000017689	*Itih3*	9584.09	-0.44	0.11	1.91E-06	2.38E-03
ENSRNOG00000013209	*Barhl1*	35.65	-2.56	0.74	2.16E-06	2.53E-03
ENSRNOG00000003899	*Krt16*	13.24	-1.15	0.29	3.84E-06	4.24E-03
ENSRNOG00000014240	*Trmt11*	209.77	0.00	0.00	4.12E-06	4.32E-03
ENSRNOG00000004713	*Kcnj16*	1050.66	-0.57	0.15	4.59E-06	4.57E-03
ENSRNOG00000066963	*LOC102552182*	9.85	1.36	0.36	5.11E-06	4.85E-03
ENSRNOG00000070919	*Foxc1*	155.14	-0.38	0.10	6.82E-06	6.17E-03
ENSRNOG00000009343	*Evpl*	30.23	0.94	0.26	9.76E-06	8.45E-03
ENSRNOG00000012566	*Kcnv2*	32.56	1.71	0.55	1.09E-05	9.03E-03
ENSRNOG00000025051	*Tal1*	243.47	-0.54	0.15	1.20E-05	9.59E-03
ENSRNOG00000015318	*Heyl*	1396.83	-0.21	0.06	1.53E-05	1.17E-02
ENSRNOG00000011533	*Irx3*	19.51	0.00	0.00	1.79E-05	1.31E-02
ENSRNOG00000019660	*Spib*	8.57	0.86	0.25	1.84E-05	1.31E-02
ENSRNOG00000011227	*Atp1b2*	48760.97	-0.11	0.03	2.00E-05	1.37E-02
ENSRNOG00000019336	*Gata3*	46.95	0.00	0.00	2.11E-05	1.40E-02

ENSRNOG00000032941	*Mab21l1*	59.42	-1.04	0.31	2.21E-05	1.41E-02
ENSRNOG00000021800	*Zfp541*	59.22	0.50	0.15	2.27E-05	1.41E-02
ENSRNOG00000060662	*Pou4f1*	2.24	-3.77	1.11	3.03E-05	1.83E-02
ENSRNOG00000017765	*Net1*	2052.09	-0.19	0.06	3.45E-05	2.02E-02
ENSRNOG00000018715	*Clec10a*	109.56	-0.49	0.15	3.73E-05	2.12E-02
ENSRNOG00000065555	*N/A*	58.82	0.34	0.10	4.30E-05	2.37E-02
ENSRNOG00000016696	*Angpt2*	104.21	-0.52	0.16	4.40E-05	2.37E-02
ENSRNOG00000002923	*Zcchc13*	3.96	-1.28	0.42	5.68E-05	2.98E-02
ENSRNOG00000033217	*Esam*	1052.57	-0.20	0.06	6.08E-05	3.10E-02
ENSRNOG00000071146	***N/A***	**34.61**	**-0.58**	**0.19**	**6.44E-05**	**3.15E-02**
ENSRNOG00000011823	*Tfap2b*	48.80	0.00	0.00	6.50E-05	3.15E-02
ENSRNOG00000007603	*Gabrr1*	44.54	-1.13	0.38	7.07E-05	3.35E-02
ENSRNOG00000016102	*Ebf3*	56.05	-1.51	0.54	7.26E-05	3.36E-02
ENSRNOG00000047158	*Selenbp1*	637.66	-0.21	0.07	8.28E-05	3.75E-02
ENSRNOG00000033609	*Irx1*	19.95	-1.25	0.45	8.90E-05	3.94E-02
ENSRNOG00000018394	*Chrdl2*	33.18	0.80	0.27	1.05E-04	4.55E-02

Forty-seven genes in the DG and 54 genes in the CA1 were found to be significantly differentially expressed between context exposure groups after FDR correction for multiple comparison (Figures 7.9 and 7.10). Only 8 of the 47 DG DEGs and 8 of the 54 CA1 DEGs showed reduced expression in rats exposed to a novel context 30 minutes prior to euthanasia compared with rats at baseline, indicating context exposure primarily results in increased expression of affected downstream genes in both DG and CA1. DEGs are displayed in Table 7.6 for DG and Table 7.7 for CA1. Thirty-four DEGs were common to both DG and CA1, with all showing consistent direction of effects between regions (Figure 7.10). Several of these DEGs that were common to both hippocampal regions such as *Fos*, *Fosb*, *Egr1*, *Egr2*, *Junb*, and *Arc* are known immediate early genes with known involvement in memory formation (Strekalova et al., 2003; Poirier, 2007; Gallo et al., 2018).

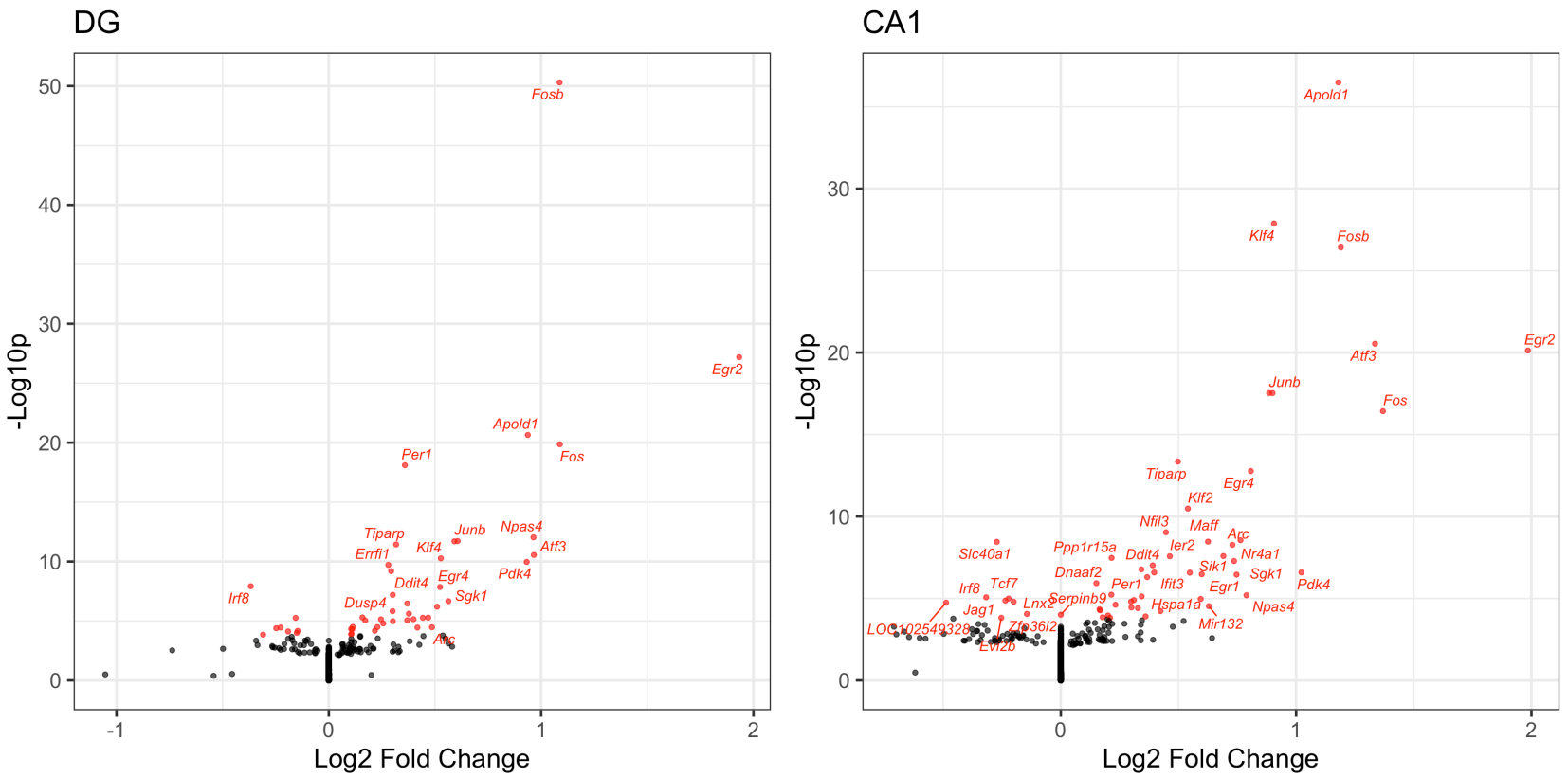

Figure 7.9. Differential gene expression in the DG (left) and CA1 (right) in animals exposed to a novel context compared with animals at baseline. Red points indicate significant DEGs at an FDR corrected $\alpha = 0.05$.

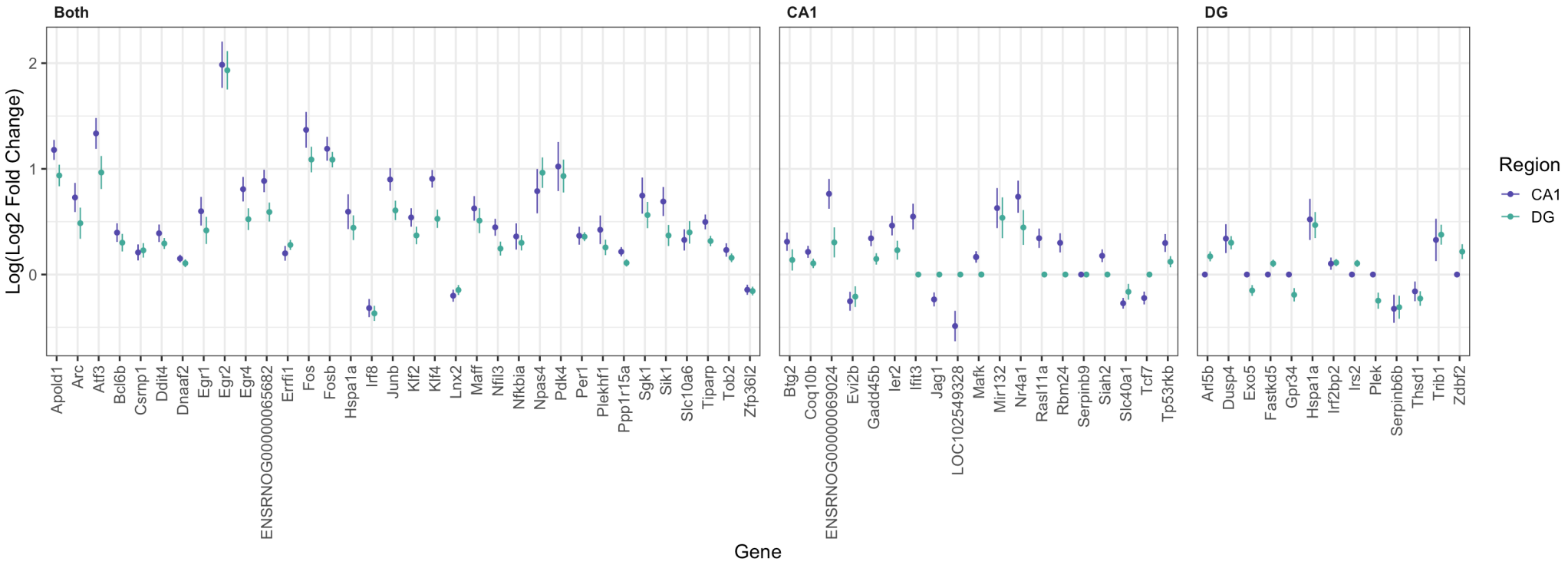

Figure 7.10. Log_2 fold change (and standard error) in context exposed relative to baseline rats in the 34 genes that were DEGs in both CA1 and DG (left), the 19 CA1 specific DEGs (middle) and the 13 DG specific DEGs (right) at FDR adjusted $\alpha = 0.05$.

