



The Role of Schizophrenia Susceptibility Genes in Associative Learning

A thesis presented for the degree of
Doctor of Philosophy

By
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Declarations

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

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Acknowledgements pertaining to the collection and contribution of patient genetic data are found in the Appendix.

Abstract

Schizophrenia is highly heritable, indicating that a large proportion of one's susceptibility to developing the disorder is attributable to genetics. Recent large-scale genomic studies have revealed that genetic variants in patients with schizophrenia affect genes involved in synaptic plasticity processes, which are required for learning and memory, including genes encoding protein complexes associated with the NMDA receptor and the postsynaptic density. Further evidence suggests that associative learning may be particularly affected, although it is unclear which components of this cognitive process are implicated in schizophrenia.

The present studies investigated the relationship between particular phases of associative learning, represented by the consolidation, retrieval and extinction of contextual fear memory in rats, with genetic variants, psychoactive drugs and postsynaptic density proteins associated with schizophrenia. I tested associative learning-related gene expression datasets for enrichment in genetic copy number variants from a large cohort of patients with schizophrenia and demonstrated that only genes associated with extinction learning are enriched in patient variants (Chapter 3). I report that fear extinction in rats was impaired by administration of the NMDA antagonist and psychotomimetic, ketamine (Chapter 4). The expression of activity-induced, postsynaptic density products of the *Homer1* gene, which has been linked to psychiatric disease, was differentially regulated in specific hippocampal subregions following extinction learning (Chapter 5), and the effect of a partial knockdown of these genes during different phases of associative learning was investigated (Chapter 6).

These results build on clinical studies linking abnormalities in associative and, specifically, extinction learning with schizophrenia and support the notion that genetic variants associated with the disorder impact particular cognitive domains. My findings are consistent with the theory that altered inhibitory-type learning processes contribute to the manifestation of schizophrenia.

Abbreviations

μl	Microliters
μm	Micrometres
ADHD	Attention deficit hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ARC	Activity-regulated cytoskeletal-associated protein
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
CA	<i>Cornu ammonis</i>
CFC	Contextual fear conditioning
CLOZUK	UK study of schizophrenic patients taking clozapine
cm	Centimetres
CNV	Copy number variant
CO ₂	Carbon dioxide
CPFE	Context pre-exposure facilitation effect
CS	Conditioning stimulus
dATP	Deoxyadenosine triphosphate
DEPC	Diethylpyrocarbonate
DG	Dentate gyrus
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ERK	Extracellular signal-related kinase
FMRP	Fragile X mental retardation protein
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
h	Hours
ID	Intellectual disability
IEG	Immediate early gene

IP	Intraperitoneal
IS	Immediate shock
ISC	International Schizophrenia Consortium
mA	Milliamps
MAGMA	Multi-marker Analysis of GenoMic Annotation
MAS5	Microarray Suite 5
MEK	Mitogen activated protein kinase kinase
MGI	Mouse Genome Informatics
mGluR	Metabotropic glutamate receptor
MGS	Molecular Genetics of Schizophrenia
MHC	Major histocompatibility complex
min	Minutes
mm	Millimetres
mRNA	Messenger ribonucleic acid
NCBI	National Centre for Biotechnology Information
NMDA	N-methyl-D-aspartate
nmol	Nanomoles
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCP	Phencyclidine
PSD	Postsynaptic density
RIPA	Radioimmunoprecipitation assay
RMA	Robust multi-array average
RNA	Ribonucleic acid
RyR	Ryanodine receptor
sec	Seconds
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
SSC	Saline sodium citrate buffer
TBS	Tris-buffered saline
TRPC	Transient receptor potential canonical channel
US	Unconditioned stimulus
VGCC	Voltage-gated calcium channel

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Chapter 1: Literature Review

1.1 Schizophrenia – current status

1.1.1 Introduction

With a prevalence of up to 1% (McGrath et al., 2008; van Os and Kapur, 2009), a 10-20 years reduction of life expectancy (Chesney et al., 2014), an 80-90% unemployment rate (Marwaha and Johnson, 2004) and an enormous cost to society (around £12 billion per year in England alone; Andrew et al., 2012), schizophrenia is a severely disabling psychiatric disorder to both the affected individuals and society. It therefore warrants the tremendous research effort which endeavours to understand the disorder and improve therapeutics. However, despite more than 60 years of research since the serendipitous advent of the first antipsychotic drugs (Charpentier et al., 1952; Laborit et al., 1952; Seeman et al., 1976), medication for schizophrenia has improved very little. The most frequently used class of antipsychotics still have the same pharmacological target (dopamine receptor D₂), are effective in only a subset of patients (Leucht et al., 2013), treat only some of the symptoms and induce a number of side-effects (Leucht et al., 2012). It has been only in the past decade that advances in our understanding of the disease have accelerated, largely due to large-scale genomics projects.

Schizophrenia is typically characterised by three clusters of symptoms: positive symptoms (e.g. delusions and hallucinations), negative symptoms (e.g. social withdrawal and impaired motivation) and cognitive symptoms (e.g. attention and working memory deficits). Psychotic symptoms usually first manifest at late adolescence or early adulthood, although many patients display social and cognitive impairments

long before the onset of schizophrenia (Lewandowski et al., 2011). However, there are at present no tests or known biomarkers to facilitate a definitive diagnosis, and since schizophrenia shares many of its symptoms with other psychiatric disorders, such as bipolar disorder, major depressive disorder, autism spectrum disorder and intellectual disability, clinical differential diagnoses rely on the use of disease classification criteria such as those defined in the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 2013). Whilst this permits consistency in diagnoses across clinics globally, the segregation of psychiatric disorders into distinct categories does not necessarily reflect their overlapping genetics and pathophysiology (Craddock and Owen, 2010).

1.1.2 Pathophysiology and pathogenesis

Whilst no specific anatomical abnormalities are common to all patients with schizophrenia (Linden, 2012), some structural and functional differences are recurrently observed. One of the most consistently reported findings is a reduction of grey matter, which is linked to an enlargement of the ventricles and worsens as the disease progresses (Vita et al., 2012; Haijma et al., 2013). Another consistent finding is altered hippocampal volume and function (Tamminga et al., 2010). The hippocampus, particularly its *cornu ammonis* (CA) subregions, is responsible for several cognitive functions that are impaired in schizophrenia, including episodic, working and associative memory (Ranganath et al., 2004, 2008; Diwadkar et al., 2008a; Hall et al., 2009). Structural alterations in the hippocampus are accompanied by molecular changes. In the CA1 hippocampal subregion of patients, a recent study found reduced postsynaptic density (PSD)95-complex proteins, reduced metabotropic glutamate receptors and

altered Homer1 scaffold proteins in the CA1 hippocampal subregion (Matosin et al., 2016), all of which are components of synapses.

Indeed, synaptic systems have been further implicated in schizophrenia in relation to dopaminergic and glutamatergic neurotransmission. A substantial body of evidence, led by the fact that current antipsychotics block dopamine D₂ receptors, implicates abnormalities in dopamine signalling in the pathogenesis of schizophrenia (Baumeister and Francis, 2002). However, two predominant sources of evidence account for a shift in the focus of research and potential therapeutics from the dopamine system to the glutamatergic system. Firstly, antagonists of the N-methyl-D-aspartate (NMDA) glutamate receptor, including phencyclidine and ketamine, induce schizophrenia-like symptoms in healthy humans and worsen those of patients (Javitt and Zukin, 1991; Krystal et al., 1994; Newcomer et al., 1999). Secondly, there is a convergence of genetic evidence upon NMDA receptor signalling in schizophrenia, discussed later. Importantly, this evidence does not negate the role of dopamine in schizophrenia and the afflicted circuitry likely involves the interaction of both systems.

The age-dependent onset, at late adolescence or early adulthood, is a contrast to many other psychiatric disorders with childhood onset, and strongly suggests that schizophrenia has a neurodevelopmental aetiology. Consistent with this notion are multiple branches of research implicating the disruption of neuronal maturation during critical developmental periods in schizophrenia (Fatemi and Folsom, 2009; Jaaro-Peled et al., 2009). Indeed, a period of synaptic overproduction and elimination accompanies the periadolescent period, which is thought to be sensitive to environmental and intrinsic insult (Andersen, 2003; Cannon et al., 2003). Specific disruptions to neuronal

development observed in schizophrenia include γ -aminobutyric acid (GABA) interneuron maturation (Beasley and Reynolds, 1997; Lewis et al., 2005; Tseng and O'Donnell, 2007), maturation of mesocortical dopaminergic projections (Akil et al., 1999, 2000), pruning of glutamate synapses (Keshavan et al., 1994; Boksa, 2012) and myelination (Hakak et al., 2001; Tkachev et al., 2003).

The incidence of schizophrenia can vary region-by-region across the globe (McGrath et al., 2008). Hence, the exposure to environmental risk factors varies with demographics. Studies of environmental risk strengthen the view that neurodevelopment represents an important period in the pathogenesis of psychiatric disease (Cannon et al., 2003; van Loo and Martens, 2007). These studies have found relationships between schizophrenia and environmental insults such as maternal stress (Khashan et al., 2008), infection (Khandaker et al., 2012) or inflammation (Knuesel et al., 2014), postnatal stress (Dvir et al., 2011), childhood adversity (Varese et al., 2012) and exposure to psychoactive substances (Malone et al., 2010). It is notable that inflammatory and other immune processes during development represent a recurrent finding and important branch of epidemiological and clinical studies in schizophrenia (Benros et al., 2012; Smyth and Lawrie, 2013). It is the interaction between environmental risk factors and an individual's genetic predisposition that ultimately cause schizophrenia to manifest (van Os et al., 2008; Karl and Arnold, 2014).

1.1.3 Genetics

Heritability estimates for schizophrenia range from 65-80% (Sullivan et al., 2003; Lichtenstein et al., 2009). This genetic contribution to the disorder does not form a Mendelian pattern of inheritance, but is instead derived from variants in a large number

of genomic regions (Purcell et al., 2009; Lee et al., 2012; Ripke et al., 2013, 2014). Despite this complexity, an increasing number of genetic risk variants are being identified from the substantial progress made recently in large-scale collaborative genomic studies. Some of the associated genetic loci are common single nucleotide polymorphisms (SNPs), individually contributing only a small amount of risk, whilst others are rare variants in the form of copy number variants (CNVs), single nucleotide variants, insertions or deletions, which may contribute a much greater amount of risk (Stefansson et al., 2008; Fromer et al., 2014; Purcell et al., 2014; Rees et al., 2014b; Ripke et al., 2014). Figure 1.1 illustrates the relationship between frequency and penetrance of genetic risk variants, due to selection pressure.

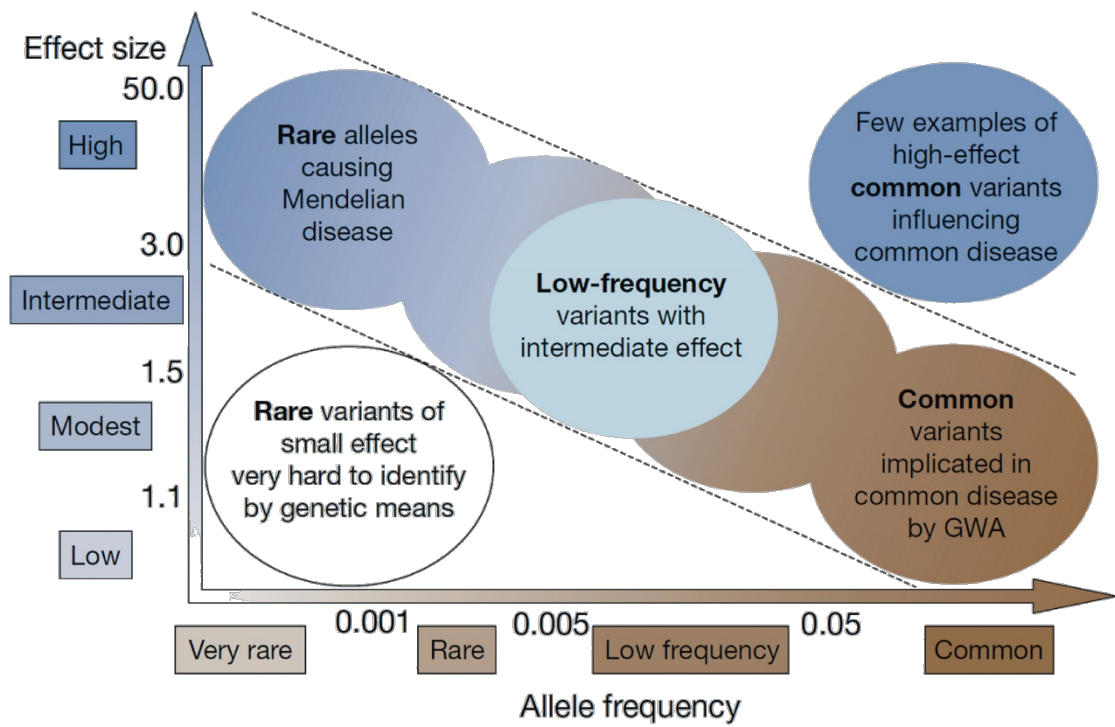


Figure 1.1 The inverse relationship between allele frequency and effect size in genetic diseases. Variants with small effect sizes may become common in a population because of weak selection pressure. Variants with larger effect sizes will therefore be rare in the population, and often *de novo*. Rare variants with small effect size are difficult to detect due to lack of statistical power. From (Manolio et al., 2009), adapted from (McCarthy et al., 2008).

1.1.3.1 Common variants

It is estimated that over 8000 SNPs contribute to schizophrenia (Ripke et al., 2013). Together, they may contribute more than 50% of overall risk (Ripke et al., 2013), although each one typically has an odds ratio for the development of schizophrenia of around 1.10 (Ripke et al., 2011, 2013, 2014). In order to obtain sufficient statistical power to reveal some of these SNPs, a Psychiatric Genomics Consortium generated meta-analyses of genetic data from large numbers of patients and control subjects. In the most recent of these analyses, involving data from approximately 37,000 cases and 113,000 controls, 108 separate loci were associated with schizophrenia with sufficient statistical significance to surpass the genome-wide multiple comparisons involved (Ripke et al., 2014).

The next step – linking these SNPs to individual genes – is not straight forward. Most SNPs have the potential to affect multiple genes, creating uncertainty as to which functional product mediates the risk encoded by a SNP. Many of the associated SNPs are within non-coding regions of DNA and several appear to affect non-protein-coding genes (Maurano et al., 2012; Ripke et al., 2014). Instead, it is likely that a large proportion of these variants affect gene expression timing or location (Kleinman et al., 2011). Furthermore, SNPs attributed to risk for schizophrenia may also contribute risk for other psychiatric disorders. Such pleiotropy exists between schizophrenia and bipolar disorder, major depressive disorders, autism spectrum disorder and attention-deficit hyperactivity disorder (Lee et al., 2013). Despite these limitations, biological insights into genetic risk from common variants is emerging (Ripke et al., 2014).

1.1.3.2 *Rare variants*

Studies of rare variants have identified an increased burden of CNVs, either deletions or duplications, in patients with schizophrenia compared to controls (Stefansson et al., 2008; Malhotra and Sebat, 2012). CNVs are thought to contribute around a tenth of the heritability attributed to common SNPs (Purcell et al., 2014). So far, 11 recurrent CNVs have been linked to schizophrenia (Rees et al., 2014a, 2014b), which each confer a relatively high risk to the disorder (Kirov et al., 2014). Odds ratios for these CNVs range from around 2 to 60 (Rees et al., 2014b, 2015). Like SNPs, CNVs are often difficult to map to specific genes, since many overlap with multiple genes and others are within non-coding regions (Rees et al., 2014a). Schizophrenia-associated CNVs also confer risk for other disorders, including intellectual disability, autism and generalized epilepsy (Malhotra and Sebat, 2012; Kirov et al., 2014). In addition, the occurrence of *de novo* CNVs through new mutations, as well as other varieties of rare variant, i.e. single nucleotide variants and indels (point mutations), also play a role in conferring risk to schizophrenia (Kirov et al., 2012; Fromer et al., 2014; Purcell et al., 2014).

1.1.3.3 *Missing heritability*

Indeed, many more common and rare genetic variants in addition to those already discovered contribute risk to schizophrenia and will require further studies, including analyses with larger sample sizes, in order to identify them. However, even taking all those variants yet to be identified into account, their respective contributions to the disorder do not completely account for the total heritability of schizophrenia (Manolio et al., 2009; Lee et al., 2011). At least some of this “missing heritability” may derive from multiplicative, rather than additive, effects of multiple genetic variants within the same

individual (epistasis; Mackay, 2014). In support of this, epistatic interactions between schizophrenia candidate genes have been observed in clinical experiments (Nicodemus et al., 2007, 2010a, 2010b, 2014). A further component of heritability may come from interactions between genetic variants and environmental input *via* epigenetic factors (Millan, 2013), whereby inherited non-genetic mediators of gene expression, such as histone modifications and DNA methylation, alter sensitivity to environmental risk factors, increasing susceptibility to disease progression (Dempster et al., 2013).

1.2 Risk variants to biological pathways

1.2.1 Introduction

Genetic discoveries do not in themselves equate to drug targets, particularly for polygenic disorders such as schizophrenia. Even though some variants may be mapped accurately to affected genes, many genes have unknown, or partially known, functions and their gene product may not be possible to target with drugs (Plenge et al., 2013). In order to use genetic findings to better understand the pathophysiology of schizophrenia and related complex disorders, it is important to find common molecular pathways affected by many genetic variants conferring susceptibility. Here, I present the evidence implicating specific biological pathways in schizophrenia from previous studies and compare the pathways implicated through common and rare variants. I also address the variety of methods used to approach pathway analyses of polygenetic data.

1.2.2 Glutamate synapses

As alluded to previously, caution must be taken when attempting to link genetic variants to specific genes. In the case of CNVs, this is due to the fact that they may span several genes. In order to circumvent this issue, studies of biological pathways represented in patient CNVs use a hypothesis-based systems approach, whereby gene sets are tested for statistical overrepresentation in case variants. This gene set enrichment analysis has been used in analyses of CNVs from large cohorts of patients with schizophrenia and healthy controls. Kirov *et al* (2012) found an enrichment of genes in the NMDA glutamate receptor complex in patient inherited and *de novo* CNVs, corroborating pre-existing NMDA hypofunction hypotheses (Olney and Farber, 1995; Kantrowitz and Javitt, 2010) and candidate gene studies (Moghaddam, 2003; Harrison and Weinberger, 2005).

This finding accompanied an overall enrichment of genes encoding components of the postsynaptic density, together lending support to the notion that excitatory glutamate synapses are involved in schizophrenia's aetiology (Kirov et al., 2012; Pocklington et al., 2014).

Further synaptic components have been linked to schizophrenia through gene set enrichment analyses in rare variants. These include interactors of the activity-regulated cytoskeletal-associated protein (ARC), which have been implicated through CNVs (Kirov et al., 2012; Pocklington et al., 2015) and point mutations (Fromer et al., 2014; Purcell et al., 2014). Arc is an immediate-early gene which is rapidly activated through local translation at the synapse and has an essential role in learning and memory (Plath et al., 2006). Studies of point mutations, through exome sequencing, again found an enrichment of the NMDA receptor complex, as well as the related PSD-95 protein complex (Fromer et al., 2014; Purcell et al., 2014).

A role for synaptic components in schizophrenia is further corroborated by genome-wide significant common variants. SNPs associated with the disorder lie within genes encoding a subunit of the NMDA receptor (*GRIN2A*), a subunit of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor (*GRIA1*), the glutamate metabotropic receptor 3 (*GRM3*) and an enzyme responsible for synthesizing the intrinsic NMDA agonist, D-serine, serine racemase (*SRR*) (Ripke et al., 2014), all of which are involved in glutamate receptor signalling.

It is important to note that, as well as glutamate signalling, components of the dopamine system have also been implicated through genetic association studies, albeit to a lesser extent. Perhaps the most interesting of these is the association with schizophrenia of

common variants within the *DRD2* gene (Ripke et al., 2014), which encodes the dopamine receptor targeted by current antipsychotic medicine. An enzyme responsible for metabolising dopamine, Catechol-O-methyltransferase, is associated with schizophrenia through the overlap of its gene, *COMT*, with a recurrent CNV located at 22q11. However, since *COMT* is not the only gene affected by this CNV, it is not clear that it is the deletion of this gene that confers risk to the disorder (Liu et al., 2002; Bassett and Chow, 2008).

1.2.3 Synaptic plasticity and calcium signalling

The emerging picture from studies of the biological pathways represented by schizophrenia-associated genetic variants is an enrichment of overlapping molecular systems, not all situated within the postsynaptic cleft, yet which are collectively involved in synaptic plasticity processes (Hall et al., 2015; Figure 1.2). One of the most consistently reported gene set enrichments in rare variants is that of targets of fragile X mental retardation protein (FMRP). This group of genes are enriched in rare disruptive and *de novo* point mutations (Fromer et al., 2014; Purcell et al., 2014) and CNVs (Szatkiewicz et al., 2014a; Pocklington et al., 2015). FMRP regulates the translation of specific mRNAs, including *ARC* (Darnell et al., 2011; Fernandez et al., 2013), through interactions with its binding partner CYFIP1, associated with schizophrenia through a CNV located at 15q11.2 (Napoli et al., 2008; Stefansson et al., 2008). Through the regulation of translation, CYFIP1 and FMRP are thought to be critical mediators of synaptic plasticity processes, specifically the regulation of dendritic complexity and spine morphology (Pathania et al., 2014). These same processes are additionally

regulated by actin filament complexes, which are enriched in schizophrenia-associated point mutations (Fromer et al., 2014).

A further group of proteins central to synaptic plasticity and implicated in schizophrenia through genetic variants is voltage-gated calcium channels. Genes coding for these proteins are enriched in case point mutations (Purcell et al., 2014), and schizophrenia-associated SNPs exist within genes *CACNA1C* and *CACNB2* (Ripke et al., 2013, 2014), encoding the subunit of Ca_v1.2 and Ca_v1.3, respectively. Along with NMDA receptors, voltage-gated calcium channels mediate calcium signalling cascades required for the activation of transcription factors and the modification of synaptic excitability (West et al., 2001; Moosmang et al., 2005), and have been shown to be central to learning and memory (Moosmang et al., 2005; White et al., 2008; Heck et al., 2014).

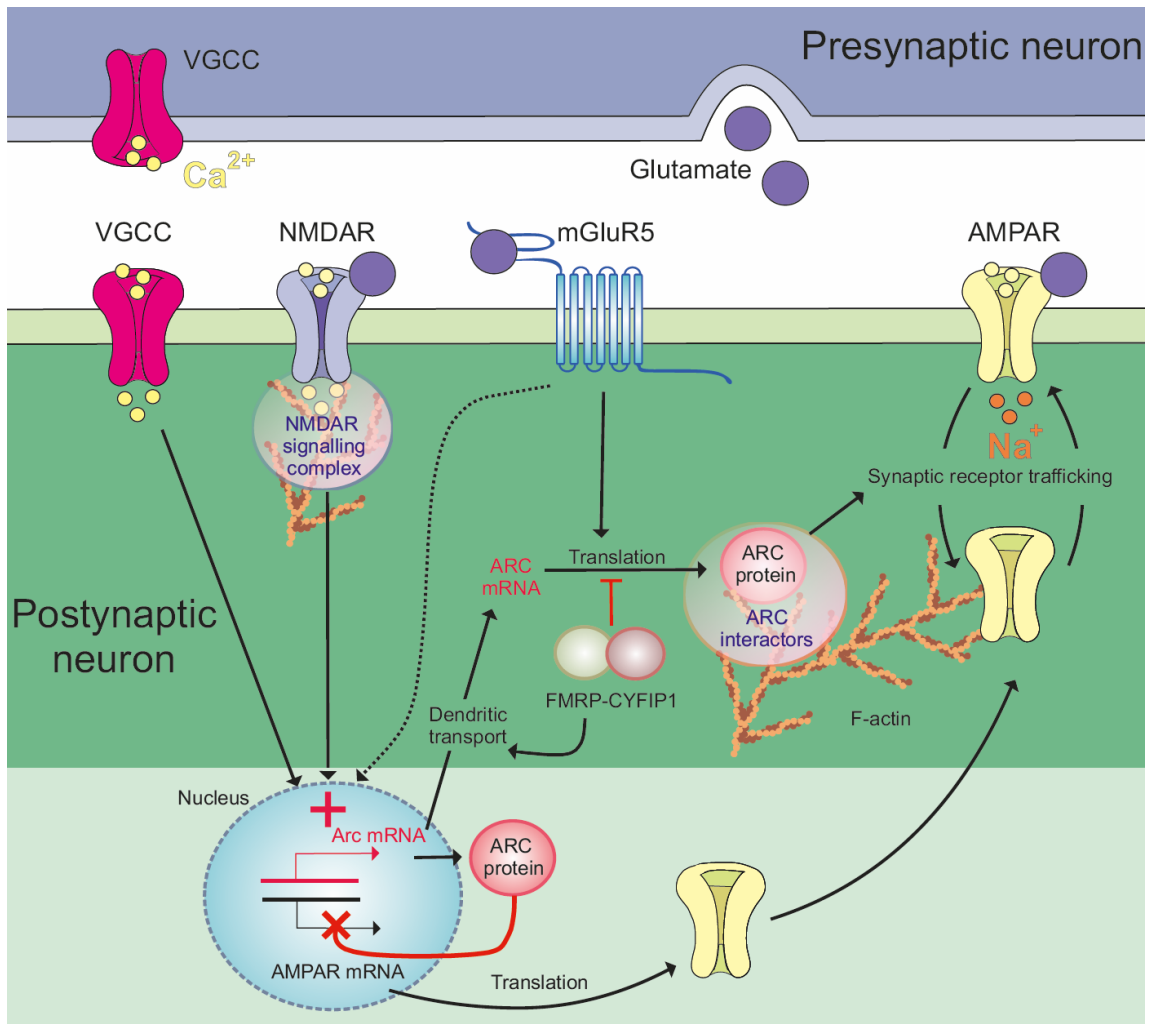


Figure 1.2 The convergence of synaptic pathways implicated in schizophrenia. Protein complexes, including the NMDA receptor complex, actin filament complex, Arc interactors, voltage gated calcium channels and targets of FMRP/CYFIP1 are associated with schizophrenia through genetic variants. Abbreviations: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), activity-regulated cytoskeletal protein (ARC), N-methyl-D-aspartate receptor (NMDAR), voltage-gated calcium channel (VGCC). From Hall et al., 2015.

1.2.4 Excitatory *and* inhibitory signalling complexes

A recent study by Pocklington *et al* (2015) replicated and extended previous findings linking excitatory glutamatergic signalling to CNVs from patients with schizophrenia, whilst providing novel evidence implicating inhibitory GABAergic systems in schizophrenia (Pocklington *et al.*, 2015). The study demonstrated that genes coding for components of GABA_A receptor complexes are enriched in patient CNVs, building on previous hypotheses of GABAergic signalling deficits and the involvement of inhibitory interneurons in schizophrenia (Lewis *et al.*, 2005, 2012; Gonzalez-Burgos and Lewis, 2012). Since alterations in the ratio of excitation to inhibition have been proposed to underlie schizophrenia phenotypes (Kehrer *et al.*, 2008), these results lend support to the notion that disruption of either glutamatergic or GABAergic signalling could create an imbalanced state, vulnerable to the development of psychosis.

1.2.5 Conclusions

The advent of large collaborative clinical studies, genome-wide association studies and sequencing brought with it high-powered evidence supporting the involvement of specific genetic variants in schizophrenia. Multiple pathway analyses with these data revealed a striking convergence of common and rare variants on genes involved in synaptic plasticity processes. These studies provide valuable guidance to future experiments aiming to further our understanding of the biological mechanisms underlying schizophrenia. However, “synaptic plasticity” is not specific enough to advise therapeutic design. One of the limitations affecting these pathway analyses is the gap in understanding of gene function and hence the incompleteness of gene ontology databases. This also creates bias in the analyses, due to the overrepresentation of those

biological pathways on which more research has been focused. Gene expression studies that capture gene sets associated with particular functionality of a tissue may yield more specific biological information in gene set enrichment analyses of disease-associated variants.

1.3 Associative learning and schizophrenia

1.3.1 Introduction

Synaptic plasticity is widely believed to underlie learning, memory and the development of neural circuits (Abbott and Nelson, 2000; Martin et al., 2000; Neves et al., 2008). However, the convergence of genetic findings on synaptic plasticity processes is not mirrored by universal memory deficits in schizophrenia. Instead, patients display impairments in specific facets of learning and memory (Stone et al., 1998; Rushe et al., 1999; Diwadkar et al., 2008a), which implies that the genetic component of schizophrenia susceptibility must impact on more specific biological mechanisms and perhaps on particular cognitive processes.

Based on the pre-existing convergence of patient CNVs on genes involved in synaptic plasticity, Pocklington *et al* (2015) investigated the enrichment of more specific genes sets, all relating to aspects of plasticity, learning and memory, in schizophrenia-associated CNVs. Among the gene sets statistically associated with case deletions, duplications or both combined, were those relating to “learning/memory/conditioning”, “contextual conditioning behaviour”, “cued conditioning behaviour” and “associative learning” (Pocklington et al., 2015). These intriguing findings highlight a specific memory system – association formation – as impacted on by CNVs in schizophrenia. They also strengthen existing hypotheses concerning dysfunctions of associative learning in the manifestation of schizophrenia phenotypes (Miller, 1976; Bazin and Perruchet, 1996; Friston, 1998; Diwadkar et al., 2008a; Hall et al., 2009).

1.3.2 Neural circuitry for aversive conditioning to a context

Since associating environmental stimuli with safety or danger is a key functionality for the survival of all mammals, it is understandable that the circuitry responsible is common among many species (Pearce and Bouton, 2001; Fanselow and Poulos, 2005). Studies of classical Pavlovian conditioning (Pavlov, 1927) in rodents have contributed to a thorough understanding of the systems involved in associative learning (Mackintosh, 1983). One of the best understood systems is that of aversive learning, typically studied through fear conditioning (Maren, 2001). In this paradigm, an innately neutral stimulus, such as a cue or context is presented to a rodent simultaneously to, or with constant temporal separation from, the presentation of an unconditioned shock stimulus. Their pairing leads to the previously neutral stimulus becoming an aversively conditioned stimulus, so that the presentation of the cue or context alone elicits a fear response akin to that exhibited following the unconditioned stimulus.

The central hub for fear conditioning is the amygdala (LeDoux et al., 1991; Phelps and LeDoux, 2005). Through projections from the thalamus, hippocampus and cortical regions, and projections to the hypothalamus, periaqueductal grey and other brainstem regions, the amygdala mediates the expression of conditioned fear responses to learned conditioned stimuli (Applegate et al., 1982; LeDoux et al., 1991; Pitkänen et al., 1997). Whilst simple auditory conditioned stimuli are processed *via* direct projections from the medial geniculate nucleus of the thalamus to the amygdala, more complex conditioned stimulus information, such as context cues, reach the amygdala after processing by the entorhinal cortex and hippocampus (LeDoux et al., 1991; Maren and Fanselow, 1995; Fanselow and Poulos, 2005; Hugues and Garcia, 2007). Hence, the hippocampus is able

to gate complex contextual fear learning and fear responses (Radulovic and Tronson, 2010).

At the molecular level, both glutamatergic and dopaminergic systems are required for the acquisition of aversive conditioning. Blockade of NMDA glutamate receptors in lateral and basal nuclei of the amygdala prevents fear conditioning (Fendt, 2001; Bauer et al., 2002; Fanselow and Poulos, 2005). Furthermore, hippocampal NMDA receptor-mediated plasticity has been shown to be necessary for the generation of context representations during conditioning to a context (Cravens et al., 2006). The interactions of these processes with dopamine signalling are additionally involved in the acquisition of conditioned associations. Mesolimbic dopamine neurons fire in response to the presentation of an unconditioned stimulus, yet following conditioning, fire in response to the conditioned stimulus (Grace, 1991). This activity serves to mediate NMDA receptor-dependent synaptic plasticity in the hippocampus and cortex (O'Carroll and Morris, 2004; Calabresi et al., 2007) and to provide feedback in the form of prediction error, valence or salience (Berridge and Robinson, 1998; Waelti et al., 2001; Kapur et al., 2005; Cooper and Knutson, 2008).

1.3.3 Associative learning in psychopathology

From a psychological perspective, new associative learning in healthy individuals is mediated by an interaction between experience and belief (Corlett et al., 2009; Fletcher and Frith, 2009). Experiences that do not challenge a belief system become predictable and can be ignored, yet experiences that contradict a belief induce new learning. Prediction error is a model used to explain the process through which these unpredictable events induce new learning, and was derived from Bayesian machine

learning approaches to the study of learning (Fletcher and Frith, 2009). It has been proposed that the instability of this system could lead to the persistence of delusional beliefs, despite the existence of conflicting evidence, in schizophrenia (Corlett et al., 2009; Fletcher and Frith, 2009).

Deficits in multiple aspects of associative learning have been recurrently reported in schizophrenia (Diwadkar et al., 2008a). Firstly, patients exhibit impairments in contextual processing (Maren et al., 2013), demonstrated through tests of AX-continuous performance (Cohen et al., 1999; Barch et al., 2003), which may reflect hippocampal and medial prefrontal cortex abnormalities in the disorder (Taylor et al., 2012). Secondly, deficits in the classical conditioning of associations have been observed, typically in test of eyelid conditional discrimination (Sears et al., 2000; Hofer et al., 2001; Marenco et al., 2003). Thirdly, patients with acute schizophrenia show evidence of impaired signalling of prediction error when existing associations are challenged (Baruch et al., 1988; Jones et al., 1992; Serra et al., 2001). Fourthly, learning of aversive stimuli is consistently reported to be abnormal in the disorder (Kosmidis et al., 1999; Jensen et al., 2008; Linnman et al., 2013).

1.3.4 Inhibitory learning of associations

A further component of associative learning frequently linked to the persistence of psychotic and cognitive symptoms in schizophrenia is the inhibitory learning of associations (Baruch et al., 1988; Swerdlow et al., 1996; Rascle et al., 2001; Serra et al., 2001; Holt et al., 2009, 2012). Three types of inhibitory learning are relevant in this regard. Firstly, latent inhibition is the process by which prolonged or repeated exposure to a neutral stimulus prior to its pairing with an unconditioned stimulus, inhibits

conditioning to the neutral stimulus (Escobar et al., 2002). Secondly, Kamin blocking describes the prevention of conditioning to a neutral stimulus due to its pairing with an unconditioned stimulus in conjunction with a further stimulus previously associated with the unconditioned stimulus (Padlubnaya et al., 2006). Finally, extinction learning occurs when a previously conditioned stimulus is presented repeatedly or for an extended period of time in the absence of the unconditioned stimulus, resulting in the reduction of memory-associated behavioural responses (Quirk and Mueller, 2008).

Deficits in each of these types of inhibitory learning have been reported in schizophrenia (latent inhibition: Baruch et al., 1988; Swerdlow et al., 1996; Serra et al., 2001; Corlett, 2012; blocking: Jones et al., 1992; Serra et al., 2001; extinction: Holt et al., 2009, 2012). Focusing on complex contextual associations, substantial evidence suggests that the hippocampus is responsible for the context-dependent gating of inhibitory fear extinction (Corcoran et al., 2005; Maren and Hobin, 2007; Zelikowsky et al., 2012), through indirect projections to inhibitory intercalated neurons of the amygdala *via* medial prefrontal cortex (Likhtik et al., 2008; Maren, 2011; Sotres-Bayon et al., 2012). It has been proposed that abnormalities in the functional connectivity within this fear extinction circuit form the neurobiological basis for impaired extinction learning in adolescence (Neumann et al., 2008; Pattwell et al., 2012), which has important implications for the development of schizophrenia in early adulthood.

1.3.5 Conclusions

The enrichment of associative learning-related genes in CNVs from patients with schizophrenia is complimentary to the convergence of schizophrenia-associated genetic variants on synaptic pathways in that it provides a hint of particular cognitive processes

affected by schizophrenia-associated variants. It is also consistent with behavioural and physiological correlates of associative learning deficits in schizophrenia. However, since associative learning is comprised of multiple components, including inhibitory processes, it is unclear which specific aspects of associative learning are implicated in schizophrenia through genetic variants.

1.4 Homer family activity-induced proteins

1.4.1 Introduction

As detailed above, there is a substantial convergence of schizophrenia-associated genetic variants on genes involved in synaptic plasticity (Hall et al., 2015). A considerable component of this is attributable to genes encoding parts of the postsynaptic density (PSD) (Kirov et al., 2012; Pocklington et al., 2014). The PSD is an architecture of specialised proteins responsible for mediating the response to converging synaptic input. Rearrangement of PSD proteins, as a consequence of changes to the pattern of neurotransmission, leads to alterations in synaptic strength, required for learning and memory (Kennedy et al., 2005; Moutin et al., 2012; Murakoshi and Yasuda, 2012). Hence the study of PSD protein dynamics represents a fundamental route towards understanding synaptic plasticity and may be central to discerning the aetiologies of psychiatric disorders such as schizophrenia (Grant, 2012).

Homer proteins are key components of the PSD and form functional links between receptors, ion channels and scaffolding proteins (Brakeman et al., 1997; Xiao et al., 1998). They share an N-terminal ligand-binding domain, which interacts with the proline-rich sequences displayed by PSD proteins involved in regulating synaptic architecture, intracellular calcium signalling and neuronal development. Whilst most transcription variants of the Homer family are constitutively expressed in mammals, two truncated forms of Homer1, Homer1a and Ania-3, are activity-induced immediate early genes (IEGs) (Fagni et al., 2002). These isoforms possess a common enabled/VASP homology 1 (EVH1) domain but lack a coiled-coil domain that permits longer Homer proteins to oligomerise with one another (Hayashi et al., 2009). As a consequence,

Homer1a and Ania-3 bind as dominant negative regulators, allowing them to disrupt interactions between long Homers and their effector proteins (Xiao et al., 1998; Kammermeier and Worley, 2007).

The expression of Homer IEGs appears to represent a powerful modulator of synaptic plasticity. The literature surrounding Homer1 IEGs indicates that their precise temporospatial expression and recruitment to active synapses is essential for the regulation of PSD protein complexes and calcium signalling in dendritic spines and may therefore be important for learning and memory (Worley et al., 2007; Okada et al., 2009a; Hu et al., 2010). Here, potential mechanisms through which Homer1 IEGs may mediate synaptic plasticity are discussed. Since most research to date has focused on either Homer1a only or short Homer1 isoforms as a collective, any distinct functions of Homer1a and Ania-3 remain somewhat hidden. Thus the majority of the content in this section addresses Homer1a function.

1.4.2 Implications for Neurological Disease

Homer1 IEGs are rapidly induced following neuronal activity in limbo-corticostriatal circuits responsible for mediating cognitive and emotional functions (Tekin and Cummings, 2002). Single nucleotide polymorphisms in the Homer1 gene have been associated with schizophrenia (Norton et al., 2003; Spellmann et al., 2011), autism (Kelleher et al., 2012), major depression (Rietschel et al., 2010a), suicide attempt (Strauss et al., 2012; Rao et al., 2016), cocaine dependence (Dahl et al., 2005) and opiate abuse (Jacobs et al., 2013). These genetic associations are not conclusive, however, and replication is needed to confirm their validity. Further evidence suggests that Homer1a may additionally be involved in the pathology of neuropathic pain (Tappe et al., 2006;

Roselli et al., 2009; Tappe-Theodor et al., 2011; Obara et al., 2013) and Alzheimer's disease (Dickey et al., 2003; Yamamoto et al., 2011). Here, I provide an overview of the evidence implicating short Homer1 proteins in autism and schizophrenia.

Perhaps the strongest evidence for the involvement of Homer IEGs in neurological disease is from studies of their association with Fragile X mental retardation protein (FMRP). The absence of expression of FMRP (coded by the *FMR1* gene) is a common single-gene cause of autism and targets of FMRP are enriched in rare genetic variants from patients with schizophrenia (Fromer et al., 2014; Pocklington et al., 2015). The binding partner of FMRP, CYFIP1, is also implicated in schizophrenia (Stefansson et al., 2008). FMRP is a negative regulator of group I mGluR-dependent protein synthesis (Weiler et al., 2004) and mice lacking *Fmr1* show enhanced long-term depression (Huber et al., 2002). It was demonstrated that mGluR5-mediated translation initiation and long-term depression are dependent on mGluR5-long Homer interactions (Ronesi and Huber, 2008; Ronesi et al., 2012), which are diminished in *Fmr1* knockout mice (Giuffrida et al., 2005). Moreover, Homer1 protein is decreased in post-mortem cerebellar vermis and superior frontal cortex of autistic patients (Fatemi et al., 2013). Instead, mGluR5 is more associated with Homer1a in mice lacking *Fmr1* (Giuffrida et al., 2005; Ronesi et al., 2012). Ronesi and colleagues generated an *Fmr1* / Homer1a (and Ania-3) double knockout in an attempt to rescue mGluR5 function. The additional deletion of Homer1a restored mGluR5-long Homer interactions, stabilized translation rates and reversed behavioural phenotypes. The abnormal translational control of specific FMRP target mRNAs or altered mGluR-mediated long-term depression in *Fmr1* knockout mice were not rescued by Homer1a deletion, however (Ronesi et al., 2012). These observations imply that a

higher ratio of short / long Homer proteins interacting with mGluR5 might be responsible for disrupting mGluR5-mediated signalling in fragile X syndrome, contributing to the disorder. Therefore, the therapeutic action provided by mGluR5 antagonists in fragile X syndrome (Pop et al., 2014) may be due to inhibition of Homer1a / mGluR5-mediated events.

mGluR5 signalling complexes are also a potential target for therapy in schizophrenia (Matosin and Newell, 2013). Critically, Homer1a and Ania-3 induction is manipulated by environmental stimuli that impact on the manifestation of psychosis (reviewed in Szumlinski and Kippin, 2008). For example, acute cocaine administration in rodents induces a pronounced elevation of Homer1a expression in the striatum, prefrontal cortex and ventral tegmental area (Brakeman et al., 1997; Zhang et al., 2007; Ghasemzadeh et al., 2009). This response is necessary for the regulation of cocaine sensitivity (Szumlinski et al., 2004; Lominac et al., 2005). Psychotomimetic NMDA antagonists and, interestingly, antipsychotics also modulate Homer1a and Ania-3 expression (De Bartolomeis et al., 2002, 2013; Polese et al., 2002; Ambesi-Impiombato et al., 2007; Iasevoli et al., 2007, 2009; Dell'Aversano et al., 2009). Furthermore, recent evidence showed that the ratio of short to long Homer1 protein may be abnormal in patients with schizophrenia. In analyses of protein expression in post-mortem hippocampus, Matosin et al (2016) showed that there was increased Homer1a, but decreased long isoforms Homer1b/c, in the CA1 region of patients compared to controls (Matosin et al., 2016). This is consistent with an earlier report of decreased Homer1 protein in the hippocampus and prefrontal cortex of patients with schizophrenia (Engmann et al., 2011). Finally, Homer1 knockout mice display several behavioural

phenotypes consistent with schizophrenia, including impaired pre-pulse inhibition, increased anxiety and enhanced locomotion in response to MK-801 or methamphetamine (Szumlinski et al., 2005), together implying that an imbalance of the competitive short / long Homer1 isoforms could contribute to the expression of schizophrenic symptoms in humans.

