Exploring the Relationship Between Schizophrenia Risk and Adult Hippocampal Plasticity

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Abbreviations

µL	Microlitre
µM	Micrometer
ABP-GRIP	AMPA-binding-protein-and-glutamate receptor-interacting-protein
AD	Alzheimer's disease
AMPAr	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
ASD	Autism spectrum disorder
BDNF	Brain derived neurotrophic factor
bp	Base pairs
CA1	Cornu ammonis 1
CA2	Cornu ammonis 2
CA3	Cornu ammonis 3
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CANTAB	Cambridge Neuropsychological Test Automated Battery
cDNA	Complimentary DNA
CFC	Contextual fear conditioning
cLTP	Chemical long-term potentiation
CNV	Copy number variant
CR	Conditioned response
CRE	cAMP-response element
CREB	cAMP-response element binding protein
Cry2	Cryptochrome Circadian Regulator 2
CS	Conditioned stimulus
Ct	Cycle threshold
DAG	Diacylglycerol
DEG	Differentially expressed genes
DG	Dentate gyrus
DGE	Differential gene expression
dNTP	deoxynucleotide triphosphates
DRB	5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole
DSM-5	Diagnostic and Statistical Manual of Mental Disorders

EC	Entorhinal cortex
Egr	Early growth factor
eIF	Eukaryotic initiation factor
eIF2	Eukaryotic initiation factor 2
E-LTP	Early long term potentiation
FDR	False discovery rate
GO	Gene ontology
GTP	Guanosine triphosphate
GWAS	Genome-wide association studies
HA	Human influenza hemagglutinin
HSF1	Heat Shock Factor Protein 1
IA	Inhibitory avoidance
IEA	Inferred from electronic annotation
IEG	Immediate early gene
IHC	Immunohistochemistry
IL-1	Interleukin-1
IP	Immunoprecipitation
IP3	Inositol triphosphate
LEC	Lateral entorhinal cortex
L-LTP	Late long term potentiation
logFC	Log-fold change
LTD	Long term depression
LTM	Long term memory
LTP	Long term potentiation
M	Molarity
mA	milliamp
MAGMA	Multi-marker analysis of genomic annotation
MEC	Medial entorhinal cortex
mGluR	Metabotropic glutamate receptor
mL	Millilitre
MP	Mammalian phenotype
MPC	Missense badness, Polyphen-2 and constraint
MRI	Magnetic resonance imaging
mRNA	Messenger RNA

MTL	Medial temporal lobe
mTOR	Mammalian target of rapamycin
NAS	Non-traceable author statement
NFAT	Nuclear factor of activated-T cells
NF-κβ	Nuclear-factor κβ
NMDAr	N-methyl-D-aspartate receptor
OCT	Optimal cutting temperature [compound]
PBS	Phosphate buffered saline
PCA	Principal component analysis
PFA	Paraformaldehyde
PGC	Psychiatric Genomics Consortium
PI	Phosphatidyl inositol
PI3K	phosphoinositide 3-kinase
PICK1	Protein-interacting-with-C-kinase
PKA	Protein kinase A
PKC	Protein kinase C
Post-traumatic stress disorder	PSTD
PP1	Protein phosphatase 1
PSD-95	Post synaptic density 95
PTV	Protein truncating variants
qPCR	Quantitative polymerase chain reaction
RCA	Inferred from reviewed computational analysis
RIN	RNA integrity number
RNA-seq	RNA sequencing
RQN	RNA quality number
SCHEMA	Schizophrenia Exome Meta-Analysis Consortium
SCR	Skin conductance response
SE	Standard error
SG	Stratum granulosum
SL	Stratum lucidum
SLM	Stratum lacunosum moleculare
SM	Stratum moleculare
SNP	Single Nucleotide Polymorphism

SO	Stratum oriens
SP	Stratum pyramidale
SR	Stratum radiatum
STM	Short term memory
TF	Transcription factor
TRAP	Translating ribosome affinity purification
TRAP-seq	Translating ribosome affinity purification sequencing
US	Unconditioned stimulus
UTR	Untranslated region
Vegfb	Vascular Endothelial Growth Factor B
x *g*	Relative centrifugal force
Zif268	Zinc finger protein 268

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Summary

Bleuler was the first to identify abnormal associations as one of the basic symptoms of schizophrenia, which has since been extensively demonstrated. Consolidation and retrieval of contextual fear conditioning (CFC) have been shown elicit differential patterns of gene expression in the hippocampus. Large-scale genomic studies have identified common and rare variants that are enriched in patients with schizophrenia, and these have been shown to impact genes involved in synaptic plasticity and associative learning. Here, CFC was used to explore how gene expression underlying associative learning may be associated with schizophrenia risk variants.

In Chapter 3, RNA sequencing was used to detect gene expression changes following retrieval and extinction of conditioned fear. Contrary to previous work, few genes were found to be significantly differentially expressed, and there was no enrichment of learning-related gene sets in schizophrenia-associated genetic variation. Next, using publicly available data, I demonstrated that gene-sets derived from a functional LTP experiment were significantly enriched in schizophrenia-associated risk variants, particularly genes expressed in CA1 excitatory neurons, as determined using cell-type specific TRAP-sequencing (Chapter 4). Finally, given that LTP processes are thought to underlie consolidation of fear memories, I used TRAP-seq to explore the gene expression profile of excitatory hippocampal neurons following acquisition of CFC. I found significantly more genes differentially expressed in excitatory hippocampal neurons compared to bulk RNA-seq, mirroring the results of Chapter 4. Genes that were significantly down-regulated 5- hours after acquisition of CFC, during the consolidation window, were enriched in immune processes, suggesting a down-regulation of the immune system during the consolidation of contextual fear. However, no enrichment in risk variants associated with schizophrenia were found.

These results build on previous literature examining gene expression following consolidation and retrieval of conditioned fear, and demonstrate the advantages of using cell-type specific sequencing in such experiments.

Chapter 1: Introduction

1.1 Schizophrenia

Dementia praecox or 'precocious madness' was a term first coined by Bénédict Morel in the 18th century to describe a degenerating mental illness which occurred in the young. In 1896, Emil Kraepelin went further, describing it as a disorder of intellectual functioning with a poor, deteriorating prognosis, distinct from manic depression in which patients had periods of remission (Kraepelin, 1896). In 1908, Eugene Bleuler renamed the condition schizophrenia, rooted from the Greek "schizein" (to split) and "phrēn" (mind), based on his belief that the "splitting of psychic functioning" was central to the disorder (Bleuler, 1908). Bleuler went on to identify 4 basic symptoms of schizophrenia: abnormal associations, autistic behaviour, abnormal affect and ambivalence (Bleuler, 1950). Altered associations were a core concept in Bleuler's theory of schizophrenia, being denoted as both a basic and primary symptom, that is, one that is present in all cases and caused directly by the neurobiological disease process ((Bleuler, 1950), reviewed in (Maatz et al., 2015)). As such, he theorised that the disease process gave rise to altered associations, which in turn were reacted to by the psyche to give rise to secondary symptoms, such as delusions and hallucinations.

Whilst diagnostic criteria, such as the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), focus on positive (for example delusions and hallucinations) and negative (for example apathy and withdrawal) symptoms (Regier et al., 2013), cognitive symptoms are widespread, with an estimated 50 to 70 % of patients having neuro-cognitive deficits in areas such as attention, learning and memory and problem solving (Kelly et al., 2000;Nuechterlein et al., 2015;Zhang et al., 2017). Further, cognitive impairments have been linked to functional outcomes (Bowie and Harvey, 2006;Nuechterlein et al., 2011;Fu et al., 2017). In a 20-year follow up cohort study, cognitive performance was found to be significantly correlated with employment status in patients with schizophrenia (Strassnig et al., 2018). Further, only 27.8 % of the cohort were in employment, and fewer still reported living independently (24.7 %), highlighting the functional difficulties that patients with schizophrenia often face. Cognitive functioning has also been associated with poor self-care in later life (Evans et al., 2003) which, alongside medical co-morbidities, may go some way to explain the lower life expectancy of patients with schizophrenia.

1.2 Associative learning

Over the past 50 years, associative learning and memory, and their neural and molecular underpinnings, have been studied using pre-clinical models. Animal and cellular models permit tight control over experimental procedures, in addition to genetic manipulation, allowing exploration of the pathways and neurobiology underlying associative learning. Furthermore, advances in methodology and genetics have allowed the exploration of whether associative learning processes are impacted in neurological and psychiatric diseases, such as schizophrenia.

Associative learning can be described as a process through which one learns the relationship between two or more stimuli. This type of learning underpins many behaviours important for survival, for example avoiding food which previously led to illness, or returning to a location that has a water source. Animals, and indeed humans, which can successfully learn and recall associations, and adapt them as circumstances change, are more likely to survive and thrive in their environments. In a now classical experiment, Pavlov demonstrated that a neutral conditioned stimulus (CS) and biologically significant unconditioned stimulus (US) could be paired to elicit a conditioned response (CR) in the presence of the CS only (Pavlov, 1927). This type of associative learning is also known as Pavlovian conditioning, and has been demonstrated to occur across a range of circumstances and stimuli (reviewed in (Rescorla, 1988)).

1.2.1 Fear conditioning

Pavlovian fear conditioning involves learning that a particular environmental stimulus or stimuli predicts a negative outcome. This has drawn much attention from researchers, both because of its potential contribution to clinical conditions such as phobias, post-traumatic stress disorder and schizophrenia, but also because the principles of Pavlovian conditioning can be transferred to pre-clinical research paradigms. Through decades of scientific research, the neurobiological underpinnings of fear conditioning, including the brain regions involved, underlying physiology and molecular correlates are well-known, making it an ideal paradigm for the investigation of learning and memory and schizophrenia.

Several rodent associative learning paradigms exist, centered around the Pavlovian principles of a CS (environmental stimulus) becoming associated with a US (negative action) such that the CS alone produces a CR (often measured as freezing behaviour). One such paradigm is cued fear conditioning in which a cue, often a tone or light, is paired simultaneously with a footshock. Upon presentation of the cue alone, rodents demonstrate robust fear responses such as freezing (Blanchard and

Blanchard, 1969), potentiated startle (Brown et al., 1951) and alterations to physiological activities such as increased heart rate (Kapp et al., 1979).

An alternative paradigm is contextual fear conditioning (CFC), whereby rodents are placed in neutral context and after a certain period of exploration (often 2-3 minutes), receive a footshock through the floor bars. As with cued conditioning, subsequent presentation of the context produces a robust fear response. Further, CFC fear memories are subject to extinction, that is, after presentation of the context in the absence of foot shock, gradually the fear response diminishes. Figure 1.1 shows a schematic of the CFC protocol used throughout this thesis. CFC can be robustly produced after 1 context-footshock pairing, reducing the confound of multiple learning points. One of the main differences between cued- and contextual- fear conditioning is the brain circuitry that supports each type of associative learning. Whilst the amygdala has been shown to support cued-fear conditioning (Helmstetter, 1992;Phillips and LeDoux, 1992b), CFC has been found to be hippocampal dependent (Phillips and LeDoux, 1992b;Daumas et al., 2005)**.** Given that the hippocampus is often found to be altered in patients with schizophrenia (see section 1.3.3), and that genetic association studies have found enrichments in hippocampal regions (see section 1.6), CFC is a good paradigm to explore the association between learning and memory and schizophrenia risk.

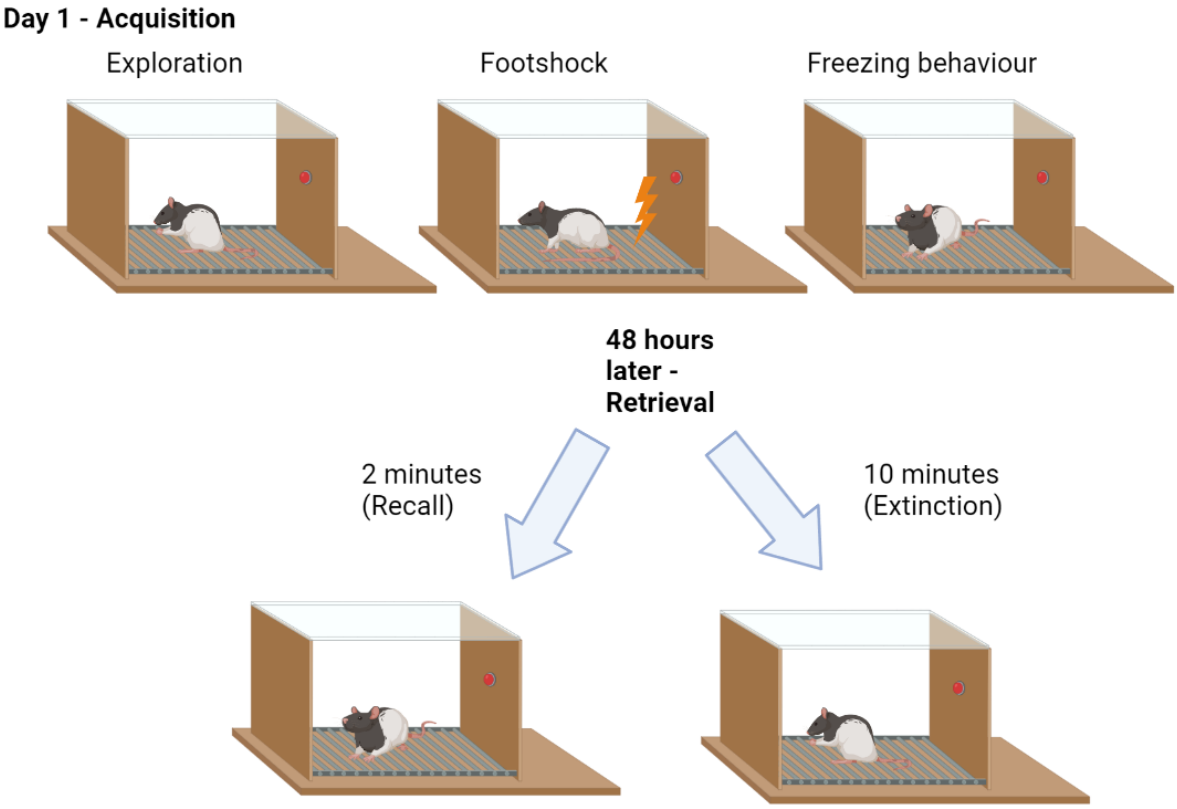


Figure 1.1. *Schematic diagram outlining the stages of the contextual fear conditioning (CFC) paradigm. At the acquisition phase (day 1), rodents are placed in the context for exploration, before a footshock is administered. Following this, rodents show fear-related behaviours, including freezing. 48 hours later, rodents are placed back in the context for either recall or extinction. In recall trials, rodents still show fear behaviours. In extinction trials, whilst rodents initially show fear behaviour, towards the later point of the trial they show exploratory behaviours again.*

1.2.2 Stages of contextual fear conditioning (CFC)

There are multiple stages underlying long term memory: acquisition, consolidation, and retrieval. Acquisition is the period of active practice during which new memories are generated. Acquisition occurs contemporaneously to the learning event and in the seconds afterwards and so, whilst the key initiating event in memory formation, is difficult to capture molecularly before consolidation occurs. Thus, the focus of this thesis is on consolidation and retrieval processes.

1.2.2.1 Consolidation

Consolidation is the process of memory stabilisation, occurring in the minutes to hours after the initial acquisition of learning, which allows the formation of a long-term memory. After initial acquisition, memories are labile, that is, can be easily disrupted, and thus the process of stabilising these memories by consolidation mechanisms is vital for long term memory (LTM) formation.