Table 7.6. Differentially expressed genes in DG between context exposure conditions. Positive Log_2 Fold Change values reflect higher expression in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. NA values correspond to Ensembl IDs of novel genes with no existing gene symbols. Genes in bold were also found to be DEGs in the CA1.

Ensembl ID	Gene Symbol	Base Mean	Log_2 Fold Change	L_2FC SE	*p*	FDR *p*
ENSRNOG00000046667	***Fosb***	**495.56**	**1.09**	**0.07**	**4.98E-51**	**8.20E-47**
ENSRNOG00000000640	***Egr2***	**267.8**	**1.93**	**0.18**	**6.45E-28**	**5.31E-24**
ENSRNOG00000007830	***Apold1***	**395.18**	**0.94**	**0.1**	**2.25E-21**	**1.24E-17**
ENSRNOG00000008015	***Fos***	**669.59**	**1.09**	**0.12**	**1.37E-20**	**5.64E-17**
ENSRNOG00000007387	***Per1***	**3940.39**	**0.36**	**0.04**	**7.88E-19**	**2.60E-15**
ENSRNOG00000020009	***Npas4***	**275.83**	**0.96**	**0.14**	**9.25E-13**	**2.54E-09**
ENSRNOG00000067725	***Junb***	**2526.93**	**0.61**	**0.09**	**1.91E-12**	**4.13E-09**
ENSRNOG00000065682	***N/A***	**2588.87**	**0.59**	**0.09**	**2.00E-12**	**4.13E-09**
ENSRNOG00000011238	***Tiparp***	**1389.1**	**0.32**	**0.05**	**3.67E-12**	**6.72E-09**
ENSRNOG00000003745	***Atf3***	**55.86**	**0.97**	**0.16**	**2.80E-11**	**4.62E-08**
ENSRNOG00000016299	***Klf4***	**591.3**	**0.53**	**0.09**	**5.38E-11**	**8.06E-08**
ENSRNOG00000009565	***Pdk4***	**1063.85**	**0.93**	**0.16**	**1.08E-10**	**1.48E-07**
ENSRNOG00000058186	***Errfi1***	**4013.15**	**0.28**	**0.05**	**1.93E-10**	**2.45E-07**
ENSRNOG00000057078	***Ddit4***	**3530.68**	**0.29**	**0.05**	**6.39E-10**	**7.53E-07**
ENSRNOG00000017869	***Irf8***	**265.36**	**-0.37**	**0.07**	**1.20E-08**	**1.32E-05**
ENSRNOG00000015719	***Egr4***	**581.73**	**0.52**	**0.1**	**1.39E-08**	**1.43E-05**
ENSRNOG00000011921	*Dusp4*	4417.96	0.3	0.06	6.35E-08	6.16E-05
ENSRNOG00000011815	***Sgk1***	**7946.02**	**0.56**	**0.12**	**2.19E-07**	**2.00E-04**
ENSRNOG00000067705	***Klf2***	**746.7**	**0.37**	**0.08**	**3.40E-07**	**2.95E-04**
ENSRNOG00000012886	***Maff***	**66.68**	**0.51**	**0.12**	**6.33E-07**	**5.21E-04**
ENSRNOG00000007390	***Nfkbia***	**1177.72**	**0.3**	**0.07**	**1.51E-06**	**1.18E-03**
ENSRNOG00000004100	*Trib1*	661.81	0.38	0.09	2.48E-06	1.86E-03
ENSRNOG00000050500	***Tob2***	**2447.22**	**0.16**	**0.04**	**5.06E-06**	**3.49E-03**
ENSRNOG00000045654	*Hspa1a*	230.65	0.47	0.12	5.22E-06	3.49E-03
ENSRNOG00000050647	***Hspa1a***	**625.35**	**0.44**	**0.12**	**5.38E-06**	**3.49E-03**
ENSRNOG00000005067	***Zfp36l2***	**2631.75**	**-0.16**	**0.04**	**5.50E-06**	**3.49E-03**
ENSRNOG00000002057	***Slc10a6***	**91.72**	**0.4**	**0.11**	**7.19E-06**	**4.29E-03**
ENSRNOG00000011668	***Nfil3***	**515.91**	**0.25**	**0.07**	**7.28E-06**	**4.29E-03**
ENSRNOG00000001189	***Sik1***	**634.4**	**0.37**	**0.1**	**8.74E-06**	**4.97E-03**

ENSRNOG00000064112	*Arl5b*	1138.75	0.17	0.05	9.08E-06	4.99E-03
ENSRNOG00000059956	***Bcl6b***	**227.61**	**0.3**	**0.08**	**1.06E-05**	**5.66E-03**
ENSRNOG00000027724	***Plekhf1***	**489.3**	**0.26**	**0.07**	**1.62E-05**	**8.36E-03**
ENSRNOG00000070206	*Irf2bp2*	3161.55	0.11	0.03	3.20E-05	1.60E-02
ENSRNOG00000033433	***Csrnp1***	**546.25**	**0.23**	**0.07**	**3.29E-05**	**1.60E-02**
ENSRNOG00000043465	***Arc***	**8291.5**	**0.49**	**0.15**	**3.40E-05**	**1.60E-02**
ENSRNOG00000012108	*Thsd1*	486.71	-0.23	0.07	3.56E-05	1.60E-02
ENSRNOG00000019422	***Egr1***	**9403.9**	**0.42**	**0.13**	**3.58E-05**	**1.60E-02**
ENSRNOG00000005214	*Plek*	239.34	-0.25	0.08	4.10E-05	1.78E-02
ENSRNOG00000023509	*Irs2*	6518.5	0.11	0.03	4.67E-05	1.97E-02
ENSRNOG00000020938	***Ppp1r15a***	**1133.97**	**0.11**	**0.03**	**5.00E-05**	**2.06E-02**
ENSRNOG00000024114	*Zdbf2*	2790.98	0.22	0.07	6.67E-05	2.68E-02
ENSRNOG00000000955	***Lnx2***	**556.9**	**-0.15**	**0.05**	**6.85E-05**	**2.69E-02**
ENSRNOG00000039759	*Gpr34*	755.51	-0.19	0.06	7.67E-05	2.94E-02
ENSRNOG00000037432	*Exo5*	269.77	-0.15	0.05	1.00E-04	3.76E-02
ENSRNOG00000028155	***Dnaaf2***	**867.19**	**0.11**	**0.04**	**1.21E-04**	**4.45E-02**
ENSRNOG00000053270	*Fastkd5*	826.69	0.1	0.04	1.31E-04	4.69E-02
ENSRNOG00000016420	*Serpinb6b*	65.11	-0.31	0.11	1.41E-04	4.95E-02

Table 7.7. Differentially expressed genes in CA1 between context exposure conditions. Positive Log_2 Fold Change values reflect higher expression in females compared with males. NA values correspond to Ensembl IDs of novel genes with no existing gene symbols. Genes in bold were also found to be DEGs in the DG.

Ensembl ID	Gene Symbol	Base Mean	Log_2 Fold Change	L_2FC SE	*p*	FDR *p*
ENSRNOG00000007830	***Apold1***	**524.2**	**1.18**	**0.09**	**3.31E-37**	**5.83E-33**
ENSRNOG00000016299	***Klf4***	**871.77**	**0.91**	**0.08**	**1.32E-28**	**1.16E-24**
ENSRNOG00000046667	***Fosb***	**696.89**	**1.19**	**0.11**	**3.84E-27**	**2.25E-23**
ENSRNOG00000003745	***Atf3***	**74.61**	**1.34**	**0.15**	**2.90E-21**	**1.28E-17**
ENSRNOG00000000640	***Egr2***	**550.4**	**1.98**	**0.22**	**7.46E-21**	**2.63E-17**
ENSRNOG00000065682	***N/A***	**5322.69**	**0.89**	**0.11**	**2.97E-18**	**7.46E-15**
ENSRNOG00000067725	***Junb***	**5263.98**	**0.90**	**0.11**	**2.97E-18**	**7.46E-15**
ENSRNOG00000008015	***Fos***	**951.91**	**1.37**	**0.17**	**3.77E-17**	**8.29E-14**
ENSRNOG00000011238	***Tiparp***	**1671.47**	**0.50**	**0.07**	**4.45E-14**	**8.71E-11**
ENSRNOG00000015719	***Egr4***	**1331.1**	**0.81**	**0.12**	**1.69E-13**	**2.98E-10**
ENSRNOG00000067705	***Klf2***	**1180.2**	**0.54**	**0.09**	**3.31E-11**	**5.29E-08**
ENSRNOG00000011668	***Nfil3***	**737.6**	**0.45**	**0.08**	**9.25E-10**	**1.36E-06**
ENSRNOG00000069024	*N/A*	1475.73	0.76	0.14	2.81E-09	3.80E-06
ENSRNOG00000012886	***Maff***	**88.68**	**0.63**	**0.12**	**3.42E-09**	**4.14E-06**
ENSRNOG00000003872	*Slc40a1*	534.8	-0.27	0.05	3.53E-09	4.14E-06
ENSRNOG00000043465	***Arc***	**16193.82**	**0.73**	**0.14**	**5.37E-09**	**5.90E-06**
ENSRNOG00000001189	***Sik1***	**854.19**	**0.69**	**0.14**	**2.60E-08**	**2.61E-05**
ENSRNOG00000067274	*Ier2*	828.11	0.46	0.09	2.67E-08	2.61E-05
ENSRNOG00000020938	***Ppp1r15a***	**1657.25**	**0.22**	**0.04**	**3.36E-08**	**3.11E-05**
ENSRNOG00000007607	*Nr4a1*	6862.13	0.74	0.15	5.21E-08	4.58E-05
ENSRNOG00000057078	***Ddit4***	**6449.02**	**0.39**	**0.08**	**9.58E-08**	**8.03E-05**
ENSRNOG00000019822	*Gadd45b*	1097.65	0.34	0.07	1.69E-07	1.35E-04
ENSRNOG00000009565	***Pdk4***	**1758.91**	**1.02**	**0.23**	**2.60E-07**	**1.89E-04**
ENSRNOG00000059956	***Bcl6b***	**323.6**	**0.40**	**0.09**	**2.62E-07**	**1.89E-04**
ENSRNOG00000022839	*Ifit3*	139.12	0.55	0.12	2.69E-07	1.89E-04
ENSRNOG00000019422	***Egr1***	**14173.09**	**0.60**	**0.14**	**3.34E-07**	**2.26E-04**
ENSRNOG00000011815	***Sgk1***	**13094.56**	**0.75**	**0.17**	**3.53E-07**	**2.30E-04**
ENSRNOG00000007387	***Per1***	**4653.88**	**0.37**	**0.08**	**5.01E-07**	**3.15E-04**
ENSRNOG00000028155	***Dnaaf2***	**1183.49**	**0.15**	**0.04**	**1.18E-06**	**7.16E-04**
ENSRNOG00000014456	*Coq10b*	1955.4	0.21	0.06	5.91E-06	3.46E-03