1.4.3 Protein Structure and Interactions

Homer proteins contain a highly conserved ena / vasodilator-stimulated phosphoprotein (ena / VASP) homology 1 (EVH1)-like domain (Irie et al., 2002; Shiraishi-Yamaguchi and Furuichi, 2007), which maintains much sequence homology with other VASP proteins responsible for regulating cytoskeletal dynamics (Reinhard et al., 2001). A neighbouring proline motif is specific to the Homer1 subfamily (Shiraishi-Yamaguchi and Furuichi, 2007). Through the EVH1 domain, Homer proteins interact with proline-rich sequences of a range of targets including group I metabotropic glutamate receptors (mGluR1 and mGluR5), inositol 1,4,5-triphosphate (IP3) receptors, Shank scaffolding proteins, ryanodine receptors, transient receptor potential canonical channels, voltage-gated calcium channels and dynamin 3 (Tu et al., 1998, 1999; Xiao et al., 1998; Beneken et al., 2000; Feng et al., 2002; Gray et al., 2003; Yuan et al., 2003; Olson et al., 2005; Hayashi et al., 2009). Long Homer isoforms (Homer1b-h, Homer2a and b, Homer3a and b), which form multimers through their (low homology) carboxy-terminal coiled-coil domains, mediate functional links between these PSD proteins, facilitating signal transduction (Kato et al., 1998a; Xiao et al., 1998; Hayashi et al., 2006).

Expression of shorter Homer1 isoforms is introduced through alternative splicing. Premature termination of transcription downstream of exon 5 creates the truncated

Homer1a and Ania-3 proteins (Figure 1.3; Brakeman et al., 1997; Soloviev et al., 2000; Bottai et al., 2002). The fifth intron of the Homer1 gene comprises sections of DNA specific to the transcription of Homer1a or Ania-3 mRNA (Bottai et al., 2002). These isoforms therefore lack a coiled-coil domain, the absence of which prevents Homer1a and Ania-3 from forming homo- or hetero-oligomers. Hence, since their discovery (Brakeman et al., 1997; Berke et al., 1998), it has been supposed that Homer1a and Ania-3 act as dominant negative regulators of long Homer function, uncoupling them from effector proteins through competition for the proline-rich sequence of the target protein (Fagni et al., 2002; Irie et al., 2002). Indeed, expression of Homer1a blocks the binding of long Homers to group I mGluR and disrupts long Homer-mGluR-mediated functions (Tu et al., 1998; Xiao et al., 1998; Kammermeier et al., 2000).

Short Homers are synthesised in the soma before vesicular transportation to dendrites (Brakeman et al., 1997; Okada et al., 2009a), which is dependent upon interactions with Group I mGluRs (Ango et al., 2000; Okada et al., 2009a). This is indicated by the dendritic colocalisation of Homer1a and mGluR5 following depolarisation, whilst a mutant form of the mGluR5 receptor that cannot bind Homer1a does not relocate from the soma to dendrites (Ango et al., 2000). Long Homer1 isoforms are also transported with group I mGluRs to dendrites (Roche et al., 1999; Ango et al., 2000), yet, in the absence of Homer1a, retain the receptor complex within endoplasmic reticulum, inhibiting mGluR5 surface expression (Roche et al., 1999; Ango et al., 2000, 2002; Coutinho et al., 2001). The induction of Homer1a through neuronal excitation then interrupts the endoplasmic retention of mGluR5, permitting their trafficking to the membrane (Ango et al., 2002).

Hence, long Homers keep a pool of group I mGluRs at the endoplasmic reticulum until released by activity-induced short Homers (Figure 1.4).

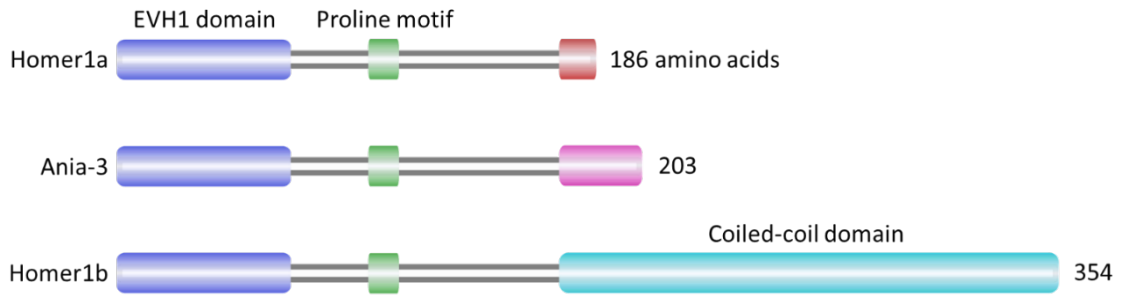


Figure 1.3 Primary structure of Homer1a, Ania-3 and Homer1b proteins. The amino-terminal EVH1 domain (blue) is conserved across all Homer proteins and permits their interaction with proline-rich sequences of target proteins. The proline motif (green; Ser-Pro-Leu-Thr-Pro) is specific to the Homer1 subfamily. At the carboxy-terminal region, long Homer isoforms, including Homer1b, contain a coiled-coil domain (cyan) required for dimerization. Short Homers, Homer1a and Ania-3, lack the coiled-coil domain and are therefore unable to form dimers.

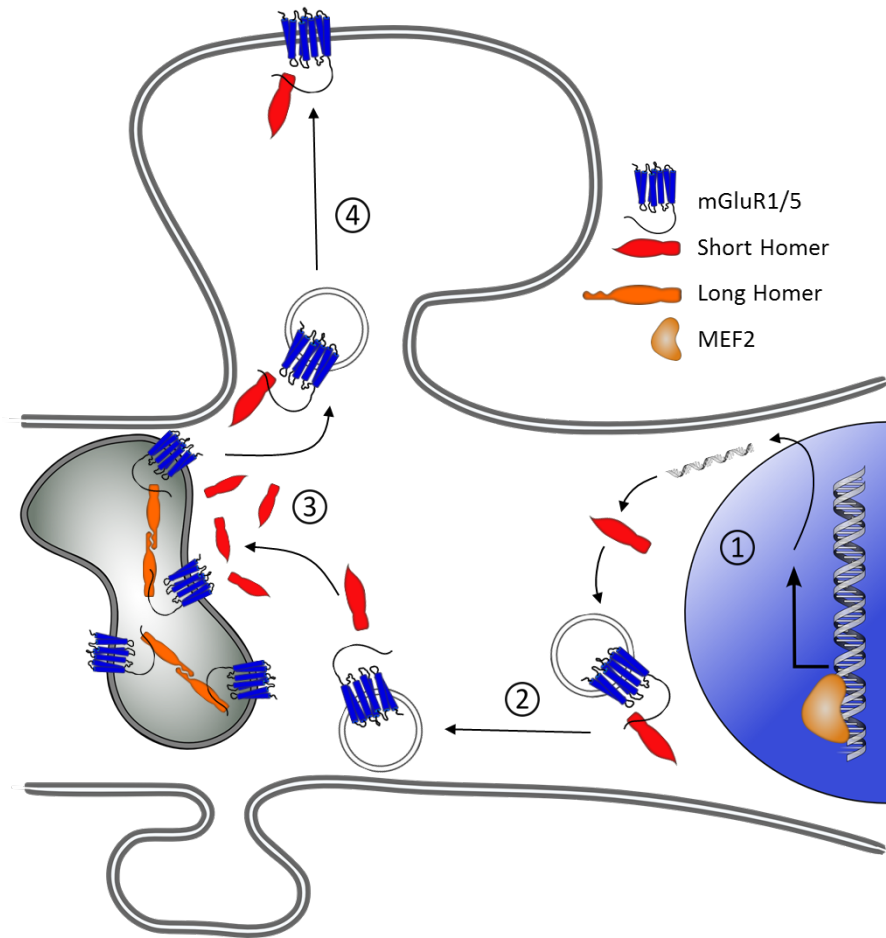


Figure 1.4 Cotransport of group I mGluRs and Homer proteins from the soma to the endoplasmic reticulum and cell membrane. (1) Transcription of short Homers is regulated by myocyte enhancer factor-2 (MEF2) transcription factors. The protein is synthesised in the soma. (2) Short Homers bind to group I mGluRs on transport vesicles and, together, they are transported in dendrites to synaptic sites. (3) Whilst long Homers retain clusters of mGluRs at the endoplasmic reticulum, short Homers reverse the intracellular retention of mGluRs, (4) permitting their trafficking to the cell membrane.

1.4.4 Homer1 IEG induction and regulation

Neuronal activity-induced expression of short Homer1 mRNA has been observed throughout brain structures known to be involved in learning and memory, including the cerebral cortex, hippocampal CA1, CA2, CA3 and dentate gyrus, striatum, and amygdala (Brakeman et al., 1997; Sun et al., 1998; Xiao et al., 1998; De Bartolomeis et al., 2002; Polese et al., 2002; Vazdarjanova et al., 2002; Ary et al., 2007; Zhang et al., 2007; Montes-Rodríguez et al., 2013). The induction of Homer1a has been investigated in vitro through neuronal depolarization with ionotropic glutamate receptor agonists, N-methyl-D-aspartate (NMDA), kainate and potassium channel blockers (Ango et al., 2000), application of brain-derived neurotrophic factor (BDNF) (Mahan et al., 2012), the use of a magnesium-free artificial cerebrospinal fluid medium (Okada et al., 2009a), gonadotropin-releasing hormone (Wang et al., 2014), traumatic injury (Huang et al., 2005; Luo et al., 2014), and evoking epileptiform activity with bicuculline and 4-aminopyridine (Li et al., 2012). Induction in vivo has been reported following electroconvulsive shock (Brakeman et al., 1997; Kato et al., 1997; Xiao et al., 1998; Bottai et al., 2002), long term potentiation (Kato et al., 1997, 1998a), exposure to psychoactive drugs such as cocaine (Brakeman et al., 1997; Zhang et al., 2007), lysergic acid diethylamide (LSD) (Nichols and Sanders-Bush, 2002; Nichols et al., 2003) and ketamine (Iasevoli et al., 2007; De Bartolomeis et al., 2013), direct dopamine D1 receptor stimulation (Berke et al., 1998), typical and atypical antipsychotic administration (De Bartolomeis et al., 2002; Polese et al., 2002; Fatemi et al., 2006; Ambesi-Impiombato et al., 2007), associative learning (Mahan et al., 2012), instrumental learning (Hernandez et al., 2006), exploration of a novel environment (Vazdarjanova et al., 2002; Marrone et al., 2008), visual experience (Brakeman et al., 1997), environmental stressors (Igaz et al.,

2004; Ary et al., 2007) and neuropathic pain (Ma et al., 2009). Importantly, it is exclusively the short, IEG isoforms of the Homer1 gene that are induced under these conditions, whilst the expression of the longer isoforms typically remains unchanged.

As alluded to previously, neuronal activation induces a switch in the transcription of the Homer1 gene to the preferential expression of short isoforms. This switch is created through an activity-dependent change in the polyadenylation site usage, altering transcription termination, and is controlled by myocyte enhancer factor 2 (MEF2) transcription factors (Bottai et al., 2002; Flavell and Greenberg, 2008; Flavell et al., 2008). With an aim to gain insight into the transcriptional machinery of Homer1a that may regulate synaptic plasticity during memory consolidation, Mahan and colleagues (2012) examined the epigenetic modulation of the Homer1a gene during BDNF-induced plasticity in vitro and in vivo. BDNF prompted an upregulation of Homer1a in primary amygdala and hippocampal cultures that was dependent upon MEK and ERK signalling. Around the Homer1 promoter region, they observed an increase in histone H3 acetylation, a marker of enhanced gene transcription, or a decrease in histone H3K9 methylation, a transcriptional repressive marker. When mice underwent Pavlovian fear conditioning, the consolidation of which involves BDNF signalling (Lee et al., 2004; Rattiner et al., 2004; Barnes and Thomas, 2008), the same Homer1 epigenetic modifications that were seen in vitro were found in the mouse hippocampal and amygdalar extracts (Mahan et al., 2012). This work demonstrates one possible mechanism through which activity-induced Homer1 transcripts may be generated during processes of learning and memory or developmental synaptic maturation to bring about the necessary plastic changes at the synapse.

A few developmental studies have shown that Homer1a is regulated with postnatal age. Given its functional location at the glutamate PSD, its interactions with NMDA receptor complexes, the critical role of glutamate synapses in the developmental refinement of neuronal connections (Komuro and Rakic, 1993; Behar et al., 1999; Ikonomidou et al., 1999; Matsugami et al., 2006; Zhang et al., 2013) and the developmental impairments caused by disrupting glutamate receptor complexes (Fredriksson et al., 2004; Clifton et al., 2013), Homer1a is in a prime position to impinge on synaptogenesis and synaptic pruning mechanisms during brain maturation. Postnatal forebrain expression of Homer1a increases from birth, peaking between 3-5 weeks (Brakeman et al., 1997). Montes-Rodríguez et al (2013) demonstrated that Homer1a is induced differentially by neuronal activity through early mammalian postnatal development. Maximal electroconvulsive shock treatment in rats only increased hippocampal Homer1a expression after the first postnatal week, indicating that Homer-related activity dependent synaptic plasticity is absent at this early stage of synaptogenesis (Montes-Rodríguez et al., 2013). The number of activity-induced Homer1a positive cells rapidly increased during the second postnatal week, reaching adult levels by P9, although hippocampal activity-induced Homer1a intra-nuclear foci intensity, representing mRNA quantity, peaked after 3 weeks, in parallel with synaptic maturation, hinting towards a role of the protein in the refinement of neuronal circuits (Yoshihara et al., 2009; Montes-Rodríguez et al., 2013). Furthermore, the balance of short to long Homer1 isoforms is an important regulator of axonal pathfinding in developing neurons (Foa et al., 2001). Unsurprisingly, disruption of Homer1a expression causes developmental impairments. These include deficits in motor coordination, learning, memory and sensorimotor gating (Szumlinski et al., 2005; Tappe and Kuner, 2006; Jaubert et al., 2007).

1.4.5 Regulation of mGluR-NMDA Complexes

At the PSD, short Homer1 proteins act to interrupt the scaffolds created by long Homer1 isoforms (Kato et al., 1998a; Xiao et al., 1998; Hayashi et al., 2006). Overexpression of Homer1a in cultured hippocampal neurons, or induction of Homer1a expression by BDNF, prompts a reduction in long Homer1c clusters, without decreasing the amount of protein (Inoue et al., 2004). Clusters of F-actin and PSD-95 are similarly reduced, through disruption of their association with Shank protein (Sala et al., 2003; Inoue et al., 2004; Hayashi et al., 2009), and dendritic spine size is decreased (Sala et al., 2003). Homer1a therefore appears to redistribute functional synaptic architecture constructed by long Homer1 proteins, in response to neuronal activity, to bring about modifications in PSD scaffolds and spine morphogenesis, indicative of synaptic plasticity (Bosch et al., 2014; Tønnesen et al., 2014).

The interaction between Homer and group I mGluRs (mGluR1 and mGluR5) has been a particular focus of functional research into Homer proteins, because of the role of Homer as an intermediary between mGluRs and their effector proteins, including the NMDA receptor (Tu et al., 1998; Bertaso et al., 2010; Sylantyev et al., 2013). Reciprocal signal transduction between group I mGluRs and NMDA receptors permits the regulation of NMDA-evoked currents by mGluR activation (Fitzjohn et al., 1996; Awad et al., 2000; Bertaso et al., 2010; Sylantyev et al., 2013), which is required for certain NMDA-dependent processes (Jia et al., 1998; Rodrigues et al., 2002). The directionality of NMDA modulation by group I mGluRs varies between studies. Whilst some studies report that group I mGluR activation augments NMDA activity (Fitzjohn et al., 1996; Awad et al., 2000; Sylantyev et al., 2013), others report inhibition (Yu et al., 1997;

Bertaso et al., 2010; Moutin et al., 2012). The emerging explanation for these opposing outcomes is that it depends upon the relative involvement of long and short Homer1 proteins.

NMDA receptors are located within the PSD, whereas group I mGluRs are typically located at extrasynaptic and perisynaptic regions (Baude et al., 1993; Lujan et al., 1996) and do not colocalize with NMDA receptors (Moutin et al., 2012). Instead, interactions between the two glutamate receptors are mediated by a Homer-Shank-GKAP-PSD95 scaffold (Naisbitt et al., 1999; Tu et al., 1999). It has been suggested that mGluRs use this long Homer-containing scaffold to facilitate NMDA activity, supported by the observation that the potentiation of NMDA receptor currents by group I mGluR agonists is blocked by postsynaptic transfection of Homer1a (Sylantsev et al., 2013). Other studies have similarly demonstrated a Homer1a-dependent inhibition of NMDA receptor activity, whilst showing that it is calcium-independent (Bertaso et al., 2010) and requires the integrity of the EVH1 domain of Homer1a (Sala et al., 2003), highlighting further the functional importance of mGluR-Homer interactions.

This model does not yet fully explain how group I mGluRs could promote the inhibition of NMDA currents in the presence of Homer1a, as reported by some studies using sustained stimulation paradigms (Bertaso et al., 2010; Moutin et al., 2012). mGluR1a-mediated Homer1a-dependent inhibition of NMDA receptors gives rise to the co-immunoprecipitation of G β -protein with NMDA NR1 subunits and is attenuated by disrupting G-proteins (Bertaso et al., 2010). Moreover, these results could be imitated by transfecting neurons with Shank3 mutants that are unable to bind Homer complexes, but not by mutants unable to bind the GKAP-PSD95 complex (Bertaso et al., 2010). These

data indicate that Homer1a may release group I mGluRs from their perisynaptic attachment to the scaffolding complex, permitting their translocation into the PSD and physical inhibition of NMDA receptors via G $\beta\gamma$ subunits. This notion is supported by a recent bioluminescence resonance energy transfer (BRET) study of cultured hippocampal neurons, which imaged spine-specific protein-protein interactions between mGluR5a and NMDA receptors only in the presence of Homer1a, prompting an inhibition of NMDA currents (Moutin et al., 2012).

Hence, group I mGluRs regulate NMDA receptor activity. Whether that regulation is facilitatory or inhibitory is determined by the mutually exclusive interaction with long or short Homer proteins, respectively. Homer1a-induced scaffold remodelling represents a switch for mGluR function upon synaptic stimulation that could be important for the initiation of plasticity processes such as long-term potentiation or long-term depression.

1.4.6 Calcium Homeostasis

The majority of the proteins that Homer is known to interact with are involved in calcium homeostasis, including mGluRs, IP3 receptors, TRPC channels, ryanodine receptors and L-type voltage dependent calcium channels. Elevation in intracellular calcium in dendritic spines modifies neuronal excitability, initiates signalling cascades and recruits new membrane proteins, ultimately adjusting synaptic strength (Berridge, 1998; Yang et al., 1999; Yeckel et al., 1999).

It has long been recognised that group I mGluRs regulate the activity of the calcium releasing IP3 receptors on intracellular endoplasmic reticulum stores via Homer and Shank protein scaffolds (Kawabata et al., 1998; Tu et al., 1998; Hwang et al., 2005; Sala et al., 2005). In neocortex pyramidal neurons, intracellular injection of Homer1a dose-

independently enhances spike-induced calcium increases through mGluR-IP3 signalling (Yamamoto et al., 2005). The same is not achieved by injection of Homer1b/c. However, the maintenance of this Homer1a-induced calcium augmentation depends instead on elevated calcium influx through L-type voltage dependent calcium channels. It was demonstrated that Homer1a lowers the threshold for calcium spikes by facilitation of the currents through these channels (Yamamoto et al., 2005).

Indeed, L-type voltage dependent $Ca_v1.2$ and $Ca_v1.3$ calcium channel subunits possess Homer binding domains on their carboxyl termini (Olson et al., 2005; Huang et al., 2007). A study of excitation-contraction coupling, which modelled calcium-induced calcium release by ryanodine receptor 2 (RyR2) and $Ca_v1.2$ in human embryonic kidney (HEK) cells, suggests that the communication between $Ca_v1.2$ at the cell surface and RyR2 on endoplasmic reticulum is mediated through the Homer-calcium channel interaction (Huang et al., 2007). Long Homer1b/c facilitates an interaction between RyR2 and $Ca_v1.2$, which decreases the sensitivity of the cell to membrane depolarization-induced calcium elevations, whereas Homer1a disassembles the interaction, increasing the sensitivity and efficiency of calcium-induced calcium release (Huang et al., 2007; Figure 1.5). These observations corroborate closely with those from neocortex pyramidal neurons (Yamamoto et al., 2005), both illustrating how activity-induced Homer isoforms permit a rise in intracellular calcium concentration.

The link between ryanodine receptors and L-type voltage gated calcium channels is not the only occurrence of Homer1 facilitating the signal transduction between endoplasmic reticulum and cell membrane proteins involved in calcium homeostasis. IP3 receptors communicate with transient receptor potential canonical (TRPC) family cation channels

within a complex assembled by Homer. Similarly to the regulation of L-type calcium channels, the disassembly of this complex increases channel activity and calcium influx (Yuan et al., 2003). TRPC-Homer-IP3R disassembly is regulated by calcium store depletion and IP3-IP3R binding (Yuan et al., 2003, 2012; Kim et al., 2006), although expression of Homer1a similarly causes complex dissociation by interrupting the coupling between long Homer isoforms and TRPC, permitting channel opening by stromal interacting molecule 1 (STIM1; Yuan et al., 2003, 2012; Dionisio et al., 2015). Homer1a has also been reported to translocate TRPC complexes to the membrane, where they are spontaneously active (Kim et al., 2006).

These studies collectively demonstrate that activity-induced Homer proteins dynamically regulate the communication between calcium signalling proteins to achieve the facilitation of calcium currents. Calcium elevations through NMDA and mGluR-coupled signalling cascades are important components of spike-timing-dependent synaptic plasticity and determinants of the directionality of changes in synaptic strength (Kampa et al., 2006; Nevian and Sakmann, 2006). Therefore, the role of Homer1a in initiating calcium entry is highlighted as a key modulator of synaptic plasticity.

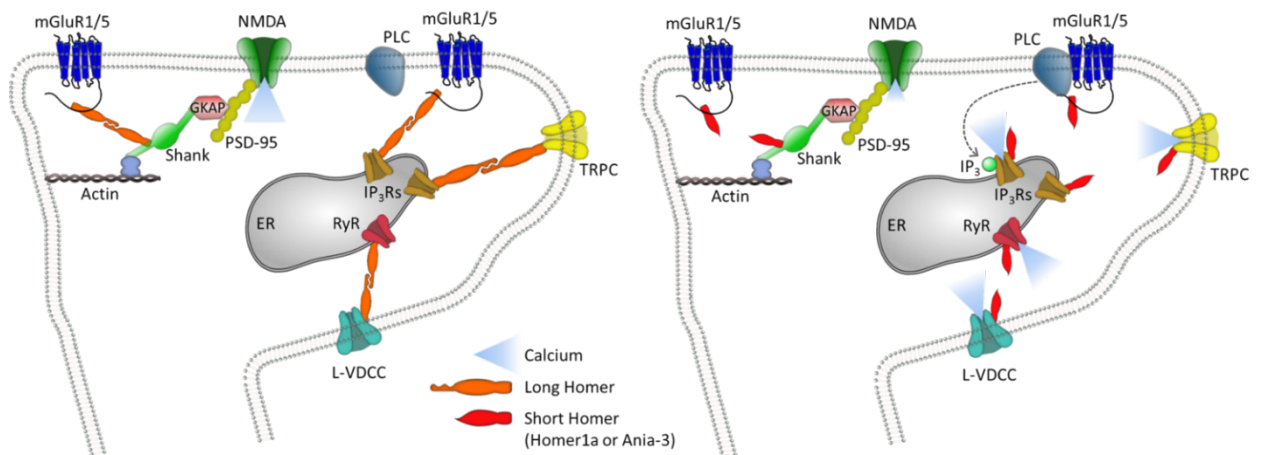


Figure 1.5 The regulation of postsynaptic calcium homeostasis by Homer proteins. Long Homer proteins (left) link calcium-regulating proteins into complexes by forming dimers through their carboxy-terminal domains. Activity-induced short Homer proteins (right) act as dominant negative regulators of long Homer complexes, disrupting the links between synaptic proteins and affecting the gating of calcium. A group I mGluR-dependent potentiation of NMDA receptors is facilitated by a long Homer-Shank-GKAP-PSD95 scaffold, which is dismantled by short Homers. Short Homer proteins may also elicit the agonist-independent activation of group I mGluRs. TRPC and L-VDCC channels form complexes with IP₃Rs and RyRs, respectively, through long Homer dimers, which limits the influx of calcium. The induction and binding of short Homers disrupts these complexes, increasing channel activity and calcium influx. ER, endoplasmic reticulum; GKAP, guanylate kinase-associated protein; IP₃R, inositol 1,4,5-triphosphate receptor; L-VDCC, L-type voltage-dependent calcium channel; mGluR1/5, metabotropic glutamate receptor 1/5; NMDA, N-methyl-D-aspartate; PLC, phospholipase C; PSD-95, postsynaptic density protein 95; RyR, ryanodine receptor; TRPC, transient receptor potential canonical channel.

1.4.7 Functional Plasticity

I have discussed how activity-induced Homer protein is expressed during synaptic plasticity and interacts with other PSD components responsible for bringing about changes in synaptic strength; I now present the evidence that short Homers are themselves functionally involved in synaptic plasticity. This work implicates Homer1a and Ania-3 in the local regulation of synaptic strength and the global scaling of synaptic excitability.

Synaptic strength may be modified by the trafficking and phosphorylation of AMPA-type glutamate receptors in the postsynaptic membrane, causing an adjustment of excitatory postsynaptic currents (Bredt and Nicoll, 2003; Derkach et al., 2007). This process is in part driven by group I mGluR activation (Snyder et al., 2001; Kelly et al., 2009). Overexpression of Homer1a in hippocampal neurons can globally decrease surface AMPA receptors (Sala et al., 2003; Hu et al., 2010; Rozov et al., 2012), reduce GluA2 subunit tyrosine phosphorylation (Hu et al., 2010) and block the maintenance of long-term potentiation (Celikel et al., 2007; Rozov et al., 2012). These effects are dependent on group I mGluR signalling, yet do not require the binding of glutamate to the receptors (Hu et al., 2010). Indeed, Homer1a has been shown to activate group I mGluRs independently of glutamate (Ango et al., 2001; Hu et al., 2010). Conversely, knocking out either the Homer1 gene or the short Homer1 isoforms results in an up-regulation of postsynaptic AMPA receptors and currents (Hu et al., 2010; Rozov et al., 2012). In contrast, one study reported an increase in synaptic AMPA receptors following Homer1a overexpression (Hennou et al., 2003), which may reflect differences in experimental protocols, whilst supporting the notion that scaffold remodelling can lead to the scaling

up or down of synapses, depending on the nature of the stimulation. Van Keuren-Jensen and Cline (2006) demonstrated in tadpole optic tectal neurons that the application of group I mGluR agonists to cells with a low Homer1a/Homer1b ratio increased AMPA currents, similarly to cells that had been removed from tadpoles after >12h of darkness. Conversely, either the exogenous expression of Homer1a, or Homer1a induction through visual stimulation for >4h, reversed the mGluR-mediated change in AMPA currents (Van Keuren-Jensen and Cline, 2006). These studies clearly demonstrate the involvement of Homer1a in AMPA receptor regulation, whilst indicating a specific role of the protein in the homeostatic scaling of synaptic strength.

The mechanism through which Homers regulate mGluR-mediated AMPA plasticity may involve their coupling to dynamin 3 in endocytic zones (Gray et al., 2003). Long Homers physically tether clathrin-positive endocytic zones near the PSD through interactions with Shank and dynamin 3, to permit local cycling and recapture of AMPA receptors (Gray et al., 2003; Zhang et al., 2007). Disruption of this link by the expression of Homer1a in hippocampal neurons frees the endocytic zones from the PSD, which leads to a decrease in the number of postsynaptic membrane AMPA receptors (Lu et al., 2007). Therefore, Homer1a induction can remove the AMPA cycling capacity of dendritic spines, regulating synaptic strengthening.

The long-term maintenance of synaptic strength for memory consolidation requires *de novo* protein synthesis and scaffold remodelling over an extended period of time following an initial synaptic event (Kandel, 2001; Lee et al., 2004). One of the intriguing aspects of Homer1a function is its expression following neuronal activity, which occurs in conjunction with the expression of Arc, another IEG essential for memory

consolidation (Vazdarjanova et al., 2002; Plath et al., 2006). Both are induced in the same hippocampal neurons by exploration of a novel environment (Vazdarjanova et al., 2002; Marrone et al., 2008), implying that their coordinated activity may be necessary for forming a memory pertaining to that environment. Further work has examined the recruitment of Homer1a to active synapses in order to select spines for plasticity. Okada and colleagues (2009) demonstrated that soma-derived Homer1a is recruited from dendrites specifically into activated spines. This only occurred if NMDA receptors were stimulated and required extracellular calcium and the nitric oxide-protein kinase G signalling pathway. Trapping of Homer1a into spines did not, however, require group I mGluR activation (Okada et al., 2009a). The recruitment of Homer1a may therefore represent a synaptic tag for subsequent spine remodelling and the targeting of *de novo* plasticity-related proteins.

A few studies have evaluated the effect of manipulating the expression of short Homer isoforms on learning and memory. Overexpression of Homer1a in the hippocampus impairs spatial working memory and spatial reference memory (Klugmann et al., 2005; Celikel et al., 2007). Short form-specific Homer1 knockout mice exhibit a deficit in cued and contextual fear conditioning (Inoue et al., 2009a; Mahan et al., 2012). These mice have normal memory acquisition and short term memory of contextual fear, but impaired fear memory consolidation and memory retention, reminiscent of Arc knockout mice (Plath et al., 2006), another IEG critical for regulating synaptic plasticity. Interestingly, re-exposing short Homer1 knockout mice to the context, and therefore reactivating the fear memory, attenuated the conditioned response, indicating a deficit in reconsolidation or memory updating (Inoue et al., 2009a). These studies form

additional corroboration to the view that activity-induced Homer proteins are critical for coordinating plasticity relevant to learning and memory. It should be noted that, since both Homer1a and Ania-3 are absent in these knockout models, delineation of their respective contributions to fear memory consolidation is lacking.

1.4.8 Conclusions

Homer1a is recruited to active dendritic spines upon stimulation and de novo Homer1a protein is synthesised somatically and transported to the PSD, thereby facilitating short- and long-term scaffold remodelling for early and late phases of synaptic plasticity. Primarily, its function is the dominant negative disruption of functional synaptic architecture constructed by long Homer1 proteins, which can result in a switch of group I mGluR function, receptor translocation or the accommodation of calcium entry. These findings suggest that the role of short Homer proteins is to transiently destabilise the PSD in order to permit scaling of the most currently or recently active synapses; or in other words, to selectively facilitate the plasticity of excitatory synapses. Disruption of Homer1a and Ania-3 has implications for neurological disease and their manipulation may represent a novel therapeutic approach.

1.5 Experimental Plan

Synaptic plasticity is presented here as a critical point of convergence in schizophrenia pathogenesis, particularly in relation to NMDA receptor and postsynaptic density protein complexes. It is noted that this may mediate specific deficits in associative learning processes. In this thesis, I further the investigation of the role of associative learning in schizophrenia through studying the impact that genes and pathways conferring risk to the disorder have on the consolidation, retrieval and extinction of contextual fear memory. My aims were as follows.

- Determine whether genes associated with particular components of associative learning are enriched in the genetic variants from patients with schizophrenia and related disorders.
- Explore the effect of the NMDA antagonist and psychotomimetic, ketamine, on the consolidation and extinction of contextual fear memory.
- Deduce the role of postsynaptic density Homer1 proteins in associative fear learning processes.

Chapter 2: General Methodology

This chapter describes the general protocol for each method used in this thesis. Details of the specific methodology used in each experiment is given within its respective chapter.

2.1 Copy number variant enrichment analysis

Enrichment analyses are used to determine whether a gene set is overrepresented within a reference gene list, compared to that which you would expect by chance. They can also be used to compare the relative enrichment of a gene set in genetic mutations from patients of a particular disease, relative to its enrichment in mutations from healthy controls. For CNVs, this can be done using a logistic regression analysis that regresses, for each CNV, case-control status of the patient on the number of genes from the gene set that the CNV overlaps with.

2.1.1 Logistic regression analysis

Gene sets were formatted as tab-delimited text files containing the human Entrez ID for each gene. CNV lists were annotated with CNV size, deletion or duplication, case-control status, chip type (Affymetrix chip ID), CNV study name, number of genes hit by the CNV and Entrez gene IDs. Using MATLAB (R2015a) or R (v 3.2.3), the number of genes in a gene set overlapped by each CNV was counted. The logistic regression analysis was performed using the general linear model (glm; binomial) function in R, as follows:

$$\text{case-control status} \sim \text{gene set hits} + \text{CNV size} + \text{total genes in CNV} + \text{study} \\ + \text{chip type}$$

P -values outputted by this analysis were converted to $-\log_{10}(P\text{-value}) \times \text{sgn}(Z\text{-value})$.

2.1.2 Permutation correction

In order to remove the effect of any background enrichment in the gene sets, some analyses were subjected to permutation correction. This adjusts the observed enrichment P -value so that it is empirically determined by a distribution of P -values obtained from randomly permuted gene sets.

Using MATLAB, all genes (after any filtering processes specific to the study) were permuted many times (e.g. 1000). Following each permutation, the top N genes were taken as a random gene set, where N is the size of the gene set used for the analysis being corrected. Each gene set was subjected to a logistic regression analysis as described above, producing a null distribution of P -values (or $-\log_{10}(P\text{-value})$). An empirical P -value is then calculated as follows:

for $y = y_1 \dots y_N$ given x

$$P = \frac{\sum_{i=1}^N z}{N} \text{ where } z = \begin{cases} 1 & y_i \leq x \\ 0 & y_i > x \end{cases}$$

where y is the null distributed P -values and x is the observed P -value.

2.2 Common variant enrichment analysis

The enrichment of a gene set in common variants is done using whole genome data analysis toolsets, such as PLINK and MAGMA. Due to the different statistical models employed by each approach, they may yield different results when applied to the same dataset.

2.2.1 PLINK SNP analysis

Gene sets were converted to genomic locations using the genome build 37.3 sequence file found at the NCBI FTP site:

(ftp://ftp.ncbi.nih.gov/genomes/MapView/Homo_sapiens/sequence/BUILD.37.3/initial_release/seq_gene.md.gz).

1000 Genomes reference files (<http://www.1000genomes.org>) were used for linkage disequilibrium calculations. Case-control SNPs were mapped to the gene set and the enrichment probability calculated using Plink's "--set-screen" function. Each analysis yielded *P*-values from two levels of statistical stringency. *P*₁ is an uncorrected *P*-value and *P*₂ is an empirical *P*-value corrected for the multiple SNPs within the set.

2.2.2 MAGMA SNP analysis

MAGMA (Multi-marker Analysis of GenoMic Annotation) uses a multiple regression model and performs single gene analysis in conjunction with gene set analysis for added flexibility.

Case-control SNP data was annotated with gene location using NCBI Build 37.3 gene locations obtained from (http://ctg.cncr.nl/software/magma/aux_files/NCBI37.3.zip). Then a gene-wide analysis was performed, using the 1000 Genomes reference files (as above) for linkage disequilibrium calculations. Gene set analyses were performed using the output from the gene-wide analysis. During this analysis, MAGMA executed a correction for false discovery rate using 100,000 data permutations.

2.3 The ethical use of animals in research

All experiments were conducted in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act (Project licenses PPL 30/3135 and PPL 30/2236; Personal license PIL I16A5E86C).

2.4 Contextual fear conditioning behavioural paradigms

2.4.1 Subjects

Subjects were adult male Lister Hooded rats from Charles River UK, weighing 250-350 g (8-14 weeks old). Upon arrival, rats were housed in pairs in conventional cages in a holding room maintained at 21°C on a 12h reversed light/dark cycle. All experiments were conducted during the dark period. Food (chow) and water were freely available and the cages were cleaned weekly. A cardboard tube was provided as environmental enrichment. Rats were habituated to their home cages for at least 5 days prior to the experiment start. Tails were labelled for identification using a permanent marker pen. The handling of animals from each experimental group was ordered pseudorandomly.

2.4.2 Contextual fear conditioning, recall and extinction

The protocol described here is a well-established paradigm for studying associative long-term memory in rodents (Lee et al., 2004; Barnes and Thomas, 2008), in which a rat learns to associate a novel context with the receipt of an electric footshock. Rats were transported from the holding room to the behaviour suite in dark transport boxes to avoid exposing the animals to the light during their dark period. Rats were placed individually into either conditioning context A or B. Conditioning contexts were 30.5 cm x 24.1 cm x 29.2 cm chambers (Med Associates, UK) with 19 floor bars raised 1.6 cm above a floor tray, all contained within a sound-attenuating cubicle 55.9 cm x 55.9 cm x

35.6 cm. During conditioning trials, rats received a 2 second 0.5 mA footshock after 2 min context exposure. The footshock was administered through the floor bars and was controlled by a Med-PC (version IV) Research Control and Data Acquisition System (Med Associates). Rats were removed from the context 1 min after footshock and transported back to their home cages.

Recall and extinction trials were conducted at the same time of day as the conditioning trial. Rats were transported to the context in the same transport boxes. In recall trials, rats were returned to the context for 2 min. In extinction trials, rats were returned to the context for 10 min. Proof of concept experiments are presented in sections 4.2-4.3.

All periods of context exposure were recorded on video (JSP Electronics, China), using Numeriscope software (Viewpoint, France), for behavioural analysis.

2.4.3 Quantification of freezing response

The freezing response of the animals was used as a metric of fear memory acquisition and recall. Files containing video recordings of context exposures were randomised programmatically in order to blind the experimenter to the experimental group of each subject, thereby minimising subjectivity during analyses. Freezing behaviour was defined as the complete and sudden cessation of movement and was recorded as a binary variable every 10 sec (freezing / not freezing), starting from the time at which the door to the conditioning context was closed. Percent freezing therefore represents the proportion of samples in which the rat was observed freezing.

2.5 *In situ* hybridization

In situ hybridization is a method of localising and quantifying specific mRNA sequences in fixed tissue sections by hybridising with labelled strands of complementary nucleotide sequences (Figure 2.1).

2.5.1 Brain extraction, coronal sectioning and paraformaldehyde fixation

Rats were killed by CO₂ inhalation at the time point of interest. They were then immediately decapitated and the brain was extracted and snap frozen on dry ice within 10 min of death. Brains were wrapped in Parafilm (Sigma-Aldrich, USA) and foil before storage at -80°C until needed.

Microscope slides used for mounting coronal brain sections were prepared by baking overnight at 180°C and allowing to cool before coating in poly-L lysine solution (2%). The slides were dried and stored at 4°C until use. A custom MATLAB program was used to design the arrangement of brain sections onto a library of microscope slides, so that there was a maximum of 6 sections per slide, each from a different subject, with pseudorandom subject selection and without position bias for any experimental group. From each subject, 114 14 µm coronal sections of the region of interest were cut using a cryostat (Leica Biosystems, Germany) and mounted on a series of glass slides. Slides were then racked into hollow glass slide racks for fixing and storage.

To fix the brain sections, slides were bathed in 4°C 4% paraformaldehyde / phosphate buffer saline (PBS; 1.3 M NaCl, 70 mM disodium phosphate, 30 mM monosodium phosphate) solution for 5 min, before washing in PBS (1 min) and 70% ethanol (4 min). Slides were then stored in 95% ethanol at 4°C until use. A minimum of 2 weeks was allowed for before performing *in situ* hybridization to allow for delipidation.

2.5.2 Oligonucleotide probe design

Single-stranded oligonucleotide probes were designed to target unique mRNA sequences. Due to the number of filtering steps required to identify a candidate oligonucleotide probe, a tool was developed in MATLAB to do this programmatically. Probes were designed to be only 45 base-pairs long to facilitate penetration into cells. Optimisation steps included choosing probes with G/C:A/T ratios close to 1 and excluding probes containing trains of the same nucleotide longer than 3. These steps permit consistency in hybridization conditions and prevent excessive secondary folding of the oligonucleotide.

On occasions where the probe is being designed to target a unique isoform, the probes are ranked by their maximum homology with the other isoforms of the same gene to allow identification of the most specific oligonucleotide probe.

2.5.3 5' end ³⁵S labelling of oligonucleotides

Oligonucleotide probes (Sigma-Aldrich) were radiolabelled with ³⁵S for detection. To do this, pure oligonucleotides were dissolved to 1 µg/µl in DEPC-treated phosphate buffer before dilution to 5 ng/µl in DEPC-treated water. This was added to the radioisotope in the following solution: 12.5% recombinant terminal deoxynucleotidyl transferase (rTDT) enzyme (Promega, USA), 20.1% 5x TDT buffer (Promega), 16.7% 5 ng/µl oligonucleotide probe, 12.5% ³⁵S-dATP (87-day half-life; Perkin-Elmer). The solution was incubated at 35°C for 1 h before diluting 4.2X in DEPC-treated water. In order to extract the labelled probe, the solution was spun for 2 min at 2000 rpm on a spin column consisting of a 1 ml syringe filled with Sephadex G-25 (Sigma-Aldrich) TNES (0.14 mM NaCl, 20 mM Tris,

5 mM EDTA, 0.1% SDS) slurry. Dithiothreitol (DTT; Sigma-Aldrich) was added to 3.8% to terminate the reaction, before storage at -20°C until use.