Intensive research over several decades has demonstrated that memory consolidation is underpinned by molecular and cellular changes, including gene expression and protein synthesis, which drive modifications in synaptic structures to support long term memory (reviewed in (McGaugh, 2000), (Kandel, 2001)). It has been shown that inhibiting protein synthesis in rats, using intraventricular injection of Anisomycin, prior to contextual fear conditioning prevented the expression of conditioned fear 24-hours, but not 30 minutes, later (Schafe et al., 1999). This has been demonstrated under several conditions (Bourtchouladze et al., 1998;Bailey et al., 1999). Further, prevention of mammalian target of rapamycin (mTOR)-mediated translation processes, thus preventing protein synthesis, has been shown to impair fear memory in the amygdala and hippocampus (Parsons et al., 2006;Gafford et al., 2011). Taken together, this provides robust evidence that protein synthesis is required for consolidation, and therefore subsequent expression, of contextual fear memories. Similarly, inhibiting *de novo* gene expression with RNA polymerase inhibitors has been shown to prevent the consolidation of contextual fear (Igaz et al., 2002;Duvarci et al., 2008), indicating that new gene expression is required to facilitate consolidation of fear memories.

The medial temporal lobe (MTL), including the hippocampus, was first associated with memory consolidation through patient H.M., who underwent MTL resection surgery as a treatment for his epilepsy. Following surgery, patient H.M. was unable to acquire new hippocampal-dependent declarative memories, suggesting an inability to consolidate short-term memories into long-term storage (Scoville and Milner, 1957). In the years following, the involvement of the hippocampus in consolidation has been well documented. In primates, hippocampal lesions have been demonstrated to impair performance in a discrimination task learned prior to surgery (Zola-Morgan and Squire, 1990). Further, hippocampal lesions occurring 1 day after CFC training have been shown to impair contextual fear behaviour when tested 7 days after surgery (Kim and Fanselow, 1992). Similarly, dorsal hippocampal lesions have been shown to produce deficits in freezing behaviour across a variety of studies (Phillips and LeDoux, 1992b;Maren et al., 1997;Phillips and LeDoux, 1994), indicating a role for the hippocampus in the consolidation of contextual fear.

1.2.2.2 Retrieval

Memory retrieval is the process of recalling previously encoded information, and can elicit behaviour optimal for the previously learned conditions. In CFC, retrieval of the previously learned association between context and footshock elicits freezing behaviour, a commonly measured expression of fear behaviour, upon re-exposure to the content alone. Re-exposure to the context can activate 2 processes: reconsolidation and extinction (Suzuki et al., 2004). During this re-exposure, previously

consolidated memories become labile again, thus susceptible to disruption (Nader et al., 2000). As such, reconsolidation is the process by which LTM are re-stabilised. In CFC, brief re-exposure to the context leads to reconsolidation of the original fear memory, and as such maintains the previously learned fear response in subsequent re-exposure trials (Blanchard, 1969). Conversely longer re-exposure, in which there is no further reinforcement of the context-shock association, initiates extinction processes and leads to the diminishing of the fear response. Like consolidation, extinction is an active form of new learning, which dominates, rather than erases, previous learned associations (Bouton, 2004;Quirk and Mueller, 2008).

Like consolidation, retrieval and extinction have been shown to depend on gene transcription and protein synthesis. In an early demonstration of this, Debiec and colleagues found that intrahippocampal infusions of the protein synthesis inhibitor Anisomycin led to impaired long-term, but not short-term, memory following reactivation of CFC, indicating impairments in reconsolidation when protein synthesis was prevented (Debiec et al., 2002). Further, Gafford and colleagues demonstrated that reconsolidation, in addition to consolidation, of CFC requires mTOR-mediated protein synthesis (Gafford et al., 2011). Gene transcription has also been shown to be required for reconsolidation and extinction. Inhibition of RNA polymerase II and III with α-Amanitin following retrieval of auditory fear conditioning led to impaired LTM in a post-retrieval test trial (Duvarci et al., 2008). (Mamiya et al., 2009). In addition, processes associated with retrieval have been shown to elicit distinct patterns of gene expression. Using microarray to quantify gene expression in the CA1 region of the hippocampus, Scholz and colleagues (2016) demonstrated that consolidation, retrieval and extinction of contextual fear memories lead to differential patterns of gene expression, with minimal overlap between conditions. Genes up-regulated after retrieval were enriched in 10 functional clusters including inflammatory response, whilst genes up-regulated after extinction were enriched in 12 functional clusters including cell-adhesion and regulation of developmental processes (Scholz et al., 2016).

Similar to memory consolidation, retrieval of fear memory is dependent on the hippocampus. Inactivation of the dorsal hippocampus with muscimol led to the disruption of extinction behaviours in a post-extinction test trial, indicating disruption to the reconsolidation process (Corcoran and Maren, 2001). Further, post-extinction electrolytic lesions to the dorsal hippocampus have been shown to eliminate extinction behaviour (Ji and Maren, 2005). Immediate early genes (IEG) such as *c-Fos*, *Arc* and *Egr1* are rapidly and transiently expressed after neural activity. As such, IEGs can be used as markers of neuronal activation to understand the brain circuitry involved in learning and memory processes. *In-situ* hybridisation has shown that *Egr1* mRNA is up-regulated in CA1, but not dentate gyrus (DG), neurons of the hippocampus following retrieval of contextual fear (Hall et al.,

2001). Further, optogenetic stimulation of hippocampal neurons previously activated during CFC led to recall of the fear memory in a neutral context (Liu et al., 2012).

1.2.3 Other brain regions implicated in associative learning

There are several other brain regions implicated in associative learning processes, most notably the amygdala. The amygdala is thought of as the central hub of fear learning, and mediates the expression of conditioned fear through projections from the thalamus, hippocampus and cortical regions, and projections to the hypothalamus and periaqueductal grey regions (Ledoux, 1991;Phelps and LeDoux, 2005;Pitkänen et al., 1997). Simple associations, for example auditory conditioned stimuli, are underpinned by direct projections from the thalamus to the amygdala, with the basolateral amygdala in particular thought to underpin auditory conditioned associations (LeDoux et al., 1990;Wilensky et al., 2006;Wolff et al., 2014) (Windels et al., 2016). Further, lesions to the anterior basal nuclei of the amygdala lead to deficits in contextual and auditory fear conditioning, whilst lesions to the posterior nuclei did not indicating specificity within the amygdala structure (Goosens and Maren, 2001) (Nader et al., 2001). Whilst direct projections to the amygdala have been proposed to underlie simple associations, associations involving more complex cues, such as context or temporal elements, have been proposed to involve processing via the hippocampus (Maren and Fanselow, 1995;Fanselow and Poulos, 2005;Kim and Jung, 2006). For example, pre-training hippocampal lesions have been shown to block the acquisition of contextual fear memory, but not tone-shock associations (Phillips and LeDoux, 1992a). Further, lesions to the nucleus accumbens, which receives input from the hippocampus, have been shown to impair contextual but not auditory fear associations, as demonstrated by reduced freezing in the shock associated context but not to the shock-associated cue (Riedel et al., 1997). Thus, whilst the amygdala is important for fear associations, within the study of contextual fear memory it is likely that the hippocampus plays an important role.

1.2.4 Associative learning and schizophrenia

Building further on Bleuler's theory that abnormal associations were central to schizophrenia, impairments in associative learning domains have been found in patients with schizophrenia. Patients with schizophrenia have been found to have impairments in learning neutral associations between objects and locations. Using an object-location task, it has been found that patients with schizophrenia had a significantly lower learning rate compared to controls (Diwadkar et al., 2008), an effect which was replicated in patients with schizophrenia, and extended to include patients with bipolar disorder, who show similar lower rates of learning (Brambilla et al., 2011). Further, using

neutral name-face pairings, it has been shown that patients with schizophrenia were impaired in learning the name-face associations, and retrieving them at a later time point (Oertel et al., 2019). In addition, first-degree relatives of patients with schizophrenia showed a tendency for reduced recall performance compared to controls, potentially indicating a prodromal recall impairment.

Holt and colleagues (2012) demonstrated that although patients were able to learn the association between context and shock, they had an impaired ability to recall extinction memories, and exhibited a larger skin conductance response to the context which had previously undergone extinction compared to the fear context, suggesting an impairment in extinction learning (Holt et al., 2012). This mirrors a previous study, in which the authors found that patients with schizophrenia showed larger skin conductance responses to a neutral context, compared to controls (Holt et al., 2009). Taken together, these results suggest that there might be differential associative learning abnormalities with neutral and aversive stimuli, as associative learning has been found to be reduced with neutral stimuli but not aversive. Given that the positive symptoms of schizophrenia are often negative, for example persecutory delusions and auditory hallucinations, it may be expected that negative valence associations are impacted differentially to neutral ones. Little research has been conducted into positive valence associative learning tasks in patients with schizophrenia. However, performance in the Eyes test, which measures recognition of emotions from sets of eyes, has not been found to differ for positive emotions, but was significantly worse than controls for neutral and negative emotional valence (Charernboon, 2020). This may indicate impairments of processing neutral and negative valences, compared to positive ones, in patients.

In healthy individuals, a model has been proposed whereby new associative learning is mediated by an interaction between experience and belief. It proposes that experiences which do not challenge one's belief system are ignored, but when belief systems are challenged new learning occurs (Fletcher and Frith, 2009). This model is underpinned by prediction error, a mathematical model proposed by Rescorla and Wagner (1972), in which a new or "surprising" event induces learning, but that once a CS-US association has been made, it becomes predictable (Rescorla, 1972). In the case of schizophrenia, it has been proposed that delusions, abnormal beliefs that persist in the face of conflicting evidence, may be a result of instability in this prediction error system. For example, if one attends to all cues in the environment and their consequences, rather than them becoming predictable and ignored as per the prediction error theory, grandiose delusional beliefs, that certain objects or specific situations are of crucial importance of significance, may form (Fletcher and Frith, 2009). Indeed, it has been shown that patients with schizophrenia show abnormal assignment of salience to stimuli (Murray et al., 2008) (Roiser et al., 2009). Further, a recent meta-analysis found that patients with schizophrenia had a significantly higher skin conductance response (SCR) to an

image not previously associated with an aversive stimuli compared to controls, indicating the formation of aberrant associations (Tuominen et al., 2021). Thus, not only are associative learning processes altered in schizophrenia, which is interesting in itself, but that aberrant association formation may underlie delusions, arguably one of the more debilitating symptoms of schizophrenia, makes it an important focus for research.

1.3 The hippocampus

The hippocampus is one of the principal sites involved in learning and memory, and has been demonstrated to be involved in a range of processes including spatial memory (Moser et al., 1993), recognition memory (Broadbent et al., 2004) and human episodic memory (Squire and Zola, 1998). Further, the hippocampus has been implicated in the acquisition and retrieval of contextual fear associations (Phillips and LeDoux, 1992a;Lee and Kesner, 2004;Corcoran and Maren, 2001).

1.3.1 Hippocampal anatomy

The hippocampus forms part of the limbic system, and has 3 distinct subregions: the dentate gyrus (DG), the hippocampus proper (cornu ammonis fields: CA1, CA2, CA3) and the subiculum (reviewed in (van Strien et al., 2009;Schultz and Engelhardt, 2014)). The DG and CA fields of the hippocampus proper are organised in a laminar fashion, each containing cell-types pertaining to their function, though there are widespread connections between the layers (Figure 1.2).

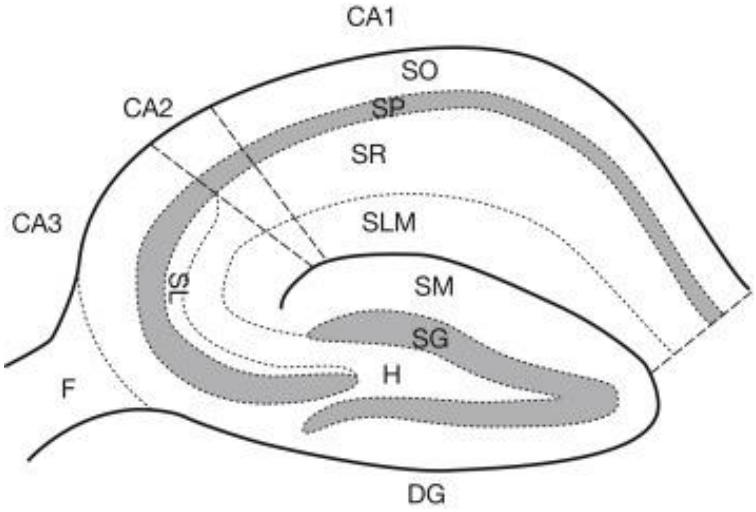


Figure 1.2. *A schematic representation of the layers of the hippocampus proper, from Noguchi et al., 2020 (Noguchi et al., 2020). DG = dentate gyrus; F = fimbria; H = hilus; SO = stratum oriens; SP = stratum pyramidale; SR = stratum radiatum; SLM = stratum lacunosum-moleculare; SL = stratum lucidum; SM = stratum moleculare; SG = stratum granulosum.*

1.3.1.1 CA1

The principal layer running through the CA1 is the stratum pyramidale (SP), which contains the soma of the excitatory pyramidal cells. These are the primary neurons of the CA1, and make up approximately 90 % of the structure (Bezaire and Soltesz, 2013). The apical dendrites of the pyramidal cells extend into the stratum radiatum (SR), and arborise in the stratum lacunosum moleculare (SLM). Basal dendrites branch out into the stratum oriens (SO) (Hammond, 2015;Noguchi et al., 2020). Further, the 4 layers of the CA1 contain cell bodies of inhibitory interneurons, which modulate the activity of the pyramidal cells.

1.3.1.2 CA3

Similar to the CA1, pyramidal cell bodies reside in the SP layer of the CA3. Axons of the CA3 pyramidal cells, called Schaffer collaterals, project to the apical dendrites of CA1 pyramidal cells in the SLM. Further, CA3 pyramidal cells project onto each other, forming local circuits. The stratum lucidum (SL) is a layer that is only found in the CA3, and contains the synapses between the mossy fibers from the DG and CA3 pyramidal cells (Hammond, 2015).

1.3.1.3 DG

The principal cell layer of the DG is called the stratum granulosum (SG) and contains a dense layer of granule cell stoma. This layer forms the stereotypical ‘v’ shape of the DG, often referred to as the

'hippocampal blades'. The suprapyramidal blade sits between the CA1 and the CA3, shown in Figure 1.2, as the topmost section of the SG, and the lower blade is the infrapyramidal blade. The axons of the granule cells are called the mossy fibres, and form the pathway to the CA3. The dendritic tree of the granule cells form the stratum moleculare (SM) (Amaral et al., 2007;Hammond, 2015).

1.3.2 Hippocampal connections

The hippocampus is connected both intrinsically and extrinsically. Figure 1.3 shows a schematic diagram of the intrinsic hippocampal connections. The main input to the hippocampal formation is from the entorhinal cortex (EC), which projects via the perforant pathway to the DG. From here, the mossy fiber pathway projects to the collateral cells in the CA3. Collateral cells in the CA3 then project via the Schaffer collateral pathway to the CA1. In addition, CA3 cells project onto themselves, forming synapses with other CA3 neurons. The CA1 pyramidal neurons then project back to the EC via the subiculum (reviewed in (Schultz and Engelhardt, 2014;Knierim, 2015)). This pathway is known as the trisynaptic loop. Whilst once thought of as a unidirectional flow of information, evidence has now shown that there is a complex pattern of connections between and within hippocampal and cortical regions (Knierim, 2015).