ENSRNOG00000020009	***Npas4***	**259.97**	**0.79**	**0.21**	**6.40E-06**	**3.63E-03**
ENSRNOG00000000956	*Rasl11a*	580.7	0.34	0.09	7.52E-06	4.13E-03
ENSRNOG00000017869	***Irf8***	**431.8**	**-0.32**	**0.09**	**8.54E-06**	**4.55E-03**
ENSRNOG00000005872	*Tcf7*	248.78	-0.22	0.06	1.02E-05	5.26E-03
ENSRNOG00000050647	***Hspa1a***	**969.56**	**0.59**	**0.16**	**1.07E-05**	**5.37E-03**
ENSRNOG00000003300	*Btg2*	1360.44	0.31	0.09	1.26E-05	6.16E-03
ENSRNOG00000007443	*Jag1*	519.88	-0.24	0.07	1.36E-05	6.46E-03
ENSRNOG00000007285	*Trp53rkb*	418.77	0.30	0.08	1.58E-05	7.24E-03
ENSRNOG00000000955	***Lnx2***	**732.96**	**-0.20**	**0.06**	**1.60E-05**	**7.24E-03**
ENSRNOG00000067191	*LOC102549328*	103.32	-0.49	0.14	1.81E-05	7.98E-03
ENSRNOG00000050500	***Tob2***	**3614.46**	**0.23**	**0.06**	**2.44E-05**	**1.05E-02**
ENSRNOG00000035516	*Mir132*	17.44	0.63	0.19	2.96E-05	1.24E-02
ENSRNOG00000046547	*Rbm24*	1341.02	0.30	0.09	3.57E-05	1.46E-02
ENSRNOG00000002057	***Slc10a6***	**119.65**	**0.33**	**0.10**	**3.88E-05**	**1.55E-02**
ENSRNOG00000065794	*Mafk*	1252.83	0.16	0.05	4.63E-05	1.81E-02
ENSRNOG00000001277	*Mafk*	1196.24	0.17	0.05	5.33E-05	2.04E-02
ENSRNOG00000027724	***Plekhf1***	**844.56**	**0.42**	**0.14**	**5.95E-05**	**2.23E-02**
ENSRNOG00000005067	***Zfp36l2***	**3713.07**	**-0.14**	**0.05**	**8.80E-05**	**3.23E-02**
ENSRNOG00000033772	*Serpinb9*	941.68	<0.01	<0.01	9.87E-05	3.54E-02
ENSRNOG00000058186	***Errfi1***	**4379.94**	**0.20**	**0.07**	**1.09E-04**	**3.85E-02**
ENSRNOG00000007390	***Nfkbia***	**1862.87**	**0.36**	**0.12**	**1.25E-04**	**4.32E-02**
ENSRNOG00000013703	*Siah2*	1161.72	0.18	0.06	1.41E-04	4.77E-02
ENSRNOG00000033433	***Csrnp1***	**770.88**	**0.21**	**0.08**	**1.52E-04**	**4.96E-02**
ENSRNOG00000014125	*Evi2b*	160.01	-0.25	0.09	1.52E-04	4.96E-02

7.3.4.3 Gene Set Enrichment Analysis

In the DG, the 80 significant *Cacna1c* genotype DEGs corresponded to 74 human analogues, which were found to be enriched for 100 significant GO terms (Table 7.8). Terms related to a wide variety of processes, but many of the most significant terms related to the extracellular matrix and related processes, as well as immune system processes and related signalling pathways. Other terms were related to response to stimuli, general cellular processes, phosphorylation, and vascular processes. As only ten genes were found to be differentially expressed in the CA1, the gene set was not large enough to conduct GSEA.

Table 7.8. Gene ontology and human phenotype terms significantly enriched within *Cacna1c* genotype significant DEGs in the DG at $p_{FDR} < 0.05$. Key: extra-cellular processes, immune processes, stimulus response, general cellular processes, phosphorylation, vascular processes, other.

Extracellular region	Extracellular matrix
Collagen-containing extracellular matrix	Extracellular exosome
Extracellular space	Extracellular membrane-bounded organelle
Extracellular organelle	Extracellular vesicle
Regulation of immune system process	Complement activation
Immune response	Response to cytokine
Positive regulation of immune system process	Defense response
Defense response to virus	Negative regulation of immune system process
Humoral immune response	Cytokine-mediated signaling pathway
Immune system process	Regulation of innate immune response
Regulation of immune response	Positive regulation of cytokine-mediated signaling pathway
Innate immune response	Immune effector process
Cellular response to cytokine stimulus	Complement activation, classical pathway
Response to virus	IgM B cell receptor complex
Positive regulation of immune response	Hexameric IgM immunoglobulin complex
Positive regulation of response to cytokine stimulus	Response to bacterium
Regulation of type II interferon-mediated signaling pathway	Cellular response to molecule of bacterial origin
Regulation of response to type II interferon	Activation of immune response
Response to other organism	Response to chemical
Positive regulation of response to external stimulus	Response to external biotic stimulus
Response to prostaglandin	Biological process involved in interspecies interaction between organisms
Cellular response to lipopolysaccharide	Defense response to symbiont

Positive regulation of response to biotic stimulus	Defense response to other organism
Cellular response to alcohol	Cellular response to chemical stimulus
Response to stimulus	Positive regulation of response to stimulus
Regulation of response to biotic stimulus	Cellular response to prostaglandin stimulus
Response to biotic stimulus	
Cell activation	Clathrin-coated vesicle
Cell periphery	Trans-Golgi network
Plasma membrane	Clathrin-coated endocytic vesicle
Cell surface	Lysosome
Membrane	Lytic vacuole
Golgi lumen	Lysosomal lumen
Cytoplasmic vesicle	Microvillus
Extrinsic component of external side of plasma membrane	Clathrin-coated endocytic vesicle membrane
Vesicle	Protein targeting to vacuole
Intracellular vesicle	Microvillus membrane
Coated vesicle	External encapsulating structure
Cell surface receptor signaling pathway	
Regulation of phosphate metabolic process	Positive regulation of phosphorylation
Regulation of phosphorus metabolic process	Positive regulation of protein phosphorylation
Peptidyl-tyrosine phosphorylation	Positive regulation of peptidyl-tyrosine phosphorylation
Regulation of protein phosphorylation	Regulation of phosphorylation
Regulation of peptidyl-tyrosine phosphorylation	Positive regulation of phosphorus metabolic process
Positive regulation of phosphate metabolic process	Peptidyl-tyrosine modification
Platelet alpha granule lumen	Blood microparticle
Circulatory system development	Regulation of tissue remodeling
Circulatory system process	Vascular process in circulatory system
Positive regulation of vascular permeability	Platelet alpha granule
Regulation of multicellular organismal process	Skeletal system development
Negative regulation of multicellular organismal process	Tube development

The 27 significant sex DG DEGs corresponded to 21 human analogues, which were found to be significantly enriched for 121 GO terms (Table 7.9), and the 46 sex CA1 DEGs corresponded to 45 human analogues, which were significantly enriched for 92 GO terms (Table 7.10). 23 of the terms were common to both DG and CA1.

Table 7.9. Gene ontology and human phenotype terms significantly enriched within sex significant DEGs in the DG. Key: epigenetic processes, sexual or wider development, transcription and translation processes, other.

Protein demethylase activity	**Demethylase activity**
Histone demethylase activity	**2-oxoglutarate-dependent dioxygenase activity**
Histone H3 demethylase activity	**Dioxygenase activity**
Histone H3K27me2/H3K27me3 demethylase activity	**Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen**
MLL3/4 complex	Histone H3K4me/H3K4me2/H3K4me3 demethylase activity
Histone H3K4 demethylase activity	Chromatin DNA binding
Histone modifying activity	Oxidoreductase activity
Histone methyltransferase complex	Telomerase activity
Methyltransferase complex	
Germ plasm	Hypoplasia of the maxilla
Pole plasm	Abnormality of the male genitalia
P granule	Abnormal male external genitalia morphology
Abnormal testis morphology	X-linked recessive inheritance
X-linked inheritance	Decreased testicular size
Cryptorchidism	Abnormality of the genital system
Y-linked inheritance	Abnormality of the testis size
Abnormal external genitalia	Abnormal reproductive system morphology
Abnormal maxilla morphology	Decreased fertility in males
Aplasia/Hypoplasia of the testes	Short phalanx of finger
Abnormality of the endocrine system	Non-obstructive azoospermia
Functional abnormality of male internal genitalia	Cleft palate
Strabismus	Depressed nasal tip
Abnormal palate morphology	Coarctation of aorta
Abnormality of skin pigmentation	Short columella
Micropenis	Abnormal spermatogenesis
Abnormal jaw morphology	Short digit
Azoospermia	Abnormal male reproductive system physiology
Abnormal conjugate eye movement	Abnormality of the dentition
Abnormal facial skeleton morphology	Broad nasal tip
Orofacial cleft	Short stature
Craniofacial cleft	Abnormality of the genitourinary system
Abnormal penis morphology	Abnormality of skull size

External genital hypoplasia
Hypoplastic male external genitalia
Pansynostosis
Abnormal lip morphology
Aplasia/Hypoplasia of the phalanges of the hand
Irregular hyperpigmentation
Abnormal oral cavity morphology
Abnormal oral morphology
Postnatal growth retardation
Male infertility
Double outlet right ventricle with doubly committed ventricular septal defect and pulmonary stenosis
Areolar fullness
Tooth malposition
Abnormal ear morphology
X-linked dominant inheritance
Anal mucosal leukoplakia
Abnormality of the anus
Abnormal localization of kidney
Infertility
Abnormal limb bone morphology
Abnormality of limb bone
Abnormal nasal tip morphology
Brachydactyly
Hypoplasia of penis
Abnormality of body height

Translation regulator activity
tRNA binding
ATP-dependent activity, acting on RNA
RNA polymerase II-specific DNA-binding transcription factor binding
Nucleic acid binding
Nucleoplasm
Catenin-TCF7L2 complex
Box H/ACA snoRNP complex
Beta-catenin-TCF7L2 complex
Box H/ACA RNP complex
Box H/ACA telomerase RNP complex
Nuclear lumen
CTPase activity
Translation regulator activity, nucleic acid binding
Organic cyclic compound binding
Localized skin lesion
Nuclear receptor binding
Transcription factor binding
RNA helicase activity
RNA strand annealing activity
Formation of translation preinitiation complex
Eukaryotic translation initiation factor 2 complex
Eukaryotic initiation factor 4E binding
Armadillo repeat domain binding
Helicase activity
RNA-directed DNA polymerase activity
Box H/ACA scaRNP complex
Translation initiation factor activity
Translation factor activity, RNA binding
Box H/ACA snoRNA binding
Ribosome binding
Hypermelanotic macule
Macule

Table 7.10. Gene ontology and human phenotype terms significantly enriched within sex significant DEGs in the CA1. Key: epigenetic processes, sexual or wider development, transcription and translation, other.