2.5.4 Hybridization of coronal brain sections

The specific activity of the labelled oligonucleotide probe was calculated by mixing a sample of it with scintillation fluid at a ratio of 1:1000 and quantifying the disintegrations per minute (dpm) using a liquid scintillation counter (Tri-Carb 2800 TR; Perkin-Elmer, USA). Coronal brain sections representing the same region along the sagittal plane were selected. Each slide of coronal brain sections was covered with a solution containing $\frac{200}{\text{Probe specific activity per } \mu\text{l}}$ μl oligonucleotide probe, 100 μl HYB buffer (50% deionised formamide, 20% 20X saline-sodium citrate buffer, 5% 0.5 M sodium phosphate, 1% 0.1 M sodium pyrophosphate, 10% 50X Denhardt's solution, 5% 4 mg/ml acid-alkali hydrolysed salmon sperm DNA, 2% 5 mg/ml polyadenylic acid, 0.1% 120 mg/ml heparin and 10% dextran sulphate) and 2 μl DTT. Control (non-specific) slides were covered in the same solution with the addition of $\frac{200}{\text{Probe specific activity per } \mu\text{l}} \times 4$ μl 5 ng/ μl unlabelled oligonucleotide probe. A strip of Parafilm (Sigma-Aldrich) acted as a coverslip on each slide, before incubation in a humid environment overnight at 42°C. Slides were bathed in 1X saline-sodium citrate buffer (SSC) at room temperature to facilitate the removal of the coverslips. The slides then underwent washes in 52°C 1X SSC (30 min), 52°C 1X SSC (30 min), room temperature 0.1X SSC (1 min), 70% ethanol (1 min) and 95% ethanol (1 min).

2.5.5 Imaging and quantification of gene expression

When dry, labelled slides were arranged in a radiographic cassette, along with a ^{14}C standard slide for calibration, sealed underneath a sheet of radiographic film (American

Radiolabeled Chemicals, USA). The duration for which the slides were left against the radiographic film depended on the intensity of the autoradiograph produced by the oligonucleotide probe (typical variation from 3-30 days) and preliminary tests were performed to optimize this duration. The film was developed using an EXOMAX x-ray processor (PROTEC, Germany) and scanned to produce a digital greyscale image (Figure 2.1).

Autoradiographs of labelled brain sections were analysed using ImageJ (NIH). Analysis was performed blind to the respective experimental groups. Densitometry was calibrated using known radioactive quantities from the ^{14}C standards. Triplets of intensity measurements were sampled from each region of interest, per hemisphere, per section. For each subject, the intensity of signal within a brain region, representing the quantity of mRNA expressed, was calculated as follows.

$$\text{mRNA expression} = \frac{\widetilde{sA} + \widetilde{sB}}{2} - \widetilde{ns}$$

where Sa and Sb are the densitometry values sampled from the specific signal of one brain region in sections A and B of a subject (Figure 2.1) and ns is the densitometry value acquired from the control (non-specific) section from the same subject. Expression values for each brain region were normalized to the mean expression of the control group.

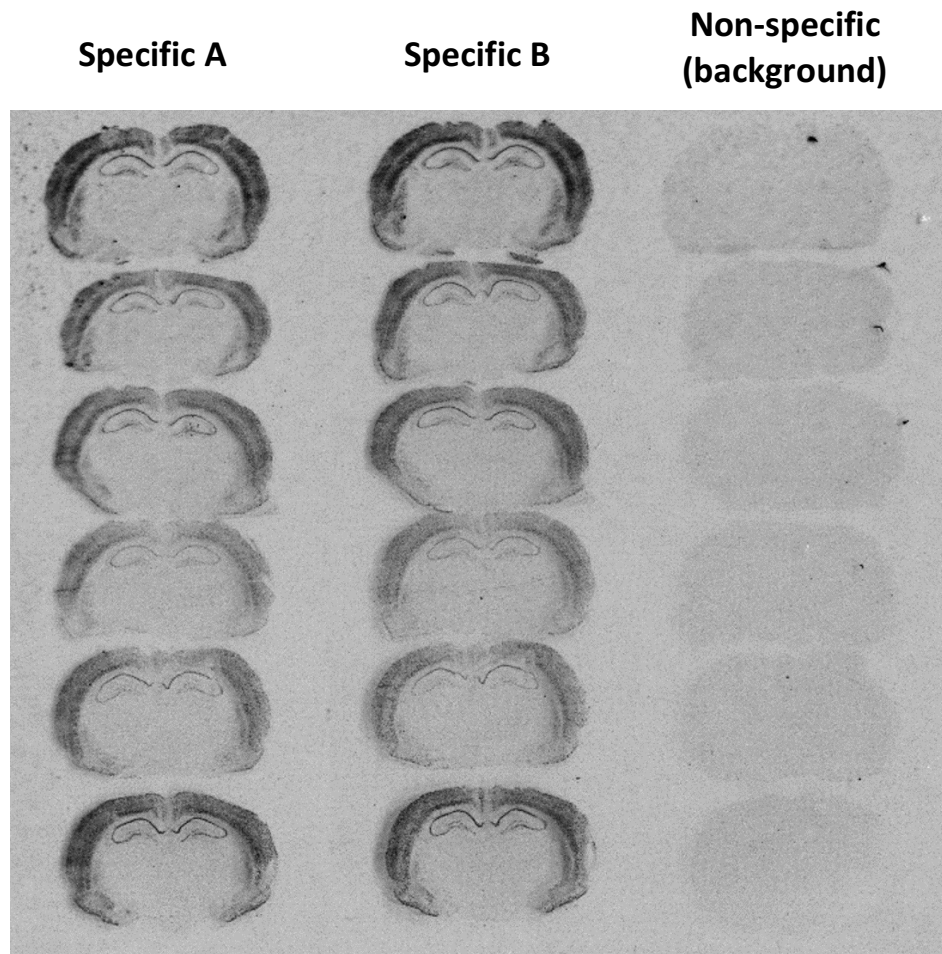


Figure 2.1 *In situ* hybridization autoradiograph from coronal rat brain sections hybridized with radiolabelled oligonucleotides specific to the Homer1a mRNA sequence. The first and second columns are experimental repeats, showing the specific binding of the oligonucleotide to its complementary mRNA sequence on two separate slides of brain sections. Sections in the third column have been hybridized with an excess of unlabelled probe, giving the background signal, which is deducted from the specific signal during quantification.

2.6 Immunoblotting

Immunoblotting was used to quantify proteins from hippocampal tissue samples obtained from behaving rats.

2.6.1 Tissue homogenisation and sample preparation

2.6.1.1 Preparation of whole hippocampal homogenates

Snap-frozen, micro-dissected hippocampi were stored at -80°C until preparation for immunoblotting. For the preparation of whole hippocampal fractions, 30-100 mg hippocampal tissue was homogenised with 4°C 10 $\mu\text{l}/\text{mg}$ radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, USA) and protease inhibitors (Roche, Germany), using a glass 3 ml pestle and mortar on ice. Homogenates were centrifuged at 1200g for 20 min at room temperature to pellet and remove nuclei. Samples were stored at -80°C until use.

2.6.1.2 Preparation of hippocampal synaptosomes

To prepare synaptosomal fractions of hippocampal tissue, 30-100 mg hippocampal tissue was homogenised with 4°C Synaptic Protein Extraction Reagent (Syn-PER; Thermo Fisher, USA) and protease inhibitors (Roche, Germany) using a glass 3 ml pestle and mortar on ice. Homogenates were centrifuged at 1200g for 10 min at 4°C . The pellet was discarded and the supernatant was further centrifuged at 15000g for 20 min at 4°C . The supernatant, which contains the cytosolic fraction, was stored for reference. The pellet was re-suspended in 1 ml/g (original hippocampal tissue) Syn-PER with protease inhibitors, and re-homogenised, before storage at -80°C .

2.6.1.3 Global protein quantification using the Bradford or BCA assay

Total protein concentration of samples homogenised in Syn-PER was calculated using the Bradford assay. A concentration gradient of bovine serum albumin (BSA; Bio-Rad, USA) was used to create a standard curve for reference. Samples were diluted 10X in Syn-PER and mixed 1:50 with Bradford reagent (Bio-Rad) in a cuvette. Optical density was determined at 595 nm.

Since the Bradford assay is sensitive to buffers containing strong detergents, total protein concentration of samples homogenised in Radioimmunoprecipitation assay (RIPA) buffer was determined using the bicinchoninic acid (BCA) assay (Pierce BCA Protein Assay Kit; Thermo Fisher, USA). Samples were diluted 10X in RIPA, before being added to BCA reagent mix 1:20 and incubated at 37°C for 30 min. Following incubation, samples were cooled rapidly to room temperature and the optical density was determined at 562 nm.

2.6.2 Western blot procedure

To separate the proteins, samples were subjected to gel electrophoresis in an electrophoresis chamber (Bio-Rad). Prior to loading onto the gel, samples were diluted to equal protein concentrations and boiled with 45% Laemmli sample buffer (Bio-Rad) and 5% β -mercaptoethanol (Bio-Rad) at 96°C for 5 min. Samples containing 10-40 μ g protein were loaded into each well of a 12% precast polyacrylamide gel (Bio-Rad), bordered by 5 μ l prestained protein standards (Bio-Rad). Electrophoresis was run at 50V for 10 min, followed by 150V for 40 min, or until the sample has traversed the gel.

Proteins were transferred to a nitrocellulose membrane (Bio-Rad), using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were blocked in a 5% milk (Amersham ECL

Prime, GE Healthcare, UK) and Tris-buffered saline with Tween (TBST; 50 mM Tris, 150 mM NaCl, 0.05% Tween 20) solution for 1 h at room temperature. Primary antibodies were diluted in 5% milk / TBST and incubated with the membrane overnight at 4°C. The membrane was washed three times in TBST for 5 min, before incubating with secondary fluorescent antibodies (LI-COR), diluted in 5% milk / TBST, for 1 hour at room temperature. The membrane was washed a final three times in TBST (twice) and TBS (once) for 5 min each. Protein bands were imaged using an Odyssey Clx Infrared Imaging System (LI-COR).

2.6.3 Quantification of protein of interest

Typically, each membrane was probed with antibodies targeting the protein of interest as well as a housekeeping gene, the expression of which is known to be unaffected by the experiment manipulation, for normalisation and quality control. Western blots were analysed using Image Studio (LI-COR). Relative protein quantities were calculated as follows.

$$\text{Protein of interest in sample (arbitrary unit)} = \frac{P_i - pB_i}{H_i - hB_i}$$

where each variable is a fluorescence intensity: P is protein of interest, H is housekeeping gene, pB is the background surrounding the protein of interest, hB is the background surrounding the housekeeping gene and i is the lane.

2.7 Intracerebral microinjection of substances

2.7.1 Surgical placement of indwelling cannula to the dorsal hippocampus

Rats were anaesthetised in an induction chamber using 5% isoflurane (Abbott, UK), before the removal of fur on the top of the head and positioning of the animal in a

stereotaxic frame (David Kopf, USA), where 2-3% isoflurane was continuously administered via a gaseous mixture of oxygen (0.8 L/min) and nitrogen (0.4 L/min). A midsagittal incision was made to expose the skull and two holes were drilled at the following coordinates from Bregma: -3.5 mm anterior-posterior, \pm 1.9 mm medio-lateral. A bilateral guide cannula (22 gauge, 3.8 mm centre-to-centre, 3 mm below pedestal; Plastics One, UK) was implanted targeting the dorsal hippocampus and secured with screws and dental acrylic (Kemdent, UK). A dummy stylet (Plastics One) was inserted into the cannula to prevent blockage. 0.02 ml 5 mg/ml Metacam was administered subcutaneously approximately 10 min prior to the end of surgery. After surgery, the rat was placed in a warm (22°C) recovery chamber for 30-60 min and given a minimum 7 days recovery before testing.

2.7.2 Bilateral hippocampal infusion

To administer substances via intracerebral microinjection, the dummy stylets were replaced with injector stylets (28 gauge, 4 mm; Plastics One) attached to two Hamilton syringes (Hamilton, Switzerland) by polyethylene tubing, fixed to a syringe pump (Harvard Apparatus, UK). Substances were infused at 1 μ l/hemisphere at a rate of 0.5 μ l/min. A further 2 min was allowed after infusion before removing the injectors to permit sufficient diffusion of the solution into the tissues.

Each subject underwent a practice infusion session using sterile PBS at least 3 days prior to testing, in order to habituate the animal to the process.

2.7.3 Histology

Nissl staining was performed on sections of brain tissue taken from rats used in surgical procedures to verify the placement of cannulae and the site of infusion.

14 µm coronal sections were cut and fixed as described in section 2.5.1. Slides were then washed as follows: distilled water (1 min), 0.1% Thionin (10 min), distilled water (1 min), 70% ethanol (1 min), 95% ethanol (1 min), 95% ethanol (1 min), 100% ethanol (1 min), 100% ethanol (1 min). Stained sections were bathed in Histoclear (National Diagnostics, UK) for at least 30 min, before being dried and covered with DePeX mounting medium (BDH Laboratory Supplies, UK) and a glass coverslip (Agar Scientific, UK).

Sections were imaged using a Leica DM2000LED microscope and the injection site was identified by finding the location of the most ventral point of the implant. Local inflammation and tissue damage was noted. Accurate bilateral dorsal hippocampal infusion was a criterion for inclusion of a subject's data in analysis.

Chapter 3: The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders

3.1 Introduction

The pathophysiology of schizophrenia is unknown, yet its high heritability implicates inherited genetic variants in its aetiology (Sullivan et al., 2003). A major branch of research seeks to identify genetic variants associated with the disorder through large-scale genomic studies. In recent years, this approach has progressively revealed a growing list of common variants, rare variants and structural variants that increase an individual's susceptibility to schizophrenia (Stefansson et al., 2008; Stone et al., 2008; Kirov et al., 2012; Ripke et al., 2013, 2014, Rees et al., 2014a, 2014b; Pocklington et al., 2015). Each genetic variant may contribute a relatively small component of risk, with rare variants typically contributing more risk than common variants, but collectively they can form a substantial burden that accounts for a large proportion of risk (Purcell et al., 2009; Stefansson et al., 2009). Some progress has been made in subsequently linking these genetic variants to individual genes and clustering affected genes into particular functional pathways (Hall et al., 2015; Kotlar et al., 2015; Owen et al., 2016). However, the insight from these analyses is limited by our current understanding of gene function and is therefore at present too broad to be of sufficient value to therapeutic design.

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It is notable that many of the genetic variants linked to schizophrenia also increase risk for the development of other disorders. For example, there is substantial overlap of the risk conferred by schizophrenia-associated common variants to bipolar disorder and major depressive disorder, and by schizophrenia-associated rare variants to intellectual disability and autism spectrum disorder (Lee et al., 2013; Doherty and Owen, 2014). This implies that there is substantial overlap between the molecular pathways involved in the pathophysiology of these phenotypically-related disorders. However, the neural circuits and cognitive domains that are consequently impaired may be very different (Millan et al., 2012).

3.1.1 A convergence of susceptibility genes on learning pathways

Despite the complex genetics of schizophrenia, pathway analyses across studies have found some points of convergence within the implicated genes. A predominant molecular convergence is that of synaptic proteins, particularly those of the postsynaptic density (Kirov et al., 2012; Fromer et al., 2014). Through analyses of inherited and *de novo* CNVs from patients with schizophrenia, Kirov et al. (2012) found an enrichment of members of the NMDA receptor complex, PSD-95 complex and activity-regulated cytoskeleton-associated protein (ARC) signalling complex (Kirov et al., 2012), all of which function at the synapse. Critically, these same protein complexes are also enriched for small variants observed in exome sequencing studies (Fromer et al., 2014; Purcell et al., 2014). A further group of genes, those targeted by fragile X mental retardation protein (FMRP), were also implicated through exome sequencing (Fromer et al., 2014; Purcell et al., 2014) and through the largest common variant study to date (Ripke et al., 2014). FMRP regulates the translation of a number of synaptic proteins

The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders (Fernandez et al., 2013) through its interactions with CYFIP1, which has been individually linked to schizophrenia through CNV studies (Stefansson et al., 2008; Kirov et al., 2012). Common variant studies of the Psychiatric Genomics Consortium have revealed associations with schizophrenia of several genes coding for synaptic proteins, including clusters of genes coding for voltage-gated calcium channel subunits and glutamate receptor subunits (Ripke et al., 2011, 2013, 2014).

Beyond the structural convergence of schizophrenia-associated genes at the synapse, further functional patterns have been noted amongst these genes. Many of the implicated synaptic proteins are key regulators of synaptic plasticity (Hall et al., 2015), both of excitatory and inhibitory systems (Pocklington et al., 2015). Pocklington et al (2015) explored the enrichment of 134 gene sets in patient CNVs from a large case-control cohort. The gene sets were derived from functional data, mostly curated in the Mouse Genome Informatics (MGI) Mammalian Phenotype database, and all related to aspects of brain function and development. As well as replicating previous findings, such as the overrepresentation of the NMDA receptor and PSD-95 complexes in patient CNVs, they also show that genes associated with particular plasticity processes, including long-term potentiation, contextual conditioning and associative learning, are enriched within CNVs from sufferers of schizophrenia (Pocklington et al., 2015). These findings are consistent with clinical studies showing deficits in associative-type learning in schizophrenia, which is thought to contribute to the manifestation of positive and cognitive symptoms (Serra et al., 2001; Corlett et al., 2009; Hall et al., 2009). However, there are multiple components to associative learning (Barnes et al., 2012; Maren et al.,

2013), and it is thus far unclear which precise learning processes are affected by schizophrenia-associated CNVs.

3.1.2 Differential regulation of consolidation, retrieval and extinction of aversive memory

Associative learning, and its constitutive components, has been studied in detail, particularly in relation to hippocampal processing of contextual fear memory (Barnes et al., 2012; Maren et al., 2013; Scholz et al., 2016). Such studies have shown that the consolidation, retrieval and extinction of contextual fear memory are dependent on distinct patterns of *de novo* gene expression, thereby recruiting different families of proteins to facilitate the particular memory process (Duvarci et al., 2008; Mamiya et al., 2009; Barnes et al., 2012; Scholz et al., 2016). Like memory consolidation, memory extinction is an active form of new learning, which occurs upon the prolonged or repeated exposure to a conditioned stimulus in the absence of a previously associated unconditioned stimulus, and results in dominance over the former associative memory (Bouton, 2004). Using microarrays to quantify gene expression in CA1 following fear memory consolidation, retrieval and extinction in rats, Scholz et al. (2016) were able to create gene sets associated with each learning stage and explore their respective molecular pathways. This data therefore provides a framework for further investigating the enrichment of associative learning-related genes in genetic variants from patients with psychiatric disorders.

Using gene sets obtained from the Scholz et al. (2016) study, I examined the hypothesis that genetic variants from patients with schizophrenia and related disorders are selectively located within genes related to specific components of associative learning.

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Specifically, I used gene set enrichment analysis to investigate whether genes differentially expressed following the consolidation, retrieval or extinction of contextual fear are enriched in CNVs from patients with schizophrenia, autism, intellectual disability or ADHD, or in the SNPs from patients with schizophrenia.

3.2 The enrichment of learning-related genes in schizophrenia-associated CNVs

3.2.1 Methods

The general methodology for enrichment analyses and contextual fear conditioning can be found in sections 2.1-2.4. The *in vivo* gene expression data used in this chapter was generated from experiments published in Barnes et al (2012) and (Scholz et al., 2016). Three independent expression datasets were generated representing genes regulated in rat CA1 associated with the consolidation, retrieval and extinction of contextual fear memory. Section 3.2.1.1, below, describes the methods employed in these previous studies.

3.2.1.1 The generation of learning-related gene expression data – previous studies

In brief, adult male Lister Hooded rats individually underwent contextual fear conditioning (see General Methods 2.4). In the consolidation protocol, awake rats (N = 8) received bilateral microinfusions of either brain-derived neurotrophic factor (BDNF) antisense (5'-TCTCCCCTTTAATGGT-3') or missense oligonucleotides (5' - ATACTTCTGTTCTTGCC-3') into the dorsal hippocampus (1 µl, 2 nmol/µl) via chronically implanted steel cannula, 90 min prior to the conditioning trial. The knockdown of hippocampal BDNF levels via antisense administration blocks the consolidation of contextual fear memory (Lee et al., 2004), therefore generating a control group. In the

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retrieval protocol, rats were replaced into the same context 48 hours after conditioning for a short recall trial (2 min). They (N = 8) received bilateral microinfusions via chronic indwelling cannulae targeting the dorsal hippocampus of either Zif268 antisense (5'-GGTAGTTGTCCATGGTGG-3') or missense oligonucleotides (5'-GTGTTCGGTAGGGTGTCA-3'; 1 μ l, 2 nmol/ μ l), 90 min prior to the short recall trial. Reducing hippocampal Zif268 levels using antisense impairs the post-retrieval memory processes that serve to maintain the expression of contextual fear memory (Lee et al., 2004; Trent et al., 2015). To generate the extinction dataset, rats were pre-exposed to two different novel contexts (Context A or Context B) over 3 days (10 min / day), prior to conditioning in either Context A or Context B. 48 hours later, rats underwent a long recall trial (10 min) in either the conditioned context (N = 6) or the unconditioned context. Those placed in the conditioned context exhibit a progressively decreased freezing response over the duration of the long recall trial, as, in the absence of the unconditioned stimulus, the association between the context and the footshock is extinguished, creating the extinction group.

All rats were killed by CO₂ inhalation 2 h after either the conditioning or recall trials and the dorsal CA1 region of the hippocampus dissected and snap frozen prior to gene expression analysis. Microarray analysis was performed using Affymetrix Rat Genome Array 230.2 (Thermo Fisher Scientific, US). Individual differential gene expression datasets associated with the consolidation, retrieval and extinction of contextual fear memory were generated using two normalisation methods: Microarray Suite 5 (MAS5) and Robust Multi-array Average (RMA). These normalisation methods each yielded a P-

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value per probe set, representing the probability of differential expression compared to the respective consolidation, retrieval and extinction control groups.

3.2.1.2 Extracting consolidation-, retrieval- and extinction-related gene sets

The initial step in the analysis was to extract gene sets from the microarray data associated with contextual fear memory consolidation, retrieval and extinction. Using Affymetrix Rat Genome Array 230.2 resources, probe sets that did not target a unique coding gene were filtered from the dataset. To permit comparison with human mutation data, rat Entrez gene IDs were converted to human homologs through the HomoloGene ID index (Box 3.1), obtained from the Mouse Genome Informatics (MGI) Vertebrate Homology table (<http://www.informatics.jax.org/homology.shtml>). Genes that do not have a unique human homolog according to this database were thus excluded.

Genes were then ranked by the significance of their differential expression. For each probe set, MAS5 and RMA P -values were combined using Fisher's method, so that each probe set was represented by a single P -value. For genes targeted by more than one probe set, these P -values were further combined using Simes P -value correction (Python 3.5.1, "StatsModels" package), thereby giving one P -value per gene. For consolidation, retrieval and extinction, genes could then be ranked by this P -value, with smaller values representing a stronger association with the particular learning phase.

In primary analyses, the top 5% learning-related genes (representing 607 genes from the consolidation, retrieval and extinction ranked data sets) were used as the gene set. In subsequent analyses, gene sets were generated from the top 1, 2, 5, 10, 15, 20 and 25% of learning-related genes, equating to 121, 243, 607, 1214, 1822, 2429 and 3036 genes

The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders respectively. For single gene analyses, each gene from the top 5% of the learning-related genes was considered separate sets.

Box 3.1: HomoloGene ID

Not all human genes are present in the rat genome, just as not all rat genes are present in the human genome. For those that do have corresponding homologs, the precise sequence of the gene may vary and therefore its function, and that of any proteins it codes for, could differ. Hence when converting genes across species, it is crucial that controls are put in place to ensure that identified homologs are likely to be functionally identical.

The HomoloGene ID is a numerical index for labelling genetic homologs across several species (including human, rat, mouse, chimpanzee, rhesus macaque, dog, cattle and zebrafish), created by the National Center for Biotechnology Information (NCBI). The input for HomoloGene comes from comparing both the gene sequence and protein structure, permitting the additional comparison of distance metrics such as molecular distance and Ka/Ks ratio. An algorithm is applied that uses thresholds of bits per position and Ks values to filter out unlikely orthologs and group together true homologs.

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3.2.1.3 Collating patient CNV data and quality control

CNV data from patients with schizophrenia and healthy controls were compiled from multiple large-scale studies, to reduce any bias incurred from the demographics of any one cohort. Data from three European datasets was used: the Molecular genetics of Schizophrenia (MGS, dbGAP phs000167.v1.p1 and phs000021.v3.p2), the International Schizophrenia Consortium (ISC) and a UK study of schizophrenic patients taking clozapine (CLOZUK; Stone et al., 2008; Levinson et al., 2011; Rees et al., 2014; Details of genotyping: Stone et al., 2008; Levinson et al., 2011; Rees et al., 2014a; Pocklington et al., 2015). Control subjects for the CLOZUK cohort contains data obtained from The Genetic Architecture of Smoking and Smoking Cessation (dbGAP phs000404.v1.p1); Genetic Epidemiology of Refractive Error in the KORA Study (dbGAP phs000303.v1.p1); High-Density SNP Association Analysis of Melanoma (dbGAP phs000187.v1.p1). In total, this dataset contains CNVs from 11917 schizophrenia cases and 16416 control subjects. CNVs from patients with melanoma were obtained from a Study of Melanoma Risk in Australia and the United Kingdom (n = 2416 following QC). 6335 control subjects were obtained from The Genetic Epidemiology of COPD (COPDGene, dbGAP phs000179.v3.p2); A Genome-Wide Association Study of Fuchs' Endothelial Corneal Dystrophy (FECD, dbGAP phs000421.v1.p); California Pacific Medical Center Research Breast Health Cohort (dbGAP phs000395.v1.p1); National Blood Donors Cohort (Wellcome Trust Case Control Consortium 2).

Each CNV was annotated with genes using the corresponding human genome reference assembly (MGS: Build 36, ISC: Build 35, CLOZUK: Build 37). Only protein coding genes were included in the annotation, as defined by the "Homo sapiens_geneinfo.txt" file

The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders within the genome reference assembly files. The resulting CNV dataset was subjected to quality control. This involved filtering out CNVs smaller than 100 kb, covered by fewer than 15 probes or having a frequency greater than 1%. These steps are consistent with recent approaches to base analyses on large, rare and reliable CNVs (Szatkiewicz et al., 2014a; Pocklington et al., 2015).

3.2.1.4 Logistic regression analysis

Enrichment analyses were performed for each learning-related gene set, comparing the number of consolidation-, retrieval- and extinction-related genes that overlap with CNVs from patients or healthy controls (Pocklington et al., 2015). This was done using the following logistic regression analysis:

$$\text{case-control status} \sim \text{gene set hits} + \text{CNV size} + \text{total genes in CNV} + \text{study} \\ + \text{chip type}$$

where “case-control status” is a binary variable that can be either 0 (control) or 1 (case), “gene set hits” is the number of times a CNV overlaps a gene from the gene set and “CNV size” (base pairs), “total genes in CNV”, “study” (i.e. CLOZUK, MGS or ISC) and “chip type” (Affymetrix chip ID) are covariates.

P-values obtained from primary analyses (top 5% learning-related genes) were adjusted by Bonferroni correction for multiple testing. *P*-values from secondary analyses (top 1-25%) were adjusted by permutation correction. *P*-values from single-gene analyses were uncorrected.

3.2.1.5 Permutation Correction

Null distributions of *P*-values for each gene set size were generated by permuting all genes 2000 times, each time taking the top 1, 2, 5, 10, 15, 20 and 25% (giving a total of

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14000 randomised gene sets) and analysing these gene sets by logistic regression analysis. Empirical enrichment *P*-values were calculated from these null distributions by finding the proportion of *P*-values within the null distribution greater than the observed *P*-value (General Methods 2.1.2).

3.2.2 Results

The consolidation, retrieval and extinction of contextual fear memory induced the expression of distinct groups of genes, with only chance levels of overlap between the top 5% of each learning condition in Fisher's exact test (consolidation vs retrieval $P=0.85$, consolidation vs extinction $P=0.39$, retrieval vs extinction $P=0.29$; Figure 3.1). Top 5% gene sets encompassed genes with differential expression *P*-values < 0.012 (consolidation), $P < 0.021$ (retrieval) and $P < 0.032$ (extinction).

In primary logistic regression analyses, using a gene set comprised of the top 5% learning-related genes, there was a marked enrichment of extinction-related genes in patient CNVs ($P = 3.9 \times 10^{-4}$; Bonferroni corrected), but not consolidation- or retrieval-related genes (consolidation: $P = 0.31$; retrieval: $P = 0.23$; Bonferroni corrected; Figure 3.2).

The enrichment of extinction-related genes was observed in duplication CNVs and deletion CNVs separately. The top 5% extinction-related genes were enriched in both CNV types (deletions: $P = 0.0034$, duplications: $P = 0.042$, Bonferroni corrected; Figure 3.1b and c). No enrichment of the top 5% consolidation- or retrieval-related genes was observed in deletions or duplications.

By expanding the analysis over a range of gene set sizes (top 1-25%), the statistical association of the learning-related gene set with case CNVs increased with the size of

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the gene set (Figure 3.3a-c). Plotting the enrichment of randomly permuted gene sets of the same sizes confirmed that the strength of the enrichment was proportional to gene set size (Figure 3.3d). This may be due to a background enrichment from all genes convertible from rat to human homologs. Hence, the permuted gene sets were used to correct the observed enrichment *P*-values. Following permutation correction, only the top 1%, 2% and 5% extinction-related genes yielded a significant enrichment in schizophrenia-associated CNVs (Figure 3.3g), whilst all sizes of consolidation- and retrieval-related gene sets showed no enrichment (Figure 3.3e and f).

Since this CNV dataset is made up of CNVs from three separate studies (MGS, ISC and CLOZUK), the existence of this finding in each independent cohort was investigated. Interestingly, the same enrichment of extinction-related genes in patient CNVs was present in each of the CNV datasets (Table 3.1). These results suggest that CNVs which increase an individual's susceptibility to developing schizophrenia impact selectively on genes involved in the extinction of associative fear memories.

To test the specificity of these findings to schizophrenia-associated CNVs, a control analysis was performed observing the enrichment of each learning-related gene set in the CNVs of patients with melanoma. Using logistic regression analysis, no enrichment of consolidation-, retrieval- or extinction-related genes in melanoma-associated CNVs was measured (consolidation $P = 0.59$; retrieval $P = 1.0$; extinction $P = 1.0$; Bonferroni-corrected; Table 3.2).

In order to explore this association between extinction-regulated genes and schizophrenia CNVs further and to identify specific genes driving the association, the contribution of individual genes was assessed. Single-gene logistic regression analysis

The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders was performed on each extinction-related gene from the top 5%. The statistical association of a gene with extinction was a significant predictor of the gene's enrichment in CNVs of patients with schizophrenia ($R^2 = 0.087$, $F_{(1,141)} = 13.49$, $P = 0.0003$; Figure 3.4). Even within the top 1% extinction-related genes, this association was maintained ($R^2 = 0.15$, $F_{(1,29)} = 5.3$, $P = 0.029$; Figure 3.4, inset).

A number of the genes contributing to this enrichment have already been implicated in schizophrenia through CNV studies (Table 3.3): MFI2, CLDN5, ATP10A, SNRPN, CGNL1 and PDZK1 (Goodbourn et al., 2014; Rees et al., 2014a, 2014b). A further three extinction-related genes have been implicated through genome-wide association studies: ERCC4, GALNT10 and SLC39A8 (Ripke et al., 2014).

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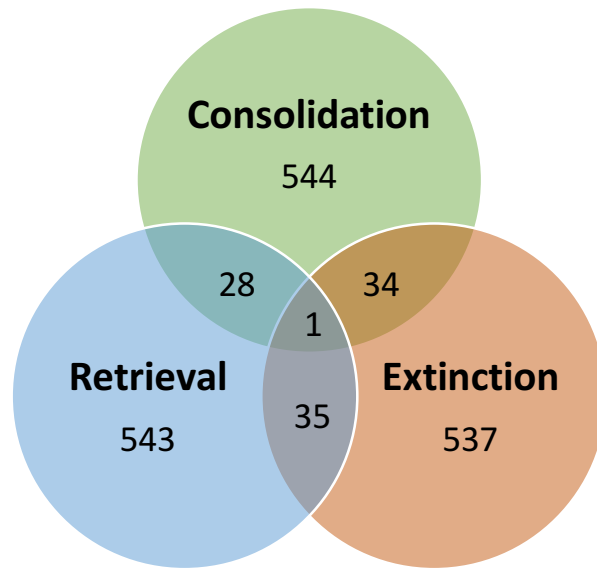


Figure 3.1 Distinct groups of genes are associated with different components of fear memory processing. Represented here is the overlap of the top 5% genes by significance of differential expression in the CA1 region of the hippocampus two hours after each learning phase. Only one gene was common to all three gene sets (ICOS). The degree of overlap is no greater than chance levels (consolidation vs retrieval $P = 0.85$, consolidation vs extinction $P = 0.39$, retrieval vs extinction $P = 0.29$ in Fisher's exact test).

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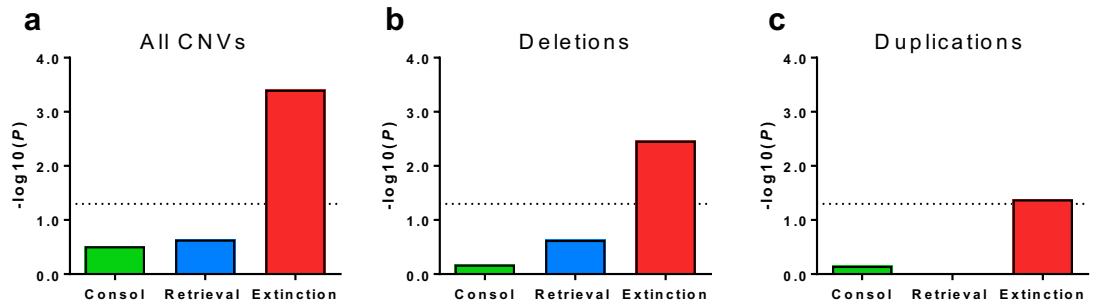


Figure 3.2 Extinction-related genes are enriched in CNVs from patients with schizophrenia. **(a)** The top 5% genes differentially expressed following fear extinction, but not consolidation (Consol) or retrieval, are overrepresented in schizophrenia CNVs vs control CNVs ($P = 3.9 \times 10^{-4}$). Bars represent $-\log_{10}(P)$ -value) after logistic regression enrichment analysis and Bonferroni P -value correction. **(b)** Deletions from schizophrenic patients alone show an overrepresentation of fear extinction-related genes ($P = 0.0034$, Bonferroni corrected). **(c)** The enrichment of fear extinction-related genes in duplications from schizophrenic patients is significant ($P = 0.042$, Bonferroni corrected) yet weaker than in deletions. **(a-c)** Dotted line represents a $P=0.05$ threshold for statistical significance

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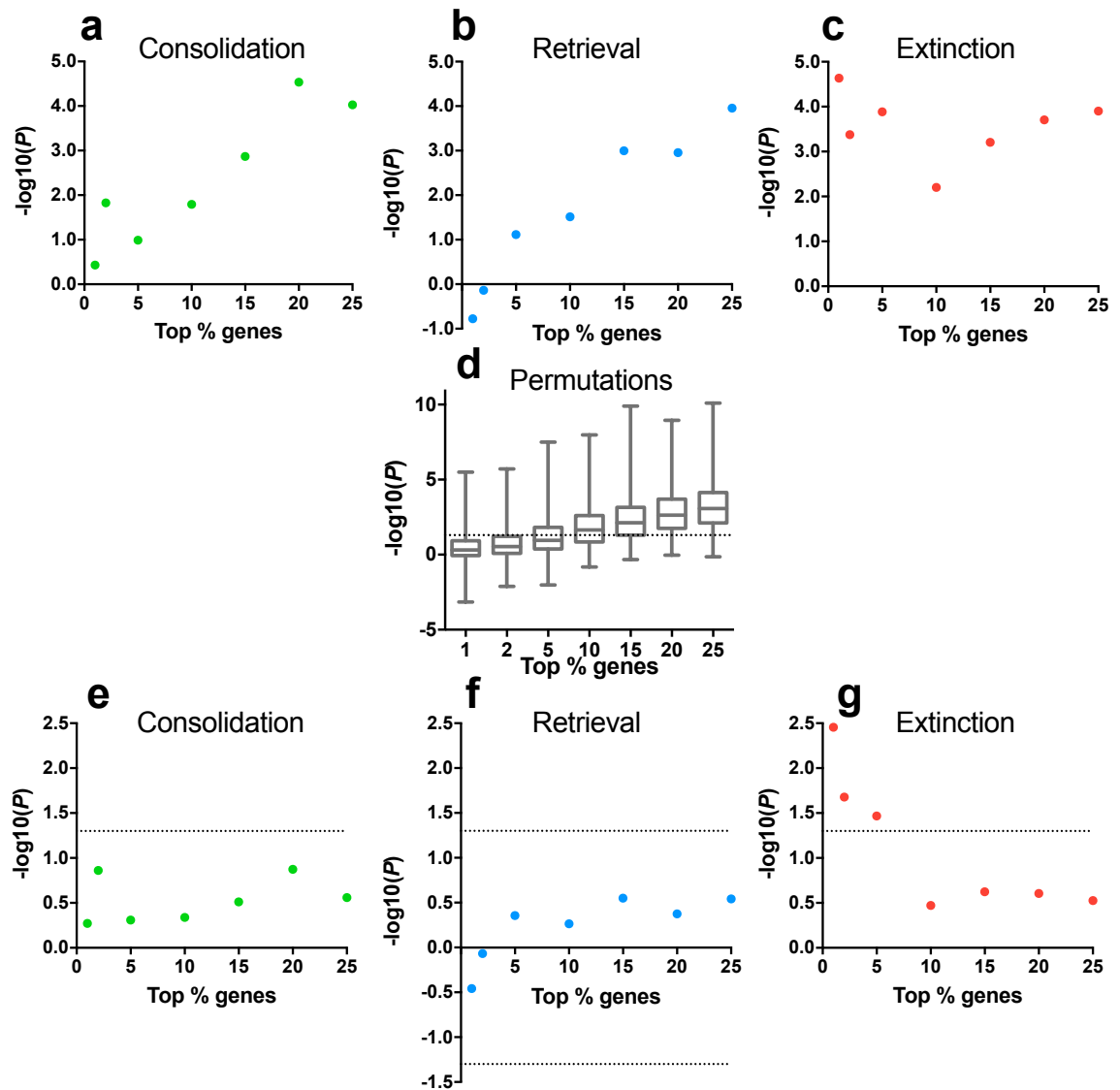


Figure 3.3 Secondary CNV enrichment analyses performed over the top 1, 2, 5, 10, 15, 20 and 25% genes associated with consolidation, retrieval and extinction of fear memory. Points represent $-\log_{10}(P\text{-value}) \times \text{sgn}(Z\text{-value})$. (a-c) Prior to P -value correction, enrichment becomes more significant with gene set size. (d) The statistical association of a gene set with case CNVs was proportional to the gene set size. All genes were permuted 2000 times, each time yielding random gene sets from the top 1-25%. This data was used to correct P -values obtained from the secondary analyses of the experiment. Boxplots display the minimum, first quartile, median, third quartile and maximum $-\log_{10}(P\text{-value}) \times \text{sgn}(Z\text{-value})$ following logistic regression analyses. (e-g) Enrichment of the top 1-25% learning-related genes following P -value adjustment by permutation correction. Only the top 1-5% extinction-related gene sets are enriched in schizophrenia-related CNVs. (d-g) Dotted line represents a $P=0.05$ threshold for statistical significance.

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Extinction gene set	CLOZUK <i>P</i>	GAIN <i>P</i>	ISC <i>P</i>
Top 1%	0.00067	0.029	0.041
Top 2%	0.0056	0.11	0.043
Top 5%	0.00030	0.18	0.11

Table 3.1 The enrichment of extinction-related genes in schizophrenia patient CNVs exists in each cohort independently. The top 1, 2 and 5% extinction-related genes were analysed for enrichment in each CNV dataset using logistic regression analysis. Sample sizes: CLOZUK 6,307 cases, 10,675 controls; MGS 2,215 cases, 2,556 controls; ISC 3,395 cases, 3,185 controls.

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Gene set	All CNVs		Deletions		Duplications	
	<i>P</i>	<i>P</i> _{adj}	<i>P</i>	<i>P</i> _{adj}	<i>P</i>	<i>P</i> _{adj}
Consolidation	0.20	0.59	0.62	1.0	0.17	0.50
Retrieval	0.50	1.0	0.34	1.0	0.68	1.0
Extinction	0.75	1.0	0.09	0.28	0.56	1.0

Table 3.2 No learning-related gene sets were enriched in the CNVs of patients with melanoma. The top 5% learning-related genes were subjected to enrichment analysis in melanoma CNVs from the CLOZUK2 control dataset, using logistic regression analysis. Displayed are uncorrected and Bonferroni-corrected (*P*_{adj}) *P*-values representing the association between consolidation-, retrieval- and extinction-related genes and all melanoma CNVs, as well as deletions and duplications separately.