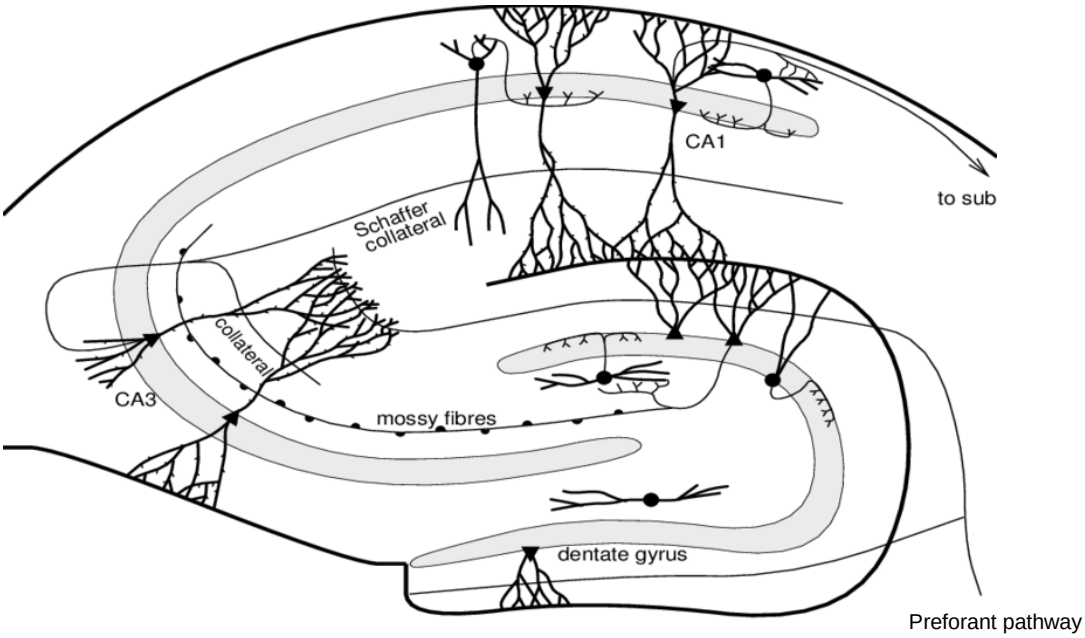


Figure 1.3. *Schematic diagram of hippocampal circuitry from Schultz et. al, (1999). The entorhinal cortex (EC), the main input to the hippocampus, projects via the perforant pathway to the DG. From here, the mossy fiber pathway projects to the collateral cells in the CA3. Collateral cells in the CA3 then either project via the Schaffer collateral pathway to the CA1, or onto themselves, forming synapses with other CA3 neurons. The CA1 pyramidal neurons then project back to the EC via the subiculum in a pathway is known as the trisynaptic loop.*

The medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC) project to the DG and CA3 regions of the hippocampus via the perforant pathway. The projections to the CA1 are segregated such that the area of the CA1 closest to the subiculum receive input from the LEC, and the area closest to the CA2 receives input from the MEC (Tamamaki and Nojyo, 1995). The EC is also organised in a laminar structure. Layer II projects to the DG and CA3, whilst layer III projects to the CA1 and subiculum. The deeper layers of the EC receive input from the hippocampus proper. In addition to the EC, the hippocampus is connected to an array of cortical and subcortical brain regions including the perirhinal cortex, the hypothalamus and the striatum, and receives neuromodulatory input from regions such as the medial septum and the locus coeruleus (reviewed in (Knierim, 2015), (Rho and Storey, 2001)). Further, it has been shown that the CA1 and CA3 regions are innervated by the basolateral amygdala complex (Pikkarainen et al., 1999), an area which is important for fear conditioning (Helmstetter and Bellgowan, 1994;Huff et al., 2005). Given the extensive connectivity between the hippocampus and other brain regions, it is a key site for the

encoding and retrieval of a range of memory processes, including episodic and working memory, and contextual fear conditioning (Ranganath et al., 2004;Phillips and LeDoux, 1992a).

1.3.3 The hippocampus and schizophrenia

The hippocampus has been associated with a range of psychiatric and neurological conditions including schizophrenia. Some of the earliest evidence linking schizophrenia to the hippocampus comes from post-mortem studies, in which altered levels of neurotransmitters such as noradrenaline and dopamine, and catalytic enzymes such as dopamine-β-hydroxylase were found (Bird et al., 1977;Winblad et al., 1979;David Wise et al., 1974). In addition, post-mortem studies have found that pyramidal cell neurons are reduced in size in patients with schizophrenia compared to control subjects (Benes et al., 1991;Arnold et al., 1995;Zaidel et al., 1997).

Since the more widespread adoption of imaging technology, particularly structural and functional magnetic resonance imaging (MRI), further evidence of hippocampal dysregulation has been found. In particular, structural MRI of patients with schizophrenia often report reduced hippocampal volume (Velakoulis et al., 1999;Shenton et al., 2001;Szeszko et al., 2003). Further, reductions in hippocampal volume is one of the most consistently reported findings from imaging studies (Tamminga et al., 2010;Haijma et al., 2013;van Erp et al., 2016;Okada et al., 2016a). In addition, it has been found that hippocampal subfield volume reductions may exhibit a dose-dependent relationship, with patients at high-risk of schizophrenia showing reduced hippocampal volumes compared to controls, and patients with schizophrenia exhibiting further reductions in hippocampal volume compared to patients at high-risk (Vargas et al., 2017). Further, it has been found that patients with early psychosis (less than 2 years duration) exhibit reduced hippocampal volumes restricted to the anterior hippocampus particularly the CA regions, whereas patients with chronic psychosis (longer than 2 years duration) exhibited volume reductions in both the anterior and posterior hippocampus, again particularly in the CA regions (McHugo et al., 2018). This suggests that hippocampal volume deficits are progressive, supporting the neuroprogressive model of schizophrenia, whereby initial glutamate dysregulation in CA1 leads to prodromal symptoms and drives the transition to psychosis (Lieberman et al., 2018).

Medication use in patients is a potential confound of imaging studies, and it can be difficult to ascertain whether changes in brain volumes are causative or secondary to other factors such as long-term medication use, or self-treating behaviours such as smoking and illicit substance use. One way of beginning to unpick the potential association is to study people at high risk of schizophrenia who do not take anti-psychotic medications. Ho and colleagues (2017), conducted a longitudinal study of people who were at ultra-high risk of schizophrenia due to genetic risk, subthreshold symptoms and

a deterioration of functioning over the previous 12 months. Importantly, exclusion criteria were past history of frank psychosis, use of anti-psychotic medications, illicit substance use and alcohol dependence, which controls for the confounds mentioned above, as much as is possible in a population study. It was found that there were no baseline differences in hippocampal CA1 volume between groups, however people whose subthreshold symptoms persisted, or who developed frank psychosis, had greater CA1 volume decreases compared to controls and people whose symptoms remitted. Further, the rate of CA1 volume decline was associated with increased symptom severity over time, suggesting that CA1 hippocampal volume decreases may be contributory, rather than secondary, to schizophrenia pathogenesis (Ho et al., 2017).

In addition to structural differences in the hippocampus, functional differences between patients with schizophrenia and controls have also been observed. Energy metabolism in neurons requires glucose uptake and increased oxygen consumption, which can be measured indirectly through cerebral blood flow or cerebral blood volume. Changes to these parameters compared to controls may indicate metabolism changes in patients with schizophrenia. Functional MRI studies have found that patients with schizophrenia have increased hippocampal activity at rest (Tregellas et al., 2014), and cerebral blood flow data also shows an increase in hippocampal blood flow in patients (Medoff et al., 2001;Malaspina et al., 2004), particularly in the CA1 (Schobel et al., 2009;Talati et al., 2014). Further, it has been found that prodromal increases in CA1 cerebral blood volume predicted clinical progression to psychosis, and correlated with positive clinical symptoms; specifically, delusional severity had the strongest association (Schobel et al., 2009). In addition, a recent study found that anterior hippocampal cerebral blood volume was increased in patients with psychotic disorder, and that this was inversely correlated with task-related activation in this region, suggesting that hyperactivity of the anterior hippocampus leads to sub-optimal recruitment of this region during a scene-processing task (Maureen McHugo et al., 2019).

Taken together, these results highlight the potential role of hippocampal impairments in schizophrenia pathogenesis.

1.3.4 Other brain regions implicated in schizophrenia

1.3.4.1 The prefrontal cortex

In addition to the hippocampus, other brain regions have also been implicated in schizophrenia, particularly prefrontal regions (Karlsgodt et al., 2010). The prefrontal cortex is involved in cognitive control, integrating new sensory information with existing information to form adaptive behavioural plans (Holt et al., 2008). As such, it is important for a range of cognitive processes that require

executive function, such as attention, impulse inhibition, and cognitive flexibility. Impairments in tasks requiring these behaviours have been demonstrated in patients with schizophrenia. The Wisconsin Card Sorting Test (WCST) is a classic measure of cognitive flexibility, in which individuals must adapt their selections to a changing set of rules that are unknown to them. It has been shown that patients with schizophrenia are impaired in this task, completing fewer rule categories than controls, needing more trials to complete the first category, and making more errors (Everett et al., 2001). Impairments in this task have been demonstrated extensively across the literature (Lysaker et al., 1995;Roffman et al., 2008;Afshari et al., 2020;Nestor et al., 2020).

Further, changes in prefrontal cortex structure have also been observed in patients with schizophrenia. Sun and colleagues conducted longitudinal MRI imaging of 16 patients with schizophrenia and healthy controls and found that patients had a significantly greater atrophy of the cortical surface of the prefrontal cortex compared to controls (Sun et al., 2009). It has also been found that patients with poorer executive functioning, as determined by lower scores on the WCST, have reduced dorsolateral prefrontal grey matter volume compared to patients with higher executive functioning (Rüsch et al., 2007). Functional changes have also been demonstrated in patients with schizophrenia. In one of the first demonstrations of this, Ingvar and Franzen demonstrated that older patients with schizophrenia had reductions in cerebral blood volume in the prefrontal cortex, which correlated with the degree of their psychosis (Ingvar and Franzen, 1974). This result has been replicated several times in the decades since (Goldberg et al., 1990;Barch et al., 2001;Schlösser et al., 2007;Eisenberg and Berman, 2010).

1.3.4.2 The amygdala

Functional impairments in amygdala function have been demonstrated in patients with schizophrenia. It has been demonstrated that patients show reduced activation of the amygdala complex during negative affect, unlike healthy controls (Schneider et al., 1998). Similarly, Phillips and colleagues found that patients with schizophrenia did not show activation of the amygdala in response to fearful facial expressions (Phillips et al., 1999). Further it has been found that observing fearful facial expressions led to increased arousal in patients with schizophrenia, but decreased amygdala activity, a finding which was more pronounced in patients with paranoid schizophrenia subtype (Williams et al., 2004).

Imaging studies have demonstrated reduced amygdala volumes in patients with schizophrenia compared to controls. In a large-scale, multi-site study of 884 patients with schizophrenia, it was found that patients had reduced amygdala volumes compared to healthy controls (Okada et al.,

2016b). Similarly, a recent meta-analysis of 94 studies of MRI, diffusion tensor imaging and functional connectivity of the amygdala in patients with schizophrenia and bipolar disorder found reductions in left, right and total amygdala volumes compared to both healthy controls and patients with bipolar disorder (Ho et al., 2019). Whilst some smaller studies have not found these reductions in amygdala volume , that large scale studies have found such reductions may suggest that amygdala volume is reduced in patients with schizophrenia.

1.4 Synaptic plasticity

In the now classical text by Donald Hebb (Hebb, 1949), it was theorised that repeated stimulation of cell *B* by cell *A* leads to metabolic changes in one or both cells and a subsequent increase in firing efficiency, a theory now widely known as Hebb's postulate. In the decades that followed, scores of research studies were undertaken exploring this phenomenon, including the molecular components of synaptic plasticity and the neuronal circuits involved. Two of the most well-studied forms of plasticity are Long-Term Potentiation (LTP) and Long-Term Depression (LTD), both of which contribute to one's ability to form and modulate the complex and long-term memories that underlie behaviours such as associative learning.

1.4.1 Long term potentiation (LTP)

LTP is defined as the persistent increase in synaptic strength following repeated stimulation of a synapse. One of the most studied forms of LTP, particularly within the hippocampus, is N-methyl-D-aspartate receptor- (NMDAr) dependent LTP in the CA3-CA1 Schaffer collateral pathway (Collingridge et al., 1983). NMDAr are ionotropic glutamate receptors composed of an NR1 subunit and one of several NR2 subunits (Hollmann and Heinemann, 1994), with the NR2 subunit regulating ion channel gating and calcium sensitivity (Monyer et al., 1992). At resting membrane potential, external magnesium ions enter the NMDAr pore due to the differing extracellular and intracellular concentration gradient (Blanke and VanDongen, 2008). Magnesium binds tightly to the NMDAr pore, preventing further ions from entering the channel (Nowak et al., 1984). Presynaptic release of glutamate binds to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAr), leading to an influx of sodium ions and depolarisation of the cell. Similar to NMDAr, AMPAr are ionotropic glutamate receptors, composed of 4 subunits encoded by the genes GRIA1-4 (Collingridge et al., 2009)**.** AMPAr are enriched at excitatory glutamatergic synapses and are highly dynamic, moving both laterally along the cell membrane and trafficked to and from the post-synaptic membrane following particular patterns of neuronal activity (Chater and Goda, 2014). AMPAr are thought to mediate fast-action excitatory neurotransmission, causing brief depolarisations lasting a few milliseconds (Blanke and VanDongen, 2008). Depolarisation of the cell causes the removal of the

magnesium block from NMDAr which, alongside glutamate binding, allows calcium ions into the cell. As such, NMDAr are known as coincidence detectors, as both pre- synaptic glutamate release and post-synaptic depolarisation are required for their activation (Seeburg et al., 1995).

The influx of calcium into the postsynaptic neuron leads to a cascade of changes which can enact long-term changes to synaptic structure. Calcium ions bind to calmodulin to form a calcium-calmodulin complex, which in turn activates several key enzymes including calcium/calmodulin-dependent protein kinase II (CaMKII). Phosphorylation of CaMKII activates the RAS-ERK signalling pathway, which leads to the exocytosis of AMPAr-containing vesicles, thus increasing the pool of receptors at the synapse (Patterson et al., 2010). In addition, CaMKII phosphorylates stargazin, which binds AMPAr to PSD-95, anchoring it to the synapse (Tomita et al., 2005;Opazo et al., 2010). Further, CaMKII binds to NMDAr, particularly the NR2B subunit for which it has a high affinity (Strack and Colbran, 1998). The formation of NMDAr- CaMKII complexes allows proximity of CaMKII to the GluR1 subunit of AMPAr, leading to the phosphorylation of AMPAr which increases their conductance (Barria et al., 1997;Derkach et al., 1999). This process is key to the induction of LTP, and switching the high-affinity NR2B NMDAr subunit for a low affinity subunit, or inducing mutations which reduce NMDAr-CaMKII binding significantly attenuates LTP induction (Barria and Malinow, 2005). The formation of calcium-calmodulin complexes activates several other receptors, including protein kinase C (PKC). PKC phosphorylates AMPAr at S831, increasing channel conductance (Roche et al., 1996). PKC also phosphorylates AMPAr at S818, which promotes AMPAr incorporation into the synapse, the blocking of which reduces LTP (Boehm et al., 2006). Together, CaMKII and PKC contribute to early- LTP (E-LTP), a transient form of LTP which does not require protein synthesis (Frey et al., 1993;Klann et al., 1993)

Late-LTP (L-LTP) is an extension of E-LTP and requires protein synthesis and gene expression to modulate the long-term structural changes that supports synaptic plasticity, including dendritic spine enlargement (Frey et al., 1988;Harris et al., 2003;Bosch et al., 2014). Increased calcium concentration inside the cell leads to the activation of calcium-sensitive adenylyl cyclase enzymes, such as AC1 and AC8, which catayse the production of cyclic adenosine monophosphate (cAMP) (Chetkovich et al., 1991;Poser and Storm, 2001). Rises in cAMP leads to the phosphorylation of kinases such as protein kinase A (PKA), which in turn activates extracellular regulated kinase (ERK) (Impey et al., 1998). Subsequently, ERK activates 90 kDa S6 kinase (RSK) family proteins (Chen et al., 1992), which phosphorylate a range of transcription factors required for gene expression, including cAMP-response element binding protein (CREB) and nuclear-factor κβ (NF-κβ) (Frödin and Gammeltoft, 1999;Anjum and Blenis, 2008). CREB binds to the cAMP-response element (CRE) of the

promotor of its target genes, leading to the initiation of the transcription of genes key for the expression and maintenance of LTP.