Demethylase activity	**Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen**
Histone H3 demethylase activity	**MLL3/4 complex**
Histone demethylase activity	**2-oxoglutarate-dependent dioxygenase activity**
Protein demethylase activity	**Dioxygenase activity**
Histone H3K27me2/H3K27me3 demethylase activity	Methanethiol oxidase activity
Sterol 14-demethylase activity	
Pole plasm	**Germ plasm**
P granule	Sensory organ development
Circulatory system development	Kidney epithelium development
Eye development	Nephron development
Visual system development	Mesenchymal cell development
Glomerular epithelium development	Nephron epithelium development
Kidney development	Animal organ development
Renal system development	Sympathetic nervous system development
Kidney vasculature development	Glomerulus vasculature development
Renal system vasculature development	Sensory system development
Glomerulus development	AF-1 domain binding
Nucleic acid binding	**ATP-dependent activity, acting on RNA**
Eukaryotic translation initiation factor 2 complex	**RNA strand annealing activity**
tRNA binding	**Translation regulator activity**
Transcription factor binding	**Formation of translation preinitiation complex**
RNA polymerase II-specific DNA-binding transcription factor binding	**RNA helicase activity**
Organic cyclic compound binding	Cis-regulatory region sequence-specific DNA binding
Transcription cis-regulatory region binding	Transcription regulatory region nucleic acid binding
RNA polymerase II transcription regulatory region sequence-specific DNA binding	Sequence-specific double-stranded DNA binding
RNA polymerase II cis-regulatory region sequence-specific DNA binding	Sequence-specific DNA binding
DNA binding	Double-stranded DNA binding
DNA-binding transcription factor activity	DNA-binding transcription factor activity, RNA polymerase II-specific
Transcription regulator activity	DNA-binding transcription activator activity
DNA-binding transcription activator activity, RNA polymerase II-specific	DNA-binding transcription repressor activity, RNA polymerase II-specific
DNA-binding transcription repressor activity	tRNA threonylcarbamoyladenosine metabolic process
Chromatin	Regulation of DNA binding
Protein-DNA complex	Positive regulation of transcription regulatory region DNA binding
Chromosome	Transcription by RNA polymerase II
Translation initiation factor activity	DNA-binding transcription factor binding
Positive regulation of RNA metabolic process	Positive regulation of DNA-templated transcription
Regulation of transcription by RNA polymerase II	Positive regulation of RNA biosynthetic process

Regulation of transcription regulatory region DNA binding	Regulation of RNA biosynthetic process
Positive regulation of transcription by RNA polymerase II	Regulation of DNA-templated transcription
Positive regulation of DNA binding	E-box binding
HMG box domain binding	
Positive regulation of macromolecule biosynthetic process	Cellular nitrogen compound biosynthetic process
Positive regulation of cellular biosynthetic process	Positive regulation of biosynthetic process
Dihydroceramidase activity	Multicellular organismal-level homeostasis
Protein-containing complex	Tissue homeostasis
Positive regulation of nucleobase-containing compound metabolic process	Keratinocyte differentiation
Anatomical structure homeostasis	Positive regulation of nitrogen compound metabolic process

The 47 significant context exposure DG DEGs corresponded to 45 human analogues, which were found to be significantly enriched for 365 GO terms, and the 54 significant context exposure CA1 DEGs corresponded to 50 human analogues, which were significantly enriched for 350 GO terms. Owing to the large number of terms, tables are not reported here. The majority of terms, 303, were common to both DG and CA1 sex DEGs. Terms related to the general themes of transcription and translation, metabolic processes, general cellular processes, apoptosis and programmed cell death. Several terms related to memory and interleukin/cytokine signalling were also included.

7.3.4.4 Gene Set Analysis

For the gene annotation step, at least one SNP was mapped to 19364 genes out of the 19427 total genes. The gene analysis step mapped 6955227 of 7035715 total SNPs in the PGC3 Schizophrenia GWAS summary statistics to 18270 valid genes from the annotation step output and ran the gene analysis using the SNPwise-mean model. Continuous gene property analysis was conducted using a background of all expressed genes in each tissue. In the DG data, 14363 valid genes were retained for the analysis as 3907 genes were not found in all data sets, whereas in the CA1, 14557 valid genes were retained with 3713 genes not found in all data sets. In the DG, the regression analysis of the association between $-\log_{10}$ *p*-values of *Cacna1c* differential expression and Z-scores of gene association with schizophrenia from the PGC3 summary statistics showed that there was a small, but highly significant negative association ($\beta = -0.07$, SE = 0.02, $p = 2.21 \times 10^{-5}$). However, in the CA1, there was no association ($\beta = 0.001$, SE = 0.02, $p = 0.909$).

7.4 Discussion

In a preliminary study of the effects of *Cacna1c* genotype on gene expression in the male rat hippocampus, no significant differences were observed, likely due to insufficient power. In order to remedy this problem, and to investigate how the factors of sex and hippocampal activation induced by exposure to a novel context may interact with *Cacna1c* genotype, a larger RNA-sequencing experiment was subsequently conducted.

7.4.1 The effects of *Cacna1c* genotype on hippocampal gene expression.

Eighty genes in the DG and 10 genes in the CA1 were found to be differentially expressed between *Cacna1c* genotypes. Although statistical power was lower in CA1 compared with DG owing to missing samples, the DEGs in CA1 were not a strict subset of DG DEGs, indicating independent effects of *Cacna1c* heterozygosity between hippocampal subregions, rather than poor power being responsible for the lower number of genes expressed in the CA1. In line with expectations, higher expression of *Cacna1c* was observed in *Cacna1c*$^{+/+}$ compared with *Cacna1c*$^{+/-}$ animals in the DG. However, this was not found to be the case in CA1. Thus, these results suggest that the *Cacna1c* heterozygous deletion model may not impact gene expression in hippocampal subregions in a uniform manner. Although the low level of *Cacna1c* differential expression in CA1 could be interpreted as flawed analyses, the patterns of differential gene expression between sexes and context exposure groups in the CA1 were similar to that of the DG, indicating intact validity of the RNA sequencing and data processing. Owing to the differing functions of the CA1 and DG regions and wider complex circuitry of the hippocampus, it may be that compensatory mechanisms that are not captured in this experiment work to increase *Cacna1c* expression in the CA1 region. Analysis of $Ca_v1.2$ protein levels in each region is required to better ascertain the functional impact of this difference.

The list of DEGs according to *Cacna1c* genotypes in the CA1 was too small to effectively carry out GSEA. However, GSEA results of the *Cacna1c* DEGs in the DG showed that several immune related terms were enriched within the gene list, such as “immune system process”, “regulation of immune system process”, “innate immune response”, “complement activation”, “cytokine-mediated signaling pathway”, “immune effector process”, “complement activation, classical pathway”, and “regulation of type II interferon-mediated signaling pathway”, amongst others. Thus, *Cacna1c* heterozygosity appears to result in alterations in a range of immune processes as opposed to a singular pathway. Numerous lines of evidence have implicated altered immune processes in neuropsychiatric disease. A range of neuroimmune factors such as interleukin (IL)-1, IL-6, tumour necrosis factor (TNF)-α, CD4+CD25+T regulatory cells (T reg), self-specific CD4+T cells, monocyte-derived macrophages, microglia and astrocytes have been implicated in depression, and are thought to play a mediating role in the neuroplastic alterations seen in the disorder (Eyre and Baune, 2012; Nettis and Pariante, 2020). Similarly, altered immune profiles have been observed in some patients with schizophrenia (Ermakov et al., 2022), and there is speculation as to whether altered immune pathways may mediate the relationship between environmental risk factors such as cannabis use, early childhood adversity and social exclusion, and later onset of psychosis (Radhakrishnan et al., 2017). Hence, these results are suggestive of aberrant immune system functioning occurring downstream of altered LTCC mediated Ca^{2+} signalling.

The GO term, “immune system process”, significantly enriched in *Cacna1c* DEGs is a large term with 1455 genes, and reflects a gene’s involvement with any aspect of immune function. 23 of the 80 *Cacna1c* DEGs were tagged with this term. Some more specific immune-related GO terms were also significantly enriched in *Cacna1c* DEGs. Two such terms were “complement activation”, consisting of 30 tagged genes, three of which were *Cacna1c* significant DEGs, and “complement activation, classical pathway”, which consists of 11 tagged genes, two of which were significant *Cacna1c* DEGs. The three *Cacna1c* DEGs tagged with complement activation were

Ighm, *C1qc*, and *A2m*, and *Ighm* and *C1qc* were also the drivers of the "complement activation, classical pathway" association.

The complement system is a highly complex system of proteins that plays a major part in both the innate and adaptive immune system. This network consists of over thirty proteins that make up three arms: the classical pathway, the lectin pathway, and the alternative pathway (Sarma and Ward, 2011). The complement system also plays a key role in the brain and components of the complement pathways can be locally synthesised. The complement system has a highly involved regulatory function in many integral brain processes including neurogenesis, synaptic plasticity, repair and removal of damaged cells, and protection from infection (Magdalon et al., 2020). Hence, disruption of the complement system is likely to have a considerable impact on brain function. Complement proteins C1q, C4 and factor B, factor H and properdin have been shown to be at higher concentrations in the periphery in depression and bipolar disorder patients in comparison with controls (Yu et al., 2023), and numerous lines of evidence suggest that there are higher levels of complement activity in schizophrenia patients compared with controls (Woo et al., 2020). Thus, these results provide supportive evidence for the hypothesis that complement dysregulation may play a mediating role in the effects of altered LTCC signalling on aberrant cognition relevant to the transdiagnostic cognitive domain of neuropsychiatric disorders. Although *Ighm* and *C1qc* were only *Cacna1c* DEGs in the DG, as has been previously discussed, *A2m* was differentially expressed in both the DG and CA1. Thus, although the effects of *Cacna1c* heterozygosity on complement activity may differ between hippocampal subregions, there appears to be evidence for a common effect.

Another specific immune pathway implicated in the GSEA of *Cacna1c* DEGs in the DG is type II interferon (IFN-II), which was also implicated as the top term in the GSEA of the WGCNA analysis (albeit not at a statistically significant level; data not reported). The two DEGs driving the enrichment of IFN-II terms were *Parp14* and *Gbp2*, both of which showed increased expression in the DG in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. Interferons (IFNs) are classified as type I or type

II, and IFN-γ is the only IFN protein within the IFN-II subclass. IFN-γ is secreted by a variety of immune cells such as CD4+ T-helper lymphocytes, CD8+ cytotoxic lymphocytes, natural killer cells, natural killer T-cells and B cells (Schroder et al., 2004). *Parp14* encodes the poly(ADP-ribose) polymerase family member 14, which is known to control the protein ADP-ribosylation action of IFN-γ, which is an important component of anti-viral signalling cascades (Ribeiro et al., 2024). *Gbp2* encodes interferon-inducible guanylate binding protein (GBP2) and plays an important regulatory role in the innate immune response to microbial pathogens, as well as the inhibition of cell proliferation (Godoy et al., 2014). Although the majority of research on GBP2 focuses on cancer as opposed to psychiatry (Yu et al., 2020; Li et al., 2022), in schizophrenia patients, *GBP2* expression has been found to be upregulated in numerous case-control studies (Merikangas et al., 2022). Similarly, both common and rare variants of *PARP14* have been implicated in a range of neuropsychiatric conditions including schizophrenia, depression, ADHD and PTSD (de Jong et al., 2016; Ganesh et al., 2019). Furthermore, the GSEA analysis by de Jong et al. (2016) of the module of genes implicated in neuropsychiatric disorders that included *PARP14* was enriched for genes involved in IFN-II signalling. Hence, these data provide further evidence for downstream impacts of altered LTCC-mediated Ca^{2+} signalling on immune processes with known relevance to neuropsychiatric disease and may indicate that *CACNA1C* risk variants play a role in driving aberrant IFN-II related immune activity.