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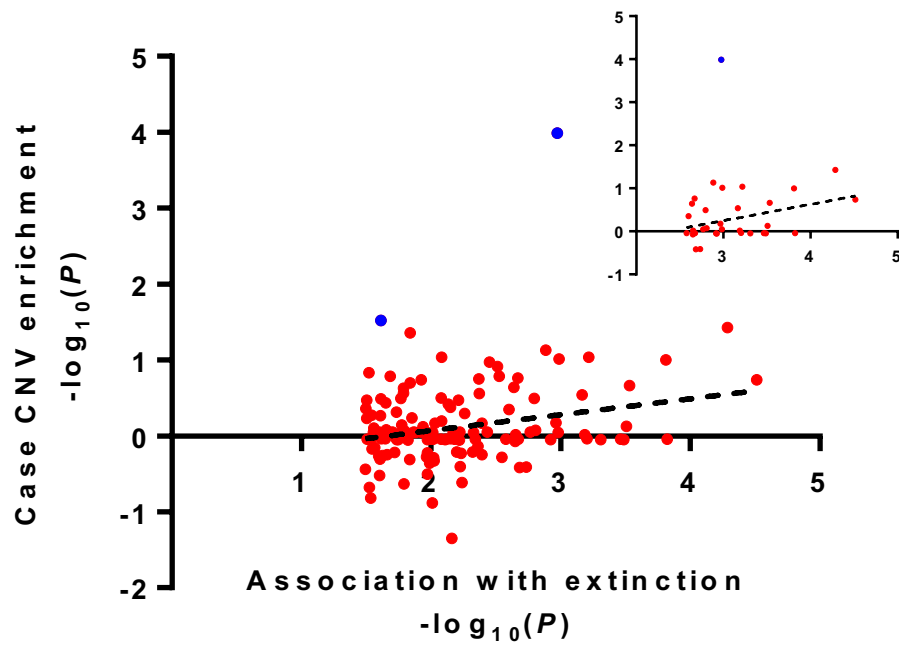


Figure 3.4 Within the top 5% extinction-related genes, those more strongly associated with extinction learning are also more enriched in schizophrenia case CNVs ($R^2 = 0.087$, $F_{(1,141)} = 13.49$, $P = 0.0003$). This is also true within the top 1% extinction-related genes ($R^2 = 0.15$, $F_{(1,29)} = 5.3$, $P = 0.029$; inset). Dots represent $-\log_{10}(P\text{-value})$ of the significance of differential expression following extinction learning regressed against $-\log_{10}(P\text{-value}) \times \text{sgn}(Z\text{-value})$ of the enrichment in case CNVs following single-gene logistic regression analysis. Outliers, which were excluded from analysis, are displayed in blue. Genes that gave no contribution to the model were excluded from this analysis and are not displayed.

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Gene symbol	Gene ID	Gene name	Region	Mutation type	Extinction <i>P</i>	Case hits	Control hits	CNV enrich <i>P</i>
ATP10A	57194	ATPase class V type 10A	15q11.2-13.3	Duplication	0.007	10	1	0.4
CGNL1	84952	Paracingulin	15q21.3	Duplication	0.001	52	29	0.0001
CLDN5	7122	Claudin 5	22q11.2	Deletion	0.002	47	14	0.2
MFI2	4241	Melanotransferrin	3q29	Deletion	0.00005	10	1	0.04
PDZK1	5174	PDZ domain containing 1	1q21.1	Del / Dup	0.004	66	57	0.1
SNRPN	6638	Small nuclear ribonucleoprotein-associated protein N	15q11.2-13.3	Duplication	0.03	11	2	0.6
ERCC4	2072	Excision repair cross-complementation group 4	16p13.12	SNP	0.004	0	0	NA
GALNT10	55568	Polypeptide N-acetylgalactosaminyltransferase 10	5p33.2	SNP	0.00009	1	0	0.89
SLC39A8	64116	Solute carrier family 39 member 8	4q24	SNP	0.03	0	0	NA

Table 3.3 Extinction learning-related genes within loci previously implicated in schizophrenia through CNV studies (deletions or duplications) and genome-wide association studies (single-nucleotide polymorphisms, SNP). Gene symbols, gene IDs (Entrez) and regions listed pertain to the Homo sapiens genome. “Extinction *P*” relates to the significance of differential expression following extinction learning. “CNV enrich *P*” relates to the significant of the single-gene enrichment in schizophrenia-associated CNVs.

3.3 Comparing the enrichment of learning-related genes in CNVs from patients with schizophrenia, autism, ADHD and intellectual disability

3.3.1 Methods

Gene sets relating to consolidation, retrieval and extinction were unchanged from above.

3.3.1.1 Collating patient CNV data and quality control

Autism-associated CNVs (2446 cases, 2640 controls) were obtained from the Autism Genome Project (Pinto et al., 2014). Intellectual disability-associated CNVs were obtained from two sources: 15767 cases from Signature Genomic Laboratories (Cooper et al., 2011) and 15749 cases from International Standards for Cytogenomic Arrays consortium (Kaminsky et al., 2011). The latter source contained CNVs associated with various intellectual and developmental disabilities, from which 608 CNVs from patients whose Subject Phenotype contained the words “Intellectual disability” were selected. Control CNVs for the intellectual disability analyses were taken from the MGS, ISC and CLOZUK control cohorts (16416 controls). ADHD-associated CNVs (1508 cases, 3057 controls) were obtained from the International Multi-Centre for ADHD Genetics (IMAGE) 2 project and a UK Cardiff Study (Thapar et al., 2015). CNVs were annotated (Pinto: Build 36, Cooper and Kaminsky: Builds 36/37, ADHD: Build 36) and subjected to quality control and filtering as above.

3.3.2 Results

CNV datasets from patients with ADHD, autism, schizophrenia and intellectual disability were subjected to the same filtering and quality control procedures, as described in the methods. Past studies have shown variations in CNV frequencies and sizes among

The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders patients with different psychiatric diseases (Malhotra and Sebat, 2012). Therefore, to make a comparison of these CNV datasets, the CNV size distributions for each disorder were examined (Figure 3.5). CNVs from patients with intellectual disability were on average much larger than CNVs from control subjects ($P < 2.2 \times 10^{-16}$, independent Mann-Whitney U test; Figure 3.5). Moreover, the frequency of large CNVs (>1 Mbp) was significantly greater in intellectual-disability CNVs compared to controls ($P < 2.2 \times 10^{-16}$, Binomial test). These observations are in keeping with previous studies (Girirajan et al., 2011), although may also be a reflection of the CNV detection methods.

The top 1-25% learning-related gene sets were subjected to logistic regression against each CNV dataset. In order to correct the P -values obtained from these analyses, the same 14000 permutation-generated gene sets were tested for enrichment in the CNVs associated with each disorder, giving distributions of P -values that were used for permutation correction. The top 1-25% consolidation-, retrieval- and extinction-related genes showed no enrichment in CNVs from patients with ADHD, autism or intellectual disability ($P > 0.05$ following permutation correction in all instances; Figure 3.6). The only significant enrichment observed was that of the top 1-5% extinction-related genes in the CNVs of patients with schizophrenia (Figure 3.6), as described previously (Figure 3.3).

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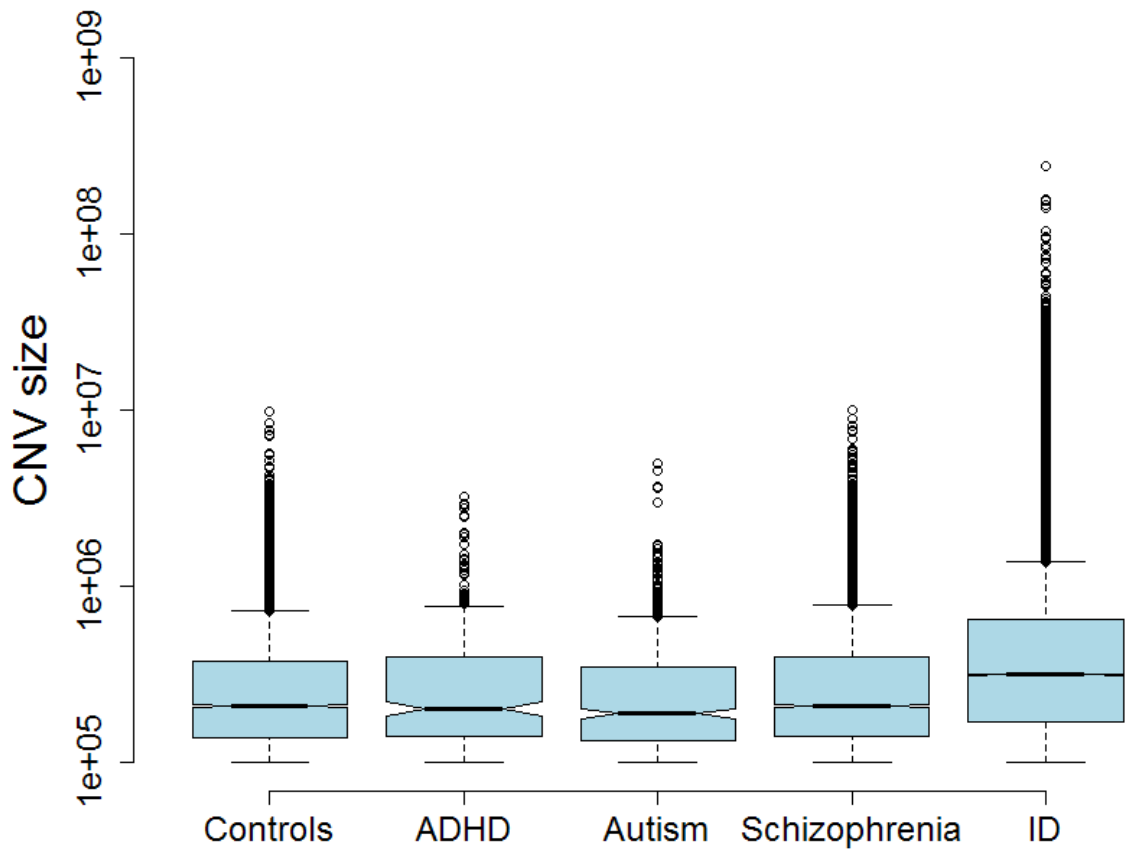
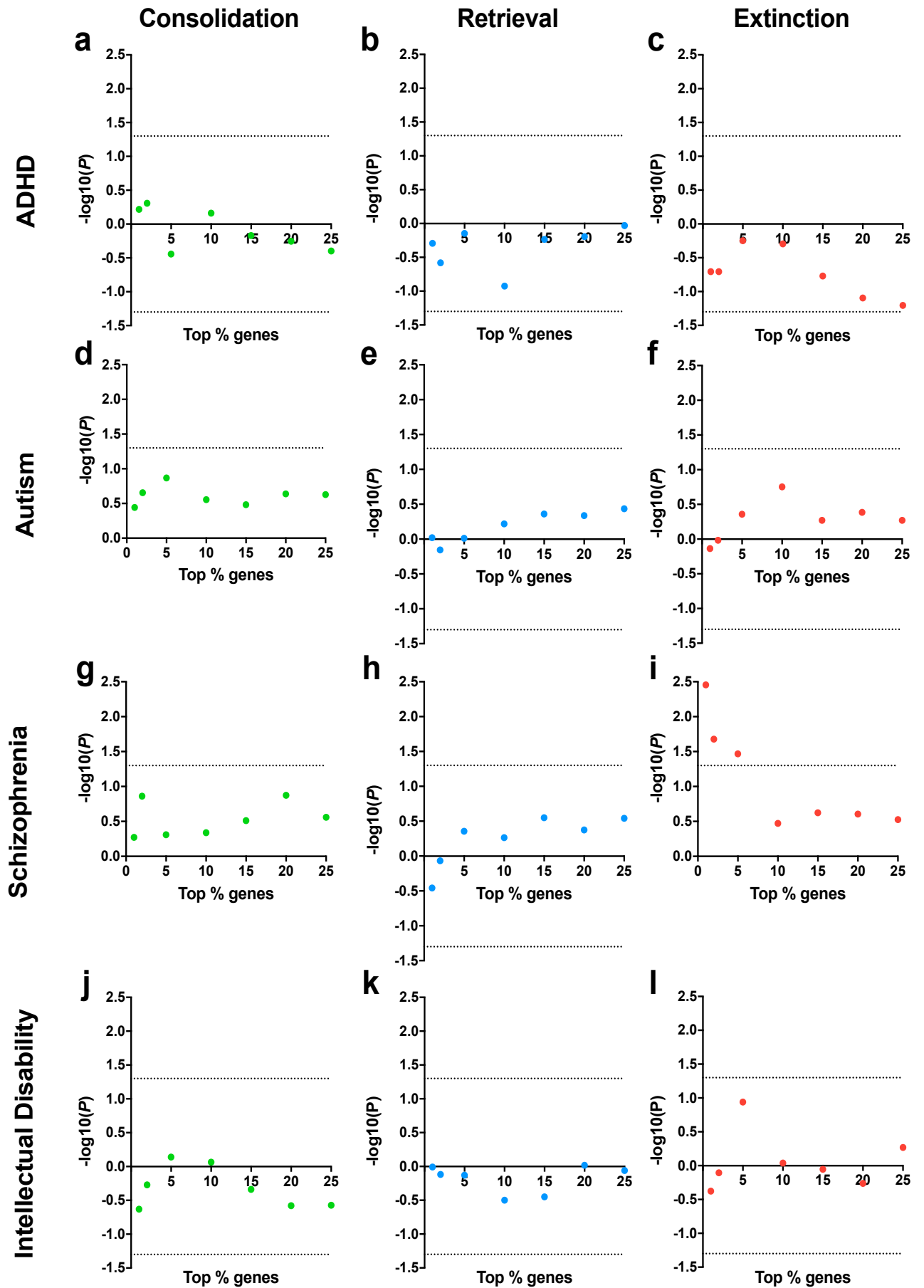


Figure 3.5 The size distribution of CNVs from patients with different psychiatric diseases. CNVs associated with intellectual disability (ID) are considerably larger than those from healthy control subjects or patients with ADHD, autism and schizophrenia. Control CNVs were taken from the ISC, MGS and CLOZUK control subjects. CNV size is plotted on a logarithmic scale following the exclusion of CNVs < 100 kb. It should be noted that variations in the CNV identification methods between CNV datasets may influence the distribution of observed CNV sizes.

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Figure 3.6 The enrichment of learning-related genes in the CNVs of patients with different psychiatric diseases. Only the top 1-5% extinction-related gene sets are enriched in schizophrenia-related CNVs. For each CNV dataset, enrichment analysis was performed over the top 1, 2, 5, 10, 15, 20 and 25% genes associated with consolidation, retrieval and extinction of fear memory. Points represent $-\log_{10}(\text{P-value}) \times \text{sgn}(\text{Z-value})$ following permutation correction. Dotted lines represents a $P=0.05$ threshold for statistical significance.

3.4 The enrichment of learning-related genes in schizophrenia-associated common variants

3.4.1 Methods

Two different analysis methods were used to investigate the enrichment of learning-related genes in schizophrenia-associated common variants: PLINK and MAGMA. For all analyses, gene sets were the top 1, 2, 5, 10, 15, 20 and 25% consolidation-, retrieval- and extinction-related genes.

3.4.1.1 Sample

SNP data from schizophrenia patients and controls was obtained from the Psychiatric GWAS Consortium (PGC) repository. The sample includes cases and controls from UK, Ireland, US, Bulgaria, Portugal, Sweden, Germany, Denmark, Norway, The Netherlands and Australia, with a total of 34,241 cases and 45,604 controls.

3.4.1.2 PLINK enrichment analysis

The SNPs were filtered to exclude those with an imputation score < 0.8 , a minor allele frequency $< 1\%$ and those within the major histocompatibility complex (MHC) region. Consolidation-, retrieval- and extinction-related genes were converted to genomic locations using the genome build 37.3 seq_gene37.3.md file. 1000 Genomes reference files (<http://www.1000genomes.org>) were used for linkage disequilibrium calculations. Plink's "--extract" function was used to reduce the genome reference data to regions covered by genes analysed in the microarray. The "--set-screen" function mapped the case-control SNPs to each gene set and calculated the enrichment probability. Each analysis yielded 2 P -values: P_1 and P_2 . P_2 is obtained following a more stringent correction. In order to correct the P -values produced by this analysis, 1000 gene sets of

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random size, consisting of genes randomly selected from the full microarray, were subjected to PLINK enrichment analysis, using a supercomputer. For each of the two levels of P -value stringency, a curve was fitted to the output from these analyses representing the expected P -value for a gene set hit by a given number of SNPs, which was used to adjust the observed P -values.

3.4.1.3 MAGMA enrichment analysis

MAGMA (Multi-marker Analysis of GenoMic Annotation) is a gene set analysis tool developed more recently than PLINK, and provides improved statistical power and speed (de Leeuw et al., 2015). The analysis uses a multiple regression model and performs single gene analysis in conjunction with the gene set analyses for added flexibility.

Case-control SNP data was annotated with gene location using NCBI Build 37.3 gene locations. Then followed a gene-wide analysis, using the 1000 Genomes reference files (as above) for linkage disequilibrium calculations. The gene set analysis was performed using the output from the gene-wide analysis. During this analysis, MAGMA executed a correction for false discovery rate using 100,000 data permutations.

3.4.2 Results

Common variant enrichment analysis performed using PLINK revealed that gene sets hit by a greater number of SNPs tended to yield a lower P -value (Figure 3.7a-d). P -values obtained from permuted gene sets fit the following models, where n is the number of SNPs within genes from a gene set.

$$-\log_{10}(P_1) = 0.0031n^{0.585}$$

$$-\log_{10}(P_2) = 0.009n^{0.4045}$$

However, there was high variability in the P -values obtained from smaller gene sets (Figure 3.7d). These models were used to correct the enrichment P -values. After correction, there was no significant enrichment of SNPs from patients with schizophrenia in the top 5% genes associated with consolidation, retrieval or extinction of contextual fear (Figure 3.7).

Results obtained from the MAGMA common variant enrichment analysis were consistent with those observations from PLINK. No learning-related gene set had a significant enrichment of patient SNPs (Figure 3.8).

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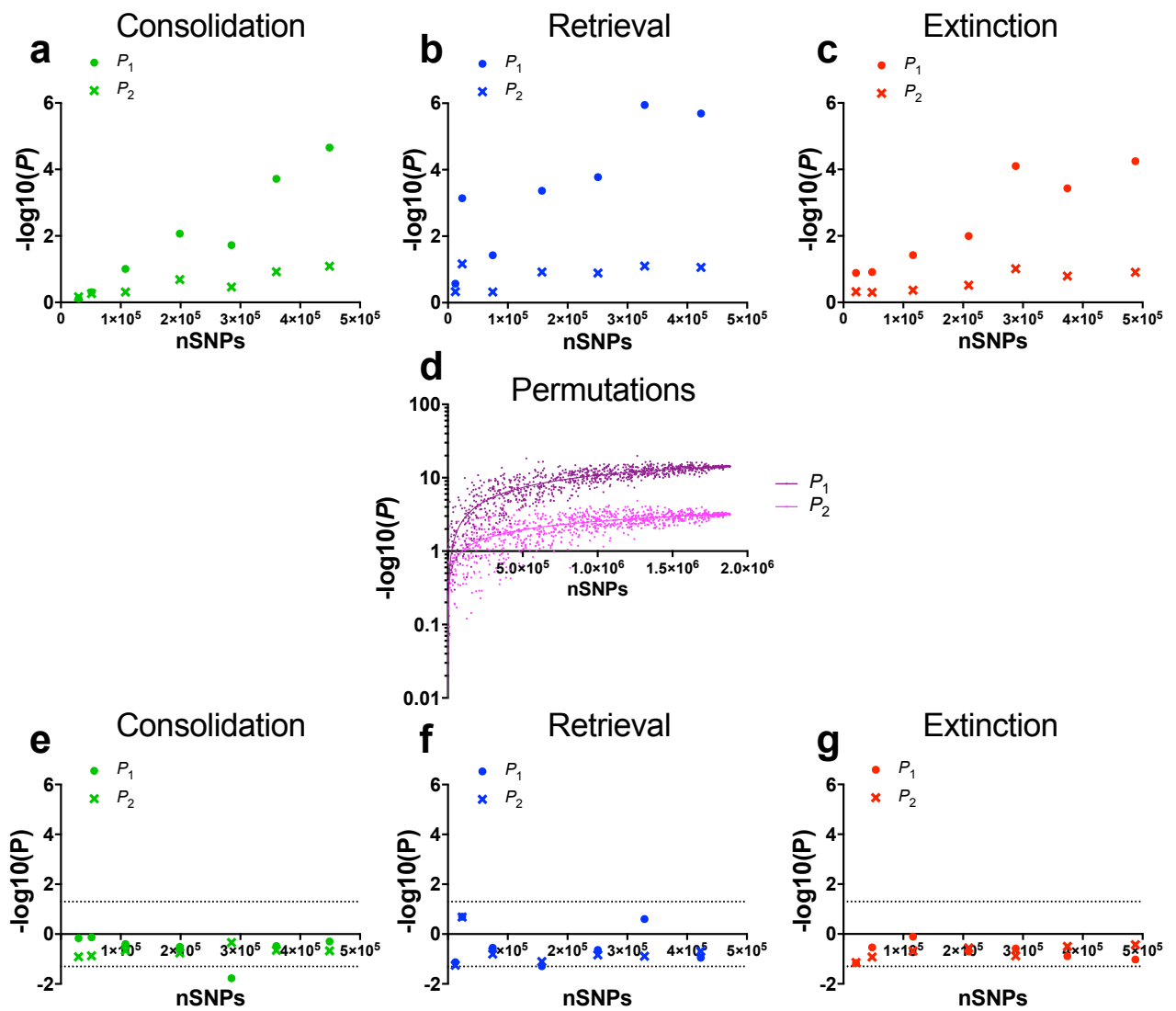


Figure 3.7 PLINK common variant enrichment analysis of the top 1-25% genes associated with consolidation, retrieval and extinction of fear memory. Gene set size is proportional to the number of SNPs hit by it. Data represented by $-\log_{10}(P_1)$ and $-\log_{10}(P_2)$. (a-c) Enrichment analysis output prior to permutation correction. (d) Permutation analysis output. 1000 gene sets of random sizes were selected from randomly permuted genes. (e-g) Permutation-corrected enrichment values. Dotted lines represent a $P=0.05$ threshold for statistical significance.

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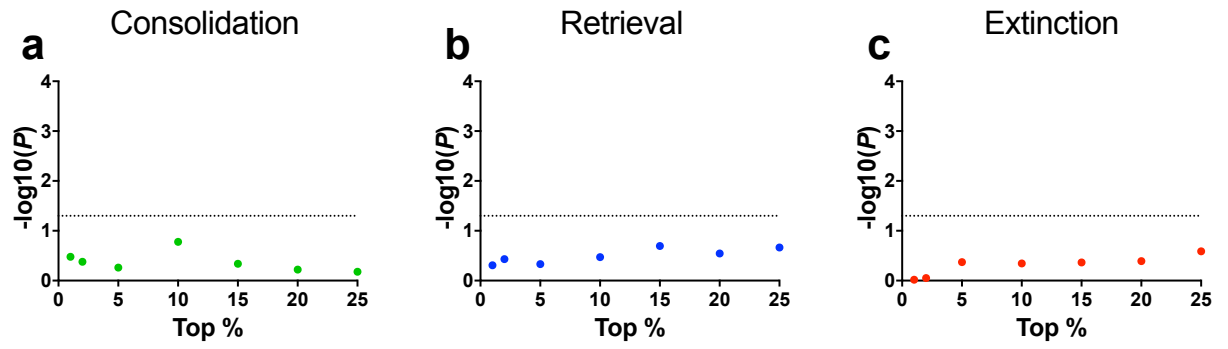


Figure 3.8 MAGMA common variant enrichment analysis of the top 1-25% consolidation-, retrieval- and extinction-related genes. No significant enrichment of any gene set was observed. Data represented by $-\log_{10}(P)$. Dotted lines represent a $P=0.05$ threshold for statistical significance.

3.5 Discussion

The results herein show that CNVs from patients with schizophrenia selectively impact on genes related to extinction learning, implying that these structural variants may confer vulnerability to the disorder by effecting molecular pathways involved in inhibitory-type learning and thereby contributing to psychotic symptoms characteristic of schizophrenia. This relationship was observed in schizophrenia-associated CNVs only; no enrichment of consolidation-, retrieval- or extinction-related genes was observed in CNVs from patients with autism, ADHD or intellectual disability, or in common variants from patients with schizophrenia.

The link between extinction-related genes in hippocampal CA1 and schizophrenia highlighted in this study is consistent with previous work showing that patients exhibit impaired extinction of fear associations (Holt et al., 2009) and furthers the hypothesis that altered associative- and, specifically, inhibitory-type learning contributes to the manifestation of schizophrenia (Serra et al., 2001; Millan et al., 2012). It also corroborates past evidence reporting irregular hippocampal responses to fearful stimuli (Schobel et al., 2009), heightened activation of CA1 (Holt et al., 2012; Talati et al., 2014) and CA1 synaptic pathology (Matosin et al., 2016) in the disorder.

These results extend past studies linking genes involved in synaptic plasticity, associative learning and contextual conditioning to CNVs from patients with schizophrenia (Kirov et al., 2012; Pocklington et al., 2015). In contrast to these previous studies, which rely on documented annotation of individual genes, the current analysis used gene sets derived from experimental quantification by microarray. This permits the integration of genes into the analysis whose function is poorly understood and overcomes the knowledge

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bias associated with traditional pathway analyses. However, this approach is vulnerable to false positives, due to the multiple testing involved in the microarray method, and incorporates only a select set of genes expressed at a certain time point (2 h) from a certain brain region (CA1).

The enrichment of extinction-related genes in case CNVs was observed both in the combined schizophrenia CNV dataset from three European case-control cohorts, and in each cohort individually, thereby providing internal replication and reinforcement of the main finding. Compared with the CLOZUK cohort the enrichment was less significant in GAIN and ISC cohorts, and the top 5% extinction gene set (used for primary analyses) was not significantly enriched in case CNVs, although this is unsurprising due to the smaller sample size of the GAIN and ISC case-control groups. Interestingly, within both the top 5% and top 1% gene sets, the association of individual genes with extinction learning was related to its single gene enrichment in case CNVs, whereby those more strongly associated with extinction learning were also more enriched in CNVs from patients with schizophrenia. This suggests that the main finding extends beyond the principle gene sets analyses to the single gene level. It should be noted, however, that the potential co-localisation of multiple genes from the same gene set within the same CNVs may bias this single gene relationship.

Extinction-related genes were enriched in case CNVs when deletions and duplications were analysed together and when they were analysed separately, implying that both structural variants impinge on extinction learning in schizophrenia. However, since the enrichment was most significant in deletions, these variants may contribute the most to inhibitory learning impairments. The same learning-related gene sets were also tested

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for enrichment in deletions and duplications from patients with melanoma. There was no enrichment of consolidation-, retrieval- or extinction-related genes in these variants when analysed together or separately. This result supports the view that the enrichment of extinction-related genes in schizophrenia-associated CNVs is disease-specific and unlikely to be a reflection of the general properties of disease-linked CNVs.

Prior to permutation correction, observed P -values were inflated by the size of the gene set used in the analysis. This was confirmed by the generation and analysis of 14000 randomly permuted gene sets, which were used to resolve the influence of gene set size. The cause of the statistical inflation is likely a background enrichment within those genes which were possible to convert to human homologs for inclusion in the gene set enrichment analyses. Hence, larger gene sets incorporated more of this background signal and tended to yield smaller P -values. Those genes which were possible to convert to human homologs are sufficiently homologous due to conservation over evolution. Since schizophrenia-related alleles are enriched in mutation-intolerant genes (Pardiñas et al., 2016), a background enrichment may have emerged from increasing the concentration of mutation-intolerant genes in the available dataset during the conversion of rat to human homologs.

I did not find a relationship between learning-related genes and CNVs from patients with autism, ADHD or intellectual disability, suggesting that the enrichment observed in schizophrenia-related CNVs does not extend to these related psychiatric disorders. This is interesting, considering the shared genetic risk and overlapping phenotypes between these disorders (Lee et al., 2013; Doherty and Owen, 2014), and implies that despite the shared genetic risk, these disorders are segregated by clusters of genetic variants

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impacting on specific cognitive processes. Indeed, the link between associative learning / fear memory and schizophrenia does not extend to autism, ADHD and intellectual disability in the existing literature (Hall et al., 2009; Holt et al., 2009, 2012; Pollard et al., 2012). Still, care should be taken when comparing the results from these cross-disorder CNV-enrichment analyses since the sample sizes varied considerably. Furthermore, different CNV identification methods were used in each study, which affects the reliability of CNV calling over the range of CNV sizes. Whilst the CNV identification methods were matched between cases and controls of the same analysis where possible, this was not true for the intellectual disability CNV cohort, since a matching control cohort did not exist, which may have created bias in the enrichment analysis.

A number of extinction-related genes driving the enrichment in schizophrenia-associated CNVs are situated within loci recurrently implicated in schizophrenia. One of the strongest contributors was the gene Claudin-5 (*CLDN5*), which lies within the recurrent CNV located at 22q11.2 and is a component of tight junctions (Escudero-Esparza et al., 2012). Interestingly, another extinction-related gene was Paracingulin (*CGNL1*) previously implicated in schizophrenia through duplications (Levinson et al., 2011; Rees et al., 2014a). Paracingulin protein is also a component of tight junctions and adherens junctions where it interacts with Rho GTPases and actin filaments (Paschoud et al., 2012). Its role in the central nervous system, however, is yet to be explored. Indeed, gene ontology analysis revealed an enrichment of genes involved in cell adhesions in the extinction-related gene set (Scholz et al., 2016).

There was no enrichment of extinction-related genes in common variants, as determined by two independent analysis methods, suggesting that these genetic

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variants do not confer vulnerability to schizophrenia by influencing molecular pathways responsible for extinction learning. It is perhaps not surprising that the same gene set is not associated with both CNVs and common variants. With the exception of some broad functional annotations, such as NMDA receptor signalling, abnormal behaviour and immune function, the overlap between pathways implicated in schizophrenia through each type of genetic variant is minimal at present (Harrison, 2015). Furthermore, common variants individually confer less risk than rare variants and are less reliably attributed to specific genes (Fromer et al., 2014; Purcell et al., 2014). Still, several of the genes linked to schizophrenia through common variants are involved in plasticity processes (Ripke et al., 2014) and further investigation of common variants in genes associated with specific types of learning and memory is warranted.

Through the integration of human genetic data with gene expression data from model organisms, these experiments build upon previous functional studies to yield a novel insight into the link between associative learning and schizophrenia. These results imply that CNVs may confer risk for schizophrenia by impairing extinction learning, therefore contributing to the development of cognitive symptoms and the persistence of delusional beliefs. The findings have implications for the role of other types of inhibitory learning in schizophrenia and corroborate current hypotheses linking inhibitory-type learning processes to the disorder

Chapter 4: Associative Learning by Contextual Fear Conditioning

4.1 Introduction

Altered associative learning and related plasticity processes have been linked to schizophrenia through genomic studies (Ripke et al., 2014; Pocklington et al., 2015) and have been proposed to contribute to the manifestation of positive symptoms (Serra et al., 2001; Corlett et al., 2009; Fletcher and Frith, 2009; Hall et al., 2009). This chapter employs the contextual fear memory paradigm in order to explore components of associative learning in the context of psychiatric disease.

4.1.1 Contextual fear conditioning and extinction learning

Fear conditioning is an evolutionarily-conserved behaviour, making it a suitably translational paradigm for studying the regulation and dysregulation of aversive associative memories (Milad and Quirk, 2012). Associative learning by fear conditioning has been extensively studied for over a century and its psychology has been well characterised (McGaugh, 1966; Maren et al., 2013). As described in the Chapter 3, three distinct component processes of learning can be discerned by the paradigm: consolidation of the fear memory, maintenance of the memory after retrieval (“reconsolidation”) and memory extinction (Pedreira and Maldonado, 2003; Lee et al., 2004). We also know that each process is accompanied by the *de novo* expression of distinct sets of genes (Duvarci et al., 2008; Mamiya et al., 2009; Barnes et al., 2012; Scholz et al., 2016).

Both the consolidation and extinction of a fear memory are considered forms of new learning (Ochs, 1968), yet relate to behaviourally antagonistic components of Pavlovian conditioning (Pavlov, 1927). Consolidation is the time-delimited process that stabilizes an association between a neutral stimulus (conditioning stimulus, CS) and an event (unconditioned stimulus, US) such as an aversive footshock association when these stimuli are contiguous, so that presentation of the CS elicits a conditioned fear response. Extinction of conditioned fear typically occurs when the subject is re-exposed to the CS in the absence of the US repeatedly or for an extended period of time. Extinction results in the loss of the conditioned response. Importantly, however, since the consolidated CS-US association can be recovered after extinction, by re-exposure to the US, for example, such studies indicate that extinction is new learning itself, which inhibits expression of the original memory rather than erasing it (Ochs, 1968; Rescorla and Heth, 1975; Bouton, 1993; Gale et al., 2004). Poor retrieval of the extinction memory, if not due to reinstatement of the conditioned fear memory, may be due to a pathological process impairing the extinction mechanisms or the recall of the CS-no US extinction memory (Quirk and Mueller, 2008).

The potential to study consolidation, retrieval and extinction processes independently in rodents using single trials makes fear conditioning a reliable and clinically relevant behavioural paradigm. Such features also make the paradigm amenable to the administration of pharmacological agents, lending it potential for the creation of models of diseases characterised by impairments in fear learning.

4.1.2 NMDA receptors in contextual fear memory and inhibitory learning

Glutamate is one of the neurotransmitter systems most frequently associated with the aetiology of schizophrenia, along with dopamine and GABA. One of the strongest sources of evidence for a role of NMDA-type glutamate receptors in schizophrenia is the observation that NMDA antagonists, such as phencyclidine (PCP) and ketamine, induce psychotic and cognitive symptoms characteristic of the disorder in healthy humans and worsen the symptoms of schizophrenia patients (Javitt and Zukin, 1991; Krystal et al., 1994; Newcomer et al., 1999). For this reason, PCP and ketamine have been used extensively in the generation of animal models of schizophrenia. Some work has been done to test the effect of these NMDA antagonists on components of associative learning, although the results have been largely inconclusive and it has principally been directed at cued tone-shock, amygdala-mediated learning, rather than single trial contextual fear conditioning mediated by the hippocampus.

Using rats, Pietersen *et al.* (2006) found that pre-training ketamine (16 mg/kg, subcutaneous) blocked fear conditioning to a tone-shock CS-US pairing and abolished the induction of amygdalar *cFos* expression (Pietersen et al., 2006). Conversely, others have found that pre-training ketamine at a lower dose (8 mg/kg, subcutaneous) or post-training with high-doses (four injections of 100/50/50/50 mg/kg spaced at 60 min intervals, intramuscular / intraperitoneal) had no effect on cued fear conditioning (Bolton et al., 2012; Groeber Travis et al., 2015). However, ketamine (8 mg/kg, subcutaneous) or microinfusion of another NMDA antagonist, 2-amino-5-phosphonovaleric acid (APV), to the prefrontal cortex profoundly impaired conditioned response in a more complex trace conditioning protocol (Gilmartin and Helmstetter,

2010; Bolton et al., 2012). Bolton *et al.* (2012) also reported that a decrease in simple contextual fear conditioning by ketamine was not statistically significant. It is likely that some of the discrepancy between these studies can be explained by dose variations or complexity of the behavioural conditioning task.

A small volume of literature exists investigating the effect of NMDA receptor manipulation on inhibitory fear learning, including extinction. High-dose ketamine administration in rats following multiple extinction trials of cued fear conditioning had no effect on subsequent freezing responses (Groeber Travis et al., 2015), while post-extinction training PCP dose-dependently impaired the extinction of cued fear memory (Pollard et al., 2012). Interestingly, pre-administration of an NMDA receptor partial agonist, D-cycloserine, dose-dependently facilitated the extinction of cued fear conditioning (Walker et al., 2002). Further evidence suggests that NMDA antagonism by ketamine (25 mg/kg, intraperitoneal) or MK-801 can inhibit a second type of inhibitory fear learning known as latent inhibition (Razoux et al., 2007; Traverso et al., 2012). Latent inhibition is a process whereby extensive prior exposure to the CS in absence of the US, before pairing of the two, masks their association upon pairing. It is plausible that ketamine acts to broadly impair inhibitory-type learning, which could be a major contributor to its psychotomimetic effects.

The amygdala is central to the expression of conditioned fear responses, through its projections to the hypothalamus, periaqueductal grey and other brainstem regions (Applegate et al., 1982; Pitkänen et al., 1997). Conversely, the hippocampus is responsible for the contextual gating of fear conditioning to an explicit conditioned stimulus (Ji and Maren, 2007; Radulovic and Tronson, 2010; Tronson et al., 2012)

through direct projections to the amygdala via the pre-limbic and infralimbic cortical areas (Hugues and Garcia, 2007). Hence, the hippocampus is able to modulate fear learning and fear responses. Since context representations are an essential component of information retrieval and logical inference (Maren et al., 2013), altered hippocampal functioning may contribute to inappropriate associative learning, which in humans could translate to delusions and paranoia characteristic of schizophrenia. Indeed, substantial evidence implicates hippocampal abnormalities and impaired contextual processing in schizophrenia (Taylor et al., 2012). Here, I measured the effects of pre- and post-training ketamine (8 mg/kg and 25 mg/kg, intraperitoneal) on the consolidation and extinction of contextual fear memory in rats to determine the sensitivity of excitatory and inhibitory associations, respectively, to ketamine.

4.2 Generating context-specific fear memory

4.2.1 Methods

General methodology used for contextual fear conditioning experiments is described at length in section 2.4.

Adult Lister hooded rats (275-325 g) were individually exposed to one of two novel but distinct contexts (Context A or Context B) for a 3 min conditioning training trial. Rats received a single scrambled footshock (0.5 mA for 2 sec) 2 min after being placed in the novel context. Rats were then returned to the home cages.

Contextual fear memory and the specificity of the memory was assessed by exposing the rats to either the same context within which the animal was conditioned, or a different context (n = 6 per group), during a short recall trial (2 min), 48 h later. Freezing

behaviour was recorded and assessed pre- and post-shock during the conditioning training and during the recall trial, as an index of fear behaviour.

4.2.2 Results

Application of footshock increased the freezing response relative to baseline (Pre US) levels ($F_{(1, 32)} = 480.4$, $P < 0.001$, repeated measures RM ANOVA; Figure 4.1). Returning the rat to the same context 48 h later induced an increase in conditioned freezing behaviour comparable to Post US freezing ($P = 0.61$, paired t-test; Figure 4.1), demonstrating the recall of a conditioned fear memory. If the rat was placed into a different context to where the animal was conditioned, the freezing compared to the post-US response was reduced ($P < 0.01$, paired t-test; Figure 4.1). Thus, this behavioural paradigm results in fear memory recall and expression in the conditioned context only.

Associative Learning by Contextual Fear Conditioning

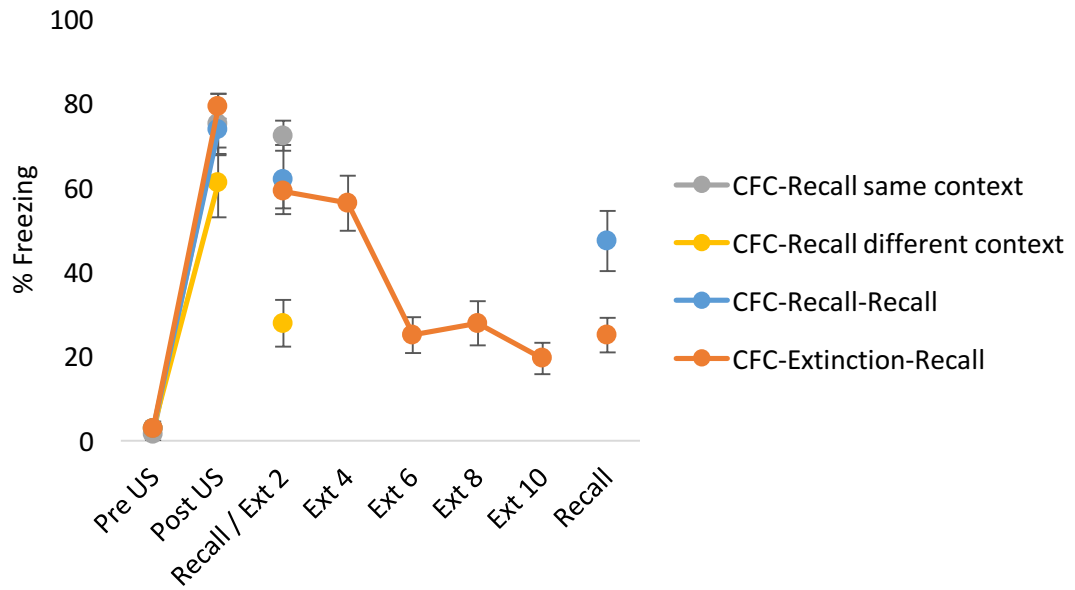


Figure 4.1 Contextual fear conditioning (CFC) to a footshock (unconditioned stimulus, US). Recall of the associative memory generated by CS-US pairing is specific to the context within which the animals is conditioned and can be extinguished by a long recall trial (10 min), but not a short recall trial (2 min). Rats placed into a novel context received a 0.5 mA footshock. Conditioning and recall trials were 48 hours apart. Freezing was sampled every 10 sec. During the extinction trial, percent freezing was calculated in 2 min bins (Ext 2-Ext 10). Data represented by mean \pm SEM of percent freezing response. $n = 6-12$.

4.3 Extinction of contextual fear memory

4.3.1 Methods

Adult Lister hooded rats ($n = 6$) were fear conditioned as above. Forty-eight h later, rats were returned into the same context for a long recall extinction training trial (10 min) in the absence of the US. To test for the extinction (loss of the conditioned response), rats were returned to the conditioning-extinction context for a 2 min recall test, 48 h after extinction training. Freezing behaviour was recorded as previously.

4.3.2 Results

Separate cohorts of fear-conditioned rats were replaced into the conditioned context for either a short recall trial (2 min) or a long recall trial (10 min). Within the long recall trial, the freezing response decreased over time ($F_{(2.859, 31.45)} = 22.21$, $P < 0.0001$, RM ANOVA; Figure 4.1). In a subsequent recall trial, 48 hours later, the freezing response was substantially reduced compared to the conditioned response (first 2 min of extinction training, Ext 2; $P < 0.0001$, t-test), which represents extinction of the CS-US association by the long recall trial. The short recall trial did not produce extinction ($P = 0.192$, t-test; Figure 4.1).