1.4.1.1 Long term potentiation and consolidation

Consolidation of fear memories and long-term potentiation (LTP) have been shown to share similar molecular mechanisms (Rogan et al., 1997;McKernan and Shinnick-Gallagher, 1997;Martin et al., 2000). In a seminal study, it was found that inhibiting NMDA receptors in the hippocampus with AP5 (alternatively known as APV) prevented consolidation of spatial working memory, and blocked LTP induction *in vivo* (Morris, 1989). Further, it has been shown that NMDAr are critical for the consolidation of inhibitory avoidance fear memories. Cammarota and colleagues blocked NMDAr with APV in dorsal CA1 immediately after training in a one-trial inhibitory avoidance task, and found a significantly decreased latency to step onto the shock-associated platform, indicating that the association had not been learned. Further, they found a significant decrease in the catalytic subunit of PKA and reductions in phosphorylated CREB, both of which are key to LTP (Cammarota et al., 2000). Similarly, Athos and colleagues demonstrated that NMDAr activation was necessary for the acquisition of contextual fear conditioning, as intra-hippocampal infusions of APV abolished the behavioral expression of conditioned fear (Athos et al., 2002).

Electrophysiology experiments provide further evidence that LTP mechanisms contribute to consolidation. McKernan and colleagues (1997) undertook whole-cell recordings in *ex-vivo* amygdala slices prepared from animals which had undergone auditory fear conditioning, and found an increase in synaptic efficacy in experimental compared to control animals, indicative of LTP(McKernan and Shinnick-Gallagher, 1997). Further, Nabavi and colleagues (2014) used optogenetics to inactivate (using LTD) and reactivate (using LTP) a previously learned auditory association, demonstrating a causal link between LTP and memory consolidation, at least under specific circumstances (Nabavi et al., 2014). Structural synaptic changes have also been reported following acquisition of CFC. Restivo and colleagues reported that apical and basal dendritic spine density increased in CA1 hippocampal neurons following CFC, but not exposure to the context alone (Restivo et al., 2009). In addition, a recent study found preliminary evidence that auditory fear conditioning promotes the formation of multi-contact synapses in the auditory cortex (Liu et al., 2021a). Taken together, these results suggest that consolidation is underpinned by similar genetic and structural changes to LTP.

1.4.2 Long term depression (LTD)

Long term depression (LTD) is characterised by a long-lasting decrease in synaptic strength and, as with LTP, modulates various aspects of cognition, including extinction of fear conditioning (Kim et al.,

2007;Dalton et al., 2008). Two main forms of LTD have been described, NMDAr-dependent and metabotropic glutamate receptor (mGluR) dependent. In NMDAr-dependent LTD, calcium influx through NMDAr leads to the activation of calmodulin (CaM) which, through a series of phosphatase cascades, activates protein phosphatase 1 (PP1) (Bear and Abraham, 1996;Kandler et al., 1998). Whilst LTP is also induced through calcium influx into the post-synatic cell, it is thought that the magnitude of calcium ion influx, moderate for LTD and large for LTP, mediates which synaptic process is activated (Mizuno et al., 2001). PP1 is a key phosphatase which dephosphorylates targets including subunits of AMPAr and post-synaptic density 95 (PSD-95). This can lead to a reduction in the conductance of AMPAr, and their eventual endocytosis at the synapse (Lüthi et al., 2004;Collingridge et al., 2004). mGluR-dependent LTD in the CA1 is mainly mediated by mGluR5 (Huber et al., 2001). Stimulation of mGluR5 leads to the hydrolysis of phosphatidyl inositol (PI) into inositol triphosphate (IP3) and diacylglycerol (DAG) which activates PKC. PKC is targeted by protein-interacting-with-C-kinase (PICK1) to phosphorylate the GluA2 subunit of AMPAr, leading to the displacement of AMPA-binding-protein-and-glutamate receptor-interacting-protein (ABP-GRIP), triggering a cascade leading to the removal of AMPAr (Jo et al., 2008;Braithwaite et al., 2002;Osten et al., 2000). Other protein kinases, including p38MAPK and phosphoinositide 3-kinase (PI3K) have been implicated in mGluR LTD (Moult et al., 2008;Rush et al., 2002;Hou and Klann, 2004). mGluR LTD can occur, at least initially, independently of both calcium signalling and protein synthesis (Fitzjohn et al., 2001;Moult et al., 2008).

1.5 Translational relevance of animal models

The use of animal models in neuroscience research allows tight environmental and experimental control of any manipulations relevant to one's area of interest. Further, the use of animals permits genetic manipulation by way of altering gene dosage or expression of certain disease-linked genes, or by using reporter lines to tag certain molecular pathways or cell types (discussed further in section 1.6). In this way, animal models are an important tool for neuroscientific research, and allow the neural mechanisms underlying processes such as learning and memory to be explored. However, the translational relevance of animal models, especially in relation to schizophrenia, which is an inherently human disease, may be called into question, particularly as many of the most debilitating symptoms, such as delusions and hallucinations, cannot be recapitulated in animal models. As such, specific facets of potential disease relevance, such as associative learning or LTP, are often studied in isolation. Whilst not ideal, and does not allow for the complex interaction of genetic and environmental factors likely at play in human disease, the use of animal models represents a compromise between experimental manipulation and control, and disease-relevance. Alternatives to

animal models may include the use of induced pluripotent stem cells taken from patients (iPSCs) which are reprogrammed to the cell type(s) of interest (Park et al., 2008). However, whilst the patients' genetic risk is recapitulated in the reprogrammed cells, translational issues still arise such as the similarity of reprogrammed cells to those *in vivo* and the fact that isolation from neural circuits may impact their functionality (Rowe and Daley, 2019). Below, factors pertaining to the translational relevance of the current study are discussed.

1.5.1 Conservation across human and rodent genomes

It has been found that around 40 % of the human genome has a homologous loci in the mouse genome, as determined by human-mouse pairwise alignment, a relatively high percentage given the differences between species (Schwartz et al., 2003). Further, using a database of almost 1200 genes from the Human Gene Mutation Database, which contains genes reported to be associated with inherited disease, 76 % were found to have orthologous genes in the rat genome, increasing to 99% when the human genes not matched to a rat ortholog were manually aligned using BLAT and BLAST. Further, 89 % of human disease-associated mis sense mutations contained the same amino acid in human and rat (Huang et al., 2004). Advances in genomic research since this time would likely result in differences if repeated at present, but nonetheless they indicate potential conservation between human and rat genomes. More recently, RNA sequencing of 6 tissue types, including brain and cerebellum, were sequenced from 10 species including human and mouse (Brawand et al., 2011). Principal component analyses highlighted that gene expression patterns separated by tissue type, rather than be species, indicating some level of conservation between humans and mice. Whilst it may be expected that large proportions of the genome may differ between species, that there is a level of genomic conservation between them, coupled with the advantages of animal modelling discussed above, means that gene expression studies within rodent species is worthwhile.

Alongside genome conservation, another consideration when comparing human to rodent gene expression is the differences in genome annotation. The human genome was first sequenced in 2000, funded by the Human Genome Project, and since then has been updated periodically by the Genome Reference Consortium (Lander et al., 2001). The latest release of the human reference genome, GRCh38, was released in 2013 and showed increases in the number of annotated genes and decreases in partially represented coding sequences compared to GRCh37 (Schneider et al., 2017). The current assembly identifies 41,722 genes and pseudo genes (NCBI, 2014a), although what percentage of the genome this equates to is unknown given that it is the official reference, and unannotated genomic regions would not be able to be identified and resolved. In addition, much of the reference genome has been sequenced from a small number of individuals, and as such likely

does not represent the full genetic diversity of humans. The mouse has been the experimental animal of choice for several decades, and as such the mouse genome is well annotated, including representation of non-transcribed pseudo-genes and haplotype specific regions (McGarvey et al., 2015). The latest release, GRCm39, contains annotation of 50,561 genes and pseudogenes, with a minimal number of sequences not aligning (1 sequence) (NCBI, 2020). Whilst historically a less popular experimental animal than mice, partially because of the advances in mouse genome manipulation techniques, rats often remain the experimental model of choice for behavioural studies due to their increased sociability, mimicking that of humans, larger brain sizes making surgery easier and more accurate, and reduced need for training compared to mice (Bryda, 2013;Ellenbroek and Youn, 2016). The latest release, Gnor_6.0, contains 39,430 genes and pseudogenes which a minimal percentage of non-alignment (0.21 %) (NCBI, 2014b). Non-alignment to curated RefSeq transcripts is a measure of genome quality, hence the low non-alignment for both mice and rats indicates that the reference genomes are of high quality. Whilst there are fewer identified genes and pseudogenes for rats compared to mice, as discussed above it is difficult to ascertain whether this is because the rat genome genuinely has fewer genes than that of the mouse, or whether there are gaps in the rat annotation, as these are the official and most widely used reference genomes. However, given that the first reference genomes were produced within a few years of each other, 2001 for humans, 2002 for mice and 2004 for rats (Lander et al., 2001;Waterston et al., 2002;Gibbs et al., 2004), and that the current reference genomes are curated by an internationally recognised research institution (National Center for Biotechnology Information), it is unlikely that large differences exist in the methods and quality of the reference genomes.

1.5.2 Similarities of the hippocampus across species

Phylogenetically, the hippocampus is one of the oldest cortical areas (Seress, 2007). Anatomically, the organisation of the hippocampus is preserved across humans, rats and mice, with the CA and DG areas having similar structural features and functional connections (Seress, 2007) (Clark and Squire, 2013) (Insausti, 1993) (Manns and Eichenbaum, 2006) (Figure 1.4, see section 1.3 for details of hippocampal connections).

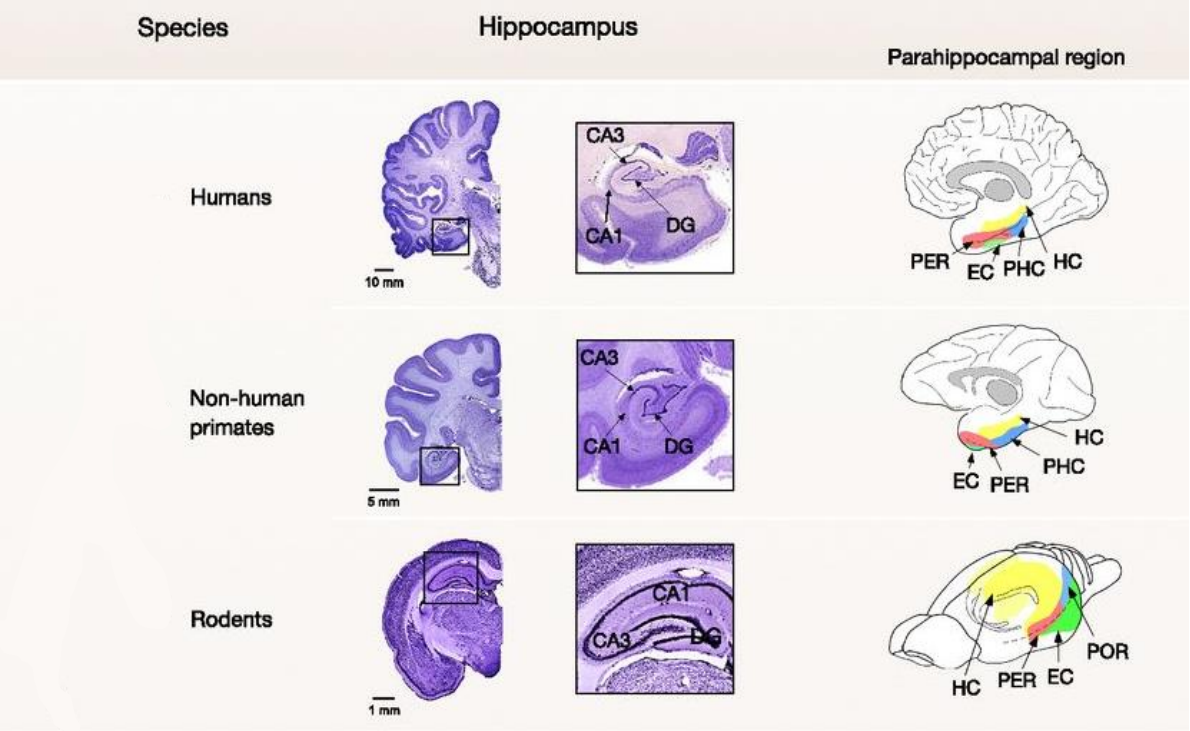


Figure 1.4. *Anatomical comparison of the hippocampus in human, non-human primates and rodents. Images show nissl staining of the the distinct subregions of the hippocampus, and can be seen to be conserved across species. Image adapted from (Allen and Fortin, 2013).*

Of particular relevance to the present work is the similarity of CA1 pyramidal neurons between species. A recent study by Benavides-Piccione and colleagues used 3D reconstruction of intracellularly injected CA1 pyramidal neurons in humans and mice to identify compare morphological features between the species (Benavides-Piccione et al., 2019). It was found that human and mouse CA1 pyramidal neurons showed several morphological similarities including the main apical shaft being thicker than other dendrites and apical dendrites having the largest segments. Whilst functionality cannot be ascertained from this study, it indicates that the cytoarchitecture of the CA1 pyramidal neurons are similar between species.

Functionally, the hippocampus has been shown to be important in learning and memory in both humans and rodents (reviewed in (Clark and Squire, 2013)). Similarly, low frequency hippocampal oscillations, thought to be important in spatial memory, have been shown to be present in both humans and rats (Watrous et al., 2013;Goyal et al., 2020). Demonstration of LTP in human hippocampus using protocols such as transcranial magnetic stimulation (TMS) is not possible, as MTL structures cannot be directly accessed using non-invasive techniques. Despite this, studies have demonstrated LTP in other regions, including the motor cortex (Esser et al., 2006). TMS studies aim to target the hippocampus through the posterior inferior parietal cortex (pIPC), which has been

shown to have robust functional connections with the hippocampus (Kahn et al., 2008). Using this method, it has been found that the application of TMS to the pIPC enhanced associative memory encoding, suggesting that LTP processes may underlie associative learning in human hippocampus (Tambini et al., 2018). An alternative method of studying LTP in human hippocampus is to conduct electrophysiological recordings from post-mortem tissue, although this is also subject to limitations such as post-mortem delay, and the mainstream use of tissue from neurological patients being used for such studies (Kramvis et al., 2018). In one demonstration of this, Testa-Silva and colleagues conducted electrophysiological recordings from the hippocampus after surgical resection in patients with treatment resistant epilepsy. Similar to rodent synapses, it was found that human hippocampal synapses showed spike-timing-dependent changes in synaptic strength following stimulation (Silva et al., 2010). Further, it has been shown that LTP can be elicited in the DG following theta-burst stimulation, and that this is NMDA-receptor dependent as perfusion of APV prior to stimulation blocked induction of LTP (Beck et al., 2000). Taken together, these results suggest that LTP mechanisms are similar in rodent and human hippocampus.

1.5.3 Recapitulation of CFC in humans

As described in section 1.2.1., CFC in rodents involves a footshock being administered after a period of exploration of a novel environment. In humans, this protocol can be modified such that the participant explores a virtual environment, before being subjected to a mild shock, often administered on their hands. This protocol has been used by Holt and colleagues (2009; 2012) to demonstrate that patients with schizophrenia have aberrant SCR responses following aversive conditioning. In this way, the CFC protocol used in the present study recapitulates the task in which patients with schizophrenia show impairments. However, altered associative learning has also been shown for more neutral associations, for example name-face associations and object-location associations. Whilst the requirement for language in the name-face associations task means it is not suitable for translational research, object-location associations can be translated. The Cambridge Neuropsychological Test Automated Battery (CANTAB) is a well-validated, cross-species translational tool containing neuropsychological tests which can be performed in both humans and rodents (reviewed in (Barnett et al., 2015)). In the Paired Associates Learning (PAL) task, participants learn the association between a visual pattern and a location on the screen, a task which has also been translated for use in mouse (Talpos et al., 2009). Therefore, whilst the current study is restricted to the recapitulation of human aversive associative learning, methodologies are available for the investigation of neutral associative learning pairs in rats and mice.