Several GO terms relating to the extracellular matrix (ECM) such as “extracellular matrix”, “collagen-containing extracellular matrix”, “extracellular region”, “extracellular space”, “extracellular vesicle” and “extracellular organelle” were among the most enriched terms within the *Cacna1c* DEGs in the DG, indicating that the impact of *Cacna1c* on downstream gene expression may also affect the brain ECM in both the DG as well as the CA1. Alongside presynaptic neurons, postsynaptic neurons, and glia, the ECM constitutes the ‘tetrapartite synapse’, a concept which reflects the importance of these four components in tandem for synaptic plasticity (Smith et al., 2015). The subgranular zone of the DG is one of two known sites of adult neurogenesis in the brain, and newly formed excitatory glutamatergic neurons

formed in this region go on to be integrated into the granule cell layer of the DG (Denoth-Lippuner and Jessberger, 2021). The highly plastic nature of adult generated hippocampal neurons makes them uniquely poised to be involved in hippocampal learning, memory and HPA regulatory functions (Levone et al., 2015). The ECM is thought to play an important role in adult hippocampal neurogenesis as signalling molecules secreted from the diffuse ECM may interact with cell surface receptors, and the ECM is able to form perineuronal net (PNN) structures which play an important part in synapse stabilisation (Cope and Gould, 2019).

The effects of *Cacna1c* heterozygosity on the complement and wider immune system, and ECM processes may not be independent of one another. Microglia are the brain's resident macrophage and play an important role in protection from pathogens and are also integral to shaping the synaptic architecture of the brain via their synaptic pruning activity. This synaptic pruning action of microglia is also known to be modulated by the complement system (Wang et al., 2019; Comer et al., 2020). As has previously been described, the ECM and glia, such as microglia, shape synaptic formation as part of the tetrapartite synapse. However, they appear to do so in conjunction with one another rather than exerting independent effects. Recent novel findings have suggested that microglia may play a role in shaping the ECM by engulfment of the ECM around synapses during synaptic remodelling (Nguyen et al., 2020; Westacott and Wilkinson, 2022). Thus, the resultant changes in complement, wider immune and ECM related genes arising from *Cacna1c* heterozygosity may function in tandem to deleteriously affect hippocampal neurogenesis and synaptic organisation within the hippocampus, with downstream consequences for hippocampal-dependent cognitive functioning.

The gene *M6pr* was the top ranked DEG in the CA1 and second ranked in DG and showed increased expression in *Cacna1c*$^{+/-}$ compared to *Cacna1c*$^{+/+}$ animals in both regions, indicating it may play a role in pathways downstream of *Cacna1c* that is not specific to hippocampal subregion. *M6pr* encodes the cation-dependent mannose-6-phosphate receptor (M6PR) which is a multifunctional protein with a major function in binding and transporting M6P enzymes to lysosomes (Gary-Bobo et al., 2007).

However, more recent evidence has shown M6PR plays an important role in the regulation of T-cell activity (Ara et al., 2018). The Consortium on the Genetics of Schizophrenia (COGS) group has previously observed an association between genetic variation at a locus spanning the *M6PR* gene and performance on the anti-saccade task (Greenwood et al., 2019), in which participants must exert voluntary and flexible control over eye movements away from a stimulus (an anti-saccade). Both human and non-human primates are more prone to error when making anti-saccades compared with saccades (eye movements towards a stimulus), and this is also a task in which schizophrenia patients are known to have poorer performance compared with controls (Munoz and Everling, 2004; Reuter et al., 2005; Subramaniam et al., 2018). This finding is of particular relevance to the deficits in LI in *Cacna1c* heterozygotes explored in this thesis, as perhaps aberrant signalling of M6PR pathways may contribute to dysfunctional attentional processes that may underlie this deficit.

Three other DEGs according to *Cacna1c* genotype were found to be common to both the DG and CA1. *F5* was ranked third in CA1 and 62nd in DG, *Rbm47* was ranked fourth in CA1 and 23rd in DG, and *A2m* was ranked fifth in CA1 and 70th in DG, and all were found to show increased expression in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. *F5* encodes the coagulation factor V protein, which is synthesised by hepatocytes and megakaryocytes (Duga et al., 2004). Disorders of coagulation have been reported in drug naïve schizophrenia patients, and a particular deficit in free protein S has been observed in patients compared with controls (Hoirisch-Clapauch et al., 2016), and evidence suggests that factor V and protein S work in synergy as cofactors to promote the anticoagulant activity of activated protein C (Dahlbäck, 1997). It has also been speculated that the dysregulation of both the complement and coagulation system may represent an integrated mechanism underlying deficits in neuropsychiatric disorders (Heurich et al., 2022). Thus, these results provide tentative evidence for aberrant LTCC-mediated Ca^{2+} signalling driving coagulation deficits that may be relevant to schizophrenia.

Rbm47 encodes the protein RNA binding motif 47 (RBM47). RNA binding motifs play prominent roles in post-transcriptional regulation of gene expression, and the majority of pathophysiological evidence for RBM47 has implicated the protein in cancer and defects in head morphogenesis. However, RBM47 also has a known role in the maintenance of immune homeostasis (Shivalingappa et al., 2021). *A2m* encodes the alpha 2-macroglobulin protein that is also known to play a key role in immune processes (Sun et al., 2023), and has been implicated in the altered immune profile observed in schizophrenia patients (Yee et al., 2017). Thus, these results suggest an impact of *Cacna1c* on immune functioning within the hippocampus across both the CA1 and DG.

Aside from the five DEGs that were common to CA1 and DG, six other genes were found to be differentially expressed between *Cacna1c* genotypes in the CA1 that were not found in DG: *Kl*, *Misp*, *Col8a1*, *Mfrp*, and *Slc4a5*. *Kl* encodes the protein Klotho which is involved in metabolic processes and has largely been studied in relation to ageing. However, Klotho is known to interact with other proteins such as FGF21 to affect HPA-axis functioning in the brain (Kuro-o, 2019). Klotho is thought to function in tandem with cortisol but with opposing direction of effects, and factors such as ageing and stress which increase cortisol, result in a decrease in Klotho (Luthra et al., 2022). The rodent analogue of cortisol, corticosterone, has been found to be increased in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals under baseline conditions (Moon et al., 2024). It is therefore surprising that *Kl* expression was found to be increased in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals in this experiment. Further work is required to replicate this finding and to ascertain the genotype difference in Klotho protein levels, and the mechanisms whereby it may interact with HPA-axis functioning in the *Cacna1c* heterozygote model.

Misp encodes the protein mitotic interactor and substrate of PLK1 (MISP), which is an important regulator of mitotic spindle positioning and orientation during mitosis (Zhu et al., 2013). Although this gene in particular has not been well studied in relation to neuropsychiatric disease, increased cell proliferation has been observed in schizophrenia patients compared with controls (Féron et al., 1999; Fan et al.,

2012). However, others have found a reduction in cell proliferation in schizophrenia patients compared with controls within the hippocampus (Allen et al., 2016). $Ca_v1.2$ and $Ca_v1.3$ have been shown to be localised to the mitotic apparatus during cell division and $Ca_v1.2$ and $Ca_v1.3$ blockade has been shown to inhibit cell proliferation (Loechner et al., 2009). The differential expression of *Col8a1*, which encodes type VIII collagen, may also be relevant to such processes as the brain ECM plays a critical role in the regulation of cell proliferation and organisation, and collagens are a crucial component of this structure. Hence, alterations in mitotic processes may result both directly, via reduced $Ca_v1.2$ expression, and indirectly, via aberrant Ca^{2+} induced transcription of genes such as *Misp* and *Col8a1*, as a result of *Cacna1c* heterozygosity.

7.4.2 The effects of sex on hippocampal gene expression.

Despite the initial hypothesis that the effects of *Cacna1c* heterozygosity may differ between sexes and context exposure groups, likelihood ratio tests comparing the linear model both with and without two-way interaction terms revealed that no genes were found to benefit from the inclusion of either the interaction between genotype and sex, genotype and context exposure group, or sex and context exposure group. The results of Chapter 5 suggest that IEG expression in the hippocampus may differ between sexes as a result of differing effects of context pre-exposure. Hence, it was anticipated that the effect of hippocampal activation induced by encoding of a novel environmental context may differ between sexes. As no genes were found to be differentially expressed according to the interaction between sex and context exposure condition, this may suggest that the effects seen in Chapter 5 are specific to the utilisation of contextual information in the formation and recall of contextual fear memories, as opposed to initial formation of contextual representations. Similarly, this may be the case for the relationship between sex and *Cacna1c* genotype. However, this study used a time point of 30 minutes after context exposure, which would likely encapsulate IEG expression, but may not reflect later

gene expression occurring downstream of IEGs in the hours following novel context exposure, which may be susceptible to sex differences.

No interactions were found between sex and *Cacna1c* genotype, indicating that at baseline, *Cacna1c* does not differentially impact hippocampal gene expression between sexes. However, it is possible that this may have occurred as a result of insufficient statistical power to detect interactions (Shieh, 2019). 27 and 46 genes were found to be differentially expressed according to sex in the DG and CA1 respectively. GSEA of the 27 DEGs according to sex in the DG revealed 121 enriched GO or HP terms within the gene list. Many terms reflected expected sex related processes such as “X-linked inheritance”, “Y-linked inheritance”, “germ plasm” and numerous terms related to genitalia development. However, several of the most enriched terms related to methylation processes, such as “protein demethylase activity”, “histone demethylase activity”, “demethylase activity”, “histone modifying activity”, and terms relating to demethylation of specific histones such as H3, H3K4, H3K4me/H3K4me2/H3K4me3 and H3K27me2/H3K27me3. Histone protein methylation is an epigenetic process with an important regulatory function of gene expression, and sex differences in methylation at the H3K4 and H3K27 sites in the hippocampus are in line with previous findings (Shen et al., 2015; Ocañas et al., 2022). H3K4 methylation has been shown to be upregulated following CFC (Gupta et al., 2010). Hence, sex differences in this process may result in differential effects of CFC according to sex on downstream gene expression. Furthermore, histone methylation has been shown to regulate *Cacna1c* expression (Baker et al., 2023). Thus, sex differences in histone protein methylation may result in differential effects of *Cacna1c* heterozygosity between males and females, particularly on processes such as CFC.

Many of the enriched GSEA terms within the DEGs according to sex in the CA1, as well as those enriched within the DEGs according to context exposure group in both the CA1 and DG related to DNA binding and transcription, such as “transcription cis-regulatory region binding”, “RNA polymerase II transcription regulatory region sequence-specific DNA binding”, “RNA polymerase II cis-regulatory region

sequence-specific DNA binding" and "transcription regulatory region nucleic acid binding". This is in line with previously reported findings of sex differences in the regulation of gene expression within the hippocampus (Ocañas et al., 2022), much of which is thought to be as a result of epigenetic mechanisms (Koss and Frick, 2017; Ratnu et al., 2017). Thus, as with the findings in the DG, the known roles of $Ca_v1.2$ mediated Ca^{2+} signalling in such processes may suggest that under circumstances such as associative contextual fear memory formation, the effects of *Cacna1c* heterozygosity on downstream gene expression and subsequent cognitive processing may differ between sexes.