4.4 Investigating the effect of ketamine on the consolidation of contextual fear memory

4.4.1 Methods

Adult male Lister hooded rats (275-325 g) underwent a single 3 min conditioning training trial using a footshock (0.5 mA for 2 sec at 2 min). This conditioning trial was preceded by the administration of the NMDA receptor antagonist, ketamine (ketamine hydrochloride, Ketaset, Henry Schein Animal Health; 8 mg/kg or 25 mg/kg,

intraperitoneal (IP)) or saline vehicle (n = 6 per group) 30 min prior to conditioning. These doses, time scales and route of administration were chosen in accordance with previous studies (Razoux et al., 2007; Bolton et al., 2012). Contextual fear memory was tested in short recall (2 min) trials in up to three subsequent time points: 2 days (Recall 1), 4 days (Recall 2) and 7 days (Recall 3) post-conditioning. Ketamine or vehicle was administered 30 min prior to the recall trials. Freezing behaviour was recorded during all training and recall trials.

4.4.2 Results

4.4.2.1 High dose ketamine pre-treatment creates a state-dependent fear memory

A footshock stimulus induced a robust freezing response in both vehicle- and 25 mg/kg ketamine-treated animals ($F_{(1, 10)} = 91.63$, $P < 0.0001$, two-way RM ANOVA; Figure 4.2a). There was no effect of treatment on freezing response ($F_{(1, 10)} = 1.003e-013$, $P = 1$, two-way RM ANOVA), indicating that ketamine-treated rats exhibit a normal level of baseline freezing and are able to acquire a contextual fear memory comparably to control subjects. In a first test phase, 48 h later, rats pre-treated with 25 mg/kg ketamine prior to conditioning displayed substantially less freezing than their control counterparts ($P < 0.0001$, t-test; Figure 4.2a), demonstrating that these animals failed to recall a fear memory and hints towards a failure to consolidate the memory in the presence of ketamine. However, when the same animals were re-exposed to the same context following the administration of the same dose of ketamine, their freezing response was not different from control levels ($P = 0.24$, t-test; Figure 4.2a). In a subsequent recall trial, without the administration of ketamine, the two groups were discernible once more: rats treated with ketamine prior to conditioning showed reduced freezing

compared to controls ($P < 0.01$, t-test; Figure 4.2a). These results suggest that fear conditioning in the presence of 25 mg/kg ketamine induces a state-dependent memory, which is only recalled upon exposure to the context following the re-administration of ketamine and hence the return to the previously-experienced state.

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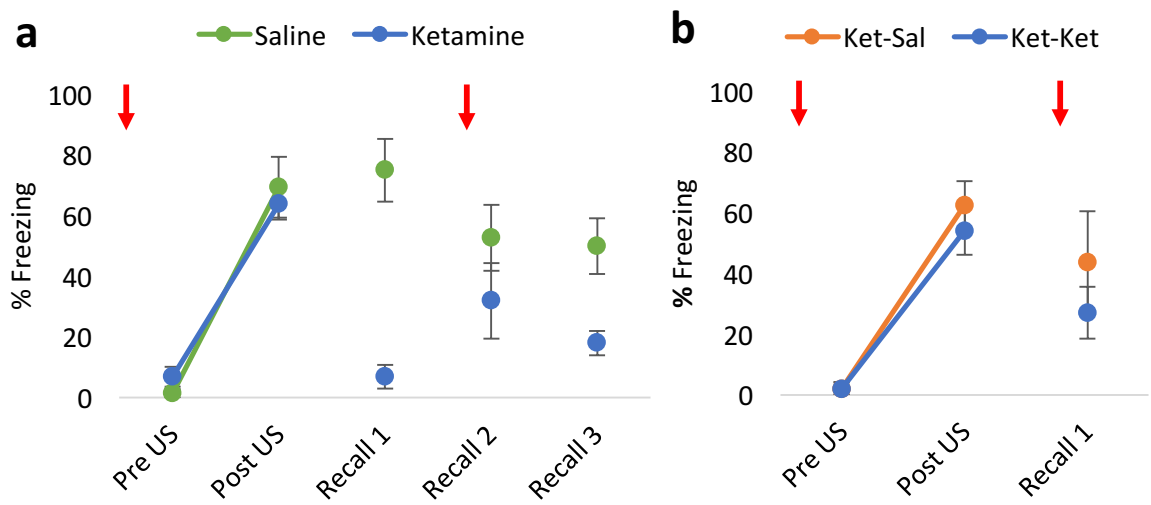


Figure 4.2 The effect of ketamine on contextual fear conditioning. (a) 25 mg/kg ketamine induced the generation of a state-dependent contextual fear memory. Ketamine (25 mg/kg) or saline vehicle were administered IP 30 min before conditioning and recall trial 2 (indicated by arrows). $n = 6$. (b) 8 mg/kg does not induce the creation of a state-dependent contextual fear memory. Ketamine (8 mg/kg) or saline vehicle was administered IP 30 min before conditioning and recall trials, indicated by arrows. $n = 4$. (a, b) Data represented by mean \pm SEM of percent freezing response..

4.4.2.2 *Low dose ketamine does not affect contextual fear conditioning*

I next sought to probe the effect of a lower dose of ketamine on memory consolidation, with the aim to avoid the induction of a state-dependent memory. Rats were conditioned following the administration of 8 mg/kg ketamine and were given a recall trial 48 h later following either 8 mg/kg ketamine or vehicle. There was no effect of treatment on the freezing response ($F_{(1, 6)} = 0.8745$, $P = 0.39$, RM ANOVA; Figure 4.2b), indicating that this dose of ketamine does not generate a state-dependent contextual fear memory, and retrieval is solely dependent on exposure to the physical context.

4.5 Investigating the effect of ketamine on the extinction of contextual fear memory

4.5.1 Methods

All rats underwent a 3 min contextual fear conditioning training trial by footshock (0.5 mA for 2 sec at 2 min), a long recall trial (10 min) 2 days after conditioning (extinction) and two short recall trials (2 min) at 4 days and 7 days after conditioning. Ketamine (8 mg/kg or 25 mg/kg) or saline vehicle were administered either 30 min before or immediately after trials ($n = 6$ per group). Freezing behaviour was recorded for all trials.

4.5.2 Results

In order to assess the effect of ketamine on the extinction of fear memory, rats underwent contextual fear conditioning followed by an extinction trial 48 h afterwards. Since 25 mg/kg induces a state-dependent memory, ketamine at this dose, or saline vehicle, was administered before both trials. In a separate cohort, 8 mg/kg ketamine or saline vehicle was administered prior to extinction. Extinction training caused a reduction in freezing behaviour over time in all treatment groups, as shown by the

difference in freezing response between the first and last 2 min period of the extinction trial (25 mg/kg $P < 0.0001$, 8 mg/kg $P < 0.0001$, RM ANOVA; Figure 4.3a, b). Recall of the extinction memory was assessed following the administration of either ketamine or vehicle. By comparing freezing during the last 2 min of the extinction trial to the first recall trial, a statistically significant interaction between treatment and learning phase was observed, at both doses (25 mg/kg $F_{(2, 15)} = 11.59$, $P < 0.001$, 8 mg/kg $F_{(3, 20)} = 3.471$, $P < 0.05$, RM ANOVA). To improve the validity of post-hoc testing, freezing responses were normalized to those exhibited during the Post-US phase (Figure 4.3c, d) and the data grouped by pre-extinction treatment (Figure 4.3e, f). In recall trials, rats treated with either 25 mg/kg or 8 mg/kg ketamine prior to extinction exhibited greater freezing compared to controls (25 mg/kg $P < 0.001$; 8 mg/kg $P < 0.01$, t-tests; Figure 4.3e, f) indicating that, at these doses, ketamine blocks the extinction of contextual fear memory. At the higher dose, subsequent administration of a different treatment given prior to conditioning reversed the elevation of freezing response (Saline vs Ketamine $P = 0.42$, t-test; Figure 4.3e), presumably due to state-dependency of the fear memory. The ketamine-induced impairment in fear extinction at 8 mg/kg persisted in a second recall trial ($P < 0.05$, t-test; Figure 4.3f).

In order to investigate whether ketamine impairs extinction learning due to its action during the acquisition of new learning or because of its action during the memory consolidation processes that occur after the trial, ketamine (25 mg/kg) or vehicle was administered immediately after conditioning and extinction trials. Post-conditioning ketamine had no effect on freezing during the first two minutes of the long recall trial ($F_{(2, 15)} = 0.4050$, $P = 0.17$, ANOVA; Figure 4.4b), indicating that there was no effect of the

drug on memory consolidation. The extinction trial caused a decrease of freezing response in all treatment groups ($F_{(1, 15)} = 53.62, P < 0.0001$, RM ANOVA). Post-extinction ketamine had no effect on total freezing during subsequent recall trials (Recall 1 $F_{(2, 15)} = 0.07557, P = 0.93$; Recall 2 $F_{(2, 15)} = 1.122, P = 0.35$, one-way ANOVA, normalised data; Figure 4.4b). These results indicate that ketamine impairs fear extinction memory only when present during the acquisition of new learning.

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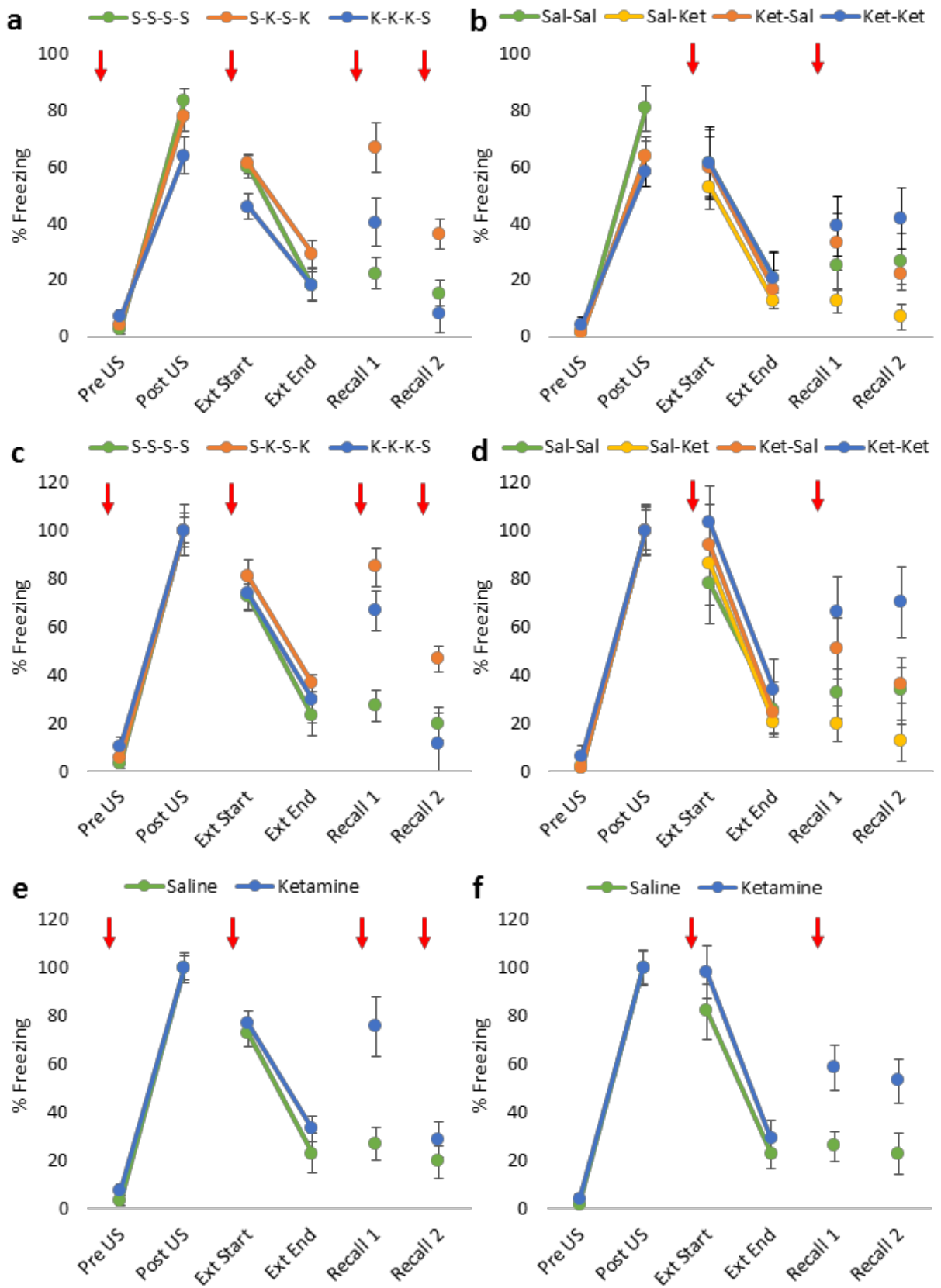


Figure 4.3 Ketamine impairs the extinction of contextual fear memory. Displayed are the first (Ext Start) and last (Ext End) 2 mins of the extinction trial (10 min). 25 mg/kg (**a, c, e**) or 8 mg/kg (**b, d, f**) ketamine or saline vehicle was administered IP 30 min before trials indicated by red arrows. $n = 6$. (**a, c, e**) In recall trials, ketamine-treated rats received either the same (Recall 1) or different (Recall2) administration to that received prior to conditioning. The same data is presented in three ways, replicated vertically: (**a, b**) Data represented by mean \pm SEM of percent freezing response. (**c, d**) Data normalised to Post-US freezing response. (**e, f**) Data grouped by pre-extinction saline- and ketamine-treatment.

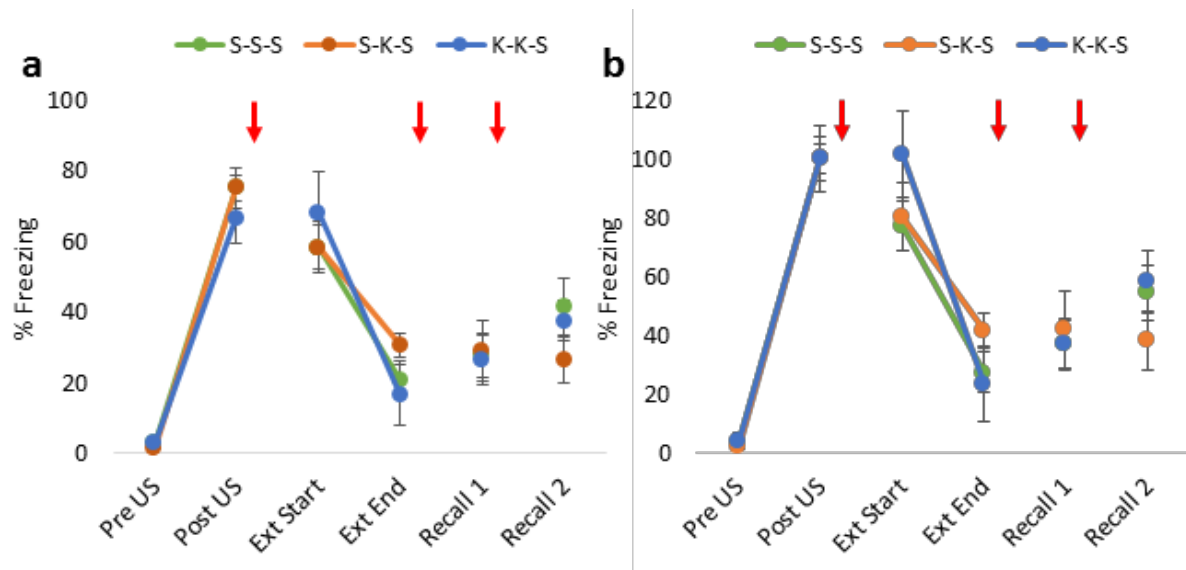


Figure 4.4 Post-trial administration of 25 mg/kg ketamine has no effect on the consolidation or extinction of contextual fear memory. Displayed are the first (Ext Start) and last (Ext End) 2 mins of the extinction trial. Ketamine (25 mg/kg) or saline vehicle was administered IP immediately after each trial, indicated by arrows. $n = 6$. (a) Data represented by mean \pm SEM of percent freezing response. (b) Data normalised to Post-US freezing.

4.6 Discussion

One of the biggest advantages of a one-trial learning paradigm for research is its amenability to pharmacological interrogation. In these experiments, a contextual fear conditioning protocol is presented as a reliable one-trial learning paradigm in which rats undergo the consolidation of contextual fear memory in a single CS-US pairing, before context-dependent retrieval of that fear memory in a recall trial 48 h later, as manifested by quantifiable freezing behaviour. A single long recall trial induced extinction of the fear memory, which was confirmed by reduced freezing in a subsequent recall trial.

The pharmacological experiments herein show that systemic administration of the NMDA antagonist, ketamine, prior to consolidation generates a state-dependent fear memory at 25 mg/kg – that is, the rats were better able to recall association information about the context when they were subjected to the same internal context to which the information was acquired in. It has been shown previously that ketamine can induce state-dependent retrieval (Oberling et al., 1996), and indeed from use at this dose and route of administration (Aguado et al., 1994). Just as this result informed subsequent experiments herein, it too has implications for the use of this dose in other studies, particularly those which fail to control for state-dependent effects. For example, the finding that 25 mg/kg intraperitoneal ketamine blocked latent inhibition of conditioned fear (Razoux et al., 2007) may be confounded by a change of internal context from CS pre-exposure to CS-US conditioning, since the study did not include a group administered with ketamine prior to both learning phases.

Ketamine did not affect the conditioned response when it was administered before or immediately after fear conditioning, indicating that the treatment had no effect on the

acquisition or consolidation of the associative memory. This is consistent with the small amount of existing literature on contextual fear learning (Bolton et al., 2012) and parallels the majority of findings from cued fear learning (Bolton et al., 2012; Groeber Travis et al., 2015). However, inconsistencies in dose, protocol and the potential for state-dependency make it difficult to compare the data.

Acute ketamine administration may elicit a worse model of psychiatric pathology than chronic administration. Amann et al used chronic ketamine to investigate the effect of prolonged NMDA receptor antagonism on contextual fear conditioning in mice. They report that whilst two weeks of daily ketamine administrations prior to conditioning had no effect on freezing response, a further two weeks of chronic ketamine impaired retrieval of the conditioned memory (Amann et al., 2009). Equally, the psychotomimetic effects of NMDA antagonists may be more pronounced when the drug is administered to coincide with a critical developmental period. Indeed, neonatal PCP treatment is frequently used as a model of schizophrenia phenotypes (Jones et al., 2011; Clifton et al., 2013). Consistent with this approach, the neonatal treatment of mice with PCP impaired conditioned fear when tested at P60 (Yuede et al., 2010). It is important to note, however, that in humans a single administration of ketamine or PCP is sufficient to induce schizophrenia-like phenotypes (Javitt and Zukin, 1991; Krystal et al., 1994; Newcomer et al., 1999).

Our results indicate that ketamine selectively impairs the extinction of contextual fear memory. This finding is consistent with previous reports that NMDA receptor activation is required for fear extinction (Burgos-Robles et al., 2007; Sotres-Bayon et al., 2007). This finding was not due to underlying effects of the treatment on baseline or conditioned

freezing responses, since there was no difference in pre-US and post-US freezing between saline- and ketamine-treated groups. Ketamine at 25 mg/kg and 8 mg/kg did not influence within-session freezing response during the long recall trial, suggesting that ketamine has no effect on the acquisition of extinction learning and instead mediates its effects by interfering with molecular processes responsible for the subsequent “consolidation” of extinction memory. However, administration of ketamine immediately after extinction training did not induce the same deficit. Therefore, extinction memory is only impaired when ketamine is present during the acquisition of extinction. This is contradictory to reports of the effect of ketamine and PCP administration following the extinction of cued fear memory (Pollard et al., 2012; Groeber Travis et al., 2015), which may highlight mechanistic differences between the involvement of NMDA receptors in the molecular processes required for hippocampal- and amygdalar-mediated extinction learning.

Due to the state-dependent effects of 25 mg/kg ketamine observed previously, extinction experiments were performed on cohorts of rats that were conditioned in the presence and absence of ketamine. The increase in fear memory retrieval in ketamine-treated rats only occurred when an equivalent pre-administration was given to that prior to conditioning, which was to be expected given the state-dependency. However, the conditioned response was elevated regardless of whether the rats were administered with ketamine prior to extinction and recall trials or just extinction, making it unlikely that the apparent extinction deficit is explained by state-dependency of the extinction memory. In support of this, 8 mg/kg ketamine, which does not induce state-dependent retrieval of contextual fear conditioning, also impaired extinction.

Whilst this is, to my knowledge, the first time that ketamine has been shown to selectively impair the extinction of contextual fear memory, the same 25 mg/kg dose of ketamine has been found to impair inhibitory fear learning in other studies. One such study examined the effect of ketamine on latent inhibition, which refers to the weakening of a CS-US association due to extensive pre-exposure to the CS in the absence of the US, before conditioning. In this study, ketamine administration prior to CS pre-exposure abolished latent inhibition of conditioned fear, which was correlated with increased extracellular glutamate in the nucleus accumbens and increased synaptic efficacy in the prefrontal-accumbens pathway (Razoux et al., 2007). The same effect was also observed following administration of the NMDA receptor antagonist MK-801 (Traverso et al., 2012). This is understandable, considering the overlap in molecular requirement for extinction and latent inhibition (Barad et al., 2004). Together, these results imply that NMDA antagonism by ketamine and related psychotomimetic drugs may have a selective effect on inhibitory fear learning processes. This is consistent with a substantial body of evidence proposing that altered inhibitory learning may contribute to the development of psychotic symptoms in schizophrenia (Serra et al., 2001; Holt et al., 2009; Millan et al., 2012), as discussed previously (Section 3.5), and reinforces the translational validity of rodent ketamine administration as a model of schizophrenia-like phenotypes.

It is feasible to suggest that rather than blocking extinction learning, the increased freezing response observed during recall is instead due to an enhancement in the retrieval of the original CS-US association. Indeed, in humans, ketamine administration leads to stronger fear conditioning (Corlett et al., 2013), which could reflect the

emergence of delusional beliefs in schizophrenia. However, when rats were administered with saline prior to extinction and 8 mg/kg ketamine prior to retrieval, the freezing response was comparable to animals treated with saline before both trials, indicating that ketamine had no effect on retrieval at this dose. Whilst the same comparison cannot be made for the higher ketamine dose, rats treated with 25 mg/kg prior to conditioning and retrieval exhibited no greater freezing response than control subjects. It appears therefore, that the effects of ketamine observed herein are due to NMDA receptor antagonism during extinction acquisition.

Chapter 5: The Regulation of Postsynaptic Density Homer1 Proteins by Associative Learning

5.1 Introduction

As addressed in previous chapters, converging genetic evidence points towards postsynaptic density proteins in the aetiology of schizophrenia (Kirov et al., 2012; Fromer et al., 2014) and particularly those involved in the regulation of associative learning (Pocklington et al., 2015). In Chapter 3, an enrichment of genes involved in a particular component of associative learning, extinction, in CNVs from patients with schizophrenia was found. Within the set of extinction-related genes was *Homer1*, a protein with key roles at the postsynaptic density and in the control of synaptic plasticity, learning and memory (see Literature Review Section 1.4). Furthermore, *Homer1* has previously been linked to several psychiatric diseases, including schizophrenia (Norton et al., 2003; Spellmann et al., 2011; Matosin et al., 2016).

5.1.1 Postsynaptic density Homer proteins in psychiatric disease, learning and memory

The *Homer1* gene codes for several isoforms, most of which are long and constitutively expressed whilst two shorter isoforms, *Homer1a* and *Ania-3*, are activity-induced immediate early genes (Fagni et al., 2002). All *Homer1* isoforms share the same binding domain, and hence have common targets (Hayashi et al., 2009), although, upon the recruitment to the postsynaptic density, short Homers disrupt interactions of the long Homers through dominant negative regulation (Xiao et al., 1998; Kammermeier and

Worley, 2007). It is understood that, through this mechanism, Homer1a and Ania-3 regulate mGluR function (Tu et al., 1999; Bertaso et al., 2010; Sylantsev et al., 2013) and calcium homeostasis (Kim et al., 2006; Huang et al., 2007; Yuan et al., 2012).

Homer1 has been linked to schizophrenia and other psychiatric disorders. Homer1 protein is decreased in the post-mortem hippocampus of patients with schizophrenia (Engmann et al., 2011) and the frontal cortex of autistic patients (Fatemi et al., 2013). A more recent study similarly found a decrease in long Homer1 isoforms in post-mortem CA1 from patients with schizophrenia, but an increase in Homer1a (Matosin et al., 2016), suggesting that these patients had a higher ratio of short to long Homer1 protein in this hippocampal subregion. Furthermore, Homer1a and Ania-3 are induced rapidly by psychoactive compounds (Szumlinski and Kippin, 2008), such as ketamine (Iasevoli et al., 2007; De Bartolomeis et al., 2013) and cocaine (Brakeman et al., 1997; Zhang et al., 2007; Ghasemzadeh et al., 2009).

Genetic variants within the *HOMER1* gene have been associated with schizophrenia (Norton et al., 2003; Spellmann et al., 2011) and other psychiatric disorders (Rietschel et al., 2010a; Kelleher et al., 2012; Strauss et al., 2012), although more recent genome-wide association studies do not find a link between *HOMER1* SNPs and schizophrenia (Ripke et al., 2014). Still, Homer1 functionally interacts with proteins recurrently linked to schizophrenia, such as FMRP (Ronesi et al., 2012; Fromer et al., 2014; Ripke et al., 2014), Arc (Vazdarjanova et al., 2002; Kirov et al., 2012) and the calcium channel subunit CaV1.2 (Huang et al., 2007; Ripke et al., 2014).

Alterations of short Homer1 expression have been shown to affect synaptic strength. Overexpression of Homer1a in the hippocampus induces a glutamate-independent

modulation of surface AMPA receptors (Hennou et al., 2003; Sala et al., 2003; Hu et al., 2010; Rozov et al., 2012), reduced GluA2 subunit tyrosine phosphorylation (Hu et al., 2010) and blockade of long-term potentiation (Celikel et al., 2007; Rozov et al., 2012). It is considered that the ratio of short to long Homer1 in dendritic spines is a key mediator of AMPA currents (Gray et al., 2003; Van Keuren-Jensen and Cline, 2006). Other studies have shown that hippocampal Homer1a overexpression impairs spatial working and reference memory (Klugmann et al., 2005; Celikel et al., 2007), whilst knocking out Homer1 short forms causes deficits in fear conditioning (Inoue et al., 2009b; Mahan et al., 2012). However, the respective roles of Homer1a and Ania-3 in learning and memory cannot yet be distinguished.

Whilst there has been substantial investigation of the induction of Homer1 isoforms following exposure to psychoactive compounds (e.g. Nichols et al., 2003; Iasevoli et al., 2007; de Bartolomeis et al., 2013) and environmental stressors (e.g. Igaz et al., 2004; Ary et al., 2007) in rodents, fewer studies have focussed on its expression after learning. Hernandez et al (2006) demonstrated that the corticostriatal expression pattern of Homer1a varies with successive trial of instrumental learning (Hernandez et al., 2006). More recently, Mahan et al (2012) reported that *de novo* Homer1a expression occurs in the hippocampus and amygdala of mice following fear conditioning (Mahan et al., 2012). However, the induction of *Ania-3* with learning, or the examination of learning phase-specific expression, such as extinction learning, of either isoform, has yet to be studied.

5.1.2 Further dissecting the molecular correlates of associative learning: the context pre-exposure facilitation effect

In order to explore the contribution from activity-induced Homer1 proteins to different components of associative fear learning, I quantified the respective expression patterns of Homer1 isoforms after the consolidation, retrieval and extinction of conditioned fear memory. To enable further interrogation of the precise role of these proteins in memory consolidation, I employed a protocol which separates the contextual fear conditioning paradigm into its constitutive parts. In the context pre-exposure facilitation effect (CPFE) protocol, learning about the context and associating the contextual memory with a footshock occur during separate, consecutive training events. Since context encoding is necessary for context conditioning (Fanselow, 1990), only rats that are pre-exposed to the same context they are subsequently given an immediate shock in undergo associative fear learning about the context. Thus, immediate early gene expression related to exposure to a novel context versus the context-shock association can be quantified independently (Fanselow, 1990; Asok et al., 2013).

5.2 Determining the regulation of *Homer1* expression by contextual fear memory conditioning

5.2.1 Methods

The general methodology for contextual fear conditioning, *in situ* hybridization and immunoblotting is described in full in sections 2.4, 2.5 and 2.6, respectively. Contextual fear conditioning behaviour described in section 5.2.1.1 was performed by Dr K Thomas and Dr S Trent. The generation of coronal brain section libraries from these animals was performed in collaboration with Miss L Sykes.

5.2.1.1 Behaviour

To investigate memory consolidation-induced gene expression of Homer1, adult Lister Hooded rats (n = 6) were subjected to a conditioning training trial (3 min novel context exposure with 2 sec 0.5 mA footshock at 2 min). Rats were killed by CO₂ inhalation 30 min, 2 h, 4 h or 24 h after conditioning (n = 6 per group), in order to capture the rapid and slower induction of mRNA species and to observe their regulation over time. Control subjects were naïve littermates, killed at the same time of day as those undergoing fear conditioning.

Whole brains were immediately dissected and snap frozen on dry ice before storage at -80°C until use in *in situ* hybridization.

5.2.1.2 In situ hybridization

Oligonucleotide probes were designed to target specific mRNA sequences of interest. Since it was necessary to target individual Homer isoforms, a tool was developed in MATLAB to select optimal nucleotide sequences for generating probes. Table 5.1 shows the oligonucleotide probes sequences that were designed to target *Homer1a* and *Ania-3*, as well as a pan-probe for the long isoforms of the Homer1 gene. The rat Homer1 mRNA sequence used has accession number NM_031707.

Coronal brain sections (14 µm) containing dorsal regions of the hippocampus were cut, thaw-mounted onto glass slides and fixed in 4% paraformaldehyde prior to dehydration in ethanol and storage in 95% ethanol at 4°C, as described. Oligonucleotide probes were end-labelled with ³⁵S-dATP, before being hybridised to the tissue sections (see section 2.5.4). For each subject, two sections were incubated with radiolabelled probe whilst one section was incubated with 100X excess of un-labelled probe, as a negative control

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(see General Methods). Bound radiolabelled probe, corresponding to mRNA quantity, was measured using radiographic film. Exposure times were as follows: *Homer1a*, 5 days; *Ania-3*, 10 days; long *Homer1*, 7 days. Films were developed and regional mRNA expression quantified by sampling the densitometry from the autoradiograph.

Target mRNA	Oligonucleotide probe sequence	Max non-specific homology
Homer1a	CATGATTGCTGAATTGAATGTGTACCTATGTGAAAATGGCAATGC	62%
Ania-3	GGTAGGGCGGAGGATTCATGACAGACAATACATGAACTTGGGCAG	53%
Long Homer1	CTCTGTCTTGTGGCTGTGCACCGCGTTTGCTTGACTACTAACACA	47%

Table 5.1 Specific 45mer oligonucleotide probe sequences targeting Homer1 isoforms. Probes were designed with a CG:AT ratio close to 1 and without long trains of the same nucleotide. Max non-specific homology denotes the maximum sequence homology with other Homer isoforms. The rat Homer1 accession number is NM_031707.

5.2.2 Results

5.2.2.1 Regional expression of Homer1

In situ hybridization yielded quantifiable autoradiographs, such as those in Figure 5.1, which show the expression of the short Homer1 isoforms, *Homer1a* and *Ania-3*, and the long isoforms (*Homer1b/c/f/g*) across coronal sections of rat brain at approximately -3.5 mm posterior to bregma. Each isoform is strongly expressed in cortical and hippocampal regions of this section, as well as some areas surrounding the basolateral amygdala. Within the hippocampus, Homer1 isoforms are expressed strongly throughout, with the exception of *Homer1a*, which shows weak expression in the dentate gyrus (Figure 5.1). These expression patterns are consistent with those observed previously, using similar methods (Iasevoli et al., 2010).

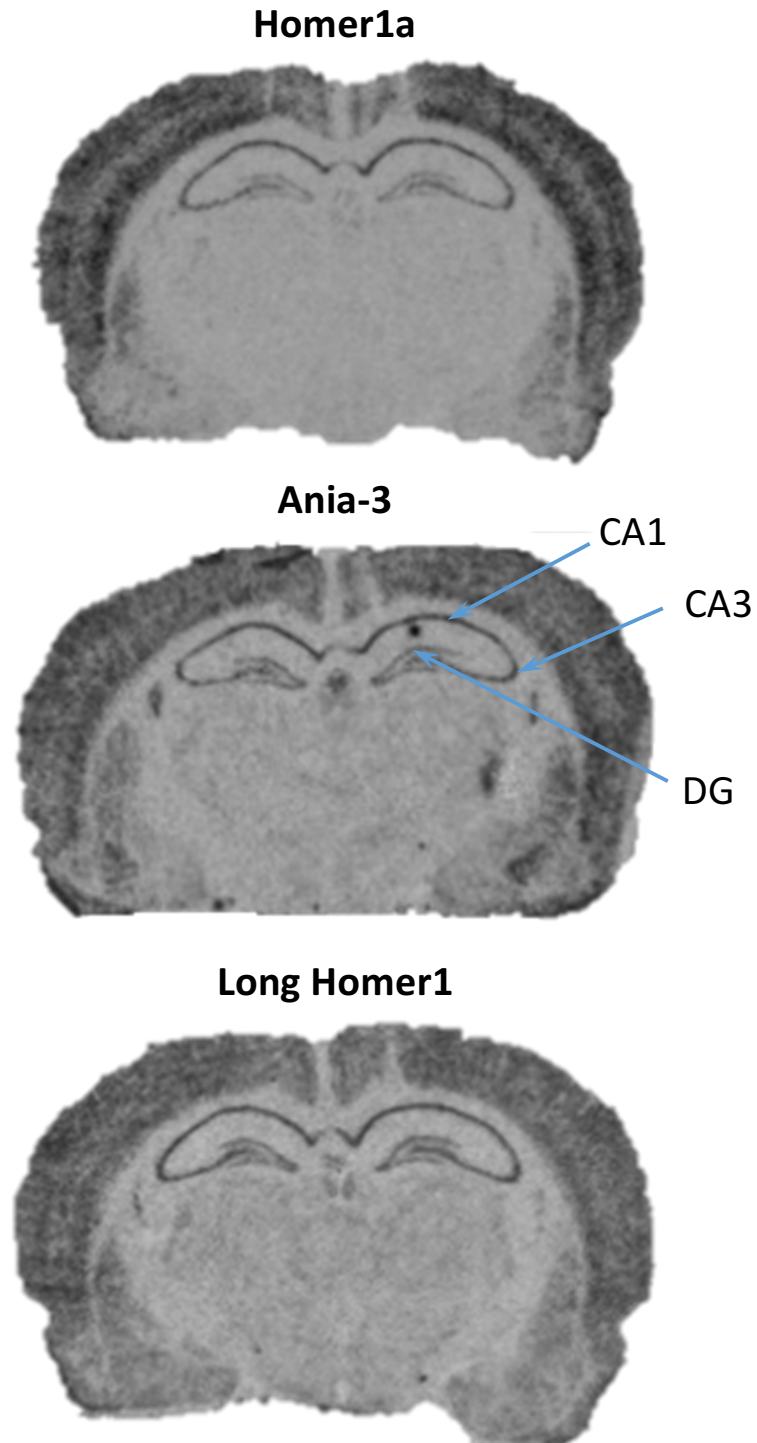


Figure 5.1 Regional expression of Homer1 isoforms. Coronal sections of rat brain taken from approximate location from bregma: -3.5 mm posterior. Short isoforms, *Homer1a* and *Ania-3*, and long isoforms, *Homer1b/c/f/g*, are expressed throughout hippocampal regions CA1, CA3 and dentate gyrus.

5.2.2.2 *Differential regulation of short and long Homer1 isoforms following contextual fear memory training*

During contextual fear conditioning training, rats displayed robust post-shock freezing, compared to baseline ($P < 0.001$, t-test; Figure 5.2). Hippocampal expression of Homer1 transcripts was visualised over 24 h following the acquisition of contextual fear memory (Figure 5.3). Quantitative analysis showed that the expression of both short Homer1 isoforms, *Homer1a* and *Ania-3*, was increased by fear conditioning, compared to naïve controls (effect of group: *Homer1a* $F_{(5, 90)} = 21.70$, $P < 0.001$; *Ania-3* $F_{(5, 90)} = 30.17$, $P < 0.001$, two-way ANOVA; Figure 5.4a,b). The expression of long Homer1 isoforms, as indicated by a pan-*Homer1b/c/f/g* oligonucleotide probe, was unchanged from naïve controls ($F_{(5, 90)} = 1.31$, $P = 0.27$; Figure 5.3, Figure 5.4c). For *Homer1a* and *Ania-3* isoforms, there was a significant effect of hippocampal subregion (*Homer1a* $F_{(2, 90)} = 6.30$, $P < 0.01$; *Ania-3* $F_{(2, 90)} = 5.34$, $P < 0.01$, two-way ANOVA), indicating that the change in *Homer1a* and *Ania-3* expression following conditioning was not uniform across the hippocampus. Post-hoc tests indicated that *Homer1a* expression was increased in the CA1 region at 30 min ($P < 0.001$, Dunnett's multiple comparisons test), 2 h ($P < 0.05$) and 4 h ($P < 0.05$) after conditioning and in the dentate gyrus at 30 min only ($P < 0.001$; Figure 5.4a), but not CA3. Conversely, *Ania-3* expression was increased in all three hippocampal regions, yet solely at the 30 min time point: CA1 ($P < 0.001$, Dunnett's multiple comparisons test), CA3 ($P < 0.05$), dentate gyrus ($P < 0.001$; Figure 5.3, Figure 5.4b). At 24 h after conditioning, neither *Homer1a* nor *Ania-3* mRNA levels differed from control in any of the regions investigated.

The Regulation of Postsynaptic Density Homer1 Proteins by Associative Learning

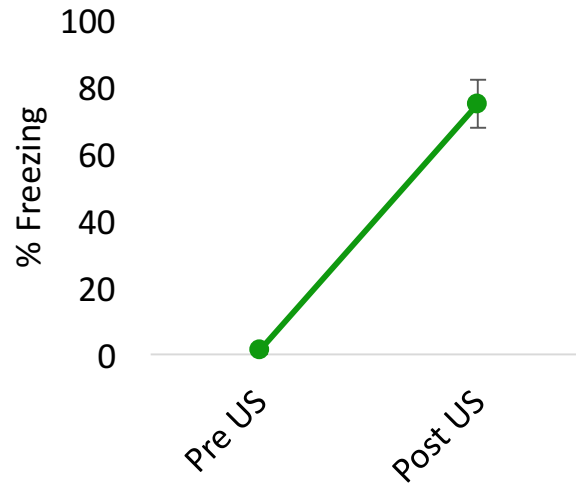


Figure 5.2 Contextual fear conditioning induced robust post-shock freezing. Rats were exposed to a novel context for 3 min and received a footshock after 2 min. Data representative of all experimental groups culled 30 min, 2 h, 4 h and 24 h after conditioning for *in situ* hybridization and immunoblotting (n = 6). Data represented by mean \pm SEM percent freezing.

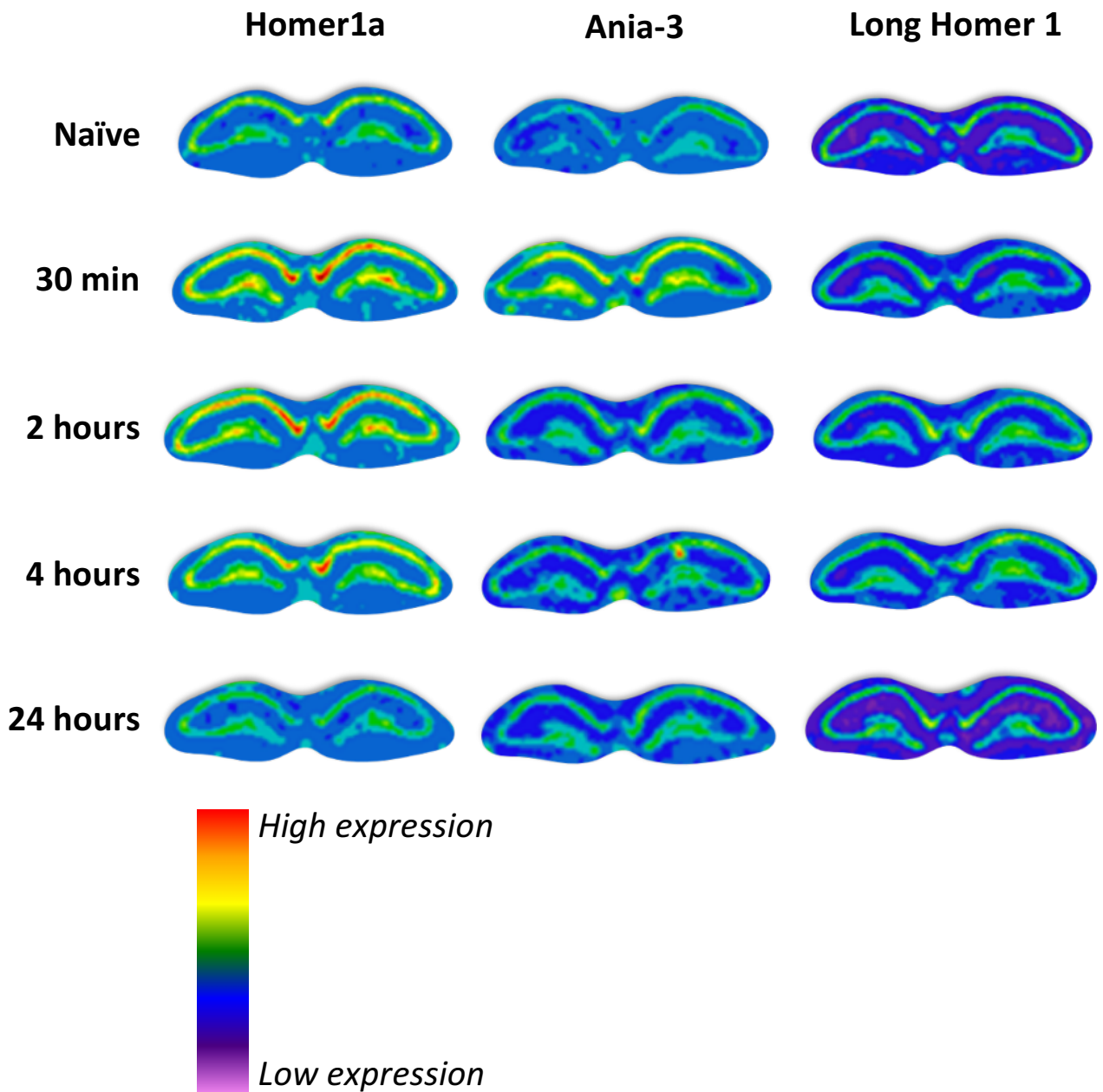


Figure 5.3 Heat map hippocampal autoradiograph of representative mRNA expression of Homer1a, Ania-3 and long Homer1 following contextual fear conditioning. False-colour heat map indicates the optical density of the autoradiograph, which represents the level of gene expression in that region. Specifically designed oligonucleotide probes were radiolabelled with dATP-³⁵S. Times indicate the duration after contextual fear conditioning that the animals were culled and the brains dissected. Note that the method does not allow one to compare the relative expression levels of genes of interest in a subregion.