1.6 Transcription and translation

1.6.1 Transcription

Transcription of messenger RNA (mRNA) and subsequent translation into proteins, underpins LTP. Transcription is the process of creating RNA from DNA, and it is mediated by RNA polymerases, particularly RNA polymerase II. Transcription is initiated by the phosphorylation of regulatory transcription factors such as CREB. Transcription factors (TFs) have DNA binding domains which recognise specific response elements in the promotors of target genes. Upon binding, TFs recruit RNA polymerase II to form a pre-initiation complex. RNA polymerase II has 3 components: a 12 subunit complex responsible for synthesising RNA, a set of 5 transcription factors responsible for promotor recognition and unwinding promotor DNA, and a Mediator unit which transduces information from activator and repressor proteins, regulating the activity of the polymerase (Boeger et al., 2005). Formation of this complex allows a single-stranded DNA template into the RNA polymerase II active site, allowing the transcription and synthesis of mRNA (Cheung et al., 2011). Transcription generates both mRNAs that are translated into proteins required for structural synaptic changes, and non-coding RNA transcripts that have regulatory roles in gene expression (Alberini and Kandel, 2014).

Transcription has been demonstrated to be important for long-term memory (LTM) of fear associations. Inhibiting RNA polymerase II, a key polymerase required for transcription, by administrating intra-hippocampal 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) led to deficits in LTM of an inhibitory avoidance task when administered immediately or 2-6 hours after training (Igaz et al., 2002). This dual-peak time window has been demonstrated extensively in relation to associative memory (Bourtchouladze et al., 1998;Cammarota et al., 2000;Taubenfeld et al., 1999)**.** Gene expression is also required for extinction of an associative memory. In a series of experiments, Vianna and colleagues demonstrated that CA1- infusion of an RNA polymerase II-inhibitor 15 minutes before a CS-no-US trial prevented extinction of the aversive CS-US association (Vianna et al., 2001) (Vianna et al., 2003). Further, genetic manipulation of CREB-mediated transcription using an inducible CREB repressor mouse line has been shown to block the reconsolidation and long-term extinction of conditioned contextual fear (Mamiya et al., 2009).

1.6.2 Translation

Translation is mediated by ribosomes, which comprise of 2 subunits: the small 40S subunit and the large 60S subunit. This process is initiated by the formation of the 43S ribosomal preinitiation complex, comprised of the 40S ribosomal subunit, a ternary complex of eukaryotic initiation factor 2

(eIF2), guanosine triphosphate (GTP) and initiator methionine transfer RNA (Met-tRNA-iMet), and other eukaryotic initiation factors (eIFs) (Merrick, 1992). This complex then attaches to the 5' end of mRNAs, mediated by eIFs, and scans the 5' untranslated region (UTR) for the initiation codon (Shatkin, 1985). Once the initiation codon is found, eIF5 hydrolyses the eIF2-GTP complex releasing it from the surface of the 40S subunit, allowing the 60S subunit of the ribosome to bind forming the 80S initiation complex (Pestova et al., 2007). Elongation factors are then recruited to the complex to carry out elongation of the peptide chain and, upon recognition of a stop codon, termination factors promote the release of the peptide chain from the ribosome (Costa-Mattioli et al., 2009). In the context of plasticity, synaptic activity can prompt the synthesis and translation of new mRNAs, or the translation of pre-existing mRNAs already located at the synapse.

Similar to transcription, translational processes have been shown to be important in memory consolidation (Davis and Squire, 1984;Helmstetter et al., 2008). Much of this research comes from inhibiting protein synthesis with compounds such as anisomycin, which interferes with translation by inhibiting peptidyl transferase, preventing formation of peptide bonds between amino acids (Grollman, 1967). Administration of anisomycin prior to contextual fear conditioning has been shown to prevent the behavioural expression of conditioned fear 24 hours later (Schafe et al., 1999). Similarly, administration of anisomycin immediately after CFC training has been shown to impair fear memory 3-, 6- and 24- hours after training (Bourtchouladze et al., 1998). Similar effects have been demonstrated for memory retrieval. Injecting anisomycin prior to re-exposure to a shock-associated context has been shown to impair long term retrieval (Debiec et al., 2002). Taken together, these results suggest that translation of mRNAs into proteins via protein synthesis mechanisms are important in the acquisition and expression of conditioned fear.

1.6.3 Genome-wide profiling

Genome-wide profiling of gene expression allows one to examine the expression of gene transcripts under the conditions of interest. Below, the genome wide profiling techniques used in this thesis are discussion, and the progression from microarray, RNA-seq, to TRAP-Seq which allows cell-type specific profiling of translating mRNAs.

1.6.3.1 Microarray

Since it's advent in the 1990s, microarrays were the cornerstone of gene expression profiling for almost two decades, being one of the first technologies to allow simultaneous derivation of the expression profiles of thousands of genes. The first reported microarray experiment was published in 1992, in which hybridisation to radiolabeled cDNA probes allowed quantification of mRNA levels (Gress et al., 1992). Since then, over 150,000 academic papers have been published reporting on the

results or analysis of microarray data (Pubmed, December 2021). Whilst several iterations of microarray exist, the underlying principle is common to all types. Total RNA is extracted from the biological sample and processed to produce fluorescent probes. These probes are then hybridised to complementary probes on the microarray chip, and fluorescence detected by a specialized scanner. The intensity of the fluorescent signal correlates to the abundance of that particular mRNA in the original biological sample (reviewed in (O'Brien et al., 2012)). There are two main types of microarray technology: cDNA microarrays in which relatively long (> 100mer) DNA molecules are immobilised on a solid surface, and oligonucleotide arrays where relatively short oligonucleotide probes (25-30mer) are immobilised on a glass surface using light-directed chemical synthesis (Schena et al., 1995;Lockhart et al., 1996;Russo et al., 2003;Chou et al., 2004). cDNA arrays are mainly used for large-scale screening, whilst oligonucleotide arrays are mainly used for gene expression studies as they also allow the detection of alternative transcripts. Affymatrix is one of the most ubiquitous microarray platforms in expression studies, whereby each gene on the chip is represented by a set of oligonucleotide probes covering the transcribed region of the gene (Jiang et al., 2008). Whilst microarrays were valuable in gaining greater understanding of gene expression under particular conditions, particularly in time before the complete sequencing of genomes, there are several limitations of the method. Firstly, microarray technologies rely on predefined transcripts, and as such may not provide a complete picture of the transcriptome. In addition, there can be variability in the fluorescence of a gene probe between samples, even technical replicates, which has a particular impact on genes expressed at a low level (Ness, 2006). At the other end of the scale, gene expression measurement is limited by signal saturation, and so may not be able to accurately quantify genes with the highest expression (Zhao et al., 2014b). Further, cross-hybridisation can occur whereby molecules bind to probes that are not their target, due to the nature of the probe design, which reduces the reliability of microarray data (Koltai and Weingarten-Baror, 2008;Reilly et al., 2006). Thus, the use of microarrays has been largely superseded by RNA-sequencing (RNA-Seq), driven both by the technical improvement it provides and the increase in cost-effectiveness over the past few years.

1.6.3.2 RNA sequencing (RNA-seq)

First published in 2005 (Margulies et al., 2005), RNA-sequencing is a high-throughput transcriptome sequencing method which enables the mapping and quantification of gene transcripts within a genome. RNA-Seq is termed a 'next generation' sequencing method, and can be performed using several platforms including Illumina, SOLiD and PacBio, which differ in their outputted read lengths, read generation capabilities and speed, amongst other factors (Van Dijk et al., 2014). Here, I focus on the Illumina sequencing platform, as it is the technology used in the present study.

Illumina sequencing can generate up to 20 billion reads per run, with read lengths of up to 250 base pairs (250 bp). Whilst many library preparation methods exist, they are based around similar principles (Berge et al., 2019). Firstly, high quality total RNA is extracted from the biological sample of interest and then the RNA is fragmented to produce shorter reads which can be sequenced (Wang et al., 2009b). Next, RNA is enriched for target RNAs by poly(A) selection or ribosome depletion, and reverse transcribed to cDNA. Adaptors, which contain functional elements required for sequencing, are ligated to the 3' and 5' end of the cDNA fragments. Finally, the cDNA library is amplified using polymerase chain reaction (PCR) in order to generate enough for sequencing. The libraries can then be cleaned using magnetic beads to remove artefacts such as adapter dimers, which can negatively impact data quality. Prepared libraries are loaded onto flow cells, and cDNA binds to short oligonucleotide sequences complementary to the ligated adaptors. Illumina uses 'sequencing by synthesis' technology, in which fluorescently labelled deoxynucleotide triphosphates (dNTPs) are incorporated into the nucleic acid chain one-by-one and then imaged (Chen, 2014). Base calling software then identifies each base based on the measured fluorescent signal intensity. Subsequent bioinformatic analysis maps and aligns the outputted reads to a reference genome, and quantifies them permitting downstream analysis including differential gene expression and pathway enrichment analysis.

1.6.3.3 Cell-type specific RNA-seq

One of the main applications of RNA-seq over the past decade within behavioural neuroscience has been to sequence the RNA within a whole region, referred to within the thesis as bulk-sequencing, thus identifying transcripts from a range of cell types. However there has been a growing movement within the field to move towards more cell-type specific RNA-sequencing which, as the name suggests, allows one to examine the transcriptome or translatome of a particular cell type in your condition of interest.

One way in which to do this is through single-cell sequencing, whereby cells are identified electrophysiologically through patch-clamping or by performing fluorescence-activating cell sorting (FACS) to isolate target cells ((Okaty et al., 2011)). RNA is then extracted from single or small groups of cells, amplified, and sequenced. Whilst this does allow identification of cell-type specific transcripts, there are several disadvantages to these methods. Due to the small amounts of RNA isolated from a single cell, these methods are prone to producing false negatives, particularly for low abundance transcripts. Further, single-cell RNA sequencing can result in sparse data, where no reads are mapped to a given gene, which presents challenges in both the bioinformatic analysis and interpretation of the results (Haque et al., 2017;Hou et al., 2020). Whilst FACS allows several cells to

be isolated, drawbacks include the method being stressful to some cell types, including mature neurons, and care must be taken to ensure the cells remain healthy (Lobo et al., 2006).

An alternative method is to use translating ribosome affinity purification (TRAP) methodology, whereby mRNAs are isolated, using immunoprecipitation, from labelled ribosomes from bulk tissue. This is achieved through the use of genetically altered mice lines, in which expression of a tagged ribosomal protein is driven in a specific cell type using cre technology (Heiman et al., 2008;Sanz et al., 2009). This means that only ribosome-associated mRNAs, implied to be those which are being actively translated, in a particular cell type are sequenced. In this thesis, the RiboTag mouse line has been used, in which a 60S ribosomal protein (*Rpl22*) has been modified such that, in the presence of cre-recombinase, the last exon is replaced with an exon tagged with human influenza hemagglutinin (HA) (Sanz et al., 2009). Immunoprecipitation against this HA tag allows mRNA from the ribosomal polysome to be isolated and sequenced (Figure 1.4).

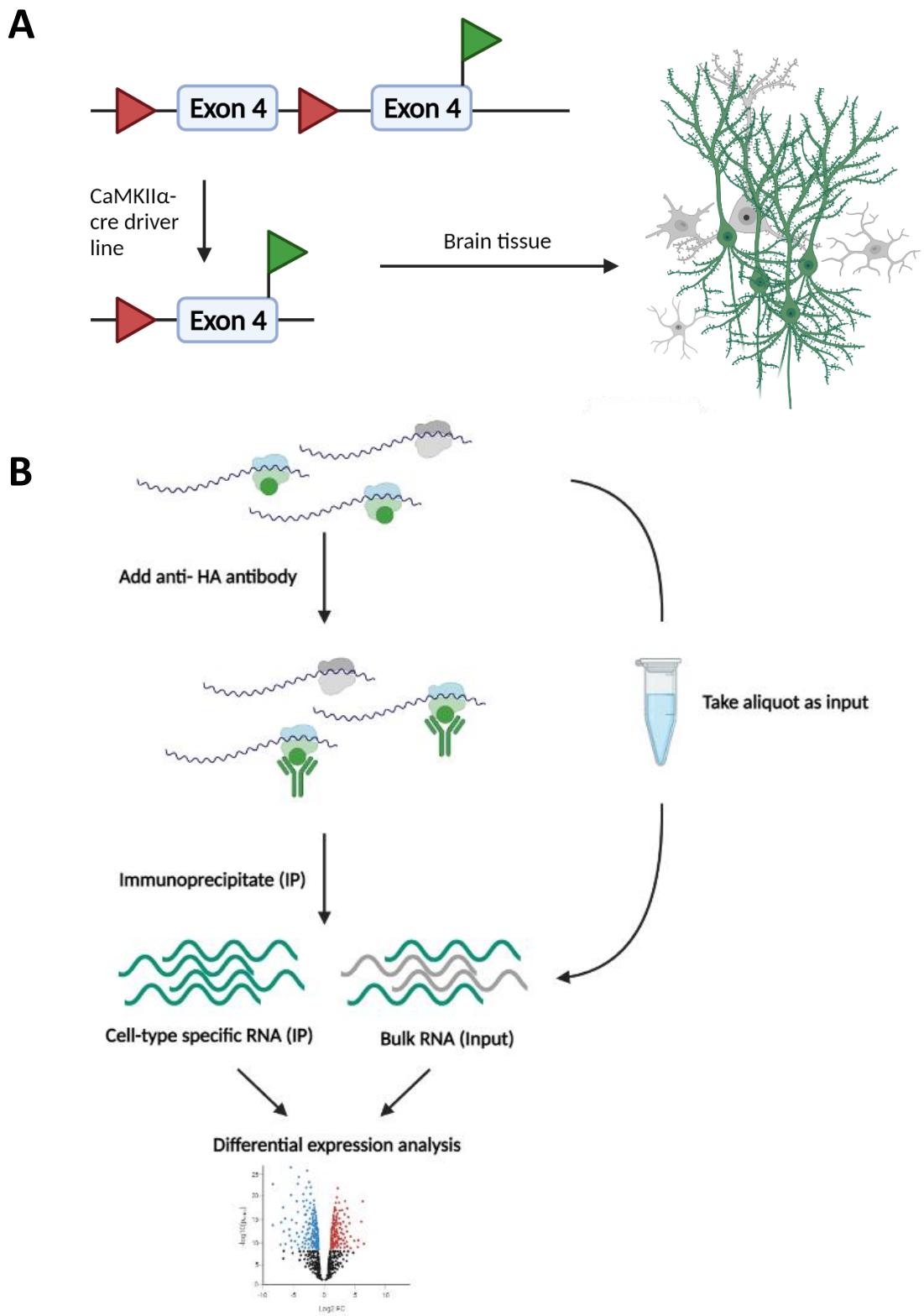


Figure 1.4. *Schematic diagram of the Translating Ribosome Affinity Purification (TRAP) methodology, which allows the isolation of cell-type specific mRNA.* ***A.*** *Schematic of the RiboTag mouse line. Exon 4 of the ribosomal protein Rpl22 is floxed so in the presence of cre-recombinase, in this case from the CaMKIIa driver mouse line, it is replaced with an exon tagged with human influenza hemagglutinin (HA), represented on this schematic by the green flag. This means that the polyribosomes of the cell-type of interest, in this case excitatory hippocampal neurons, are labelled (green) whilst other cell types (grey) are not.* ***B.*** *Initially, homogenised tissue contains both labelled and non-labelled polysomes, and an aliquot of this is taken as input for bulk RNA sequencing. An anti-HA antibody is added to the remaining lysate, and immunoprecipitated to isolate the polysomes from the cell-type of interest. RNA is isolated from the cell-type specific and input lysate, and sequenced to look at differential gene expression in specific cell-types (IP) and all cell-types (bulk). Adapted from (Sanz et al., 2009) and (Sanz et al., 2019).*

1.7 Genetics of schizophrenia

Over the past decade, genomic studies have been at the forefront of the schizophrenia research landscape, and several types of variation that may lead to increased risk of schizophrenia have been studied. These can be broadly categorised into 2 main types: common variation and rare variation.