7.4.3 The effects of context exposure on hippocampal gene expression

Based on the previously observed deleterious effects of *Cacna1c* heterozygosity on LI, it was anticipated that an interaction would be observed between the effects of genotype and context exposure condition, which was not found to be the case. However, as with the findings relating to sex, this may relate to the fact that *Cacna1c* heterozygosity is not exerting its effect during context encoding, but during the subsequent utilisation of contextual representations in the formation of associations such as contextual fear memories. Hence, future work ought to carry out RNA sequencing after contextual fear memory formation, extinction and recall, to fully characterise the possible interactions between *Cacna1c* genotype, sex and context exposure during these processes.

Forty-seven genes in the DG and 54 genes in the CA1 were found to be differentially expressed between context exposure groups, most of which were common to both hippocampal regions. Several such genes were IEGs, such as *Fos*, *Arc*, *Fosb*, *Junb*, *Erg1* and *Erg2*, all of which were found to be increased in rats exposed to the novel context. This is in line with expectations, as IEGs are known to play an important role in the hippocampal encoding of context (Kim and Cho, 2020; Pettit et al., 2022), as has previously been discussed in Chapter 5. GSEA revealed over 300 terms

enriched within the gene lists for DG and CA1 DEGs between context exposure groups, which also showed substantial overlap. This large number of terms likely reflects the inclusion of such IEGs that have known involvement in a broad range of downstream processes and thus are tagged with a high number of terms. Many of the GSEA terms related to gene transcription and protein translation, as well as metabolic processing. This is also consistent with IEG activity increasing downstream transcription and protein production, and this increased cellular activity is known to be facilitated by heightened glucose metabolism (Lundgaard et al., 2015).

7.4.4 The association between hippocampal differential gene expression and gene association with schizophrenia.

Gene set analysis conducted using MAGMA showed that in the DG, there was a small but significant negative association between -$\log_{10}$ *p*-values of differential gene expression between *Cacna1c* genotypes, and Z-score of PGC3 schizophrenia GWAS significance of each gene. However, no association was observed in the CA1. In a GWAS, a significant correction for multiple comparison is imposed to control for type-I error, as like RNA-sequencing, GWAS are exploratory and genome-wide. Thus, to overcome this correction, those variants that achieve genome-wide significance are likely to be those that are the most deleterious. Biological systems are highly complex and contain numerous genes and proteins with complex and dynamic interactions. Upstream genes such as *CACNA1C* will have a broad affect and influence a large array of downstream proteins. As proteins with a wide net of influence on downstream genes and proteins are more likely to be deleterious, they will subsequently be more likely to be GWAS hits. However, there is a single large genetic mutation in the *Cacna1c* heterozygous model, that will influence specific downstream pathways that may have relevance to neuropsychiatric disorders, but in themselves may not achieve genome wide-significance in GWAS. Thus, the negative association observed may result from a difference in the types of genes that result

from each analysis. Biological systems are not so simplistic, with unidirectional upstream and downstream genes and proteins, and there is a considerable level of bidirectional influence. However, if there is a bias towards directionality, this may result in the small but significant negative association between genes most likely to arise in the schizophrenia GWAS and those that show differential expression in the *Cacna1c* heterozygous deletion model. It is likely that an association was not seen in the CA1 as the model appears to have a smaller influence in this region compared to the DG. This does suggest, however, that *Cacna1c* heterozygosity does not appear to be functioning in a multiplicative way by affecting genes that in themselves are also likely to be GWAS significant independent of *CACNA1C*. However, limited manipulations were conducted in this experiment, hence these results may differ after more explicit learning events.

7.4.5 Conclusion

Taken together, these results suggest that *Cacna1c* heterozygosity results in altered downstream gene expression within both the DG and CA1, with subtle differences in effects between the two hippocampal subregions analysed in this study. Analysis of the DEGs in each region suggest that a variety of pathways may be implicated involving aspects of the immune system such as complement and IFN-II signalling, as well as functioning of the ECM, that may have deleterious consequences for synaptic architecture and plasticity processes, upon which hippocampal forms of associative learning and memory depend. Furthermore, it is highly plausible that these effects do not occur independently of one another and may constitute an integrated mechanism of hippocampal dysfunction. The nature of this study is highly exploratory, hence future work is required to probe the specific mechanisms whereby *Cacna1c* heterozygosity results in such deficits, and specifically how this affects the formation of contextual fear memories. Although no interactions were observed between *Cacna1c* genotype, sex and context exposure, the main effects of sex and context exposure on hippocampal gene expression implicate genes and associated

processes that under circumstances such as contextual fear memory formation and recall, may be susceptible to effects of aberrant $Ca_v1.2$ mediated Ca^{2+} signalling. Thus, further work assessing *Cacna1c* heterozygosity on gene expression within the hippocampus that assesses differential effects between specific cell types and populations, as well as in response to CFC, will be highly informative in understanding the underlying neurobiological mechanisms whereby *CACNA1C* risk variants affect hippocampal dependent cognitive processes relevant to neuropsychiatric disease, and how this may differ between sexes.

Chapter 8: General Discussion

8.1 Summary of Findings

Previous data from the lab group has shown increased fear memory generalisation (unpublished data) and deficits in LI (Tigaret et al., 2021) in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals. However, these experiments were only conducted in males. Thus, this thesis aimed to explore whether the effects of *Cacna1c* heterozygosity on such hippocampal dependent associative fear learning processes differed between sexes. Recent findings of the lab group have also shown altered HPA-axis responsivity including increased peripheral plasma corticosterone in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ rats (Moon et al., 2024). Hence, this thesis also aimed to assess whether deficits in associative fear learning would be accompanied by increased plasma corticosterone. Owing to limited existing evidence regarding the effects of sex on LI of CFC, a further aim was to establish whether sex differences would occur in the relationship between context PE duration and subsequent CFC in wild-type animals, as well as to assess accompanying IEG expression in a range of brain regions related to LI. To further examine these effects, an additional aim was to assess the effect of both sex and *Cacna1c* heterozygosity on gene expression in the DG and CA1 regions of the hippocampus.

The effect of *Cacna1c* heterozygosity and sex on hippocampal dependent associative fear learning:

- *Cacna1c*$^{+/-}$ animals did not generalize contextual fear memories to a neutral context when the recall sessions for each context were 24 hours apart.
- However, reanalysis of existing data in the research group suggests they may generalise to a neutral context if they have recently experienced a fear memory recall (same day).

- Previously, male *Cacna1c*$^{+/-}$ rats showed reduced LI compared with male *Cacna1c*$^{+/+}$ rats when a single 4-hour PE session was used (Tigaret et al., 2021). Here, a deficit was seen in both sexes in *Cacna1c*$^{+/-}$ relative to *Cacna1c*$^{+/+}$ rats when three sessions of 20-minutes of PE over three days was used.

The impact of *Cacna1c* heterozygosity and sex on plasma corticosterone concentration associated with hippocampal dependent associative fear learning:

- In the CFC generalisation experiment (Chapter 3), *Cacna1c* genotype was not found to influence plasma corticosterone levels associated with memory recall in either the CFC or neutral context.
- In the LI of CFC experiment (Chapter 4), sex differences were seen in the effect of *Cacna1c* genotype on plasma corticosterone concentration. Female *Cacna1c*$^{+/+}$ rats showed increased corticosterone concentration in the no-PE compared with PE group, but no differences were seen in female *Cacna1c*$^{+/-}$ rats. However, male *Cacna1c*$^{+/-}$ rats showed increased corticosterone concentration in the no-PE compared with PE group, but no differences were seen in male *Cacna1c*$^{+/+}$ rats.

The existing literature relating to sex differences in LI:

- Most studies investigating LI in rodents that were conducted in both sexes assessed the LI of cued rather than contextual conditioning, and the majority did not show sex differences.
- However, some sex differences were seen in the effects of administration of substances such as 17ß-oestradiol (Arad and Weiner, 2010b), polyI:C (Piontkewitz et al., 2011) and methamphetamine (Wang et al., 2012) among others, as well as experiences such as juvenile handling (Weiner et al., 1985, 1987; Shalev et al., 1998) on LI.

- Most studies in humans relating to schizophrenia or schizotypy comparing LI between sexes did not show sex differences.
- Few studies in the existing literature have examined LI of contextual rather than cued conditioning at either a behavioural or neurobiological level.

Sex differences in the relationship between context PE duration and subsequent CFC:

- In wild-type animals, males show the expected pattern of reduced fear conditioning with greater durations of context PE. However, in females, the relationship is not as clear, with the highest level of behavioural fear expression in the intermediate PE duration compared with high and low PE duration groups.
- Behavioural sex differences in the relationship between PE duration and fear conditioning were reflected in IEG expression in the hippocampus and prelimbic mPFC, particularly in females.
- Females in the intermediate PE group showed higher *Cfos* expression in the CA1 and DG of the hippocampus and PL mPFC compared with high and low PE groups. The same pattern was also observed in *Arc* expression in the PL mPFC. The inverse pattern of higher expression in the low and high PE groups compared to the intermediate groups in *Bdnf-IX* expression in the CA1 in females was seen.
- Differences were only seen in males in higher *Cfos* expression in the DG in the low PE compared with high PE group.

The effect of sex and *Cacna1c* heterozygosity on hippocampal gene expression:

- *Cacna1c* gene expression is differentially affected between hippocampal subregions in the *Cacna1c* heterozygous deletion model, with a highly significant reduction of *Cacna1c* gene expression in the DG, but not in the CA1.

- *Cacna1c* heterozygosity leads to increased expression of genes associated with processes including neuroimmune functions and the brain ECM, with a greater impact in the DG compared with CA1.
- The effects of *Cacna1c* heterozygosity on hippocampal gene expression did not differ between sexes or whether rats were exposed to a novel context. However, both sex and context exposure were associated with changes in gene expression in genes that were associated with functions such as epigenetic processing, transcription and translation.
- In the DG, significance of differential gene expression between *Cacna1c* genotypes was negatively associated with the degree of involvement in schizophrenia, as implicated by the PGC3 schizophrenia GWAS.

8.2 The impact of *Cacna1c* heterozygosity on hippocampal dependent cognition

One of the primary aims of this thesis was to investigate the impact of *Cacna1c* heterozygosity on hippocampal dependent associative fear learning, and the cognitive processes of contextual fear memory generalization and the LI of CFC were specifically assessed. LI was found to be impaired in *Cacna1c*$^{+/-}$ compared with *Cacna1c*$^{+/+}$ animals of both sexes, a finding which had only previously been assessed in males. However, contextual fear memory generalization was not found to differ between *Cacna1c* genotypes. Similarly, the degree of contextual fear memory learning when measured immediately post-US or at later recall did not differ between *Cacna1c* genotypes. Rather, reanalysis of the original evidence from the research group which suggested increased generalization of contextual fear memories in *Cacna1c*$^{+/-}$ relative to *Cacna1c*$^{+/+}$ rats showed that this was specific to animals who had previously undergone a recall session in the fear conditioning context earlier the same day.