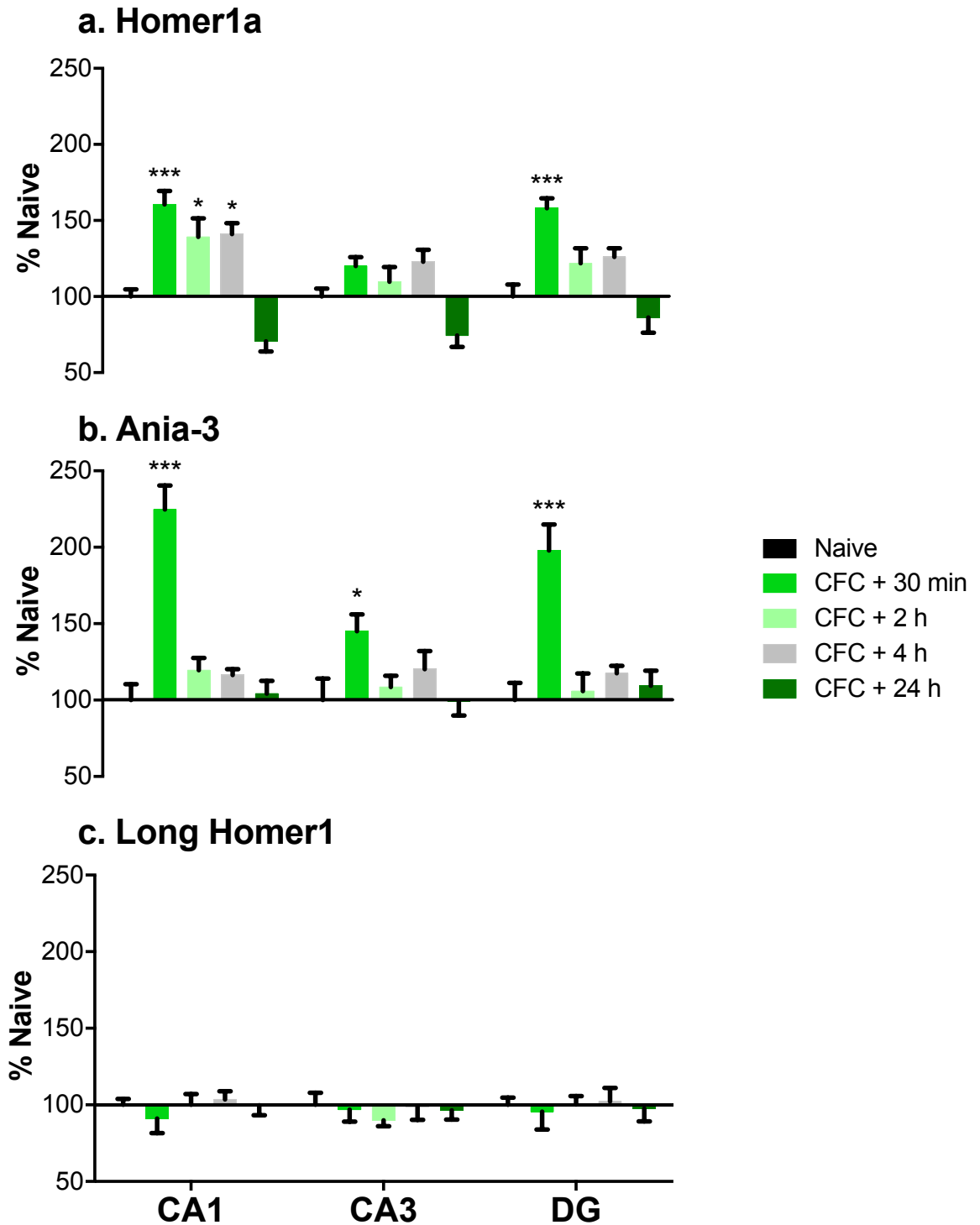


Figure 5.4 Hippocampal mRNA expression of Homer1a, Ania-3 and long Homer1 following contextual fear conditioning (CFC). mRNA expression from the CA1, CA3 and dentate gyrus (DG) regions is represented by mean \pm SEM standardised optical density values, standardised to naïve controls (100%). Brains were dissected 30 min, 2 h, 4 h or 24 h after conditioning. $n=6$ per group. Asterisks represent significance of group vs. naïve control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

5.3 The regulation of short Homer1 isoforms by novelty exposure

5.3.1 Methods

In order to determine Homer expression following uniquely the exposure to novelty, Lister Hooded rats ($n = 6$) were placed in a novel context for 3 min without the administration of a footshock. Subjects were killed by CO₂ inhalation 30 min after context exposure. These were compared to a cohort of naïve rats ($n = 6$), as well as rats which had undergone a conditioning training trial and killed at the same time point ($n = 6$). Whole brains were immediately dissected and snap frozen on dry ice before storage at -80°C. *In situ* hybridization, targeting *Homer1a*, *Ania-3* and long *Homer1*, was performed as above.

5.3.2 Results

The previous experiments show that both *Homer1a* and *Ania-3* are induced in the hippocampus by contextual fear conditioning. Since this procedure involves the exposure of the animal to a novel context, and past studies have shown *Homer1a* to be upregulated by novelty alone (Vazdarjanova et al., 2002), the expression of *Homer1a* and *Ania-3* was compared 30 min after novelty and fear conditioning in separate cohorts. I again show that contextual fear conditioning induced the expression of both short Homer1 isoforms, compared to naïve controls (effect of group: *Homer1a* $F_{(1, 30)} = 20.26$, $P < 0.001$; *Ania-3* $F_{(1, 30)} = 27.08$, $P < 0.001$, two-way ANOVA). Exposure to a novel context for 3 min was sufficient to induce the expression of *Homer1a* (effect of group: $F_{(1, 30)} = 35.95$, $P < 0.001$, two-way ANOVA) and *Ania-3* ($F_{(1, 30)} = 25.98$, $P < 0.001$), but not the long Homer1 isoforms ($F_{(1, 30)} = 2.62$, $P = 0.12$). Novelty exposure increased *Ania-3* expression in each hippocampal subregion (CA1 $P < 0.001$, CA3 $P < 0.05$, DG $P < 0.05$,

Tukey's multiple comparisons test; Figure 5.5b), while *Homer1a* was induced in the CA1 ($P < 0.001$) and dentate gyrus ($P < 0.001$), but not in CA3 ($P = 0.43$; Figure 5.5a). The change in hippocampal *Ania-3* expression did not differ between the fear conditioned and novelty-exposed groups. Interestingly, novelty exposure alone induced a greater expression of *Homer1a* in the dentate gyrus than fear conditioning ($P < 0.05$, Tukey's multiple comparisons test).

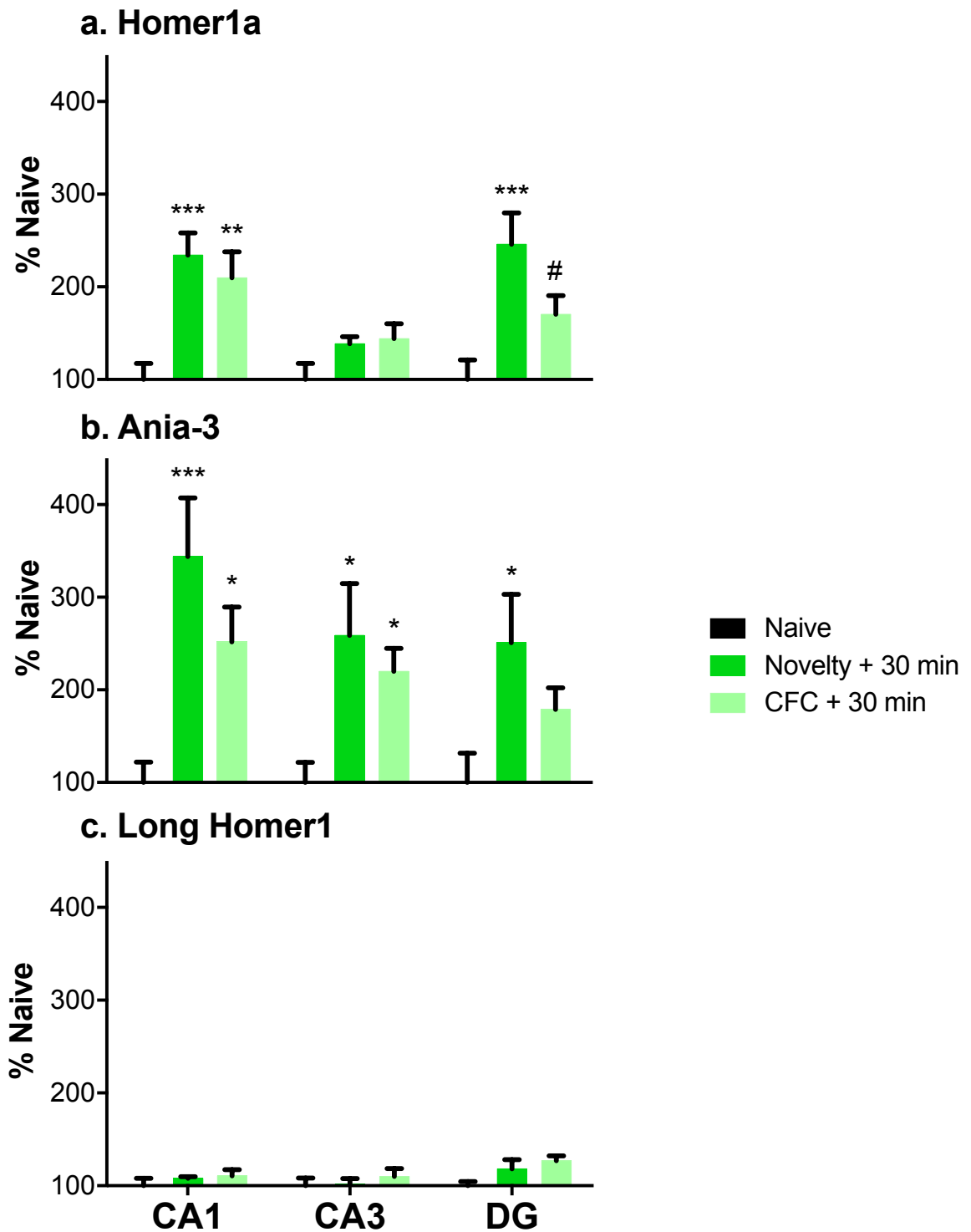


Figure 5.5 mRNA expression of Homer1a, Ania-3 and long Homer1 following exposure to a novel context or contextual fear conditioning (CFC). mRNA expression is represented by mean \pm SEM standardised optical density values, standardised to naive controls (100%). Brains were dissected 30 min after context exposure. $n = 6$ per group. Asterisks represent significance of group vs. naive control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Hash symbol represents significance of group vs. novelty ($^{\#}P < 0.05$).

5.4 Determining the regulation of Homer1 expression using the Context Pre-exposure Facilitation Effect (CPFE) behavioural model of contextual fear conditioning

5.4.1 Methods

In an immediate shock paradigm, Lister Hooded rats were exposed to one of two different contexts, context A or context B, for 20 min/day for three consecutive days, in order to familiarise the animals to one context. On the fourth day, rats were placed into either their familiar context or the novel context. One group (n = 6) was placed into the familiar context (context pre-exposure facilitation effect, or CPFE group) and received a footshock (0.5 mA, 2 sec) immediately and then removed. Two groups (n = 6 per group) were placed into the novel context for the same short duration: one group received a footshock whilst the other group did not. Groups were counterbalanced between the two contexts. Rats were killed by CO₂ inhalation 30 min after the final context exposure. Control subjects were naïve littermates. Whole brains were rapidly dissected and snap frozen on dry ice before storage at -80°C. *In situ* hybridization, targeting *Homer1a*, *Ania-3* and long Homer1, was performed as above.

5.4.2 Results

In order to determine whether *Homer1a* or *Ania-3* are induced specifically by associative learning or by the novelty exposure associated with the standard contextual fear conditioning paradigm I used a context pre-exposure facilitation effect (CPFE) procedure to further dissect the regulation of short Homer1 isoforms by training. Training was split into three components: brief exposure to a novel context with no presentation of the unconditioned stimulus (Novelty); immediate footshock given upon exposure to a novel context (Novelty IS); brief exposure to a familiar context with an immediate footshock

(CPFE). Only the CPFE procedure is sufficient to generate an associative context-footshock fear memory (Fanselow, 1990). Thirty minutes following training, the induction of *Homer1a* and *Ania-3* within the hippocampus was subregion-dependent (effect of brain region: *Homer1a* $F_{(2, 60)} = 11.62$, $P < 0.001$; *Ania-3* $F_{(2, 60)} = 4.60$ $P < 0.05$). Compared to naïve controls, the expression of both *Homer1a* and *Ania-3* was increased following each stimulus, irrespective of context-footshock pairing, but only in the CA1 region (*Homer1a*: Novelty $P < 0.001$, Novelty IS $P < 0.001$, CPFE $P < 0.01$; *Ania-3*: Novelty $P < 0.05$, Novelty IS $P < 0.01$, CFPE $P < 0.05$, two-way ANOVA with post-hoc Dunnett's multiple comparisons test; Figure 5.6). These results imply that short Homer1 isoforms are induced in some regions of the hippocampus by constitutive events of the fear conditioning procedure, but not by associative learning *per se*. Long Homer1 isoforms remained unchanged in all experimental groups.

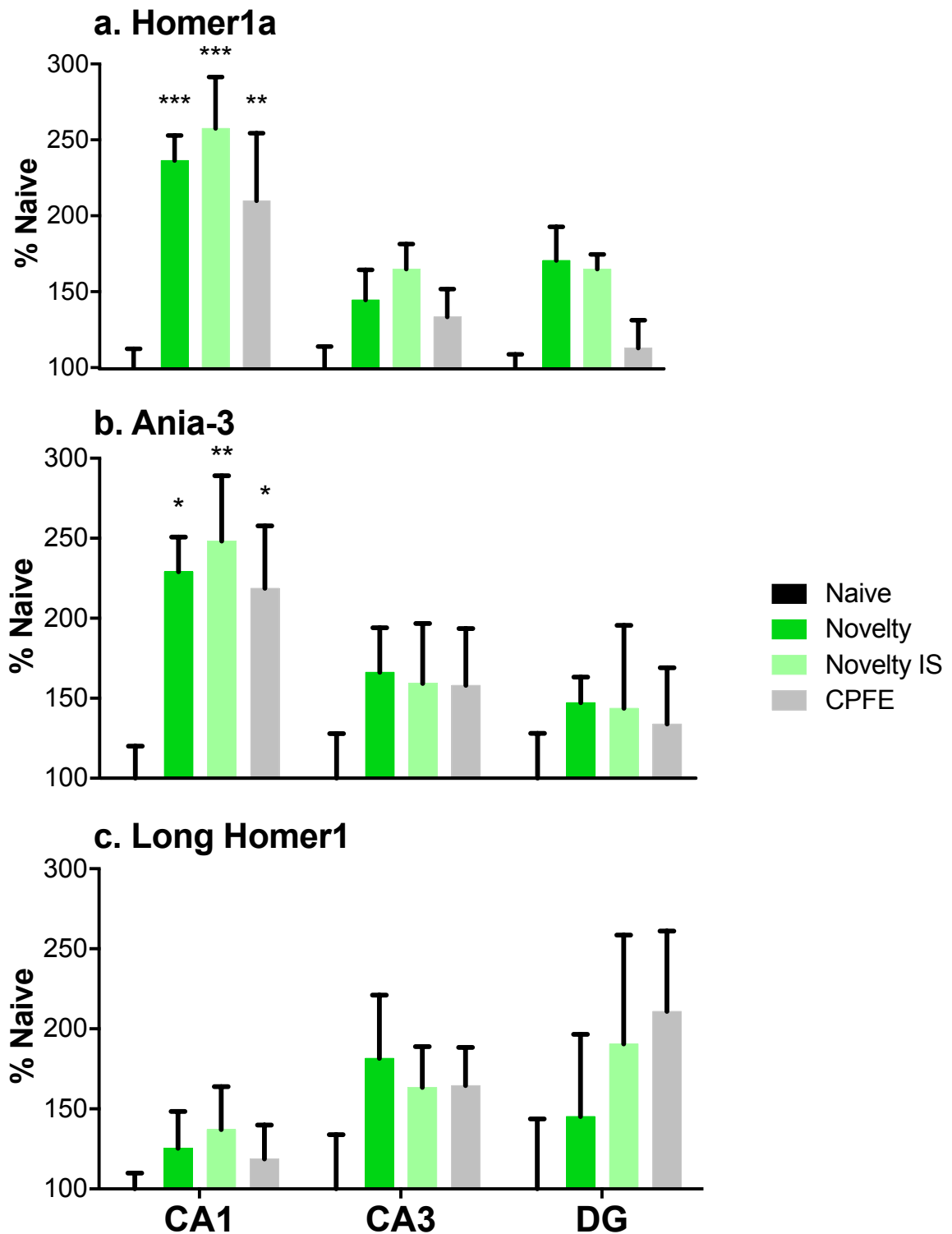


Figure 5.6 Hippocampal mRNA expression of Homer1a, Ania-3 and long Homer1 following a brief exposure to a novel context (Novelty), brief novel context exposure with an immediate footshock (Novelty IS) and brief exposure to a familiar context with an immediate footshock (context preexposure facilitation effect, CPFE). mRNA expression is represented by mean \pm SEM standardised optical density values, standardised to naïve controls (100%). Brains were dissected 30 min after context exposure. $n = 6$ per group. Asterisks represent significance of group vs. naïve control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

5.5 Differential expression of short and long Homer1 isoforms following contextual fear memory retrieval and extinction

5.5.1 Methods

Associative fear memory retrieval and extinction behavioural testing and subsequent generation of a coronal brain section library were performed previously by Dr K Thomas. Rats were subjected to a conditioning training trial followed by either a short recall trial (2 min) or a long recall trial (10 min), 48 h later. Rats were killed by CO₂ inhalation 30 min or 2 h after testing (n = 6 per group). Conditioned control rats underwent a conditioning training trial and were killed 48 h later (n = 6). Whole brains were rapidly dissected and snap frozen on dry ice before storage at -80°C. *In situ* hybridization, targeting *Homer1a*, *Ania-3* and long Homer1, was performed as above.

5.5.2 Results

In order to investigate whether short Homer1 isoforms are induced in the hippocampus by other contextual encoding processes, gene expression was quantified after the recall (retrieval) and extinction of contextual fear. All groups displayed robust post-shock freezing (effect of time: $F_{(1, 33)} = 385.5$, $P < 0.001$, two-way repeated measures ANOVA) and extinction training induced a within-trial reduction of freezing ($P < 0.001$, paired t-test; Figure 5.7). Re-exposure to the context modified the expression of *Homer1a* (effect of group: $F_{(4, 147)} = 12.05$, $P < 0.001$, two-way ANOVA) and *Ania-3* ($F_{(4, 147)} = 20.75$, $P < 0.001$). For all three targets, there was no effect of brain region on gene expression, nor was there an interaction effect. Following a 2 min recall trial, which is not sufficient to induce extinction (Barnes and Thomas, 2008), *Homer1a* expression was increased at 30 min ($P < 0.05$, Dunnett's multiple comparisons test) and at 2 h in CA1 ($P < 0.001$) and at 2 h only in the dentate gyrus ($P < 0.001$; Figure 5.8a). Conversely, *Ania-3* expression was

upregulated at 30 min in all three hippocampal regions: CA1 ($P < 0.01$, Dunnett's multiple comparisons test), CA3 ($P < 0.05$) and dentate gyrus ($P < 0.01$; Figure 5.8b), but not at 2 h.

Extinction induced a similar pattern of *Homer1a* and *Ania-3* expression. *Homer1a* expression was increased in all hippocampal regions at 30 min (CA1 $P < 0.01$, CA3 $P < 0.01$, DG $P < 0.05$, Dunnett's multiple comparisons test) and 2 h ($P < 0.001$ all instances; Figure 5.8a) after extinction training. Again, *Ania-3* expression was solely increased at 30 min, and in all regions (CA1 $P < 0.001$, CA3 $P < 0.05$, DG $P < 0.001$; Figure 5.8b). There were no significant differences between the recall- and extinction-induced expression of either short Homer1 isoform (two-way ANOVA). Despite a significant modulation of the expression of the longer Homer1 isoforms by context re-exposure ($F(4, 93) = 12.05$, $P < 0.01$, two-way ANOVA), there were no post-hoc differences observed in multiple comparison tests (Dunnett; Figure 5.8c).

The Regulation of Postsynaptic Density Homer1 Proteins by Associative Learning

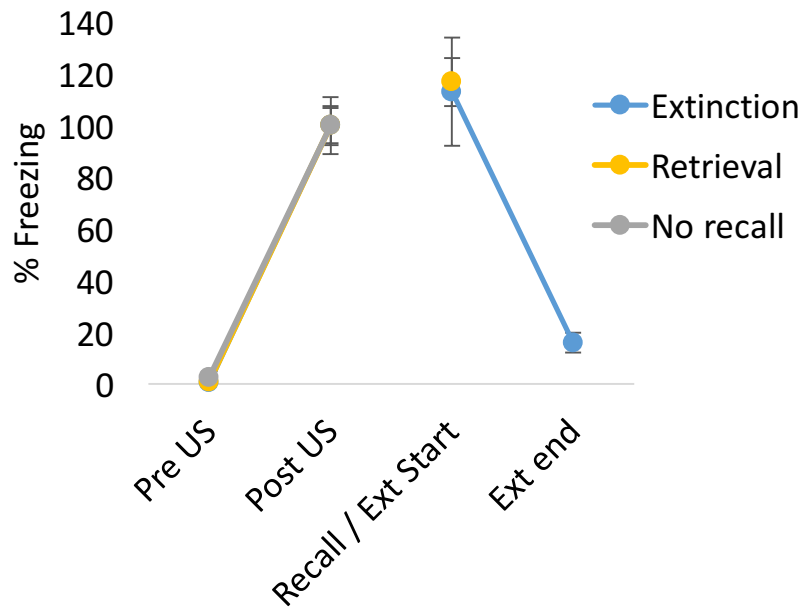


Figure 5.7 In the retrieval and extinction of contextual fear memory, rats displayed robust conditioned freezing and an extinguished response after long recall (10 min). Retrieval and extinction (Ext) training took place 48 h after conditioning. Control, “no recall” animals were conditioned and culled 48 h afterwards. Data representative of experimental groups culled 30 min and 2 h after retrieval and extinction for *in situ* hybridization or immunoblotting (n = 12). Data represented by mean \pm SEM percent freezing normalised to post-unconditioned stimulus (US) levels.

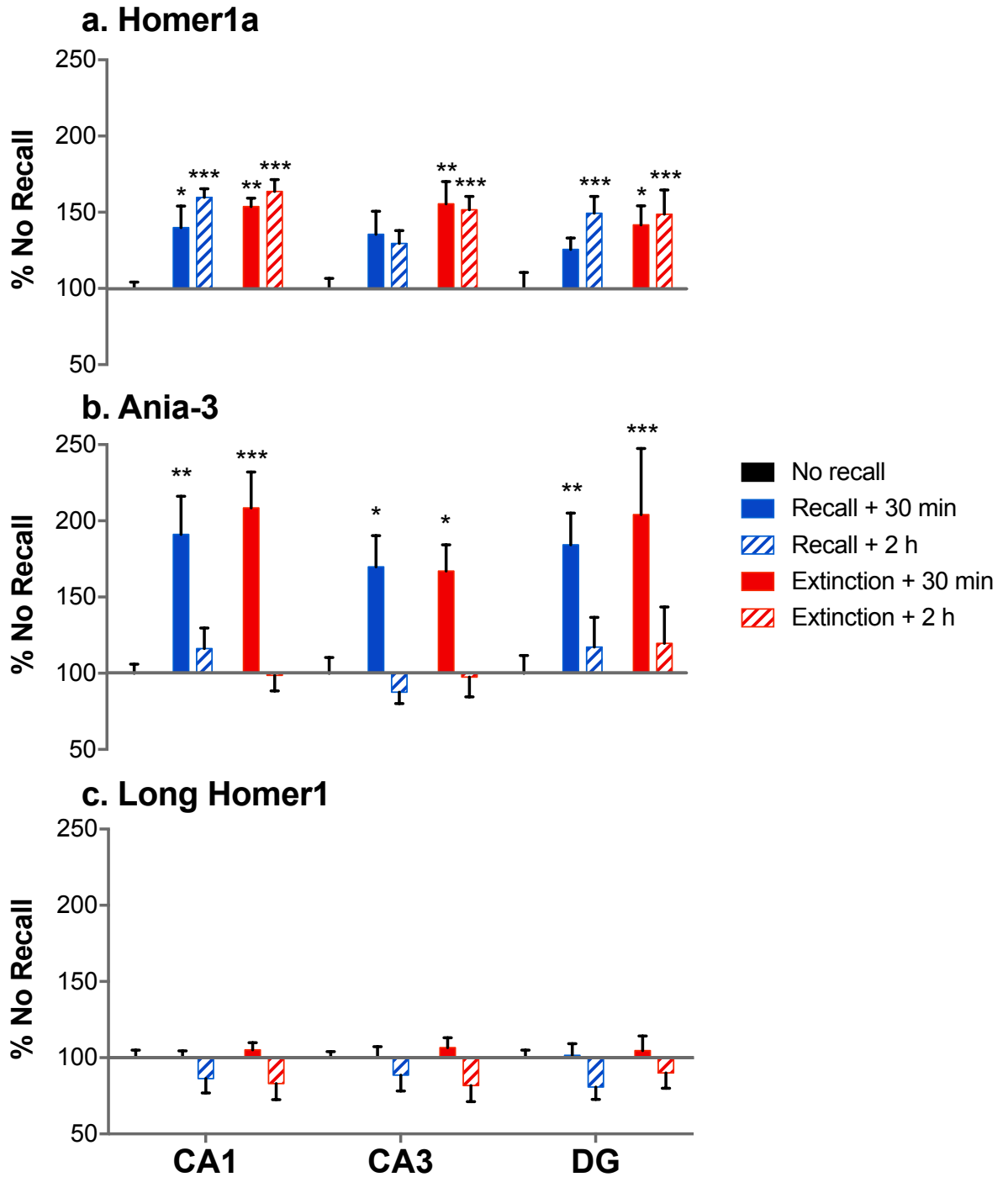


Figure 5.8 mRNA expression of Homer1a, Ania-3 and long Homer1 in the hippocampus following the recall or extinction of contextual fear memory. mRNA expression is represented by mean \pm SEM standardised optical density values, standardised to no recall controls (100%). Brains were taken 30 min or 2 h after re-exposure. $n = 6-12$ per group. Asterisks represent significance of group vs. no recall control (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

5.6 Quantification of Homer1a protein following contextual fear conditioning and extinction

5.6.1 Methods

5.6.1.1 Behaviour

For protein quantification assays, separate cohorts of rats underwent either contextual fear conditioning or conditioning training with 10 min recall trial 48 h later, in order to measure protein changes associated with the consolidation and extinction of fear memory, respectively. Rats were then killed 2 h (n = 6 per group) or 4 h (n = 6 per group) after training. Conditioning only control subjects were naïve rats and extinction controls underwent conditioning training but no recall (killed 48 h post-conditioning).

Rats were killed by CO₂ inhalation and whole hippocampi were rapidly micro-dissected and snap frozen before storage at -80°C until Western blotting.

5.6.1.2 Immunoblotting

For each subject, the hippocampus from one hemisphere was used to produce whole tissue hippocampal homogenates and the contralateral hippocampus was used to produce synaptosomal fractions. Protein concentration per sample was measured using the BCA assay (whole tissue homogenates) or the Bradford assay (synaptosomes). Samples were diluted to standardise the protein concentration added to gels for electrophoresis and 15 µl of each diluted sample (typically containing 10-40 µg protein) was loaded to a lane. Proteins were transferred to a nitrocellulose membrane and blocked in 5% milk solution (Amersham ECL Prime, GE Healthcare, UK). The membrane was incubated with anti-Homer1a polyclonal rabbit antibody (160013, Synaptic Systems, Germany), 1:1000, and either anti-GAPDH monoclonal mouse antibody (ab8245, Abcam,

UK; whole tissue homogenates), 1:5000, or anti-Calnexin polyclonal rabbit antibody (ab22595, Abcam; synaptosomes), 1:5000, overnight at 4°C. The anti-Homer1a antibody targets the following amino acid sequence at the C-terminus of the Homer1a protein: HRYTFNSAIMIK. Since an antibody targeting Ania-3 does not exist at present, protein quantification could only be performed for Homer1a. After washing in TBST, the membrane was incubated with 1:10000 goat anti-rabbit (680 nm) and donkey anti-mouse (GAPDH; 800 nm) or donkey anti-rabbit (Calnexin; 800 nm) fluorescent secondary antibodies (LI-COR), for 1 h at room temperature and washed in TBST again. Protein bands were imaged and quantified using an infrared imaging system. Homer1a quantity was standardised to the housekeeping gene of the same lane.

5.6.2 Results

With the aim to gain a greater insight into the functional induction of short Homer1 isoforms following phases of contextual fear learning, immunoblotting was used to quantify protein in samples of rat hippocampus following the consolidation and extinction of contextual fear.

There was no effect of contextual fear conditioning on Homer1a protein in whole hippocampal fractions ($F_{(1, 18)} = 4.265$, $P = 0.054$, two-way ANOVA; Figure 5.9a). In contrast, extinction learning significantly modified Homer1a protein ($F_{(1, 20)} = 7.114$, $P < 0.05$). Post-hoc tests showed that extinction increased Homer1a protein at 4 h ($P < 0.05$, Sidak's multiple comparisons test), but not at 2 h ($P = 0.63$; Figure 5.9c).

Since Homer1a functions predominantly at the postsynaptic density, the same protein quantification assays were performed on synaptosome fractions of hippocampal samples from the same animals (i.e. the contralateral hippocampus). No change in

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Homer1a protein was observed in hippocampal synaptomes following contextual fear conditioning ($F_{(1, 20)} = 0.2576$, $P = 0.62$; two-way ANOVA; Figure 5.9b) or extinction ($F_{(1, 20)} = 0.0824$, $P = 0.78$; two-way ANOVA; Figure 5.9d).

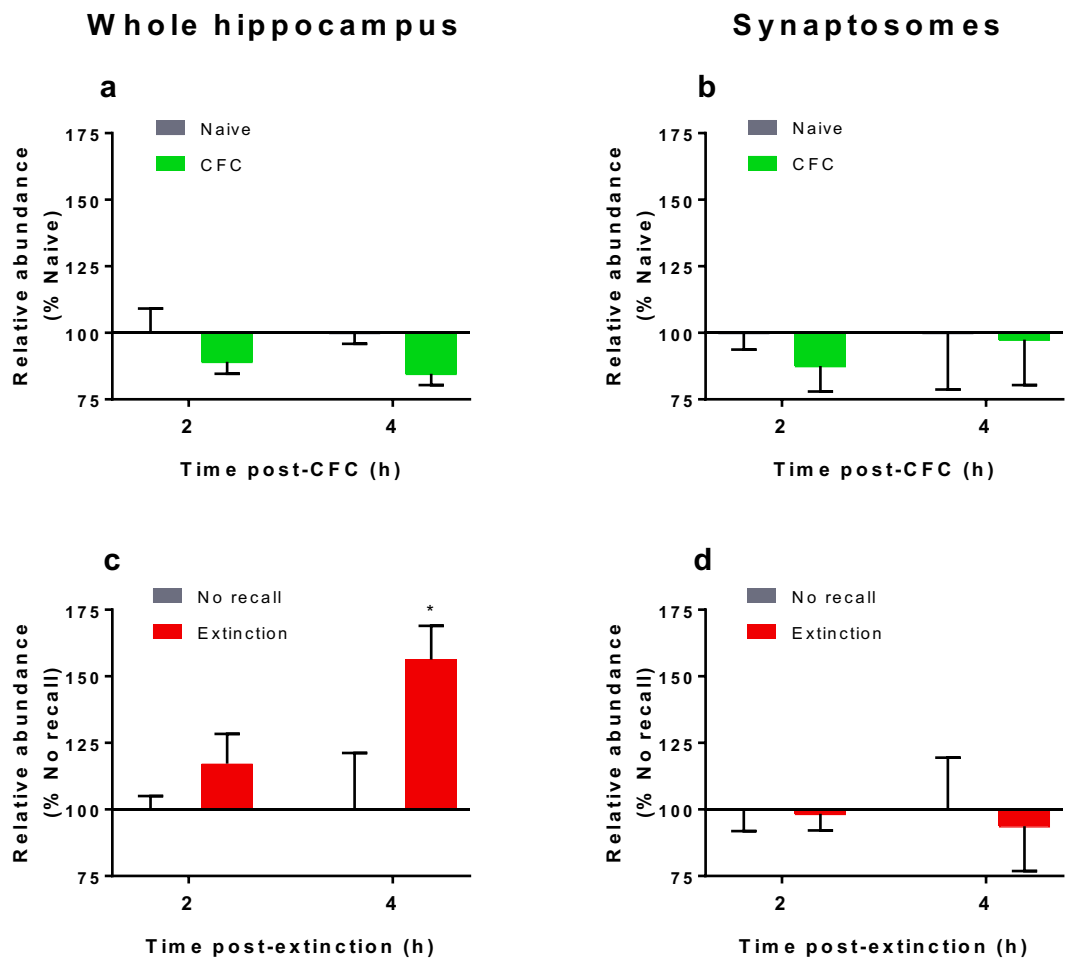


Figure 5.9 Hippocampal Homer1a protein following contextual fear conditioning and extinction. Rat hippocampal samples were homogenised as either whole tissue fractions (a, c) or synaptosomal fractions (b, d). Homer1a protein quantity is represented by mean \pm SEM optical density values, standardised to controls (100%). Brains were dissected 2 h or 4 h after fear conditioning or extinction. $n = 6$ per group. Asterisks represent significance of group vs. naïve or no recall control ($*P < 0.05$).

5.7 Discussion

The present findings indicate that the expression of short Homer1 isoforms in the hippocampus is correlated with the processing of a Pavlovian association between the conditioned and unconditioned stimuli concurrent with the contextual fear conditioning paradigm. Within this, the relative expression of *Homer1a* and *Ania-3* within different subregions of the hippocampus depended on the type of memory processing involved. Crucially, in some instances, the region-specific regulation of *Homer1a* differed to that of *Ania-3*. Long forms of Homer1, which were identified as a group with one oligonucleotide probe, were not regulated by contextual fear conditioning, retrieval or extinction in any hippocampal subregion or time point measured, consistent with its previously reported constitutive expression (Fagni et al., 2002).

Short forms of Homer1 mRNA were increased in CA1 following contextual fear conditioning, hinting at a role for Homer1a and Ania-3 in associative memory formation. Equally, *Homer1a* and *Ania-3* were regulated by 3 min novel context exposure, which, in the formation of a context representation, is considered an associative learning event (Fanselow, 1990; Matus-Amat et al., 2004). Increased hippocampal Homer1a after Pavlovian fear conditioning and novel context exposure has been demonstrated previously (Vazdarjanova et al., 2002; Mahan et al., 2012). However, I show that an increase in short Homer1 CA1 expression is induced by arousal, irrespective of a context-footshock pairing. Both Homer1a and Ania-3 mRNA was elevated in CA1 following exposure to the novel context alone or footshock alone. Upon breaking down the components of contextual fear conditioning in the CPFE experiments, expression levels in the only group that engages associative learning, the CPFE group, are not different

from the brief novelty exposure or immediate shock groups. Therefore, the regulation of short Homer1 isoforms in CA1 may be related to the activity associated with the processing of external stimuli, rather than the consolidation of associative fear memory.

The regulation of *Homer1a* and *Ania-3* in the dentate gyrus after contextual fear conditioning and novelty exposure is more likely to be related to the Pavlovian associative events of contextual memory formation. The expression of both isoforms was increased in the dentate gyrus following fear conditioning training, but not after an immediate footshock or brief novelty exposure, suggesting that short Homer1 expression solely occurs during the pairing of the two. This is consistent with the view that the dentate gyrus mediates the formation of conjunctive contextual representations (Morris et al., 2013). The lack of regulation in this subregion with CPFE may be due to the temporal separation of context representation and context-footshock pairing.

Whilst *homer1a* and *Ania-3* expression was induced by the same stimuli, their regulation after contextual fear conditioning exhibited temporal and spatial differences. The most striking of these was the duration for which the elevated expression level was detectable. Regulated *Ania-3* expression was considerably more transient than *Homer1a*, in all hippocampal subregions. This observation, which is previously unreported, implies that the post-transcriptional regulation of these two activity-induced isoforms occurs through different mechanisms. It is possible that the discrepancy occurs due to differences in metabolic processing, yet may also have functional implications for the subsequently translated proteins.

Following contextual fear conditioning, only *Ania-3* was regulated in CA3. The same was observed following brief fear memory retrieval. This regional discrepancy between *Homer1a* and *Ania-3* implies that the two isoforms are independently regulated and are likely to play different functional roles in long-term contextual fear memory. Since CA3 is posited to participate in the encoding of new representations, pattern completion and spatial short-term memory (Ji and Maren, 2008; Kesner and Rolls, 2015), it may be hypothesised, based on the current observations, that *Ania-3* has a more predominant role in such processes than *Homer1a*.

Interestingly, in contrast to the lack of changes in CA3 expression following contextual fear conditioning and retrieval, *Homer1a* was upregulated in CA3 by extinction learning, suggesting that the expression of short Homer1 isoforms in CA3 is dissociated by the type of associative memory process. This is the first time that evidence for a role of short Homer proteins in extinction learning has been presented, and may represent a key distinction between *Homer1a* and *Ania-3* function. It also implicates Homer1 in specific cognitive processes relevant to schizophrenia and other psychiatric disorders. The differential upregulation of *Homer1a* in CA3 and dentate gyrus may be related to the respective pattern completion and pattern separation processes of these two subregions (Rolls, 2016) that serve to encode the context-no footshock extinction memory (Ji and Maren, 2008) distinct from the context-footshock memory supporting the fear response.

The expression of mRNA does not guarantee its translation to functional protein. Using Western blot assays, I observed an increase in *Homer1a* protein in whole hippocampal samples 4 h after extinction learning, but not in synaptosomal fractions or after fear

conditioning. Since short Homers are synthesised in the soma before recruitment to active dendritic spines (Brakeman et al., 1997; Okada et al., 2009b), the lack of protein change in the synaptosomal fraction after extinction may indicate that recruitment to dendritic spines is yet to occur at the time points observed. However, previous evidence suggests that Homer1a can be recruited into spines within 4 h (Okada et al., 2009b). It is more likely that these observations are due to the recruitment of Homer1a to too few synapses in particular subregions of the hippocampus for detection within fractions of all synaptosomes from the entire hippocampus, yet sufficient surplus translation in somata for detection in whole hippocampal fractions, by Western blot. The absence of increased Homer1a protein following fear conditioning may also reflect a failure to detect small changes in protein with this method, yet may also reflect functional differences between Homer1a and Ania-3. Since an antibody against Ania-3 does not exist at present, it was not possible to compare the protein expression of the two short Homer1 isoforms. Considering the differential expression of the mRNA in each subregion of the hippocampus, future experiments should be designed to determine region-specific short Homer1 protein expression after components of fear learning. This approach may also facilitate the detection of more subtle protein changes from concentrating functionally relevant tissue.

In conclusion, the present results build on previous studies showing that short, activity-induced Homer1 immediate early genes are involved in learning and memory. I present novel findings showing that regulation of these short Homer1 variants accompany the retrieval and extinction of associative fear memories, and replicate previous findings demonstrating their expression after consolidation. I also demonstrate for the first time

that Homer1a and Ania-3 are differentially regulated in the hippocampus during learning processes and may function differently in long-term contextual fear-memory. It is thus far unclear whether Homer1a and/or Ania-3 expression is required for processes of associative fear learning in the hippocampus. These data pave the way for the exploration of the respective roles of short Homer1 isoforms in the consolidation and extinction of contextual fear memory. This is the subject of Chapter 6

Chapter 6: The Role of Short Homer Proteins in Fear Memory Consolidation and Extinction

6.1 Introduction

The previous chapter highlighted the differential regulation of Homer1 isoforms in the hippocampus by contextual fear conditioning, retrieval and extinction. I showed that hippocampal *Homer1a* and *Ania-3* expression, but not long *Homer1* expression, is increased following each of these phases of associative learning, yet the respective expression pattern of each isoform in subregions of the hippocampus is regulated independently. Moreover, an increase in Homer1a protein was only observed following extinction learning. However, it is still unclear whether the expression of either short Homer1 isoform is required for associative learning. Little is known about the functional differences between Homer1a and Ania-3 and it is feasible that their roles are accommodated by both proteins and therefore redundant. However, their distinct activity-regulated expression profiles suggest that Homer1a and Ania-3 may function separately in separate processes.