1.7.1 Common variation

Common variation encapsulates risk from common variants, such as Single Nucleotide Polymorphisms (SNPs), that individually may have a low effect size, but that contribute to one's polygenic risk. As the name suggests, SNPs are single nucleotide base changes that can occur in coding, intergenic or non-coding regions of the genome (Van Dijk et al., 2014). SNPs that occur in coding regions are typically biallelic, that is, have two observed alleles: one reference allele and one variant allele. In order to be classed as a SNP, the variant must occur in more than 1 % of the population. There are thought to be at least 10 million SNPs in the genome, accounting for the majority of the variation between individuals (Robert and Pelletier, 2018).

The advent of genome sequencing technologies has led to a boom of genome-wide association studies (GWAS), linking genetic variants with a range of disease phenotypes. One of the first genome wide associations with schizophrenia was reported in 2006, finding one SNP (rs752016 within the *PLXNA2* gene) which was consistently affected in cases across GWAS and family-based samples (Mah et al., 2006). Based on only 320 cases and 325 controls, it is far from the large-scale studies reported now, with the latest Psychiatric Genomics Consortium (PGC) wave based on over 76,000 cases (Trubetskoy et al., 2022). Advances in sequencing technologies, and the creation of world-wide consortia, led to a landmark study published in 2014 which identified 108 genome-wide significant loci, 83 of which had not previously been reported (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014). Associations that mapped to genes of potential interest included *DRD2*, a target of therapeutic treatments for schizophrenia, and *GRIN2A*, an NMDA receptor subunit which when blocked with ketamine, mimics some schizophrenia symptomology (Aalto et al., 2005;Tomiya et al., 2006). Since this publication, efforts have continued to undertake large-scale association studies. In 2018, a meta-analysis was conducted on the PGC sample and new cases from the CLOZUK study, which identified 145 associated loci, 50 of which had not previously been reported (Pardiñas et al., 2018). In the latest release of the PGC data, 287 distinct associated loci were reported, with 120 corresponding genes having been identified through functional genomic data (Trubetskoy et al., 2022).

Whilst identifying associated genomic loci remains an important goal for schizophrenia genomic research, it can be difficult to establish the functional or therapeutic relevance of identified loci. Nonetheless, by combining genomic and bioinformatic approaches, some progress has been made towards understanding how SNPs may impact molecular pathways, thus potentially influence schizophrenia risk. A relatively early study, using the International Schizophrenia Consortium population, identified an enrichment of "Cell adhesion molecules" in schizophrenia-associated SNPs (O'Dushlaine et al., 2011). Further, using protein-protein interaction modelling, it was found that proteins encoded by genes at schizophrenia-associated loci form a highly interconnect network, enriched for genes involved in nucleosome assembly (Luo et al., 2014). More recently, it was found that schizophrenia-associated SNPs were enriched in 'regulation of synaptic plasticity' and 'regulation of neuron differentiation' pathways, highlighting a convergence on the synapse (Schijven et al., 2018). Further investigation of this association found that there was significant enrichment in pathways representing dopaminergic and cholinergic synapses, and long-term potentiation. Associated loci from the most recent PGC3 data were enriched in genes with high expression in excitatory neurons in the hippocampus, particularly the CA1, CA3 and granule cells of the DG, and cortical inhibitory interneurons, but not glia or microglia (Trubetskoy et al., 2022). This further supports the idea that schizophrenia impacts neuronal function.

Linkage disequilibrium score regression has shown that common variation in schizophrenia is positively correlated with other psychiatric phenotypes, including bipolar disorder, autism spectrum disorder and major depressive disorder (Brainstorm et al., 2018). The association between schizophrenia and bipolar disorder appears to be driven by bipolar type I, with type II being more associated with major depressive disorder (Stahl et al., 2019). Recently, schizophrenia risk gene *CACNA1C* was identified as a significant pleotropic gene, also showing association with bipolar disorder (Reay and Cairns, 2020). This highlights the complex genetic architecture underlying psychiatric conditions.

1.7.2 Rare variation

Whilst common variation may exert risk through a multiplicative effect of several variants of low effect size, rare variation encapsulates risk from variants with moderate effect sizes, namely copy number variants (CNVs) and ultra-rare protein-coding variants.

1.7.2.1 Copy number variants (CNVs)

CNVs are defined as a segment of DNA, 1 kb or larger, that varies in copy number in comparison to the human reference genome (Redon et al., 2006). Copy number variation typically manifests as either a deletion, one less copy number, or duplication, an additional copy number, of a

chromosomal region. Whilst copy number variation contributes to 'normal' genetic diversity, it has been estimated that approximately 12 % of the genome is subject to copy number variation, several variants have been identified as harbouring increased risk for psychiatric phenotypes, including schizophrenia. One of the most well-known of these is deletion of the 22q11.2 region, with around 25 % of carriers diagnosed with schizophrenia, thus being the strongest known genetic risk factor for schizophrenia (Murphy et al., 1999;McDonald-McGinn et al., 2015). Other CNV loci which increase risk for schizophrenia include 1q21.1, 2p16.3 (*NRXN1*), 3q29, 15q11.2 and 16p11.2 (Kirov et al., 2008;Kirov et al., 2012;Marshall et al., 2017).

With the exception of *NRXN1*, CNVs often span 10s of genes, and so the mechanism by which they increase schizophrenia is complex. Combining genetic and proteomic approaches, it has been shown that case CNVs are enriched for members of the NMDA receptor family compared to control CNVs, highlighting the potential impact of CNVs on synaptic function (Kirov et al., 2012). Further, by combining genomic CNV data with functional data from the MGI mammalian phenotype database, Pocklington and colleagues identified an enrichment of genes involved in GABAergic neurotransmission in case CNVs, as well as confirming the enrichment of NMDA receptor genes (Pocklington et al., 2015). The authors also identified associations relating to learning and memory, including "contextual conditioning behaviour" and "associative learning".

Previous work has highlighted the interaction between common and rare variation in increasing schizophrenia risk. Using data from the PGC, it has been shown that schizophrenia patients that carry CNVs have a lower overall polygenic risk score (PRS) compared to patients without CNVs, which was proportional to the effect size of the CNV (Bergen et al., 2019). However, in a recent study comparing individuals with 22q11.2 deletion syndrome, with and without schizophrenia, it was found that those with schizophrenia had higher polygenic risk compared to individuals who had 22q11.2 deletion syndrome, but not schizophrenia (Cleynen et al., 2020). It may be that the differing study population, may have led to the differing results here, but serves to reinforce the complex and difficult-to-ascertain genetic architecture of schizophrenia.

1.7.2.2 Ultra-rare coding variants

Exome sequencing allows the identification of ultra-rare protein-damaging mutations, such a loss-of-function and mis-sense genetic variants (Samocha et al., 2014). Loss-of-function protein damaging mutations include frameshift mutations, which cause a change in the mRNA coding frame, and stop variants which involve a gain or loss of stop codons (Pagel et al., 2017). These can result in degraded mRNA or protein, or render them non-functional. Mis-sense genetic variants result in changes to the amino acid encoded at the affected location in the protein which, in some cases, leads to altered

function of the protein. Mis-sense variants have been further categorised according to Missense badness, Polyphen-2 and constraint (MPC) score, with variants with scores greater than 2 having been shown to be enriched in neurodevelopmental disorder cases (Samocha et al., 2017). Analyses of ultra-rare coding variants in exome sequencing studies have found such variants in ARC and NMDA receptor protein complexes in schizophrenia cases, and in the alpha subunits of voltage-gated sodium channels (Rees et al., 2019). Recently, the Schizophrenia Exome Meta-Analysis Consortium (SCHEMA) published analysis of data from more than 24,000 schizophrenia cases, reporting 10 genes with exome-wide significant protein-damaging coding mutations (Singh et al., 2022). These included *GRIN2A*, a gene encoding an NMDA receptor subunit which has previously been implicated in studies of common variation (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014), and *GRIA3*, which encodes an AMPA receptor subunit. Further, antagonism or knockout of the subunit which *GRIN2A* encodes, GluN2A, has been linked to hippocampal memory deficits (Radiske et al., 2021;Kannangara et al., 2015), supporting previously presented evidence that hippocampal memory processes may be affected in schizophrenia.

1.8 Summary

In summary, previous work has demonstrated associative learning impairments in patients with schizophrenia, particularly that they show aberrant physiological and behavioural responses to contexts which have previously undergone extinction (Holt et al., 2009;Holt et al., 2012). Further, it has been theorised that delusions experienced by patients with schizophrenia may be a result of aberrant association formation, making it an important focus for research (Fletcher and Frith, 2009). Associative learning has been shown to be dependent on the hippocampus (Phillips and LeDoux, 1992a;Ji and Maren, 2005), a brain region which has been shown to be structurally and functionally altered in patients with schizophrenia (Ho et al., 2017;Tamminga et al., 2010;Haijma et al., 2013;van Erp et al., 2016;Okada et al., 2016a). CFC is an associative learning paradigm in which shock is associated with a particular context. It is a task with translatability between rodents and humans, as the rodent task can and has been adapted for use with patients, whereby the shock is presented whilst navigating a virtual context. Thus, when examining the association between genes expressed during learning and memory and schizophrenia, CFC represents a good paradigm due to it's recapitulation between rodents and humans, the clinical relevance of associative learning, and that the brain structure underlying it is also impacted in human disease. From a methodological point of view, contextual fear memories can be generated in a single-trial, thus has a discrete learning point, allowing gene expression changes to be assigned to a particular time-point.

There are several main stages of fear memories, including acquisition, consolidation and retrieval, the latter of which can be further sub-divided into recall and extinction. Both initial consolidation of fear memories after acquisition, and reconsolidation of memories through retrieval, are dependent on gene expression and protein synthesis in the hippocampus (Schafe et al., 1999;Parsons et al., 2006;Igaz et al., 2002;Debiec et al., 2002;Gafford et al., 2011). Further, LTP-like changes have been reported following consolidation of fear memories, suggesting that LTP underlies the consolidation of CFC (Rogan et al., 1997;McKernan and Shinnick-Gallagher, 1997;Martin et al., 2000). The development of large psychiatric genetic consortia have accelerated the discovery of common variants which are associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014;Pardiñas et al., 2018;Trubetskoy et al., 2022). Further, rare variant studies have identified several copy number variants which confer increased risk for the development of schizophrenia (Kirov et al., 2008;Kirov et al., 2012;Marshall et al., 2017). Several identified genetic variants impact learning and LTP- related genes, including those which encode NMDA receptor subunits, and are enriched in functional pathways related to synaptic plasticity processes (Schijven et al., 2018). In addition, genes impacted by schizophrenia-associated CNVs are enriched in learning and memory phenotypes, including “contextual conditioning behaviour” and “associative learning” (Pocklington et al., 2015). Further, our laboratory has previously reported that genes associated with extinction memory were enriched in genes impacted by schizophrenia-associated CNVs (Clifton et al., 2017b).

1.9 Aims

Given that our laboratory previously linked genes expressed during extinction with schizophrenia-associated CNVs (Clifton et al., 2017b), and that both common and rare variation studies have found associations with LTP and learning related processes, the overall aim of this thesis was to further explore the enrichment of plasticity-related genes in schizophrenia risk. In relation to LTP, whilst it has been demonstrated that genes encoding LTP-associated proteins, such as NMDA receptor subunits, are impacted by common and rare variation for schizophrenia, this has not been demonstrated using a functional experiment.

CFC is presented here as an avenue through which to explore how the molecular processes underlying associative learning may be associated with schizophrenia risk variants. This task was used as it recapitulates an associative learning task in which patients with schizophrenia showed deficits, and is hippocampal dependent, a brain region which has shown consistent structural and functional changes in patients. The work is presented chronologically, in the order the experiments occurred in, as such the retrieval experiment is presented first, rather than consolidation

experiment. In addition, the retrieval experiment, as a replication of Clifton et al., (2017b), formed the foundation of the subsequent work and as such it was logical to present this first.

My aims were as follows:

- To test and extend previous findings that genes expressed during extinction of CFC are enriched for schizophrenia risk variants (Chapter 3).
- Quantify patterns of gene expression following long term potentiation (LTP), with particular focus on CA1 excitatory neurons, and test for association with genetic variants from patients with schizophrenia, schizophrenia-related disorders, and appropriate control disorders (Chapter 4).
- Explore the gene expression profile induced in the consolidation window of CFC in excitatory hippocampal neurons, and test for psychiatric genetic association (Chapter 5).

Chapter 2: General methods

Animal work and methodological techniques applicable to several results chapters, such as RNA sequencing, are presented here. Laboratory techniques used only in specific experiments are presented within their respective chapter.

2.1 Animals

2.1.1 Rats

Adult male Lister hooded rats (250 – 350 grams) were obtained from Charles River UK for testing. Upon arrival, rats were housed in pairs and kept on a 12-hour reverse light-dark cycle, in which the lights turned off at 10:00. This allowed testing to occur in the dark cycle, which is when rats are most active. Rats were habituated to their home cages for a minimum of 14 days to allow for adjustment to the altered light cycle. Throughout the experiment, rats were allowed free access to food and water. A cardboard tube and chew stick were provided for enrichment.

2.1.2 Mice

Homozygous T29.1 CaMKIIα-cre mice (The Jackson laboratory [(Luo et al., 2020)]) and homozygous RiboTag mice (The Jackson laboratory [029977]), aged 8 weeks, were obtained from The Jackson Laboratory.

2.1.2.1 Housing

Mice were housed 2- 3 in a cage in single-sex littermate groups, with wooden shavings, a clear plastic tube, a cardboard tube, a chew stick and paper nestlet. They had free access to food and water throughout any experimental protocols. They were housed on a 12:12 hour light cycle (lights on 08:00-20:00). The temperature in the room was kept at between 19- 21 °C, with humidity between 45 - 60 %.

2.1.2.2 Breeding

Due to the coronavirus pandemic, within-genotype breeding of T29.1 CaMKIIα-cre and RiboTag mice was conducted for 2 generations, in order to maintain the line whilst restrictions were in place. Mice were bred in trios, with 2 females and 1 male per cage. Mice were transferred to larger breeding cages in a specific breeding room, with sawdust, cotton nestlets, 2 cardboard tubes and a chew stick.

Once the females were pregnant, males were removed and singly housed in standard caging. Offspring were weaned between 21 and 28 days of age.

For experimental breeding, homozygous female T29.1 CaMKIIα-cre mice were bred with homozygous male RiboTag mice to produce offspring heterozygous for each allele (RiboTag x CaMKIIα-cre). Expression of Cre recombinase has been reported in several structures in the male germline, and so to avoid germline deletion of the floxed allele female CaMKIIα-cre mice were used, as recommended by The Jackson Laboratory (Luo et al., 2020).

2.1.2.3 Genotyping

Genotyping of parental and offspring lines was conducted externally by Transnetyx. Primer sequences are listed below (Table 2.1). All offspring were heterozygous for the RiboTag allele, and were Cre positive.