Both LI and contextual fear memory generalisation tasks involve the formation of context-US and context-no US associations. In contextual fear memory generalisation, the context-no US association is formed in the context that is distinct from the CFC environment, whereas in LI it is the same context in which the animal undergoes CFC, but the animal experiences and learns about this context in a distinct PE training session prior to CFC. Taken together, these findings suggest that heterozygosity does not appear to result in a broad and profound deficit in hippocampal dependent contextual associative fear learning. Rather, *Cacna1c* heterozygosity impacts the competition between context-no US and context-US associations to affect the fear response. Specifically, in contextual fear memory generalization, the recently recalled context-US association in a similar context interferes with the recall of the context-no US memory that was previously learned in the neutral context. The finding of intact fear memory specificity from Chapter 3 indicates that the deficit in *Cacna1c*$^{+/-}$ animals is specific to this interference effect rather than broader heightened generalisation that would always result in an increased fear response to neutral but similar contexts to the CFC context. In LI, when animals are returned to a context in which they have undergone CFC but were also pre-exposed, there is competition between the previously learned context-no US association acquired during PE and the context-US association acquired during CFC. The fact that gene expression induced by context exposure was not found to differ between *Cacna1c* genotypes in the RNA-sequencing analysis also suggests that *Cacna1c* heterozygosity does not result in a deficit relating to the initial learning of contextual information but may instead affect how this information is later reconciled with the context-US association either during contextual fear memory formation or recall.

8.3 The impact of sex on hippocampal dependent cognition

The first experiment of Chapter 4 indicated that the threshold for LI may differ between males and females as 2x10-minutes of PE over two days was insufficient to

induce LI in wild-type males, but did induce LI in wild-type females. Subsequently, a literature review (Chapter 5) was conducted to assess whether this finding was validated by the existent literature. The majority of studies found that assessed LI in both sexes did not observe differences between males and females (e.g., Caldarone et al., 2000; Maes, 2002; Mizuno et al., 2006; Vorhees et al., 2015). However, it was established that scant work had assessed whether sex differences occurred in the LI of CFC, as the majority of studies assessed LI of cued conditioning, and most also did not conduct analysis in animals of both sexes.

In Chapter 6, possible sex differences in the LI of CFC were investigated in more detail by using three different durations of PE to see how the relationship between context PE duration and subsequent CFC differed between sexes. In males, the expected relationship was observed, that longer durations of PE resulted in reduced fear expression at subsequent recall, and fear memories were also more resistant to extinction in animals with the shortest PE duration. However, in females, it was the intermediate duration of 3x20m that had the greatest fear response. Thus, the expected relationship of greater context PE and lower CFC (Miller et al., 2022) was not observed in females. As previously discussed, oestrous state of the females in this experiment was not ascertained. Hence, it may be the case that the intermediate PE duration group were overrepresented by a specific oestrous state, driving the increased fear response. Nevertheless, these findings support the hypothesis of sex differences in the relationship between PE duration and subsequent fear conditioning. Thus, the use of previously learned information relating to the neutrality or safety of a context when later conflicting information arises during CFC does not appear to function identically between males and females. The majority of the literature relating to the LI of cued conditioning did not support sex differences. Thus, the sex differences observed here indicate that the hippocampal processes upon which LI of CFC depends may be sexually dimorphic.

Several effects of sex were also observed in the experiments conducted in the *Cacna1c* heterozygote deletion model. In both Chapters 3 and 4, males were found to freeze more and have lower locomotor activity compared to females. Furthermore,

in Chapter 4, greater activity was observed in the PE compared with non-PE group in female animals, which was not found in males. This is suggestive of female animals responding with more cautious movement when they had not been pre-exposed to the conditioning context compared with females that had been pre-exposed, indicating greater fear. However, this is not necessarily suggestive of altered associative fear learning between sexes, rather that differing behavioural strategies are employed between sexes in response to fear (Bauer, 2023), as has previously been discussed.

8.4 Does the impact of *Cacna1c* heterozygosity on hippocampal dependent cognition differ between sexes?

As previously discussed, psychotic and mood disorders such as schizophrenia, bipolar disorder and depression bear sex differences in several aspects such as age of onset, symptom profile and responsivity to treatment. Hence, it was hypothesised that sex differences would occur in the impact of risk genes such as *CACNA1C*, and thus anticipated that sex differences would be observed in the *Cacna1c* heterozygous deletion model. The findings of this thesis were limited in their support for this hypothesis. Contextual fear memory specificity was found to be intact in *Cacna1c*$^{+/-}$ animals of both sexes, and the LI deficit that has previously been observed in *Cacna1c*$^{+/-}$ males (Tigaret et al., 2021) was observed in *Cacna1c*$^{+/-}$ females in this thesis. Furthermore, the impact of *Cacna1c* heterozygosity on gene transcription in either the DG or CA1 was not found to interact with sex. Thus, in broad terms, the impact of low *Cacna1c* gene dosage appears to be similar between sexes.

However, several subtle interactions between sex and *Cacna1c* genotype were observed. Although a deficit was not observed in contextual fear memory specificity in *Cacna1c*$^{+/-}$ animals, female *Cacna1c*$^{+/-}$ animals were found to freeze more than female *Cacna1c*$^{+/+}$ animals, with this genotype effect not observed in males. As

previously discussed, the greater propensity for freezing in males compared with females is thought to reflect alternative behavioural strategies in response to fear (Bauer, 2023). Thus, *Cacna1c* heterozygosity may either increase the fear response to CFC specifically in females, or alternatively alter their behavioural response to be more in line with males. This finding was not observed in the LI experiment of Chapter 4. Hence, further work is required to ascertain its validity.

In the *Cacna1c* LI experiment of Chapter 4, the effect of *Cacna1c* genotype on plasma corticosterone concentration in response to either pre-exposed or non-pre-exposed animals was found to differ between sexes. Specifically, *Cacna1c*$^{+/-}$ females showed higher corticosterone concentration in the no-PE group compared with the PE group, whereas *Cacna1c*$^{+/-}$ males showed the opposite pattern of higher corticosterone concentration in the PE compared with non-PE group. No differences in corticosterone concentration between PE groups were seen in *Cacna1c*$^{+/+}$ animals of either sex. This finding suggests that the impact of *Cacna1c* genotype on the HPA-axis response to contextual fear learning when reconciling context-no US and context-US associations differs between sexes. As with the interaction between sex and *Cacna1c* genotype in freezing behaviour discussed above, this finding was not observed in any other experiment, and thus further work must be carried out to validate this result. However, these differences in the effect of *Cacna1c* heterozygosity between sexes suggest that LTCC-dependent Ca^{2+} signalling may interact with other systems involved in task-specific aspects of hippocampal dependent cognition which may be sexually dimorphic.

8.5 Implications for psychotic and mood disorders

Risk variants of *CACNA1C* have been robustly associated with schizophrenia, bipolar disorder and depression – disorders with known sex differences in aspects such as age of onset, symptom profile and prevalence (Viguera et al., 2000; Diflorio and Jones, 2010; Falkenburg and Tracy, 2014; Kiliçaslan et al., 2014; Fernandez-

Pujals et al., 2015; Crawford and DeLisi, 2016; Menculini et al., 2022). Cognitive dysfunction is a hallmark of both psychotic and mood disorders (Marazziti et al., 2010; Lima et al., 2018; Heinz et al., 2019), and LTCCs are known to be involved in the cellular and molecular basis of cognitive functioning, via their involvement in synaptic transmission and structural plasticity processes such as LTP (Sridharan et al., 2020; Navakkode et al., 2022). The hippocampus is also a site of known importance for learning and memory processes that have been implicated in neuropsychiatric disease (Chepenik et al., 2012; Allen et al., 2016; Lucassen et al., 2016; Wegrzyn et al., 2022). Thus, it was thought that *CACNA1C* risk SNPs may play a role in the transdiagnostic dimension of cognitive dysfunction, and improved understanding of the mechanisms whereby altered *CACNA1C* gene dosage lead to deficits in hippocampal dependent associative learning may lead to the identification of pharmacological treatment targets for neuropsychiatric disease. Subsequently, the purpose of this thesis was to assess the impact of low *Cacna1c* gene dosage on hippocampal dependent associative fear learning, and to assess how this may differ between sexes.

The behavioural findings of this thesis support the idea that reduced *CACNA1C* expression may lead to subtle deficits in contextual associative fear learning as demonstrated by numerous findings of LI impairments in schizophrenia and schizotypy, albeit with heterogeneous findings (Myles et al., 2023). This is in line with our understanding of *CACNA1C* as a common risk locus that does not definitively confer disease. Such cognitive deficits may not prove pathological in all or even most individuals, reflected by the low odds ratios of *CACNA1C* risk SNPs. Conversely, in the polygenic model of neuropsychiatric disease, SNPs at such loci have a cumulative effect and interact with environmental risk factors such as early life adversity (Lipner et al., 2022; Yao et al., 2023; Uy and Gotlib, 2024) and cannabis use (Godin and Shehata, 2022; Jefsen et al., 2023) through both additive and epigenetic mechanisms (Richetto and Meyer, 2021). Furthermore, whether pathology will manifest as one specific diagnosis or another is also influenced by such factors, and the biological relevance of such discrete diagnoses is still debated (Cuthbert and Morris, 2021). However, these findings do suggest that the biological

pathways that mediate the effects of *CACNA1C* risk SNPs on neuropsychiatric disease may relate to the influence of neuroimmune and ECM processes on the brain's synaptic architecture.

The findings of sex differences in the LI of CFC as well as associated IEG expression in wild-type animals suggest that in healthy individuals, the mechanisms facilitating the use of previously learned information regarding the salience of the environment in a subsequent fear response differ between sexes. However, the findings of this thesis are limited in explaining the causal mechanisms of this difference. There was limited evidence for broad differences in the effect of *Cacna1c* heterozygosity on hippocampal dependent cognition between sexes. However, it is plausible that basic sex differences in the mechanisms that mediate the integration of previously learned information regarding the salience of context during subsequent contextual fear learning may lead to a differential impact of neuropsychiatric risk genes with known involvement in such processes, such as *CACNA1C*.

8.6 Limitations

The data produced in this thesis constitute several novel and informative findings regarding the impact of sex and *Cacna1c* heterozygosity on hippocampal functioning. However, several limitations must be considered when interpreting these findings.

No animal model can attempt to fully encapsulate complex and heterogenous neuropsychiatric conditions such as schizophrenia, bipolar disorder, and depression, and should not aim to do so. Thus, the aim of the *Cacna1c* heterozygous deletion model is to specifically model the mechanism of *CACNA1C* risk variants altering dosage of the gene. However, as discussed in Chapter 1, the evidence regarding the impact of *CACNA1C* risk variants on *CACNA1C* expression is mixed regarding

direction of effect, and there is evidence to suggest that the impact may not be uniform across brain regions or risk variants. Indeed, it was found in Chapter 7 that even within the hippocampus, the degree of mutation penetration is not uniform with reduced expression in *Cacna1c*$^{+/-}$ relative to *Cacna1c*$^{+/+}$ rats in the DG, but not CA1. Thus, how well this diversity within the rodent model matches that of humans is not known. Furthermore, recent evidence suggests that the transcriptomic profile of *CACNA1C* is highly complex within the brain (Hall et al., 2021), which may mean that specific transcripts are differentially affected by *CACNA1C* genotype. Thus far, no analysis of the transcriptomic diversity of the *Cacna1c* heterozygous deletion model has been conducted. Hence, the degree to which the human transcriptomics of *CACNA1C* are modelled in this rodent line are not known. Neuropsychiatric disorders are also highly polygenic, with numerous genes exerting deleterious effects that in combination lead to psychopathology (Ruderfer et al., 2014; Neale and Sklar, 2015; Bigdeli et al., 2022), whereas in this rodent model, no other genetic modifications are present. Thus, how *CACNA1C* interacts with other risk variants also ought to be modelled in future studies.