Few studies have manipulated the expression of short *Homer1* isoforms *in vivo* in order to provide insight into their role in learning and memory. Klugmann et al (2005) overexpressed Homer1a in the dorsal hippocampus of rats using adeno-associated virus vectors. These animals exhibited impaired encoding in a novel object recognition task and impaired spatial reference memory in the Morris water maze task, both of which

are hippocampal-dependent tasks (Klugmann et al., 2005). A later study also reported that Homer1a overexpression in the dorsal hippocampus impaired spatial working memory in a Y-maze task (Celikel et al., 2007). Most recently, Banerjee *et al* (2016) showed that overexpression of Homer1a in the basal and lateral amygdala of rats induced deficits in cued fear conditioning and social interaction (Banerjee et al., 2016). Knocking out Homer1 short forms also affected fear learning. Homer1a/Ania-3 double knockout mice exhibited impaired cued fear conditioning (Mahan et al., 2012). In a study of contextual fear, whilst the acquisition and short-term recall of fear memory was normal in short form-specific Homer1 knockout mice, long-term fear memory was impaired, suggesting that these animals have a deficit in the consolidation of contextual fear memory (Inoue et al., 2009c). These mice also displayed poor fear memory retention and reconsolidation (Inoue et al., 2009c). Hence, the expression of short Homer1 isoforms appears to be necessary for hippocampal-dependent learning. However, these studies do not address the potential divergence of the functions of Homer1a and Ania-3 in fear learning, nor their roles in fear memory extinction, another form of associative learning important for the flexibility of behavioural responses to conditioned stimuli (Bouton, 2004).

I thus investigated the effect of knocking down hippocampal short Homer1 on the consolidation and extinction of contextual fear memory. To do this, I delivered antisense oligonucleotides specific to each short Homer1 isoform *via* intracerebral microinjection to acutely knockdown their expression during specific training phases. We have successfully used this approach to show the causal relationship between the expression of specific genes and memory formation and maintenance (e.g. Lee et al., 2004; Trent

et al., 2015). The CA1, CA3 and dentate gyrus hippocampal subfields are distinguished dorsally and ventrally by distinct connectivity, gene expression patterns and functions (Strange and Dolan, 1999; Bannerman et al., 2004, 2014; Strange et al., 2014). Whilst the ventral regions are associated with motivational and emotional behaviour, common to other parts of the temporal lobe, the dorsal hippocampus is principally involved in learning and memory in relation to navigation, locomotion and exploration (Czemirski et al., 2009; Fanselow and Dong, 2010). Hence, the experiments herein targeted *Homer1a* and *Ania-3* in the dorsal hippocampus.

Antisense knockdown is a well-established technology that has been used to further understanding of gene function (Stephenson and Zamecnik, 1978). More recent advances in the technology has made them suitable for therapeutic procedures in humans and have shown promise for the treatment of neurodegenerative disorders (Evers et al., 2015). Antisense oligonucleotides are synthetic single-stranded nucleotide sequences, which are able to bind to RNA and block their translation into protein. They are ideal for customisation and specific targeting of individual mRNA variants. Compared to alternative knockdown techniques, antisense oligonucleotides hold advantages in their use in acute *in vivo* studies, in part due to their short duration of action (< 24 h) and superior specificity (Achenbach et al., 2003; Watts and Corey, 2012).

6.2 Knockdown of *Homer1a* by intracerebral microinjection of antisense oligonucleotides

6.2.1 Methods

Detailed methodology concerning the intrahippocampal microinjection of substances can be found in General Methods chapter section 2.7.

6.2.1.1 Antisense design

A specific antisense oligonucleotide sequence, 20 bases long, was designed to target *Homer1a* using strict filtering criteria. Motifs known to weaken antisense activity were avoided (i.e. GGGG, ACTG, AAA and TAA), whilst motifs known to strengthen antisense activity were favoured (i.e. CCAC, TCCC, ACTC, GCCA and CTCT; Matveeva et al., 2000). Sequences with a high GC content (> 50%) were favoured (Matveeva et al., 2000; Chan et al., 2006). Sfold (<http://sfold.wadsworth.org/>) tool was used for target accessibility predictions, binding energy optimisation and target visualisation. Regions of the mRNA with a high degree of secondary folding were also favoured. Oligonucleotide candidates were subjected to BLAST analysis (www.ncbi.nlm.nih.gov/blast/) to remove those sequences complimentary to other, non-specific mRNA targets. Selected antisense oligonucleotides were synthesised (Sigma Genosys, UK) with phosphorothioate linkages between bases 1 and 2 and bases 19 and 20, to increase the stability of the oligonucleotides in the tissue, and PAGE purified.

The mRNA coding sequence of *Homer1a* has high homology to other Homer1 isoforms. Therefore, to generate an antisense oligonucleotide with high specificity to *Homer1a*, I targeted the untranslated region of the mRNA strand, which is unique to the Homer1a transcription variant. An intronic sequence flanking the Homer1a gene continues as a three prime untranslated region in the mRNA for approximately 4.4 Kb (Brakeman et al., 1997; Kato et al., 1998b; Bottai et al., 2002). The Homer1a antisense oligonucleotide selected (5'-AGAAGTCTGACCTGGGATTT-3') targeted this region as shown in Figure 6.1. A missense oligonucleotide sequence was designed for administration to control animals. This sequence was comprised of the same nucleotides as the antisense

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oligonucleotide, yet was not complimentary to any mRNA sequences in BLAST analysis. Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>) analysis was performed to ensure that the missense and antisense oligonucleotide sequences would not bind to one another.

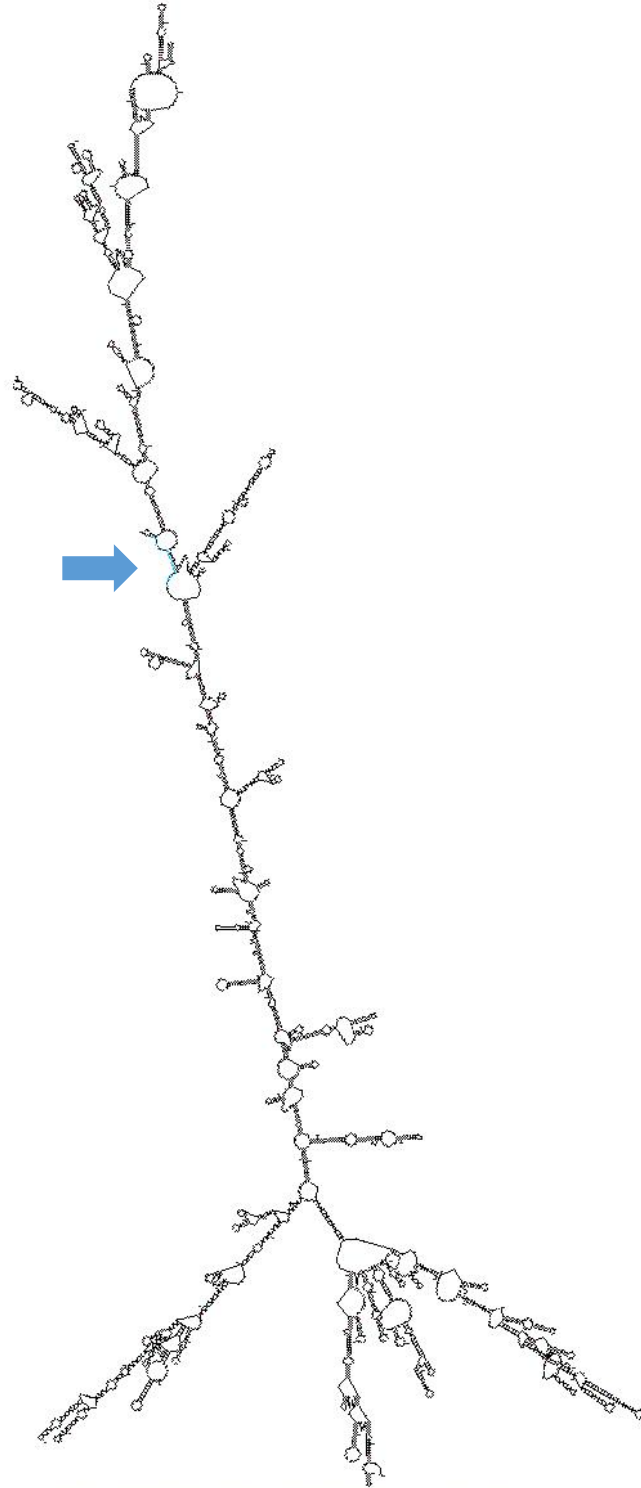


Figure 6.1 Homer1a antisense oligonucleotide target region within the predicted structure of Homer1a mRNA. Arrow indicates target location. Oligonucleotide sequence = 5'-AGAAGTCTGACCTGGGATTT-3'. Sfold (<http://sfold.wadsworth.org/>) tool was used for target accessibility predictions, binding energy optimisation and target visualisation.

6.2.1.2 Surgery

6 adult male Lister hooded Rats underwent surgery for the placement of an indwelling cannula into the dorsal hippocampus. Anaesthetised, rats were positioned in a stereotaxic frame and two holes drilled through the skull at the following coordinates from Bregma: -3.5 mm anterior-posterior, \pm 1.9 mm medial-lateral. A bilateral steel guide cannula (Plastics One, Bilaney Ltd, UK) was implanted through the holes, targeting the dorsal hippocampus (3 mm below the pedestal). A dummy stylet extending 1 mm from cannula tips was inserted into the cannula until use for injections to preserve cannula patency.

6.2.1.3 Intracerebral microinjection and behaviour

Homer1a antisense oligonucleotides were diluted to 2 nmol/ μ l in 0.01 M sterile PBS and kept under 4°C. Awake rats were administered with unilaterally with antisense oligonucleotides and/or missense oligonucleotides *via* intracerebral microinjection. To do this, dummy stylets were replaced with injectors and connected to Hamilton syringes via polyethylene micropore tubing (PE50), and infused 1 μ l *Homer1a* antisense into one hemisphere and 1 μ l of the *Homer1a* missense oligonucleotide to the other hemisphere (n = 6), at a rate of 0.5 μ l/min. Injectors were left in place for 2 min to prevent back diffusion of the injectate from the target region. At least one week after recovery from surgery, infusions were performed 90 min before contextual fear conditioning training, thereby allowing for sufficient time for the oligonucleotides to penetrate the tissue and enter cells (Lee et al., 2004; Barnes et al., 2012). The behavioural intervention was used to stimulate the expression of *de novo Homer1a* expression, thereby permitting the intra animal assessment of the effects on *Homer1a* mRNA by the antisense oligonucleotide

on Homer1a protein in the dorsal hippocampus. Specifically, rats were placed into a novel context for 3 min and received a footshock (0.5 mA, 2 sec) after 2 min. Rats were housed in their home cages between microinfusion and behavioural manipulations.

6.2.1.4 Immunoblotting

Antisense knockdown was quantified by Western blot analysis of Homer1a protein. Rats were culled by CO₂ inhalation 4 h after conditioning and each dorsal hippocampus was micro-dissected and snap frozen. Whole cell fractions of the dorsal hippocampi were made using RIPA buffer and a Western blot assay performed as described in General Methods section 2.6. The membrane was incubated with anti-Homer1a polyclonal rabbit antibody (160013, Synaptic Systems, Germany), 1:1000, and anti-GAPDH monoclonal mouse antibody (ab8245, Abcam, UK; whole tissue homogenates), 1:5000, overnight at 4°C. Secondary antibodies were 1:10000 goat anti-rabbit (680 nm) and donkey anti-mouse (800 nm) fluorescent secondary antibodies (LI-COR), for 1 h at room temperature. Relative Homer1a intensity was standardised to the housekeeping gene of the same lane. A paired statistical analysis was used to compare the quantity of Homer1a protein in hippocampi from ipsilateral and contralateral hemispheres to the antisense injection site.

6.2.2 Results

Rats subjected to contextual fear conditioning displayed robust post-shock freezing compared to baseline ($P < 0.001$, t-test; Figure 6.2). Intracerebral microinjection of Homer1a antisense oligonucleotides into the dorsal hippocampus 90 min prior to contextual fear conditioning decreased Homer1a protein by approximately 20%

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compared to rats treated with missense oligonucleotides 4 h after training ($P < 0.05$, two-tailed t-test; Figure 6.3).

The Role of Short Homer Proteins in Fear Memory Consolidation and Extinction

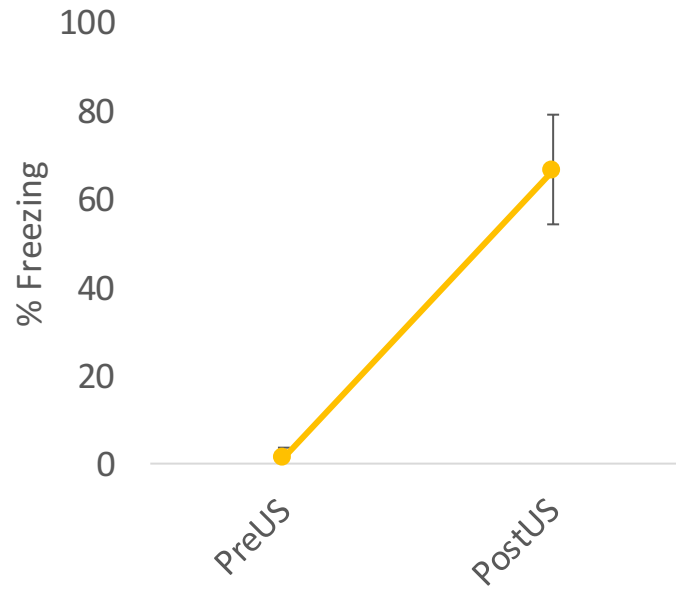


Figure 6.2 Rats administered with unilateral intrahippocampal infusions of *Homer1a* antisense oligonucleotides exhibited robust post-shock freezing. Rats were exposed to a novel context for 3 min and received a footshock after 2 min (n = 6). Rats were culled 4 h after conditioning for hippocampal dissection and immunoblotting. Data represented by mean \pm SEM percent freezing.

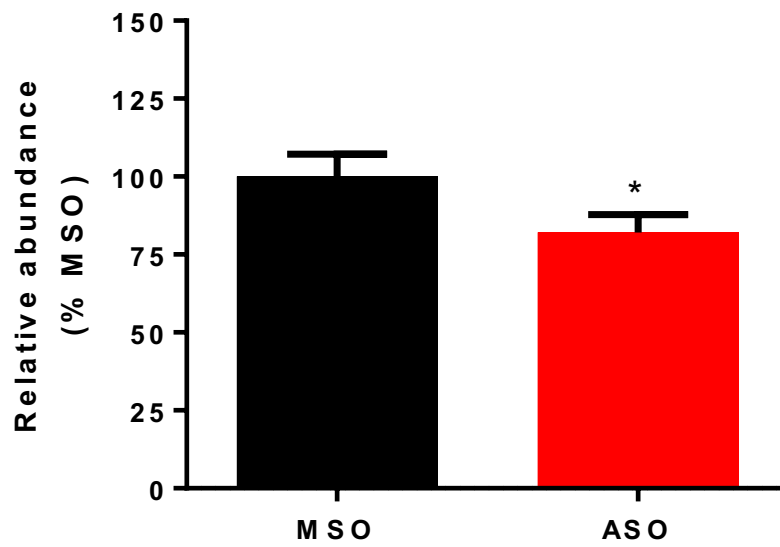


Figure 6.3 Homer1a protein knockdown in whole cell lysates of the dorsal hippocampus by Homer1a antisense oligonucleotides (ASO). Data are expressed as mean \pm SEM relative abundance in Western blot compared to protein level following administration of Homer1a missense oligonucleotides (MSO) to the contralateral hemisphere. Rats were administered 90 min prior to contextual fear conditioning and dorsal hippocampi were micro-dissected 4 h after conditioning. $n = 6$. * $P < 0.05$.

6.3 The role of Homer1a in the consolidation of contextual fear memory: antisense-targeted knockdown in the dorsal hippocampus

6.3.1 Methods

6.3.1.1 Intracerebral microinjection and behaviour

14 adult male Lister Hooded rats were given surgically implanted indwelling cannulae to the dorsal hippocampus, as described above. Rats received a bilateral infusion of 1 μ l 2 nmol/ μ l Homer1a antisense or missense (n = 7) oligonucleotides at a rate of 0.5 μ l / min, 90 min before a contextual fear conditioning trial (see above). Rats were returned to their home cages and subjected to 2 min context re-exposure recall trials 48 h and 4 weeks later. Freezing behaviour was recorded and quantified offline throughout all trials.

6.3.1.2 Histology

After behavioural testing, rats were killed by CO₂ inhalation and the brains were dissected and snap frozen for histology. This enabled verification of cannula placement positioning. The region of the brain around the site of the implant was sectioned coronally at 14 μ m, taking every fifth section and thaw mounted onto glass microscope slides prior to counterstaining in 0.1% β -Thionin, followed by dehydration using ethanol, clearing using Histoclear and coverslipping using DePeX mountant. The sections were then imaged at 50X using a bright field microscope to identify and record the injection site. Subjects were only included if the ventral-most part of the cannula tracts terminated bilaterally in the dorsal hippocampus and there was no sign of damage to adjacent brain structures or gross ventricular enlargement.

6.3.2 Results

6.3.2.1 Verification of injection site

Cannula histology verified the bilateral placement of cannulas to the dorsal hippocampus in all 14 rats used for *Homer1a* knockdown prior to contextual fear conditioning (Figure 6.4). All injection sites were situated between -3.14 mm and -3.80 mm posterior to Bregma bilaterally within the dorsal hippocampus. One rat was excluded from the MSO group due to detachment of the cannula prior to behaviour testing.

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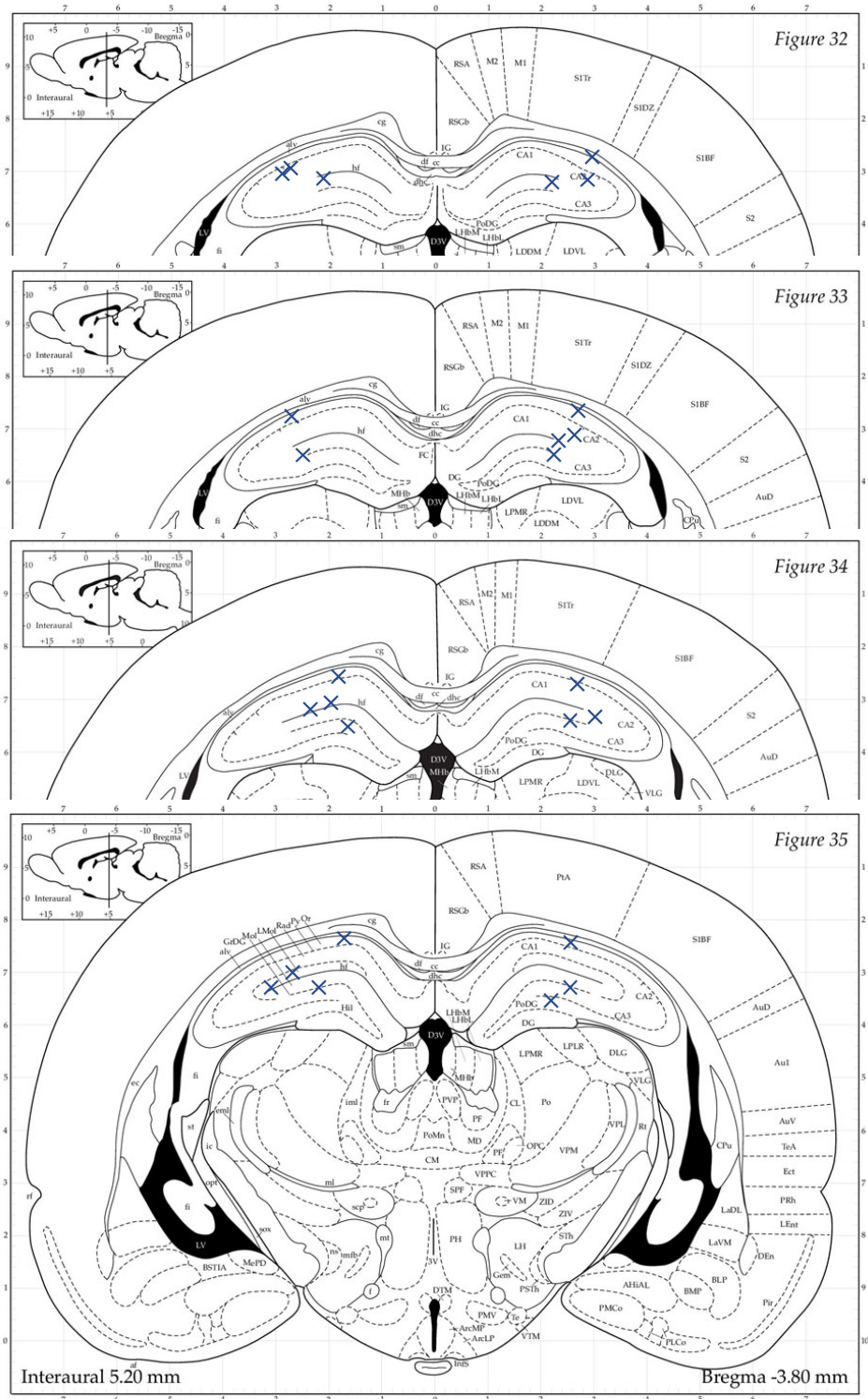


Figure 6.4 *Homer1a* oligonucleotide injection sites prior to contextual fear conditioning. Blue crosses mark the ventral-most point of the cannula placement, determined by histology. Paxinos and Watson (1997) rat brain atlas images shown from -3.80 mm to -3.14 mm posterior to Bregma.

6.3.2.2 The effect of Homer1a knockdown on the consolidation of contextual fear memory

Rats treated with intracerebral microinjection of Homer1a antisense or missense (control) oligonucleotides into the dorsal hippocampus 90 min prior to contextual fear conditioning exhibited normal levels of pre- and post-shock freezing (main effect of time: $F_{(1,11)} = 178.8, P < 0.0001$, two-way repeated measures ANOVA; Figure 6.5). There was no difference in pre- and post-shock freezing between antisense- and missense-treated groups (pre US $P = 0.92$, post US $P = 1.00$; post-hoc Sidak's multiple comparison test). In recall trials 48 h and 4 weeks after conditioning, there was no effect of treatment on conditioned freezing ($F_{(1,11)} = 0.02891, P = 0.87$, two-way repeated measures ANOVA; Figure 6.5), indicating that the administration of Homer1a antisense to the dorsal hippocampus had no effect on the consolidation of contextual fear.

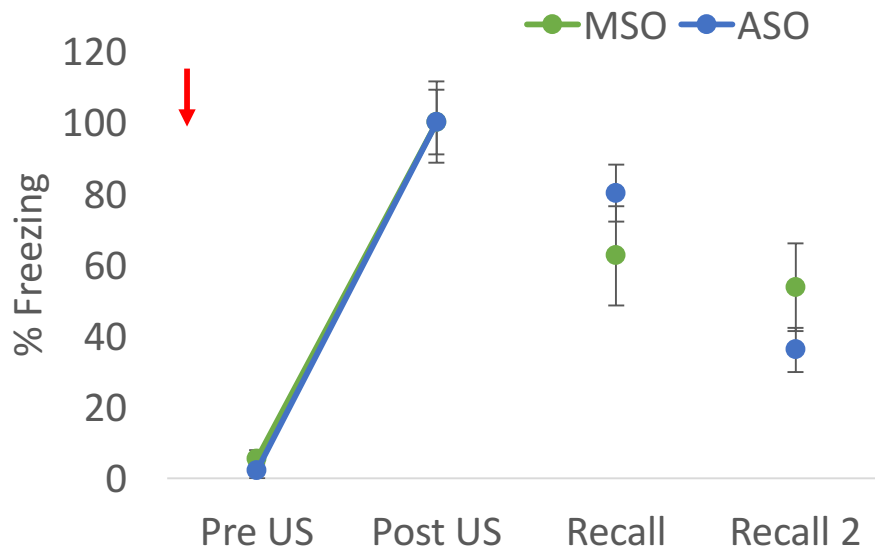


Figure 6.5 Antisense-mediated knockdown of *Homer1a* had no effect on the consolidation of contextual fear memory. *Homer1a* antisense (ASO) or missense (MSO) oligonucleotides were administered to the dorsal hippocampus by intracerebral microinjection 90 min before fear conditioning, indicated by red arrow. Recall (2 min context re-exposure) trials took place 48 h and 4 weeks after conditioning. Data are represented by mean \pm SEM percent freezing normalised to post-unconditioned stimulus (US) levels. $n = 6$ (MSO) or 7 (ASO).

6.4 The role of Homer1a in the extinction of contextual fear memory

6.4.1 Methods

14 adult male Lister Hooded rats were surgically implanted with indwelling cannula targeting the dorsal hippocampus, as described before. All rats similarly underwent contextual fear conditioning. 48 h later, rats received bilateral intra-hippocampal infusions of 2 nmol/ μ l Homer1a antisense (n = 6) in a total volume of 1 μ l or cognate missense sequences (n = 5) at a rate of 0.5 μ l/min, 90 min before extinction training. A third, smaller group (n = 3) received 1 μ l phosphate buffer saline (PBS) vehicle administered at the same rate, to control for any effects of the oligonucleotide infusion. Extinction training consisted of a 10 min recall trial in the conditioned context. All rats then underwent a recall trial 48 h later. At the start of a subsequent recall trial, 7 days later, rats were given a 2 sec 0.25 mA reminder footshock, which itself is insufficient to induce fear conditioning in naïve rats and serves to dissociate extinction from amnesia (Trent et al., 2015). A final recall trial took place at 4 weeks after fear conditioning. Freezing behaviour was quantified throughout all trials (see above).

After behavioural testing, rats were killed by CO₂ inhalation and the brains were dissected and snap frozen for histology, as described.

6.4.2 Results

6.4.2.1 Verification of injection site

Cannula histology verified the bilateral placement of cannulas to the dorsal hippocampus in all animals (Figure 6.6). Bilateral injection sites were situated between -3.14 mm and -3.60 mm posterior to Bregma, within the dorsal hippocampal region.

The Role of Short Homer Proteins in Fear Memory Consolidation and Extinction

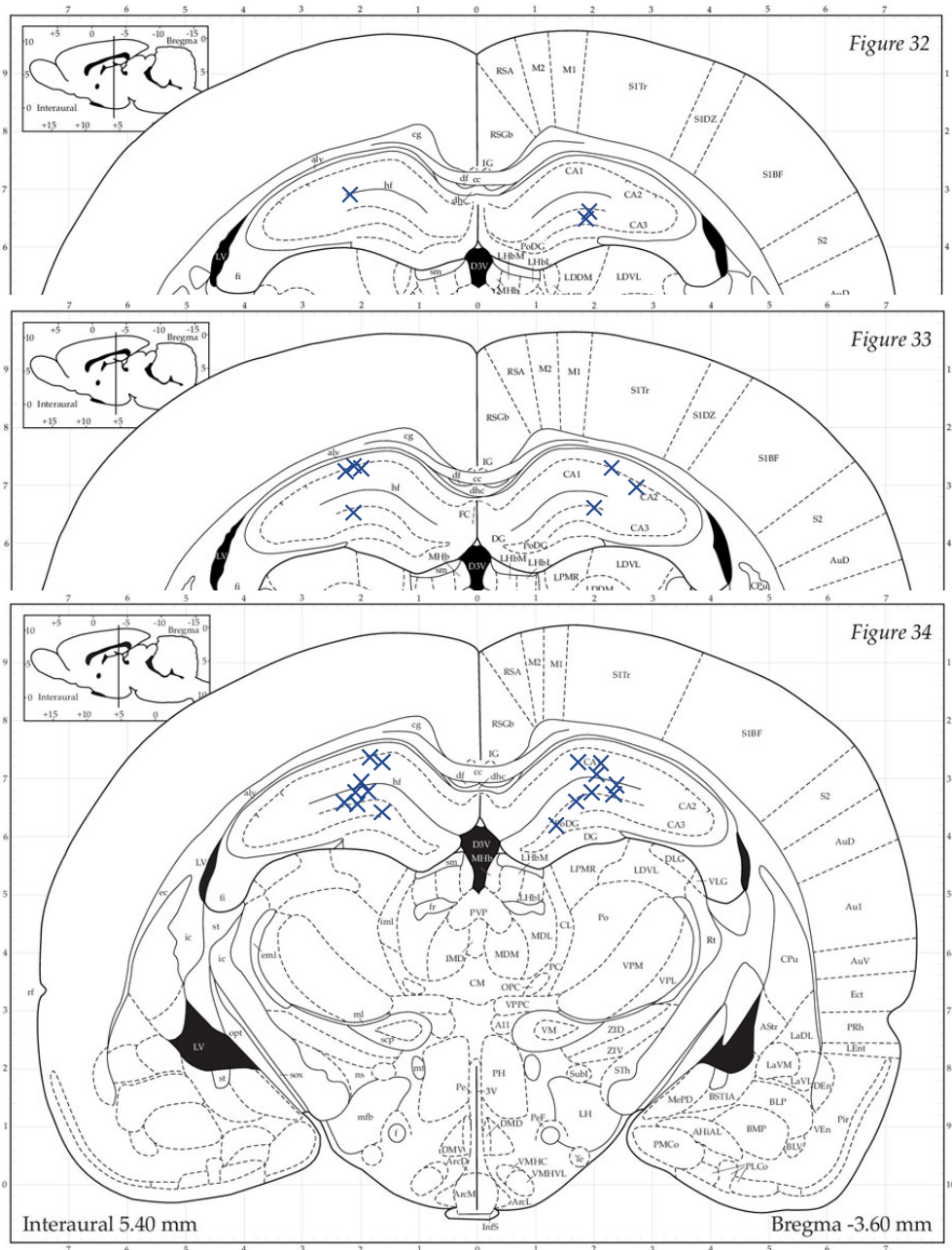


Figure 6.6 Homer1a oligonucleotide injection sites prior to extinction training. Blue crosses mark the ventral-most point of the cannula placement, determined by histology. Paxinos and Watson (1997) rat brain atlas images shown from -3.60 mm to -3.14 mm posterior to Bregma.

6.4.2.2 The effect of Homer1a knockdown on the extinction of contextual fear memory

In a comparison of the level of freezing at the start and end of the extinction trial in all groups, there was lower freezing at the end of the trial (main effect of time: $F_{(1, 11)} = 7.342$, $P < 0.05$, two-way repeated measures ANOVA; Figure 6.7), and there was no interaction between time and treatment group ($F_{(2, 11)} = 0.01594$, $P = 0.98$), showing normal within-trial extinction of the conditioned response in all groups. A main effect of treatment was observed ($F_{(2, 11)} = 5.518$, $P < 0.05$), characterised by lower conditioned freezing responses in the missense-treated group, although there were no differences between the treatment groups at the start or end of the extinction trial ($P > 0.05$, post-hoc Sidak's multiple comparisons test). Low levels of conditioned freezing were seen during a recall trial 48 h after extinction training and there was no effect of treatment ($F_{(2, 11)} = 0.4627$, $P = 0.64$, one-way ANOVA; Figure 6.7), indicating that extinction was consolidated in all treatment groups and that Homer1a knockdown in the dorsal hippocampus had no effect on extinction learning. In a subsequent recall trial, a low-intensity reminder footshock was used to test the strength of the original fear memory. Analysed together, the freezing response after the reminder was increased compared to the first recall trial (main effect of time: $F_{(1, 11)} = 11.52$, $P < 0.01$, two-way repeated measures ANOVA), although there was no interaction with treatment group ($F_{(2, 11)} = 1.455$, $P = 0.28$). No difference between the treatment groups was observed in this second recall trial ($F_{(2, 11)} = 2.089$, $P = 0.17$, one-way ANOVA), nor a third recall trial at 4 weeks post-conditioning ($F_{(2, 11)} = 1.437$, $P = 0.28$).

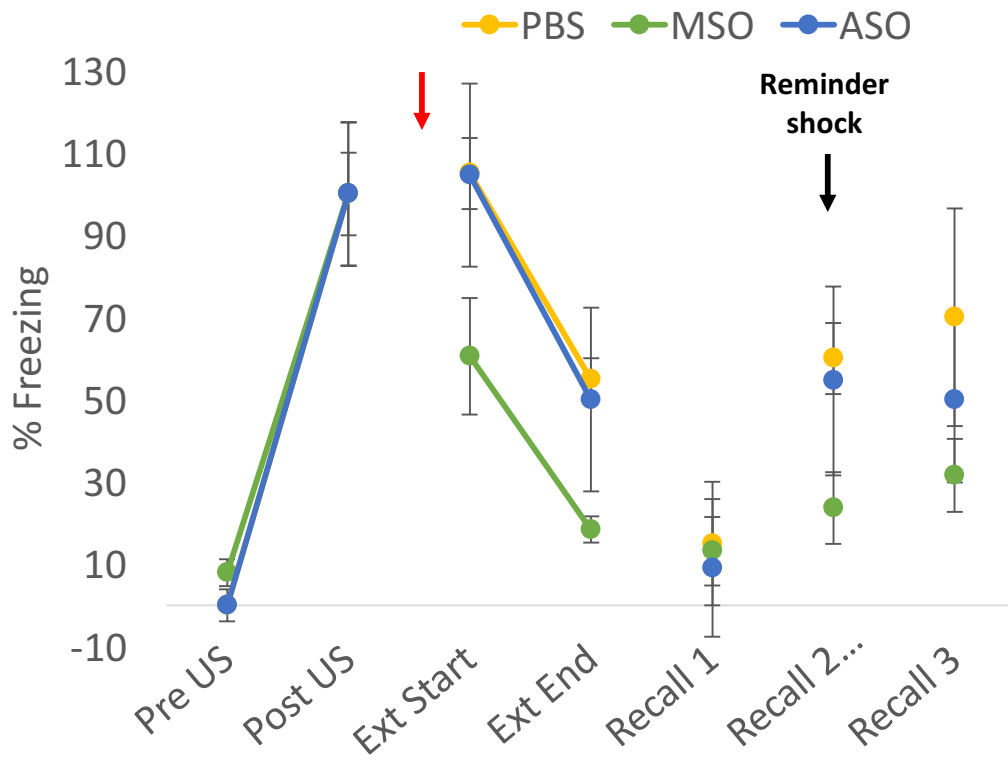


Figure 6.7 Antisense-mediated knockdown of Homer1a in the dorsal hippocampus did not influence the extinction (Ext) of contextual fear memory. Homer1a antisense (ASO) or missense (MSO) oligonucleotides or phosphate buffer saline (PBS) was administered to the dorsal hippocampus by intracerebral microinjection 90 min prior to extinction training, indicated by the red arrow. Recall trials (2 min) took place 48 h, 7 days and 4 weeks after extinction training. A reminder shock was given at the beginning of the second recall trial, as indicated. Data represented by mean \pm SEM percent freezing normalised to post-unconditioned stimulus (US) levels. $n = 3$ (PBS), $n = 5$ (MSO) or $n = 6$ (ASO).

6.5 The effect of Homer1a and Ania-3 double knockdown on the extinction of contextual fear memory

6.5.1 Methods

6.5.1.1 Antisense design

An Ania-3 antisense oligonucleotide was designed to target the three prime untranslated region of *Ania-3*, which shows little homology to the equivalent region of *Homer1a* (Bottai et al., 2002). The same criteria used for the design of *Homer1a* antisense were used in filtering candidate oligonucleotides for *Ania-3*. Since *Homer1a* and *Ania-3* antisense were to be co-infused, Clustal Omega analyses were performed to ensure that the two antisense oligonucleotides and also the two missense oligonucleotides would not interact when in solution together. The selected *Ania-3* antisense oligonucleotide sequence (5'-GATTACATAGCCAAGAGATC-3') was only fully complementary to *Ania-3* mRNA and only 20% homologous to *Homer1a* mRNA and 30% homologous to long *Homer1* variants (Figure 6.8).

No Ania-3-specific antibody currently exists, therefore making it impossible to verify knockdown of Ania-3 by the targeting antisense oligonucleotide using Western blot.

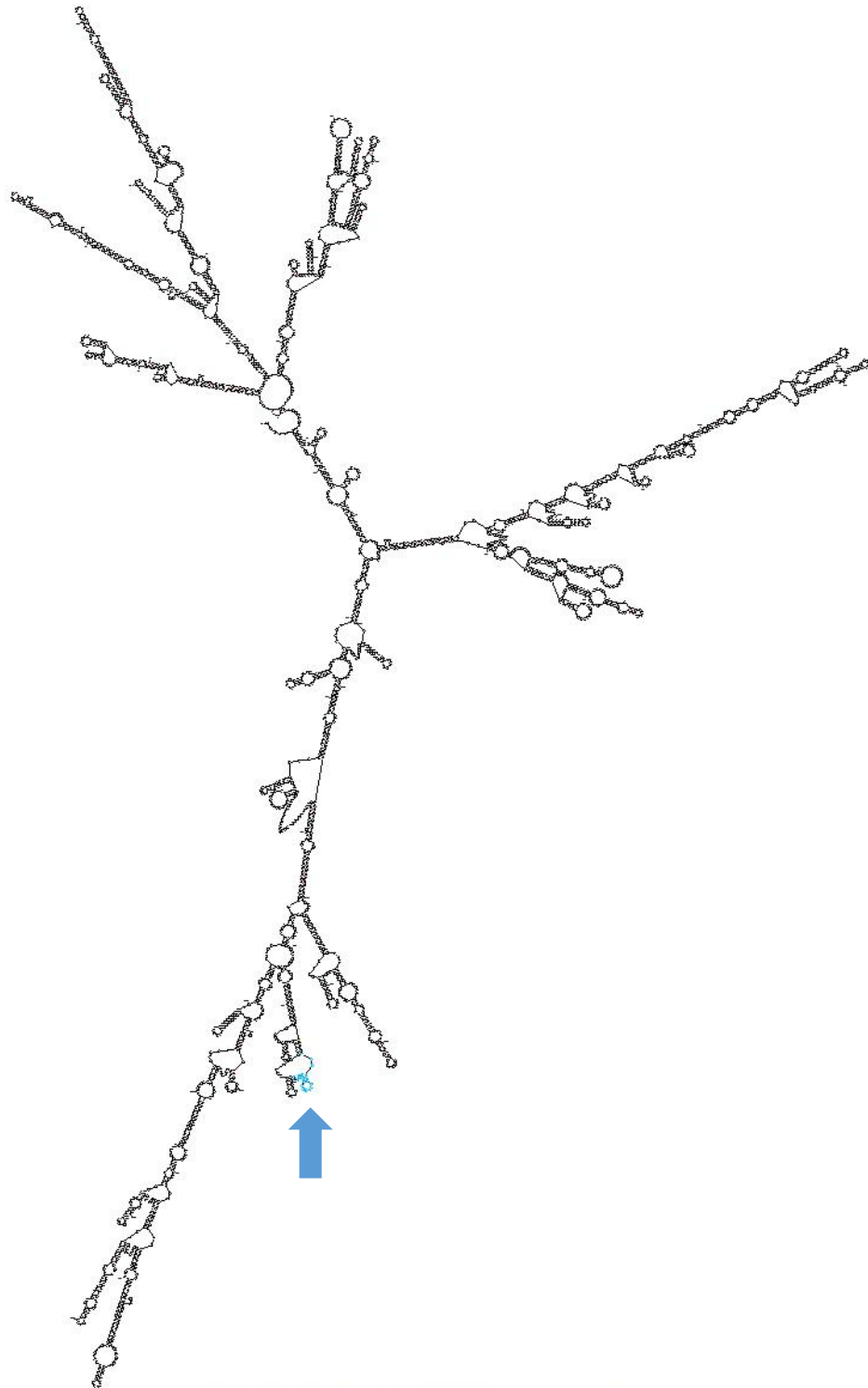


Figure 6.8 Ania-3 antisense oligonucleotide target region within the predicted structure of *Ania-3* mRNA. Arrow indicates target location. Oligonucleotide sequence = 5'-GATTACATAGCCAAGAGATC-3'. mRNA structure prediction and target visualisation performed using Sfold (<http://sfold.wadsworth.org/>) tool.

6.5.1.2 Intracerebral microinjection and behaviour

14 adult male Lister Hooded rats were surgically implanted with indwelling cannulae targeting the dorsal hippocampus, as before. *Homer1a* and *Ania-3* antisense oligonucleotides were mixed to create a solution containing 2 nmol/ μ l of each antisense. The two missense oligonucleotides were mixed in the same manner. Rats were first subjected to a contextual fear conditioning trial. 48 h later, rats received bilateral intra-hippocampal infusions of 1 μ l Homer1a/Ana-3 antisense mix or missense mix (N = 7) at a rate of 0.5 μ l / min, 90 min before extinction training. After extinction, rats then underwent three recall trials 48 h, 7 days and 4 weeks afterwards. Freezing behaviour was quantified throughout all trials as previously described.

After behavioural testing, rats were killed by CO₂ inhalation and the brains were dissected and snap frozen for histology, as described.

6.5.2 Results

6.5.2.1 Verification of injection site

Cannula histology verified the bilateral placement of cannulas to the dorsal hippocampus in all 14 rats (Figure 6.9). Injection sites were bilaterally situated between -3.30 mm and -3.80 mm posterior to Bregma.