Probe	Forward primer sequence	Reverse primer sequence
CRE	TTAATCCATATTGGCAGAACGAAAACG	CAGGCTAAGTGCCTTCTCTACA
Rpl22-1 Floxed	CAGAGCCAGTGCTCTAACAAGGCA	CGAAGTTATCGGTCTCGACTGCTGA
Rpl22-1 Wildtype	CTGTGCGGTCTTTCTCTAGTGGTCTC	GTCTATCACACATCCCTAGCTTGGCA

Table 2.1. *Primers used by Transnetyx for genotyping of CaMKIIα-cre x RiboTag offspring.*

2.1.3 Governance

All experiments were conducted in accordance with the United Kingdom 1986 Animals (Scientific Procedures) Act, under project licence 3013135 or P0EA855DA, and personal licence IB8AC6F43.

2.2 Contextual fear conditioning

Contextual fear conditioning (CFC), in which rodents learn to associate a context with an electric footshock, is a well-established method for studying associative learning (Lee et al., 2004b)**.** Trials were conducted at the same time each day and counterbalanced between groups as circadian rhythms have previously been found to influence gene expression (Gerstner and Yin, 2010).

2.2.1 Rats

Rats were handled the day before undergoing CFC. Rats were transported to the behavioural testing room individually in cardboard transport boxes to prevent exposure to light during the dark phase. Each rat was placed into one of two standard conditioning chambers, contained within a sound-

attenuating box. The conditioning chambers (Med Associates Inc.) measured 30.5 cm (L) x 24.1 cm (W) x 21.0 cm (H), and had 19 floor bars raised 1.6 cm above the floor tray. The sound attenuating box measured 55.9 cm (L) x 55.9 cm (W) x 35.6 cm (H). In conditioning trials, rats were allowed free exploration of the conditioning chamber for 2 minutes, before a 2 second 0.5 mA footshock was delivered through the floor bars. Footshock delivery was controlled by a Med-PC (version IV) Research Control and Data Acquisition System (Med Associates Inc.). Rats remained in the chamber for a further 1 minute before being removed and placed back in their transport boxes and returned to their home cage. Test chambers were cleaned between each animal with 50 % ethanol. 48 hours later, rats underwent either no recall, recall or extinction trials. For recall and extinction trials, rats were transported in the same transport box and returned to the same conditioning chamber as in the previous conditioning trial. In recall trials, rats remained in the chamber for 2 minutes, and in extinction trials rats remained in the chamber for 10 minutes, before being returned to their home cages. Rats in the no recall condition remained in their home cages as a control. Using the protocol presented here it is possible that the context could act as a cue, as the context recognition was not tested. Thus, whilst it is referred to throughout this thesis as contextual fear conditioning, this caveat should be considered. Discussion about how context specificity could have been tested is in the General Discussion.

One alternative control could have been an immediate shock condition, in which rats are placed into the context and immediately shocked and then removed. In this way, the effects of shock are controlled for, but the rats do not learn the association between the context and the shock, and as such do not display freezing behaviour. This was piloted, however in our hands, the rats still showed freezing to the context in the recall trial. This is likely due to the length of time it took to place the rats into the context, shut the door, exit the room to administer the shock (as per the set up in the experimental room), and re-enter to remove the rats from the context. An additional person assisting would have reduced this time, but was not available at the time of the initial experiment and not permitted during later experiments because of the COVID-19 pandemic. In addition, removing the “no recall” rats from their home cages and transporting them to the experimental room, but not returning them to the context, would have controlled for stress-related transfer effects, but the transportation may have acted as a cue for the recall of the learned association (Rudy et al., 2002). As such, the “no recall” condition as presented here represented the most practical control condition for these experiments, although I acknowledge that some of the gene expression may have been induced by the transfer to the experimental room.

2.2.2 Mice

2.2.2.1 Handling

Mice have increased baseline anxiety compared to rats, and so more extensive handling was required prior to CFC. Prior to the behavioural experiment, naïve mice were habituated to handling over a period of 2 weeks. Each time, mice were exposed to 5 minutes handling in their home cage. For the initial 3 days, mice were handled according to the following regime. For 2 minutes, a gloved hand was placed flat in the cage so the mice could sniff and explore it. For the subsequent 2 minutes, a gloved hand was raised level to the top of the cage, so that the mice could explore whilst getting used to the glove. This stage was added during a pilot experiment, where it was found that some naïve mice were reluctant to explore a flat gloved-hand in their cage. For the final minute, mice were transferred using handling tubes into a clean cage and back again, to get them used to being transported. For the next 4 days, a similar regime was followed but the time the gloved hand was placed in the cage was reduced to a total of 3 minutes (1-minute flat, 2-minutes lifted) and the tube handling time was increased to 2 minutes. After the first 7 days, handling was conducted every other day. It involved transporting the mice from their home cage to a clean, second cage in a frosted acrylic tube (different to that usually in their home cages) and being transferred between cages by hand. A different type of tube was used so that they did not associate being transported in and out of the CFC apparatus with the clear tube in their home cage. Mice were held lightly at the base of their tail and transferred onto a flat hand, after which the grip on the tail was loosened whilst they were transferred and then placed into the 2nd cage.

2.2.2.2 Contextual fear conditioning

Contextual fear conditioning chambers (Med-associates) were contained within sound-attenuating chambers. The walls of the chambers had either horizonal stripes, spots, or were transparent. Mice were placed into the box using the frosted acrylic tube they had been habituated to, and the door closed. The trial began when the house light turned on. Mice were allowed to explore the context for 3 minutes, before receiving a 2 second, 0.7 mA scrambled foot shock through the floor bars. After the shock, mice were left in the context for a further 1 minute, before the house light turned off signalling the end of the trial. Mice were removed from the chamber using the frosted acrylic tube. During pilot, this was found to be the easiest way to remove the mice, as mice naturally want to hide, and as such it prevented the mice from jumping out of the chamber when the door opened. Control animals remained in their home cages in the holding room.

To pilot recall of the conditioned fear memory, 24 hours after training, pilot mice (*n* = 5) were placed back into the same context for 3 minutes, without footshock.

2.2.3 Quantification of freezing behaviour

All sessions were recorded using cameras positioned centrally above the chambers to allow for the subsequent scoring of freezing behaviour. Freezing behaviour was defined as the absence of movement except that required for breathing, and was manually sampled every 5 seconds for the duration of the session. Whilst automated software presents advantages over manual scoring, namely that it is not subject to human observation, unfortunately it was not available as it is cost prohibitive to set up. Freezing scores were converted into percentages, representing the proportion of sampled time points in which the animal was observed freezing.

2.2.4 Culling method

2- or 5- hours post-behaviour, animals were culled by rising concentration of CO_2, confirmed by checking cessation of circulation as per the Animals (Scientific Procedures) Act 1986. CO_2 was administered in 2 stages: at a low flow rate of 20% of the chamber volume for 4 minutes to render the animal unconscious, then at a higher flow rate of 50% of the chamber volume for a further 3 minutes. There is concern within the literature that inhalation of CO_2 as a culling method may subject the animal to suffering (Leach et al., 2002). However, there is evidence to suggest that exposure to CO_2 at a low flow rate, as used in the present study, produces unconsciousness without increases in stress responses (Burkholder et al., 2010;Powell et al., 2016).

For rats, the alternative Schedule 1 method would be lethal injection of euthatal, as ASPA prohibits dislocation for larger rodents, and decapitation is a regulated procedure, which was not on the project licence. Lethal injection of euthatal, whilst quicker, is likely to have caused distress to the animals, especially ones that were not regularly handled, as injection has been shown to increase measures of stress responses (Cloutier and Newberry, 2008;Deutsch-Feldman et al., 2015). Further, for optimal stress reduction and improved animal welfare, injection of rats should be carried out by 2 people, which was not possible in the COVID-19 pandemic.

For mice, alternative Schedule 1 methods would be lethal injection of euthatal, or dislocation of the neck. Injection of euthatal in mice is subject to the same limitations above, namely that injections are stressful, particularly in mice that are not routinely used to human handling. Whilst dislocation of the neck is instantaneous, there is a risk that the technique required to do so damages the brain,

which would have impaired subsequent tissue collection. As such, CO_2 represented the best way to cull the animals for the present experiments, despite the potential confounds discussed above.

2.3 Laboratory techniques

2.3.1 Microdissection of hippocampus

In order to extract specific regions of the hippocampus, the brain micro punch method was employed (Palkovits, 1973). Firstly, the brain matrix (World Precision Instruments [RBMS-600C]) was cooled by placing it on a thermal block (Corning [432074]) set in wet ice for approximately 10 minutes. Each rat brain was placed in the cooled brain matrix such that ventral side was visible. Feather blades (Agar Scientific [AGT5115]) were placed every 1 mm, before pressure was applied to slice through the brain. The blades were removed from the matrix and each brain slice was gently transferred to chilled slides (VWR [631-1551]). The slices were flash frozen by placing the slides on a second thermal block set in dry ice. Specific regions of the hippocampus (dorsal- CA1, -dentate gyrus and -CA3) were extracted bilaterally using a 0.75 mm micropunch (Stoelting [57395, 57400]). Location of the punches was determined using a rat brain atlas (Paxinos and Watson, 2004), see Figure 2.1. Immediately after extraction, tissue punches from dorsal CA1 (hereafter referred to as CA1) were expelled into a lysing matrix tube (MP Biomedicals [116913100]). Punches were submerged in RLT buffer plus (Qiagen AllPrep DNA/RNA micro kit [80284]) with β-Mercaptoethanol added in a 100:1 ratio (Bio-Rad [1610710]) and placed on wet ice. Following this, punches were homogenised for 12 seconds at 5000 rpm using a tissue homogeniser (Precellys [P00669-PR240-A]), and the RNA extraction protocol followed (see 2.3.2). Punches from other brain regions were expelled into chilled microcentrifuge tubes and stored at -75 °C for long-term storage.

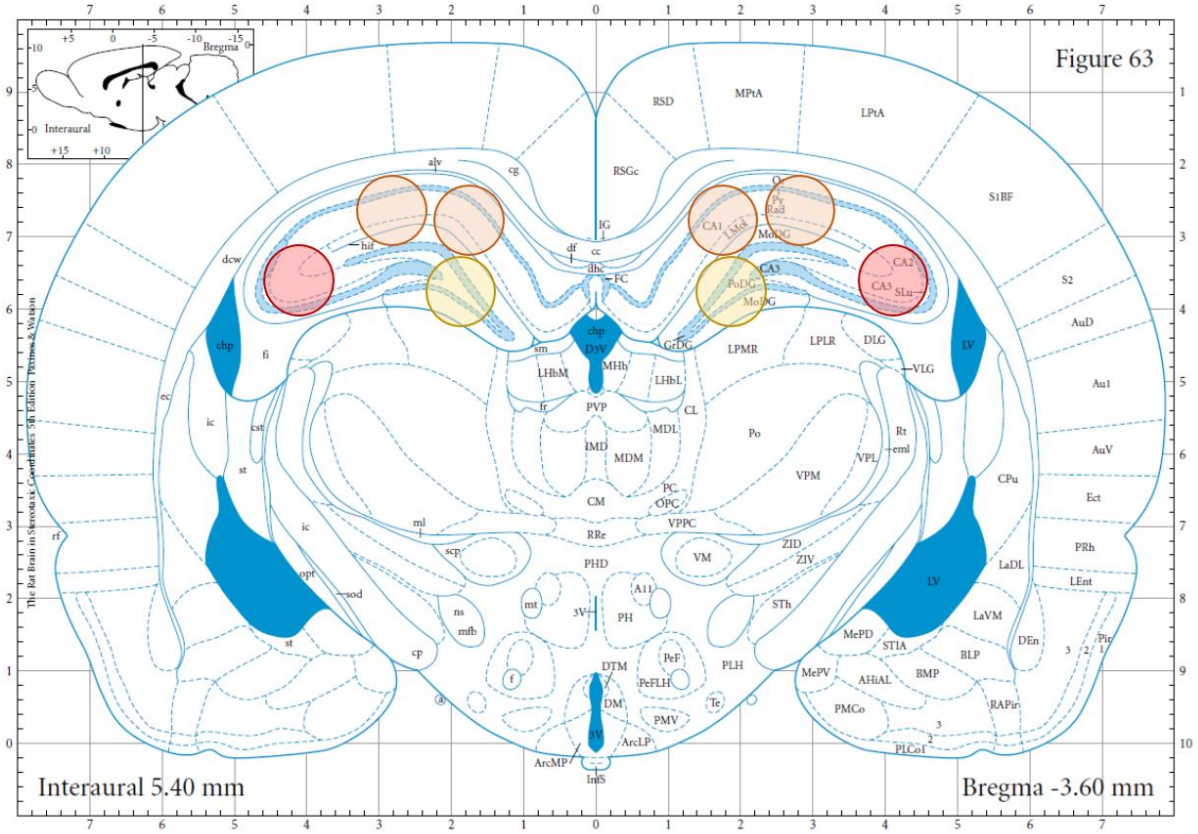


***Figure 2.2**. Schematic diagram showing the location of the 0.75 mm diameter punches taken from coronal sections to isolate specific hippocampal sub-regions: dorsal CA1 (orange), dorsal CA3 (red), and dorsal dentate gyrus (yellow). Image from "The rat brain in stereotaxic co-ordinates" (Paxinos and Watson, 2006).*

2.3.2 RNA isolation

RNA was purified from brain tissue using etiher the Qiagen AllPrep DNA/RNA Micro kit (Qiagen [80284]), the Qiagen RNeasy Micro UCP kit (Qiagen [73934]) or Qiagen RNeasy Micro kit (Qiagen [74004]), depending on experiment. As such, the protocols are detailed in the methods section of the appropriate results chapters.

2.3.3 RNA integrity analysis

RNA integrity analysis was performed by Central Biotechnology Services (CBS, Cardiff University) using the Agilent 2100 bioanalyser system (Agilent [G2939BA]). An RNA integrity number (RIN) between 1 and 10 was obtained for each sample, where 10 represents the least degraded RNA.

2.3.4 Library Preparation

The 2- and 5- hour retrieval libraries were prepared by the author and sequenced in-house by the Core Team, as detailed below. The CFC-acquisition RNA seq libraries were prepared and sequenced externally by GeneWiz, after RNA extraction by the author.

2.3.4.1 RNA preparation

RNA concentration was quantified fluorometrically with the Qubit RNA high sensitivity Assay (Thermo Fisher Scientific [Q32852]). The Qubit working solution was prepared by diluting 1 µL of RNA reagent in 199 µL of RNA buffer (per sample or standard) and vortexing to mix. The standards were prepared by mixing 190 µL of the Qubit working solution with 10 µL of standard. The samples were prepared by mixing 199 µL of Qubit working reagent with 1 µL of sample. Standards and samples were prepared in thin-walled PCR assay tubes (Thermo Fisher Scientific [Q32856]), compatible with the Qubit fluorometer. Prepared standards and samples were vortexed briefly and spun down, and then incubated for 2 minutes before running on the Qubit fluorometer. 90 ng of RNA was prepared in 50 µL of nuclease free water (Ambion [AM9937]) in a 96- well plate (Thermo Fisher Scientific [4346906]) immediately prior to library preparation.

2.3.4.2 mRNA capture

Libraries were constructed with the KAPA mRNA HyperPrep kit for Illumina® Platforms (KAPA Biosystems [08098115702]). For an overview of this workflow, see Figure 2.3.2.