Behavioural experiments were conducted to assess the impact of both sex and *Cacna1c* heterozygosity on hippocampal dependent associative fear learning and associated biological processes. Freezing was used as the primary behavioural measure of fear alongside measurement of quadrant crossings which was used to infer changes in locomotor activity. Both measures have known sex differences, with freezing thought to be more readily used as a fear response in males, and females generally being more active than males (Graham et al., 2009; Bauer, 2023). Thus, attempts to infer sex differences in cognitive processes will likely be clouded by analysing proxy measures that in themselves differ between sexes.

Peripheral plasma corticosterone concentration was used as a measure of HPA-axis activity in Chapters 3 and 4. However, this is a very general measure and does not encapsulate the full scope of the HPA-axis. The hippocampus plays an important negative-feedback role in the homeostatic regulation of the HPA-axis, with corticosterone binding to GRs and MRs within the hippocampus to trigger the

suppression of further downstream corticosterone release (Heck and Handa, 2019). Hence, the changes in the dynamic expression of these receptors in brain regions of interest such as the hippocampus and mPFC would have provided useful supplementary information regarding HPA-axis changes associated with cognitive function. Similarly, other hormones involved in the HPA-axis response such as corticotropin-releasing hormone and adrenocorticotropic hormone could have been measured.

In Chapter 6, RNA expression was measured in specific IEGs in the hippocampus, as well as a wider network of brain regions involved in LI and in Chapter 7, RNA expression of the full transcriptome was assessed. Both studies aimed to infer how *Cacna1c* heterozygosity and sex impacted hippocampal gene expression. The method used to extract each brain region was to take coronal slices of the brain, then use a tissue punch to take 1.5mm diameter punches of areas that mapped to the region of interest in a rat brain atlas. The regions chosen were subregions of larger areas such as the DG and CA1 of the hippocampus and PL and IL of the mPFC, and as such were very small. The brain atlas mapping method is relatively crude as not all landmarks seen in the atlas are visible in unstained brain slices. Hence, it can only be said that the brain regions are enriched for the region of interest, rather than that they were that region in isolation. Furthermore, as RNA was extracted from bulk tissue in all experiments, there is no cell-type specificity of the results, such as any differentiation between neuronal subtypes, and neurons and glia. Thus, it is not possible to attribute downstream functions to specific cell types, and important differences in gene expression between variables may be missed if they are cell-type specific and diluted by other non-affected cell types (Shen-Orr et al., 2010).

When tissue was extracted from the DG and CA1, this was only taken from dorsal rather than ventral sections of the hippocampus. This decision was made as the dorsal hippocampus is known to have greater involvement in memory and spatial aspects of cognition (Fanselow and Dong, 2010), which was thought to have greater relevance to the LI and novel context exposure manipulations used in Chapter 6 and

7 respectively. However, the ventral hippocampus has been extensively implicated in emotional processing and stress (Fanselow and Dong, 2010). As one of the aims of this thesis was to assess the impact of *Cacna1c* heterozygosity on HPA-axis processing, additional analysis of gene expression changes in the ventral hippocampus would have furthered this aim. Furthermore, while tissue was extracted from DG and CA1 at the same time, owing to logistical constraints, library preparation and sequencing of the DG and CA1 RNA was conducted separately. Thus, it would not be possible to directly statistically compare the gene expression between DG and CA1 regions as differences may be confounded by factors related to the experimental set up. Subsequently, this limits the ability to interpret the differences seen in gene expression between *Cacna1c* genotypes in expression between each hippocampal region.

The findings relating to gene expression changes could also be extended by measuring changes at later time points than 30-minutes after fear memory recall (Chapter 6) or novel context exposure (Chapter 7). The 30-minute time point was relevant to capture changes in IEG expression. Such IEGs have pleiotropic effects and downstream long-term memory processes depend upon their activation (Zheng et al., 2009; Gallo et al., 2018). Hence, they provide valuable information regarding activity and regional involvement. However, downstream gene expression changes occur over hours and days after learning events (Cavallaro et al., 2002), and identifying and quantifying such changes would provide further useful information as to the downstream impact of *Cacna1c* heterozygosity. Structural synaptic plasticity events relating to learning and memory are known to occur over a longer time scale including consolidation processes occurring during sleep (Pronier et al., 2023). Thus, longer time scales of analysis would elucidate how such processes differ between sexes and *Cacna1c* genotypes. Furthermore, longer time scales would allow for analysis of changes in protein concentration which would provide important complementary information to the findings in RNA. Not all RNA is translated into protein, and numerous post-transcriptional modifications of RNA influence its subsequent fate (Nachtergaele and He, 2017).

One of the primary aims of this thesis was to assess the impact of sex on hippocampal dependent associative fear memory. Several informative sex differences were observed in both the *Cacna1c* heterozygous deletion model as well as in wild type animals. However, owing to logistical constraints related to training in the required techniques, none of the animals were assessed for oestrous phase. LI has been shown to differ according to oestrous phase and gonadal hormones are known to affect gene transcription in the hippocampus (Gegenhuber et al., 2022). Hence, this information would have been useful in determining whether sex differences were attributable to activational effects of gonadal hormones as opposed to organisational effects or effects of sex chromosomes.

The final experimental chapter of this thesis used the most recent PGC schizophrenia GWAS to establish whether the degree of association with schizophrenia for each gene was associated with the significance of differential gene expression between genotypes of the *Cacna1c* heterozygous deletion model. There is a long-standing issue within the GWAS literature of predominating European samples, and 95% of all GWAS samples are thought to be of European ancestry (Troubat et al., 2024). The PGC3 schizophrenia GWAS has a greater degree of genetic diversity. However, 80% of the samples were still of European ancestry. Owing to differing LD structures between ancestries, LD reference samples that are representative of the ancestries in the GWAS must be used in MAGMA. Thus, as this represented the largest component of the GWAS, only the European data from the GWAS and the European 1000 Genomes reference sample were used in the analysis. Thus, it is a limitation of this analysis that the findings are not necessarily generalisable to other ancestries. Furthermore, the pertinence of *CACNA1C* to the genetic architecture of schizophrenia also comes from this majority European GWAS. When multi-ancestry GWAS have been performed in haematological traits, numerous novel SNPs have been revealed compared with European-only samples (Troubat et al., 2024). However, there is evidence to suggest that the genomic architecture of schizophrenia is similar between ancestries, but this has only compared East Asian and European populations (Lam et al., 2019).

8.7 Suggestions for future work

The experiments assessing the sex differences in the relationship between context PE duration and subsequent CFC were conducted as an initial probe into the hypothesis that there may be sex differences in LI. As previously discussed in the limitations, oestrous phase of female animals was not ascertained. Thus, future work ought to disentangle the contribution of gonadal hormones both at an activational and organisational level, as well as the contribution of sex chromosomal influences on associative fear learning. Animal models such as the 'four core genotypes' model in which XX and XY rats with gonads of either sex have been developed (Arnold et al., 2023), and would be valuable in assessing the impact of different aspects of sexually dimorphic biology. Furthermore, additional behavioural experiments ought to be conducted to determine the specific circumstances in which sex differences are seen in the LI of CFC, which may help to elucidate how competing context-no US and context-US associations are resolved differentially between sexes. This may then help to improve understanding of how aberrant salience processes that may underpin the formation of psychotic phenomena differ between sexes.

Much of the analyses conducted in this thesis, particularly in the last two experimental chapters, was highly exploratory in nature. Consequently, informative and novel findings were produced, but with significant room for future work to expand upon these results and to test more specific hypotheses. The RNA-sequencing analysis produced a large volume of data, upon which further analysis could still be conducted. Although bulk tissue was used, post-hoc cell-type deconvolution methods ought to be employed to determine the cell-type specificity of the differential gene expression (Chu et al., 2022; Fan et al., 2022; Cobos et al., 2023). This may also reveal new DEGs as weaker cell-type specific signals may currently be diluted in bulk analysis.

The only behavioural manipulation conducted in the RNA-sequencing experiment was exposure to a novel context. This manipulation was used in the first instance to assess whether *Cacna1c* affected gene expression changes in the hippocampus

induced by basic learning of a new spatial environment, which may have helped to reveal whether deficits in processes such as LI in *Cacna1c*$^{+/-}$ rats are as a result of differences in the encoding of context. As context exposure was not found to interact with CFC, future work ought to assess how hippocampal gene expression may differ between *Cacna1c* genotypes after CFC. This may provide richer information in addressing the hypothesis that cognitive deficits arise as a result of the integration of previous experiences of context in fear learning.

The cellular signalling cascade that facilitates LTCC-induced E-TC from stimulation at the synapse to gene transcription at the nucleus involves the CREB, CaMKII and ERK/MAPK pathways (Ma et al., 2023). ERK and CREB synapse-to-nucleus signalling have been shown to be disrupted in *Cacna1c*$^{+/-}$ rats of both sexes (Tigaret et al., 2021). ERK signalling has also been shown to be responsive to HPA-axis activity in the hippocampus (Ferland et al., 2014). Thus, owing to the results of this thesis demonstrating effects of *Cacna1c* heterozygosity on HPA-axis activity, future work could specifically assess whether dysfunction in ERK signalling in *Cacna1c*$^{+/-}$ animals occurs as a result of changes in HPA-axis activity. Currently, the profile of HPA-axis activity in response to contextual fear learning in the *Cacna1c* heterozygous deletion model has not been fully characterised. Thus, using a greater range of measures such as MR and GR expression as well as the dynamic expression of corticosterone during contextual fear learning may elucidate this further.

The RNA-sequencing findings implicated genes related to the ECM and immune related processes in the differential gene expression observed in the hippocampus. Consequently, future work ought to use imaging techniques to assess how ECM structure may differ between *Cacna1c* genotypes. Furthermore, as previously discussed, the ECM is known to interact with glia, including microglia, to influence synaptic architecture in the brain (Nguyen et al., 2020). A number of immune processes were implicated in the GSEA, including complement and IFN-II signalling, both of which are known to interact with microglia to shape synaptic structure (Li et al., 2020; Wang et al., 2020). Thus, further imaging work ought to be conducted to

assess differences in microglial activity between *Cacna1c* genotypes, and how this interacts with brain ECM structure.

8.8 Conclusions

Both *Cacna1c* heterozygosity and sex show effects on hippocampal dependent associative fear learning by affecting the integration of previously learned information in contextual fear processing. *Cacna1c*$^{+/-}$ animals also show increased gene transcription in genes with functions related to neuroimmune processing and the brain ECM, and sex differences were seen in genes with functions related to epigenetic histone protein modifications, among others. *Cacna1c* heterozygosity also appears to affect males and females similarly. However, specific sex differences in the impact of low *Cacna1c* gene dosage were observed in both behavioural and HPA-axis response measures. These findings suggest that *CACNA1C* risk variants may confer increased risk for psychotic and mood disorders by affecting contextual associative fear learning processes mediated by the hippocampus, possibly via affecting neuroimmune, brain ECM and HPA-axis processes.

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