The Role of Short Homer Proteins in Fear Memory Consolidation and Extinction

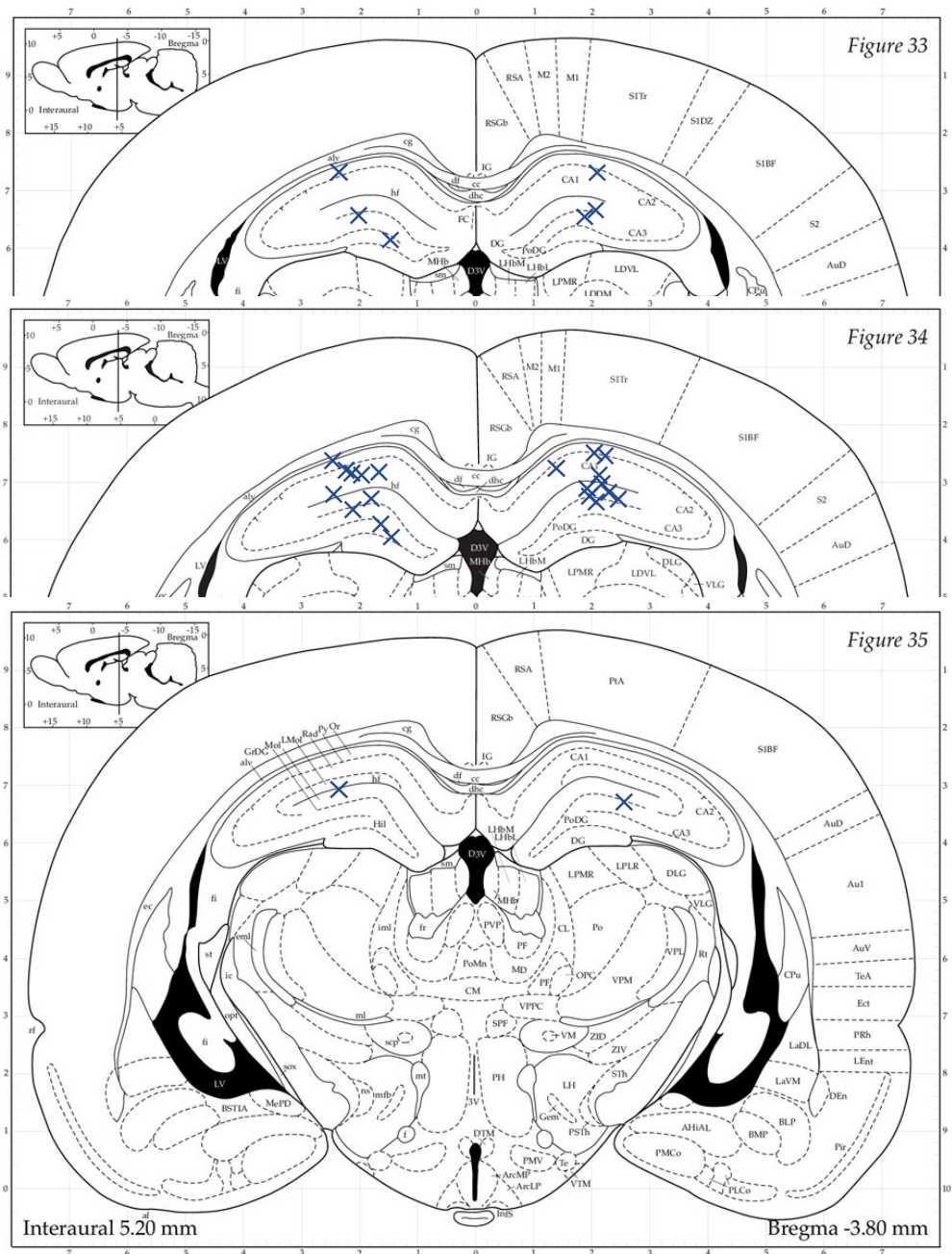


Figure 6.9 Homer1a / Ania-3 antisense injection sites prior to extinction training. Blue crosses mark the ventral-most point of the cannula placement, determined by histology. Paxinos and Watson (1997) rat brain atlas shown from -3.80 mm to -3.30 mm posterior to Bregma.

6.5.2.2 Behavioural effect of double knockdown

A double knockdown of Homer1a and Ania-3 in the dorsal hippocampus was performed during extinction learning to account for any residual support of extinction from Ania-3 in effect in the previous experiment. 48 h after contextual fear conditioning, Homer1a/Ania-3 mixed antisense or missense oligonucleotides were administered 90 min before extinction training, which caused a decrease in within session conditioned freezing response (main effect of time: $F_{(1, 12)} = 21.73$, $P < 0.001$, two-way repeated measures ANOVA; Figure 6.10). There was no interaction between time and treatment group ($F_{(1, 12)} = 0.3735$, $P = 0.55$), nor was there a main effect of treatment group ($F_{(1, 12)} = 0.5268$, $P = 0.48$). Three recall trials were performed to test the extinction memory at 3 later times. Homer1a/Ania-3 antisense infusions had no effect on the freezing response in any of these trials compared to the missense infusion group (48 h $P = 0.25$, 7 days $P = 0.26$, 4 weeks $P = 0.94$; t-tests; Figure 6.10).

The Role of Short Homer Proteins in Fear Memory Consolidation and Extinction

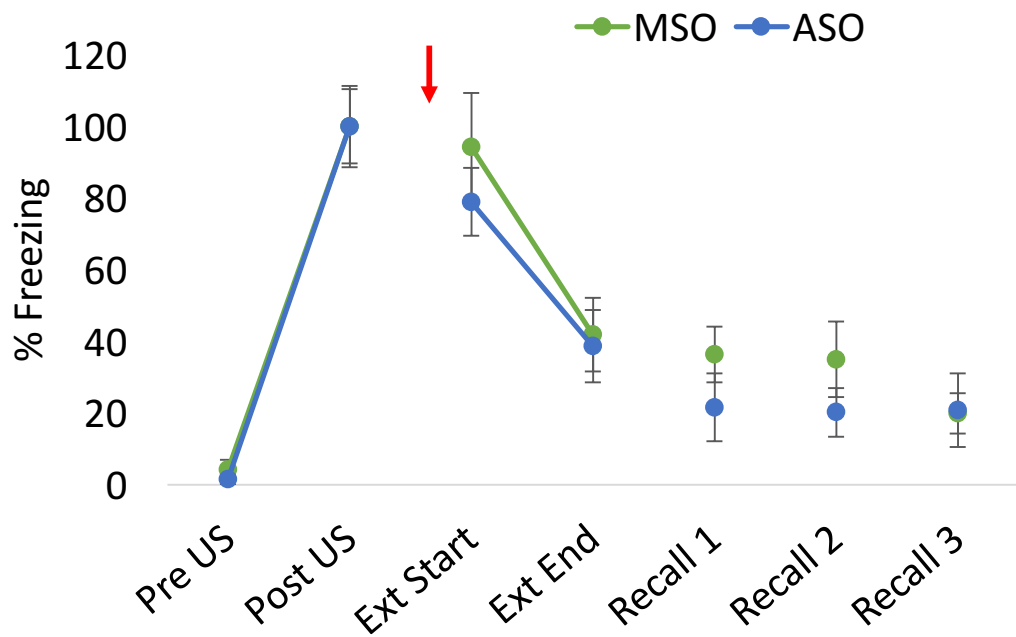


Figure 6.10 Homer1a / Ania-3 double antisense infusion into the dorsal hippocampus prior to extinction training had no effect on the extinction (Ext) of contextual fear memory. Homer1a/Ania-3 antisense oligonucleotide mix (ASO) or missense oligonucleotide mix (MSO) was administered to the dorsal hippocampus by intracerebral microinjection 90 min prior to extinction training, indicated by the red arrow. Recall (2 min) trials took place 48 h, 7 days and 4 weeks after extinction training. Data represented by mean \pm SEM percent freezing normalised to post-unconditioned stimulus (US) levels. $n = 7$ per group.

6.6 Discussion

The results presented in the current chapter show that the knockdown of Homer1a in the dorsal hippocampus by microinjection of antisense oligonucleotides had no effect on the consolidation or extinction of contextual fear memory. Furthermore, the administration of antisense sequences targeting both short Homer1 variants, *Homer1a* and *Ania-3*, had no effect on extinction learning. These findings question the changes in mRNA which parallel the consolidation and extinction of contextual fear memory (Chapter 5) and demand evaluation of the success of the knockdown technique.

Unlike these observations, previous studies have shown the manipulation of short *Homer1* expression to impair fear conditioning (Inoue et al., 2009c; Mahan et al., 2012; Banerjee et al., 2016). However, these studies used different behavioural models and methods for altering Homer1 expression in different brain regions. Banerjee et al (2016) investigated the effect of amygdalar chronic overexpression of Homer1a using viral vector on the consolidation of cued fear conditioning (Banerjee et al., 2016). Mahan et al (2012) also studied cued fear conditioning, yet following the global knockout of both short Homer1 isoforms. Both these studies suggest that Homer1a plays a crucial role in amygdala-dependent fear learning processes which may employ short Homer1 proteins differently to the dorsal hippocampus. They also leave the role of Ania-3 in cued fear conditioning under question. Inoue et al (2009) demonstrated that short form-specific Homer1 knockout mice exhibit normal acquisition but impaired consolidation of contextual fear conditioning. It is possible that this effect was due to altered short Homer1 expression in the amygdala, since contextual fear conditioning is dependent on the hippocampus and amygdala (Phillips and LeDoux, 1992). The effect of knockout on

fear memory consolidation may also be mediated by anxiogenic effects (Szumlinski et al., 2005) or developmental impairments in the hippocampus or other regions.

The disruption of both short Homer1 variants in the dorsal hippocampus may be necessary to block the consolidation of contextual fear. The effects of knocking down one isoform may be compensated by the other sufficiently for association formation. Further studies should explore the effect of knocking down both Homer1a and Ania-3 simultaneously by administering intrahippocampal antisense against *Homer1a* and *Ania-3* prior to contextual fear conditioning.

The present study is the first to explore the role of short Homer1 proteins in extinction learning. It is also the first to investigate the functional contributions of Homer1a and Ania-3 independently. I observed no effect on extinction by the intracerebral administration of antisense against either Homer1a alone or Homer1a and Ania-3 simultaneously. This suggests that the expression of short Homer1 isoforms in the dorsal hippocampus is not required for the extinction of contextual fear. It is possible that the antisense oligonucleotides used were ineffective at generating sufficient knockdown to influence behaviour. I demonstrated that the *Homer1a* antisense induced a knockdown of Homer1a protein after contextual fear conditioning, but this was only by 20% compared to missense-treated rats. This is below the commonly used definition of successful knockdown of 50% or greater (Achenbach et al., 2003; Vickers et al., 2003; Chan et al., 2006). The extent of knockdown of Ania-3 during extinction training using my custom designed antisense oligonucleotide could not be determined because there are currently no Ania-3 antibodies to quantify protein levels by Western blotting. The effectiveness of antisense oligonucleotides is influenced by several factors, including

secondary structure, serum stability and binding energy (Chan et al., 2006), which cannot be predicted with certainty. Hence, a proportion of designed antisense strands have weak activity and provide poor inhibition of the target mRNA, regardless of specificity. It is imperative that the effectiveness of each antisense sequence at reducing target protein levels is determined empirically.

If indeed the translation of short Homer1 variants is not necessary for the extinction of conditioned fear, it demands the question of why both the mRNA of Homer1a and Ania-3, and the protein of Homer1a, are increased following extinction training in subregions of the dorsal hippocampus, as presented in Chapter 5. It is possible that, through protein homeostasis, short Homer1 variants are metabolised during plasticity processes and become replaced through *de novo* gene expression but are not functionally central to the process. However, this explanation seems unlikely, or at least insufficient, considering the specificity of Homer1a recruitment to CA3 following extinction learning (Chapter 5).

In order to gain better insight into the role of Homer1a and Ania-3 in associative learning processes, future experiments may employ alternative knockdown strategies with an aim to achieve improved protein knockdown. As well as designing new antisense oligonucleotide sequences, these may be compared to RNA interference methods. Whilst neither method has been demonstrated to be conclusively advantageous *in vivo* (Watts and Corey, 2012), some studies have shown that short interfering RNA has superior potency over single-stranded antisense techniques (Grünweller et al., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003). The use of gene expression silencing techniques may be complimented by disruption of the translated protein (Campbell and

Bennett, 2016). In particular, green fluorescent protein (GFP)-tagged protein degradation techniques have been used effectively in conjunction with RNA interference to generate a more effective depletion of protein levels (Caussinus et al., 2011; Brankatschk et al., 2014; Dunst et al., 2015). Greater knockdown of Homer1 variants through such methods may provide conclusive evidence for their roles in fear memory consolidation and extinction, although the validation of their effectiveness will be limited by the availability of Homer1 short-form specific antibodies.

Our results do not allow us to conclude whether Homer1a and Ania-3 are required in the dorsal hippocampus for the consolidation or extinction of contextual fear memory. Data currently indicate that Homer1a and Ania-3 are likely to overlap functionally and therefore exhibit functional redundancy. However, their differential expression patterns with fear learning suggest that their roles in the hippocampus may diverge. The advent of an antibody against Ania-3 may help to delineate the respective roles of the short Homer1 isoforms. Further experiments may benefit from extending knockdown techniques to RNA interference or protein degradation methods.

Chapter 7: General Discussion

In this Chapter, I highlight the main findings from each results chapter presented herein and consider the primary implications of the collective results. I then discuss how these results inform future research directions and address any general limitations to the methodology and interpretation of the data. Below, the principle findings from each results chapter are summarised.

7.1 Results Chapter Summaries

7.1.1 The Enrichment of Learning-Related Genes in Genetic Variants from Patients with Schizophrenia and Related Disorders

- The enrichment of consolidation-, retrieval- and extinction-related genes in CNVs from patients with schizophrenia, autism, intellectual disability and ADHD, and in SNPs from patients with schizophrenia was investigated.
- There was an enrichment of extinction-related genes in CNVs from patients with schizophrenia, which was common to both duplications and deletions, but no enrichment of learning-related genes in CNVs from patients with other psychiatric disorders, or melanoma.
- I note that some extinction-related genes have been individually implicated in schizophrenia previously.
- There was no enrichment of consolidation-, retrieval- or extinction-related genes in single nucleotide polymorphisms from patients with schizophrenia.

7.1.2 Associative Learning by Contextual Fear Conditioning

- Contextual fear conditioning was induced by a single pairing of a novel context with a footshock stimulus, and was subsequently extinguished by 10 min re-exposure to the context without the footshock.

- Administration of a higher dose (25 mg/kg), but not a lower dose (8 mg/kg), of ketamine prior to fear conditioning induced the generation of a state-dependent fear memory.
- Neither dose affected the consolidation of contextual fear memory, which is consistent with published data.
- Both low and high dose ketamine blocked the extinction of contextual fear memory when administered prior, but not immediately after, extinction training, consistent with effects of ketamine on other inhibitory learning processes observed in past studies.

7.1.3 The Regulation of Postsynaptic Density Homer1 Proteins by Associative Learning

- I measured the expression pattern of *Homer1a*, *Ania-3* and long Homer1 isoforms following different components of associative fear learning.
- Short, activity-induced isoforms, *Homer1a* and *Ania-3*, but not the long isoforms, were regulated in the hippocampus by novel context exposure, contextual fear conditioning, retrieval and extinction.
- Whilst the induction of both short isoforms was rapid and transient, *Ania-3* levels returned to baseline earlier (by 2 h post-conditioning) than *Homer1a* (sustained for at least 4 h), irrespective of hippocampal subregion.
- Regulation of *Homer1a* in CA3 occurred solely following extinction training.
- Changes in *Homer1a* and *Ania-3* mRNA were indistinguishable following novelty or CPFE, although an increase in *Homer1a* protein was only detected 4 h after the extinction, but not the consolidation, of fear memory, in whole hippocampal homogenates.

7.1.4 The Role of Short Homer Proteins in Fear Memory Consolidation and Extinction

- Antisense oligonucleotides complimentary to *Homer1a* or *Ania-3* mRNA were designed and administered via intracerebral microinjection bilaterally to the dorsal hippocampus of rats prior to contextual fear conditioning and prior to extinction of contextual fear memory.

- Homer1a antisense produced a 20% knockdown in hippocampal Homer1a protein levels after fear conditioning, but had no effect on the consolidation of contextual fear memory.
- The administration of Homer1a and Ania-3 antisense simultaneously had no effect on fear extinction.
- The weak antisense activity of the Homer1a oligonucleotide and the lack of antibody against Ania-3 to test the effectiveness of the Ania-3 antisense sequence occludes evidence that might demonstrate that the synthesis of *de novo* short Homer1 proteins is necessary for processes of associative fear learning.

7.2 The role of extinction learning in psychopathology

A primary point of convergence from the results presented in this thesis is the implication that extinction learning processes are important in the development of schizophrenia, specifically those concerning the extinction of associative fear memories. Firstly, in studying the relationship between associative learning-related gene sets and genetic variants from patients with schizophrenia, I found an enrichment of extinction-related genes in patient CNVs, suggesting that the risk conferred by these rare variants may be attributable to effects on extinction learning. Secondly, the NMDA antagonist, ketamine, blocked the extinction of contextual fear, perhaps revealing an origin to the drug's psychotomimetic properties. Thirdly, products of the *Homer1* gene, which is central to synaptic plasticity and has been linked to psychiatric disease (Spellmann et al., 2011; Luo et al., 2012), are differentially regulated in specific hippocampal subregions following extinction learning.

Extinction is widely interpreted to be a process of new inhibitory associative learning, rather than the erasure of the original association between conditioned and unconditioned stimuli (Bouton, 2004; Quirk and Mueller, 2008). This is due to the

observation that the extinguished conditioned responses can become renewed or reinstated following manipulations such as a change of context or exposure to the unconditioned stimulus, or over time (spontaneous recovery) (Rescorla and Heth, 1975; Bouton, 1993). Through competitive processes, the inhibitory extinction memory dominates over the conditioned association with the unconditioned stimulus (Bouton, 2004). An important characteristic of extinction is its context-dependency (Bouton, 1993; Brooks and Bouton, 1994). There is substantial evidence suggesting that the regulation of fear expression after extinction using contextual stimuli is mediated by the dorsal hippocampus (Corcoran and Maren, 2001, 2004; Bouton et al., 2006). Such studies lend support to a model in which the dorsal hippocampus is responsible for the contextual gating of inhibitory associations (Bouton and Bouton, 1994; Maren and Holt, 2000), which provides a common mechanism for both extinction learning and another form of inhibitory associative learning, latent inhibition (Puga et al., 2007). Both these processes use contextual retrieval cues to delineate conflicting memories concerning a particular conditioned stimulus, influencing behaviour (Ji and Maren, 2007).

Both extinction and latent inhibition are impaired in patients with schizophrenia (Baruch et al., 1988; Jentsch and Taylor, 2001; Serra et al., 2001; Holt et al., 2009, 2012). Together with the results presented herein, these studies strongly implicate abnormalities in inhibitory-type learning in schizophrenia. Interestingly, yet perhaps unsurprisingly, these inhibitory learning processes are physiologically mediated through inhibitory cellular and neurotransmitter systems. During fear extinction learning, the hippocampus regulates the activity of projections from the ventromedial prefrontal cortex to inhibitory intercalated cells of the amygdala (Vertes, 2004; Likhtik et al., 2008;

Orsini et al., 2011). The clustering of GABA_A receptors in the amygdala occurs following extinction training, increasing inhibition within this structure (Chhatwal et al., 2005) and administration of a beta-carboline antagonist of GABA_A receptors blocks the expression of extinction (Harris and Westbrook, 1998). It is notable that parvalbumin-positive inhibitory interneurons are heavily implicated in schizophrenia (Lewis et al., 2012; Boley et al., 2014) and genes encoding components of the GABA_A receptor complex were enriched in schizophrenia-associated CNVs in a recent large-scale CNV study (Pocklington et al., 2015). This dysregulation of inhibitory and excitatory inputs to the amygdala, or elsewhere in fear memory circuitry, will have profound effects on stimulus regulated fear expression.

Indeed, it is not only hippocampal regions that are required for extinction learning. The hippocampal-prefrontal-amygdala extinction circuit has been extensively studied (for reviews see Ji and Maren, 2007; Quirk and Mueller, 2008; Milad and Quirk, 2012). In functional studies in rodents, the infralimbic prefrontal cortex is considered to be functionally homologous to the ventromedial prefrontal cortex in humans for the facilitation of extinction (Kalisch, 2006; Sierra-Mercado et al., 2011). Table 7.1 displays the effects of systemic, hippocampal, infralimbic and amygdalar manipulations on fear extinction.

Manipulation	Fear conditioning protocol	Effect on extinction	Reference
Systemic			
TrkB agonist	Cued	↑ acquisition	(Andero et al., 2011)
NR2B blockade	Cued	↓ acquisition	(Sotres-Bayon et al., 2007)
NMDA agonist	Cued	↑ consolidation	(Walker et al., 2002)
Cannabinoid agonist	Contextual	↑ consolidation	(Pamplona et al., 2008)
FGF2	Cued	↑ consolidation	(Graham and Richardson, 2009)
Oxytocin	Contextual	↓ consolidation	(Eskandarian et al., 2013)
NMDA antagonist	Contextual	↓ consolidation	Chapter 4
Hippocampus			
Hippocampal BDNF KO	Cued	↓ acquisition	(Heldt et al., 2007)
GABA _A R agonist	Cued	↓ acquisition	(Corcoran et al., 2005)
Histone deacetylase inhibition	Contextual	↑ consolidation	(Stafford et al., 2012)
NMDAR agonist	Cued	↑ consolidation	(Ledgerwood et al., 2003)
<i>Zif268</i> knockdown	Contextual	↓ consolidation	(Lee et al., 2004)
Actin remodelling	Contextual	↓ consolidation	(Fischer et al., 2004)
MEK inhibition	Contextual	↓ consolidation	(Fischer et al., 2007)
Translation inhibition	IA	↓ consolidation	(Vianna et al., 2003)
SRC inhibition	IA	↓ consolidation	(Bevilaqua et al., 2005)
P38MAPK inhibition	IA	↓ consolidation	(Rossato et al., 2006)
CAMKII inhibition	IA	↓ consolidation	(Szapiro et al., 2003)
NMDAR antagonist	IA	↓ consolidation	(Szapiro et al., 2003)
Infralimbic cortex			
CB1R agonist	Cued	↑ consolidation	(Lin et al., 2009)
BDNF	Cued	↑ consolidation	(Peters et al., 2010)
GABA _A R antagonist	Contextual	↑ consolidation	(Thompson et al., 2010)
M-K ⁺ channel block	Cued	↑ consolidation	(Santini and Porter, 2010)
CB1R antagonist	Cued	↓ consolidation	(Lin et al., 2009)
GABA _A R agonist	Contextual	↓ consolidation ↓ retrieval	(Laurent and Westbrook, 2009)
mGluR5 antagonist	Cued	↓ consolidation	(Fontanez-Nuin et al., 2011)
Dopamine D2 antagonist	Cued	↓ consolidation	(Mueller et al., 2010)
Dopamine D4 antagonist	Cued	↓ consolidation	(Pfeiffer and Fendt, 2006)
Amygdala			
MAPK inhibition	Cued	↓ acquisition	(Herry et al., 2006)
NR2B antagonist	Cued	↓ acquisition	(Sotres-Bayon et al., 2007)
mGluR1 antagonist	Cued	↓ acquisition	(Kim et al., 2007)
GABA _A R antagonist	Contextual	↑ consolidation	(Berlau and McGaugh, 2006)
Translation inhibition	Cued	↓ consolidation	(Lin et al., 2003)
BDNF knockdown	Cued	↓ consolidation	(Chhatwal et al., 2006)

Table 7.1 Effects of systemic, hippocampal, infralimbic and amygdalar manipulations on the extinction of conditioned fear memory. Abbreviations: tropomyosin receptor kinase B (TrkB); N-methyl-D-aspartate receptor (NMDAR); fibroblast growth factor 2 (FGF2); NMDAR subunit 2B (NR2B); brain-derived neurotrophic factor receptor (BDNF); γ -aminobutyric acid A receptor ($GABA_A$ R); mitogen-activated protein kinase (MEK); sarcoma kinase (SRC); p38 mitogen-activated protein kinase (p38MAPK); calcium-calmodulin protein kinase II (CAMKII); cannabinoid receptor type 1 (CB1R); M-type potassium channel (M-K⁺ channel); metabotropic glutamate receptor 1 (mGluR1); inhibitory avoidance (IA).

Abnormal functional connectivity within the hippocampal-prefrontal-amygdalar circuit has been proposed to form the neurobiological basis for alterations in extinction learning observed during adolescence (for review see Baker et al., 2014). This has important implications for the development of schizophrenia, since the emergence of symptoms typically occurs in late adolescence and early adulthood. Clinical studies have demonstrated that adolescents are resistant to the acquisition of extinction when compared to children and adults (Haddad et al., 2011; Pattwell et al., 2012). These findings are reflected in adolescent rodents, which present impairments in the acquisition (Pattwell et al., 2012) and consolidation (McCallum et al., 2010; Kim et al., 2011; Pattwell et al., 2012) of extinction.

The adolescent period is a “critical period” for neuronal development inasmuch as it is accompanied by a surge in synaptic maturation, which may involve above-normal levels of synapse formation and subsequent elimination (Feinberg, 1982; Paus et al., 2008; Pantelis et al., 2009). Hence, this period represents a vulnerable developmental stage to the effects of environmental insult (McCormick et al., 2010; Romeo, 2013). Rats subjected to chronic stress in early adolescence display deficits in extinction learning which persist into adulthood (Toledo-Rodriguez and Sandi, 2007; Zhang and Rosenkranz, 2013). Furthermore, rats exposed to early-life stress exhibit accelerated maturation of extinction learning, whereby extinction is impaired pre-adolescence but comparable to adults during adolescence (Callaghan and Richardson, 2012). It has been proposed that these age-specific effects of stress on extinction processing may be due to an imbalance between the support of fear extinction and fear expression (Eiland et al., 2012) and/or hypersensitivity of the hippocampus in its regulation of the hypothalamic-pituitary-

adrenal axis (McCormick, 2010). One study showed that chronic stress in adolescent rats led to dendritic retraction in the hippocampus and prefrontal cortex, yet dendritic hypertrophy in the amygdala (Eiland et al., 2012). Interestingly, patients with schizophrenia exhibit reduced ventromedial prefrontal cortex activity and amygdalar hyperactivity during extinction training (Holt et al., 2012).

Phenotypically, schizophrenia is not the only psychiatric disorder for which extinction learning is relevant. Anxiety disorders such as posttraumatic stress disorder are thought to be characterised by strong associations between traumatic events and specific sensory cues, which are resistant to extinction (Pitman, 1988). Importantly, genetic variants conferring risk to schizophrenia also contribute risk to other psychiatric disorders (Malhotra and Sebat, 2012; Kirov et al., 2014), raising the possibility that the link between CNVs and extinction learning, presented herein, has further relevance to other disorders. Furthermore, there is evidence suggesting that ketamine exacerbates posttraumatic stress in accident victims (Schönenberg et al., 2005, 2008), which may originate from effects on extinction learning, as observed in Chapter 4.

Due to the resistance to extinction learning exhibited by patients with posttraumatic stress disorder, current treatments include exposure-based cognitive behavioural therapy (Kar, 2011) and novel therapeutic interventions aim to exploit the function of circuits involved in inhibitory-type learning (Yehuda and LeDoux, 2007; Maren and Holmes, 2015; Singewald et al., 2015). If fear extinction processing is abnormal in schizophrenia, it is plausible that the same approaches may have therapeutic potential in schizophrenia. Whilst extinction does not typically result in the erasure of conditioned memories, some studies suggest that the precise timing of extinction training can be

used to prevent the return of conditioned fear in both rodents and humans (reviewed in Quirk et al., 2010). Furthermore, some evidence suggests that the partial NMDA receptor agonist, D-cycloserine, may be used in conjunction with cognitive behavioural therapy to normalise extinction impairments (Davis, 2011; Hofmann et al., 2013).

7.3 Generalisability and limitations

Evidence implicating altered associative learning in schizophrenia comes from clinical studies (Miller, 1976; Bazin and Perruchet, 1996; Friston, 1998; Serra et al., 2001; Diwadkar et al., 2008b; Jensen et al., 2008; Holt et al., 2012) and, as of more recently, genetic studies (Hall et al., 2009; Pocklington et al., 2015), which include the results presented herein. The apparent role of associative learning has been incorporated into logical inference models of how false beliefs such as delusions and hallucinations may come about (Corlett et al., 2009; Fletcher and Frith, 2009). It is important to note, however, that other cognitive domains have also been associated with schizophrenia through clinical studies, including attention, working memory, episodic memory, social cognition and procedural learning (Millan et al., 2012). The robust, yet broad, implication of synaptic plasticity processes in schizophrenia (Hall et al., 2015) is of equal relevance to these other cognitive processes, as it is to associative learning. Pocklington et al (2015) reported that sets of genes annotated with the terms “associative learning” and “contextual conditioning behaviour”, are enriched in CNVs from patients with schizophrenia. However, the same study also implicates some other cognitive domains, such as “spatial working memory”, “spatial learning” and “temporal memory”, whilst schizophrenia-relevant cognitive domains such as episodic memory and procedural learning were not represented by gene set analyses (Pocklington et al., 2015). This

limitation is not due to poor experimental design, however, but originates from the bias in gene ontology databases towards those cognitive domains most studied and therefore incorporated into the annotation of gene sets. It should therefore be acknowledged that the evidence implicating specific components of associative learning in schizophrenia, reported here, do not occlude the importance of additional cognitive processes in the development of the disorder.

There is considerable overlap between psychiatric disorders, particularly with regards to the genetic variants from which their risk is conferred (Lee et al., 2013; Kirov et al., 2014). Included in this is the association of *Homer1* with autism spectrum disorder and major depression as well as schizophrenia (Rietschel et al., 2010b; Spellmann et al., 2011; Kelleher et al., 2012). The assumption that psychiatric disorders can be defined as distinct diseases, each with separate aetiology and pathophysiology, has been said to impede mental health research (Craddock and Owen, 2010). Hence, it would not be appropriate to consider the relevance of these findings to be restricted to schizophrenia alone. Instead, the role of associative learning, and specifically extinction learning, should be interpreted in the context of a range of psychiatric disorders, particularly those in which CNVs play a role.

Several of the experiments I have described centre around the measurement of gene and protein expression in tissue extracted from rats, following behavioural paradigms. This approach is accompanied by some limitations. Firstly, it restricts expression data to specific time points chosen for the extraction of tissue. Since the temporal pattern of regulated gene expression can vary greatly, any conclusions drawn from the data are relevant only to those times observed. In order to increase the temporal resolution of

the expression data, it requires a multiplicative increase in the use of animals and demands higher stringency in statistical testing. Secondly, linking gene expression with a particular cognitive process preceding it relies on carefully designed control groups with which to make quantitative comparisons. Completely avoiding the observation of gene expression changes associated with non-specific aspects of a behavioural paradigm is often impossible, particularly with gene expression arrays, which must be analysed with an awareness of a proportion of false positives and false negatives. Thirdly, the magnitude of detectable differential gene or protein expression is limited by the tools used. The method favours the observation of large expression changes, but makes it more difficult to conclusively demonstrate no change from control. This was compounded by the lack of availability of short-form specific Homer1 antibodies for protein quantification, the most notable being the absence of antibodies against Ania-3.

7.4 Future directions

7.4.1 Specific future investigations

There are unresolved research questions concerning the roles of short Homer1 proteins, not just in associative learning, but also in further synaptic plasticity processes relevant to schizophrenia. I have discussed the opportunities for studying the function of Homer1a and Ania-3 in the consolidation and extinction of contextual fear memory, through the use of alternative knockdown strategies. Additional studies may investigate Homer1 expression in models of schizophrenia and related disorders. Homer1 interacts with a number of proteins strongly implicated in schizophrenia and/or autism spectrum disorders through large-scale genomic studies, including SHANK1-3, CaV1.2 and F-actin

(Sala et al., 2003; Inoue et al., 2004; Huang et al., 2007; Hayashi et al., 2009; Ripke et al., 2013, 2014; Fromer et al., 2014; Purcell et al., 2014; Rees et al., 2014a). It is notable that rats lacking the gene encoding CaV1.2, *CACNA1C*, exhibit impairments in inhibitory learning, as assessed through the latent inhibition of contextual fear conditioning (Sykes et al., 2016, unpublished). Further synaptic complexes implicated in schizophrenia through genetic variants functionally overlap with Homer1, such as FMRP and CYFIP1 (Ronesi et al., 2012; Fromer et al., 2014; Szatkiewicz et al., 2014b), as well as members of the MAGUK protein family, PSD-95 and PSD-93 (Sala et al., 2003; Kirov et al., 2012; Fromer et al., 2014; Purcell et al., 2014). Through expression and protein interaction studies in model organisms, the role of Homer1 proteins in the mechanisms through which these genetic variants confer risk for psychiatric disease may be explored.

The integration of human genetic studies with experimental gene expression studies in rodents, presented in Chapter 3, demonstrates considerable potential for this approach in the investigation of biological pathways effected by genetic variation. In comparison to pathway analyses based upon gene ontology databases, which are limited by the prior functional annotation of genes, this method incorporates all genes in the analysis, regardless of prior knowledge. This form of gene set enrichment analysis could be used to probe the impact of genetic variants associated with schizophrenia, and other disorders, on discrete synaptic plasticity processes. For example, this approach could be used to investigate the enrichment of genes associated with other inhibitory learning processes, such as latent inhibition, in schizophrenia-associated variants. Alternatively, it may be used to explore the contribution of hebbian and non-hebbian type synaptic plasticity processes to psychiatric disease (Turrigiano and Nelson, 2004; Wondolowski

and Dickman, 2013). Furthermore, by analysing genes differentially expressed in cells or animals following the genetic alteration of psychiatric disease risk genes, or the exposure to environmental insult, this method may help to validate the disease relevance of model organisms. All such experiments will need to include considerations of the relative advantages of different gene expression quantification techniques. With recent advances in technology and the reduction in cost of RNA sequencing, this may now be a favourable technique for the acquisition of high quality gene expression data, over microarrays (Ozsolak and Milos, 2011).

7.4.2 General future directions

Whilst the recent years have seen a substantial advance in our understanding of both genetic variants and learning systems implicated in schizophrenia, the translation of these findings to disease pathogenesis and, ultimately, applications in the clinic will require extensive further research.

It is likely that we will see further large-scale genomic studies of increasing size, in order to identify additional rare and common variants associated with schizophrenia. However, it is of equal importance that future research focuses on the function of those genes through which genetic variants confer risk to the disorder. One way this could be explored is through the characterisation and partitioning of genes sets highly enriched in case variants. This may include the investigation of regional and developmental expression patterns, or evolutionary origin. It is this type of information that would substantially improve gene ontology databases used in pathway analyses. Another approach is the generation of cell and animal models of alleles with high penetrance. This is facilitated by technological advances in genome engineering, notably the advent

of clustered regularly interspaced short palindromic repeats (CRISPR) (Cong et al., 2013). However, whilst these methods are useful for exploring the effects of individual risk variants, they may be less informative of the biological systems implicated in schizophrenia pathogenesis. This is where environmental models may be advantageous; particularly the study of gene-environment interactions will be critical to understanding how at-risk individuals develop schizophrenia. Together with studies into gene-gene epistatic interactions, this research will contribute to exposing the “missing heritability” associated with schizophrenia and other psychiatric disorders.

7.5 Conclusions

Genetic evidence increasingly suggests that genetic risk variants associated with schizophrenia impact on specific cognitive processes. One of these cognitive processes may be the extinction of aversive associative memories, particularly with regards to rare CNVs. The association between extinction and schizophrenia strengthens the existing link between inhibitory learning and schizophrenia and compliments recent findings implicating inhibitory circuits in the disorder. Imbalance between inhibitory and excitatory systems may represent a key abnormality contributing to the progression of schizophrenia, and may be modelled by psychoactive compounds, such as ketamine, which blocks extinction learning. There is a clear role for synaptic proteins, particularly those of the postsynaptic density, in associative learning processes relevant to schizophrenia. Whilst further studies are required to delineate the contributions of individual schizophrenia-associated synaptic proteins in these processes, current evidence warrants the continued investigation of the Homer1 family in this regard. Future research will also benefit from further use of bioinformatics to combine

experimental expression studies with human genomic data, with an aim to better characterise specific biological pathways underlying psychiatric disorders.

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Appendix

Acknowledgments for CNV datasets

We acknowledge the contribution of data from the Database of Genotypes and Phenotypes (dbGaP): A) Genetic Epidemiology of COPD (COPDGene) Funded by the National Heart, Lung, and Blood Institute. dbGaP Study Accession: phs000179.v3.p2. The principal investigators were James D Crapo (National Jewish Health, Denver, CO, USA) and Edwin K Silverman (Brigham and Women's Hospital, Boston, MA, USA). The study was run at the National Heart, Lung and Blood Institute, Bethesda, MD, USA and funded by the National Institutes of Health, Bethesda, MD USA (U01HL089897, U01HL089856). B) A Genome-Wide Association Study of Fuchs' Endothelial Corneal Dystrophy (FECD). dbGaP Study Accession: phs000421.v1.p. The principal investigators were Natalie Afshari (Duke University, Durham, NC, USA), John Gottsch (Johns Hopkins University, Baltimore, MD, USA), Sudha K Iyengar (Case Western Reserve University, Cleveland, OH, USA), Nicholas Katsanis (Johns Hopkins University, Baltimore, MD, USA), Gordon Klintworth (Duke University, Durham, NC, USA) and Jonathan Lass (Case Western Reserve University, Cleveland, OH, USA). Co-investigators were Simon Gregory (Duke University, Durham, NC, USA) and Yi-Ju Li (Duke University, Durham, NC, USA). The study was funded by the National Eye Institute, National Institutes of Health, Bethesda, MD, USA (R01EY016482, CWRU, PI: Sudha Iyengar; R01EY016514, DUEC, PI: Gordon Klintworth; R01EY016835, JHU, PI: John Gottsch). Genotyping was carried out at the Center for Inherited Disease Research (CIDR), Johns Hopkins University, Baltimore, MD, USA and was funded by the National Institutes of Health, Bethesda, MD, USA. HHSN268200782096C, NIH contract "High throughput genotyping for studying the genetic contributions to human disease"; HHSN268201100011I, NIH contract "High throughput genotyping for studying the genetic

contributions to human disease". C) California Pacific Medical Center Research Breast Health Cohort. dbGaP Study Accession: phs000395.v1.p1. The principal investigator was Elad Ziv (University of California, San Francisco, CA, USA). Co-investigators were Steven Cummings (California Pacific Medical Center Research Institute and University of California, San Francisco, CA, USA), Karla Kerlikowske (University of California, San Francisco, CA, USA) and John Shepherd (University of California, San Francisco, CA, USA). The study was run at the National Cancer Institute, National Institutes of Health, Bethesda, MD, USA and was funded by the National Institutes of Health, Bethesda, MD, USA (P01 CA107584; R01 CA120120). Genotyping was carried out at Johns Hopkins University Center for Inherited Disease Research (CIDR), Baltimore, MD, USA and was funded by the National Institutes of Health, Bethesda, MD, USA (HHSN268200782096C, "NIH contract High throughput genotyping for studying the genetic contributions to human disease"; HHSN268201100011I, "NIH contract High throughput genotyping for studying the genetic contributions to human disease"). D) Study of Melanoma Risk in Australia and the United Kingdom. dbGaP Study Accession: phs000519.v1.p1. The principal investigator was Nicholas Hayward (Queensland Institute of Medical Research, Brisbane, QLD, Australia). The study was funded by the National Cancer Institute of the National Institutes of Health, Bethesda, MD, USA (R01CA088363). Genotyping was carried out at the Center for Inherited Disease Research (CIDR), Johns Hopkins University, Baltimore, MD, USA and funded by the National Institutes of Health, Bethesda, MD, USA (HHSN268201100011I). E) Genome-Wide Association of Schizophrenia Study, (GAIN). dbGaP accession phs000021.v3.p2. Funding for this study was provided by the National Institute of Mental Health (R01 MH67257, R01 MH59588, R01 MH59571, R01 MH59565, R01 MH59587, R01 MH60870, R01 MH59566, R01 MH59586, R01 MH61675, R01 MH60879, R01 MH81800, U01 MH46276, U01 MH46289 U01 MH46318, U01 MH79469, and U01 MH79470) and the

genotyping of samples was provided through the Genetic Association Information Network. The principle investigator was Pablo V. Gejman, Evanston Northwestern Healthcare (ENH) and Northwestern University, Evanston, IL, USA. F) Genome-Wide Association of Schizophrenia Study, (MGS_nonGAIN). dbGaP accession phs000167.v1.p1. Samples and associated phenotype data for the MGS_nonGAIN study were collected under the following grants: NIMH Schizophrenia Genetics Initiative U01s: MH46276 (CR Cloninger), MH46289 (C Kaufmann), and MH46318 (MT Tsuang); and MGS Part 1 (MGS1) and Part 2 (MGS2) R01s: MH67257 (NG Buccola), MH59588 (BJ Mowry), MH59571 (PV Gejman), MH59565 (Robert Freedman), MH59587 (F Amin), MH60870 (WF Byerley), MH59566 (DW Black), MH59586 (JM Silverman), MH61675 (DF Levinson), and MH60879 (CR Cloninger). G) Genetic Architecture of Smoking and Smoking Cessation dbGAP accession phs000404.v1.p1. Funding support for genotyping, which was performed at the Center for Inherited Disease Research (CIDR), was provided by 1 X01 HG005274-01. CIDR is fully funded through a federal contract from the National Institutes of Health to The Johns Hopkins University, contract number HHSN2682007 82096C. Assistance with genotype cleaning, as well as with general study coordination, was provided by the Gene Environment Association Studies (GENEVA) Coordinating Center (U01 HG004446). Funding support for collection of datasets and samples was provided by the Collaborative Genetic Study of Nicotine Dependence (COGEND; P01 CA089392) and the University of Wisconsin Transdisciplinary Tobacco Use Research Center (P50 DA019706, P50 CA084724). H) High- Density SNP Association Analysis of Melanoma: Case–Control and Outcomes Investigation. dbGaP accession phs000187.v1.p1. Research support to collect data and develop an application to support this project was provided by 3P50CA093459, 5P50CA097007, 5R01ES011740 and 5R01CA133996. I) Genetic Epidemiology of Refractive Error in the KORA Study. dbGaP accession phs000303.v1.p1. Principal investigators: Dwight

Stambolian, University of Pennsylvania, Philadelphia, PA, USA; H. Erich Wichmann, Institut für Humangenetik, Helmholtz-Zentrum München, Germany, National Eye Institute, National Institutes of Health, Bethesda, MD, USA. Funded by R01 EY020483, National Institutes of Health, Bethesda, MD, USA.

Samples from the WTCCC2 study were downloaded from <https://www.ebi.ac.uk/ega/> and include samples from the National Blood Donors Cohort, EGAD00000000024 and samples from the 1958 British Birth Cohort, EGAD00000000022. Funding for these projects was provided by the Wellcome Trust Case Control Consortium 2 project (085475/B/08/Z and 085475/Z/08/Z), the Wellcome Trust (072894/Z/03/Z, 090532/Z/09/Z and 075491/Z/04/B) and NIMH grants (MH 41953 and MH08 3094). For the CLOZUK sample, we thank Novartis for their guidance and co-operation. We also thank staff at The Doctor's Laboratory, in particular Lisa Levett and Andrew Levett, for help and advice regarding sample acquisition. We acknowledge Kiran Mantripragada, Lesley Bates, Catherine Bresner and Lucinda Hopkins for laboratory sample management. Finally, we thank the participants and clinicians who took part in the Cardiff COGS study.