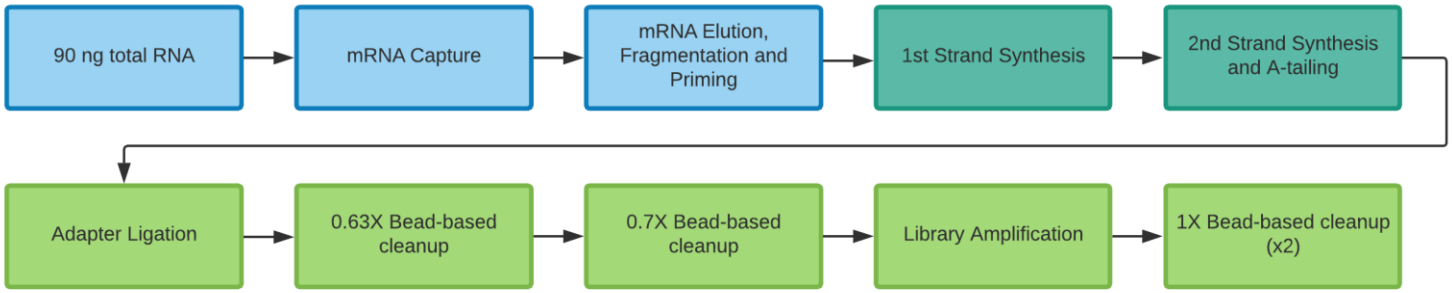


***Figure 2.3**. Overview of the KAPA mRNA HyperPrep protocol. Processes highlighted in blue represent RNA steps. Processes highlighted in teal represent RNA:cDNA hybrid steps. Processes highlighted in green represent DNA steps.*

The first step of the protocol was mRNA capture using olido-dT beads. The mRNA capture beads were equilibrated to room temperature, and then resuspended thoroughly by gentle pipetting in order to minimise foaming. The beads were then washed by transferring 52.5 µL per sample into a 1.5 mL microcentrifuge tube and placing on a tube magnet (Invitrogen [12321D]) until the supernatant was clear. The supernatant was then removed and replaced with an equal volume of mRNA bead binding buffer, removed from the magnet and resuspended by gentle pipetting. This process was repeated again, before 50 µL of the washed beads were added to the previously prepared RNA sample (see 2.3.2). The first mRNA capture step was performed in a thermocycler (BioRad [S1000]) as detailed in Table 2.2.

Step	Temperature	Duration
1st mRNA capture	65 °C	2 minutes
Cool	20 °C	5 minutes

***Table 2.2.** Parameters for 1st mRNA capture step of mRNA library preparation.*

After the 1st mRNA capture step, the plate was placed on a plate magnet (Invitrogen [12027]) and incubated until clear. The supernatant was then removed and the beads were resuspended in 200 µL of mRNA bead wash buffer. The plate was then placed back on the magnet and incubated until clear. The supernatant was removed and the beads were resuspended in 50 µL of RNase free water. The 2nd mRNA capture step was performed as detailed in Table 2.3.

Step	Temperature	Duration
2nd mRNA capture	70 °C	2 minutes
Cool	20 °C	5 minutes

***Table 2.3**. Parameters for 2nd mRNA capture step of mRNA library preparation*

Following this, 50 µL of mRNA bead binding buffer was added and the plate was incubated in the thermocycler at 20 °C for 5 minutes. Incubating the plate in the thermocycler rather than on the bench ensured that the temperature remained constant and even across wells. During the incubation period, the 1x Fragment, Prime and Elute buffer was prepared by adding an equal volume (11 µL per sample plus 10 % excess) of 2x Fragment, Prime and Elute buffer and RNase free water into a microcentrifuge tube. After the incubation period, the plate was placed on the magnet and the supernatant removed and the beads resuspended in 200 µL of mRNA bead wash buffer. The plate was placed back on the magnet and incubated until the supernatant was clear. The entire volume of the wash buffer was removed and discarded. As carryover of the mRNA bead wash buffer can inhibit 1st strand synthesis, a smaller volume pipette was used to remove any remaining supernatant.

2.3.4.3 mRNA Elution, Fragmentation and Priming

The beads from the previous step were resuspended in 22 µL of the 1x Fragment, Prime and Elute buffer. The fragmentation step was performed for 8 minutes at 94 °C in the thermocycler to achieve a mean library insert size of 100 – 200 base pairs (bp). During fragmentation, the 1st strand synthesis master mix was prepared (see Table 2.4). In order to prevent hybridisation of poly (A)-rich RNA to the capture beads, the plate was placed immediately onto the magnet following fragmentation. It was incubated until the liquid was clear, and then 20 µL of supernatant containing the eluted, fragmented and primed RNA was transferred into a new plate for further processing. The plate containing the mRNA capture beads was discarded.

Component	Volume per library
1st strand synthesis master mix:	
1st strand synthesis buffer	11 µL
KAPA script	1 µL
Total master mix volume	**12 µL**
2nd strand synthesis and A-tailing master mix:	
2nd strand marking buffer	31 µL
2nd strand synthesis and A-tailing enzyme mix	2 µL
Total master mix volume	**33 µL**
Adapter ligation master mix:	
Ligation buffer	40 µL
DNA ligase	10 µL
Total master mix volume	**50 µL**
Library amplification master mix:	
KAPA HiFi HotStart ReadyMix (2X)	27.5 µL
Library amplification primer mix (10X)	5.5 µL
Total master mix volume	**33 µL**

Table 2.4. *Master mix compositions for library preparation, detailing the components required, the volume per library and the total volume for each master mix.*

2.3.4.4 1st strand synthesis

The eluted, fragmented and primed RNA was placed immediately onto ice, and 10 µL of the previously prepared 1st strand synthesis master mix was added. The 1st strand synthesis step was performed as detailed in Table 2.5.

Step	Temperature	Duration
Primer Extension	25 °C	10 minutes
1st strand synthesis	42 °C	15 minutes
Enzyme inactivation	70 °C	15 minutes

Table 2.5. *Parameters for 1st strand synthesis step of mRNA library preparation.*

During the 1st strand synthesis, the 2nd strand synthesis master mix was prepared (see Table 2.4). Following 1st strand synthesis, the plate was placed immediately onto ice and subsequently proceeded to 2nd strand synthesis.

2.3.4.5 2nd strand synthesis and A-tailing

30 µL of the 2nd strand synthesis master mix was added to the 1st strand synthesis product, and the 2nd strand synthesis step was performed as detailed in Table 2.6.

Step	Temperature	Duration
2nd strand synthesis	16 °C	30 minutes
A- tailing	62 °C	10 minutes

Table 2.6. *Parameters for 2nd strand synthesis step of mRNA library preparation.*

The plate was placed immediately onto ice, and then proceeded to adapter ligation.

2.3.4.6 Adapter ligation

Either single-index adaptors (KAPA Biosystems [KK8700], Chapter 3), or unique dual-index adaptors (KAPA Biosystems [KK8727], Chapter 5) were used. Single-indexed adapters were diluted to 1 µM in the provided adapter dilution buffer. Unique dual-index adaptors were diluted to 1.36 µM. 5 µL of diluted adaptor was added to the 2nd strand synthesis product. Each sample to be pooled together required a different adapter index. The adapter ligation master mix was prepared (see Table 2.4), and 45 µL added to the 2nd strand synthesis product with diluted adapter. The plate was incubated at 20 °C for 15 minutes in the thermocycler, before proceeding to 1st post-ligation clean-up.

2.3.4.7 1st Post-ligation clean-up

A 0.63X bead-based clean-up was performed by adding 70 µL of KAPA pure beads to the adapter-ligated DNA from the previous step. The plate was incubated at room temperature on the bench for 10 minutes to allow the DNA to bind to the beads. The plate was then placed on the magnet and incubated until the supernatant was clear. 175 µL of the supernatant was removed and replaced with 180 µL of 80 % ethanol. Keeping the plate on the magnet, it was incubated for 30 seconds, and then the ethanol was removed and replaced with 180 µL of 80 % ethanol. The plate was again incubated for 30 seconds before the ethanol was removed. In order to remove all of the ethanol, after this second step residual ethanol was removed with a smaller volume pipette. Keeping the plate on the magnet, the beads were dried for 4 minutes, until they were matt in appearance. Care was taken not to over dry the beads, which can lead to reduced yields. Following this, the plate was removed from the magnet and the beads resuspended in 50 µL of 10 mM Tris-HCL (pH 8.0 - 8.5). The plate was incubated at room temperature for 2 minutes to elute the DNA off the beads.

2.3.4.8 2nd Post-ligation clean-up

Following the 2-minute incubation, 35 µL of PEG/NaCl solution (equilibrated to room temperature) was added and the plate incubated for 10 minutes to bind the DNA to the beads. The plate was then placed on the magnet and incubated until the supernatant was clear. 175 µL of the supernatant was removed and replaced with 180 µL of 80 % ethanol. Keeping the plate on the magnet, it was incubated for 30 seconds, and then the ethanol was removed and replaced with 180 µL of 80 % ethanol. The plate was again incubated for 30 seconds before the ethanol was removed. In order to remove all of the ethanol, after this second step residual ethanol was removed with a smaller volume pipette. Keeping the plate on the magnet, the beads were dried for 4 minutes, until they were matt in appearance. The plate was then removed from the magnet and the beads were resuspended in 22.5 µL of 10 mM Tris-HCL (pH 8.0 – 8.5). The plate was incubated for 2 minutes to elute the DNA off of the beads. The plate was placed on the magnet and incubated until the supernatant was clear. 20 µL of supernatant containing the DNA was then transferred to a new plate. Following this, the plate was stored at 4 ^{o}C overnight for subsequent library amplification.

2.3.4.9 Library amplification

30 µL of the library amplification master mix (see Table 2.4) was added to the purified, adapter-ligated DNA from the previous step. The library was then amplified using the parameters detailed in Table 2.7.

Step	Temperature	Duration	Cycles
Initial denaturation	98 ^{o}C	45 seconds	1
Denaturation	98 ^{o}C	15 seconds	14 (Chapter 3, 2-hour timepoint) 12 (Chapter 3, 5-hour timepoint)
Annealing	60 ^{o}C	30 seconds	
Extension	72 ^{o}C	30 seconds	
Final extension	72 ^{o}C	1 minute	1

Table 2.7*. Parameters for library amplification step of mRNA library preparation.*

2.3.4.10 Library amplification clean-up

Following amplification, a 1X bead-based library clean-up was performed. 50 µL of KAPA pure beads, equilibrated to room temperature, were added to the amplified library and incubated for 10 minutes.

Following this, the plate was placed on the magnet and incubated until clear. 95 µL of the supernatant was removed and replaced with 180 µL of 80 % ethanol. Keeping the plate on the magnet, it was incubated for 30 seconds, and then the ethanol was removed and replaced with 180 µL of 80 % ethanol. The plate was again incubated for 30 seconds before the ethanol was removed. In order to remove all of the ethanol, after this second step residual ethanol was removed with a smaller volume pipette. Keeping the plate on the magnet, the beads were dried for 4 minutes. It was then removed from the magnet and the beads were resuspended in 52.5 µL of Tris-HCL (pH 8.0 – 8.5). The plate was incubated for 2 minutes at room temperature, and then placed back on the magnet. 50 µL of supernatant was transferred to a new well. The 1X bead based clean-up was performed again as above, with 2 minor modifications. The beads were resuspended in 22.5 µL of Tris-HCL (pH 8.0 – 8.5) rather than 52.5 µL, and 20 µL of supernatant was transferred to a new plate rather than 50 µL. Prepared libraries were stored at – 20 ^{o}C until use.

2.3.5 Sequencing

2.3.5.1 Bioanalysis

DNA concentration was quantified fluorometrically using the Qubit dsDNA high sensitivity assay (Thermo Fisher Scientific [Q32851]). The assay was performed as described in 2.3.4.1, using the equivalent high sensitivity dsDNA reagents. Where the concentration was greater than 10 ng/ µL, the sample was diluted in 10 mM Tris-HCL (pH 8.0 – 8.5) to be within the range of the bioanalyser assay.

In order to measure the size distribution and quality of the prepared libraries, they were analysed on the Agilent bioanalyser system using the high sensitivity DNA assay (Agilent [5067-4627]). Prior to running the assay, the gel-dye matrix was prepared according to the manufacturer's instructions. Briefly, 15 µL of the dye concentrate was added to the high sensitivity DNA gel matrix, and the solution vortexed for 10 seconds. It was then transferred to the provided spin filter and centrifuged for 15 minutes at 2240 *g*. The gel-dye matrix was stored in the dark at 4 ^{o}C until use. Prior to use, the gel-dye matrix was equilibrated to room temperature for 30 minutes. The chip was loaded in accordance with the manufacturer's instructions. Briefly, the chip was first placed in the chip priming station and 9 µL of the gel-dye matrix was added to the well marked "G" (see Figure 2.4). The chip priming station was then closed and the plunger of the syringe lowered until it was held securely by the clip. After 60 seconds, the syringe clip was released and the chip priming station opened, and 9 µL of the gel-dye matrix was added to the remaining wells labelled "G". 5 µL of the high sensitivity DNA marker was added into the ladder well and each of the sample wells. 1 µL of ladder was then added to the ladder well, and 1 µL of sample was added to each of the sample wells. The chip was vortexed for 60 seconds

at 2400 rpm in a chip vortex (IKA [0003617000]). After vortexing, the loaded chip was immediately inserted into the bioanalyser and the assay run.

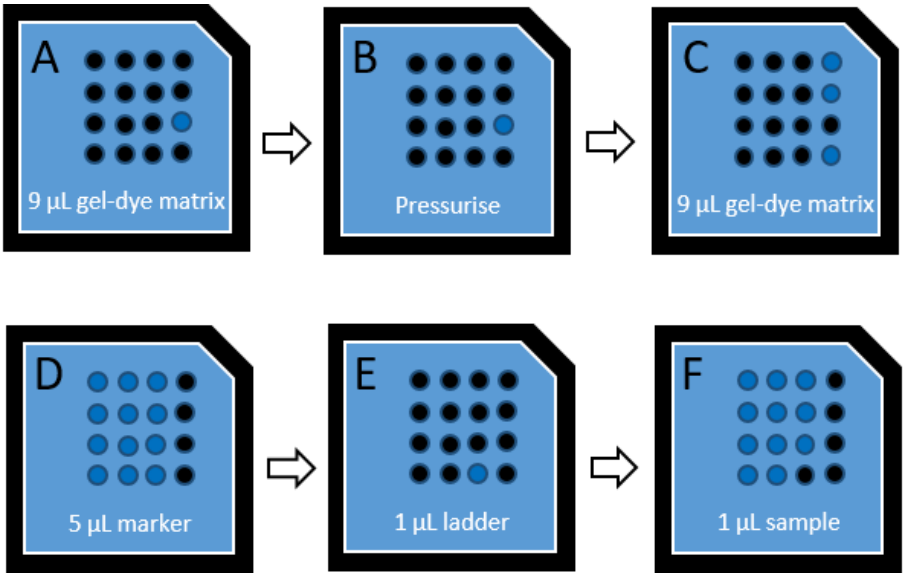


Figure 2.4. *Schematic overview of chip loading protocol for the high sensitivity DNA assay used to measure distribution and quality of the prepared libraries.* ***A.*** *First, the gel- dye matrix was added to the appropriate gel well, highlighted in dark blue.* ***B.*** *Pressure was applied to the well using the chip priming station.* ***C.*** *Gel dye matrix was added to the remaining gel wells.* ***D.*** *Marker was added to the sample and ladder wells.* ***E.*** *Ladder was added to the ladder well.* ***F.*** *Sample was added to the sample wells.*

2.3.5.2 Final DNA quantification and pooling

DNA was quantified using the Qubit dsDNA high sensitivity assay. All samples were quantified at the same time to minimise the effects of variation in the values of the standards on the DNA concentrations. The appropriate volume of sample to be added to the pool was calculated using the following formula:

$$\frac{V(f)x\ C(f)}{n\ x\ C(i)}$$

Where $V(f)$ was the total volume required, $C(f)$ was the final molarity, which remained constant between samples, n was the number of samples in a pool, and $C(i)$ was the molarity of each sample. Samples were then pooled and diluted in Illumina resuspension buffer (Illumina [15027913]). Sample pools were stored at – 20 ^{o}C for approximately 2 weeks before sequencing.

2.4 Bioinformatic Analysis

2.4.1 From raw reads to gene counts

After illumina sequencing was complete, raw reads were exported and uploaded onto a high-performance computing platform, on which the bioinformatic processing was completed, as outlined below.

Firstly, reads were trimmed of adaptor sequences and low-quality bases using Trimmomatic (Bolger et al., 2014) (Chapter 3) or Trim Galore! (Krueger, 2012) (Chapter 5) with the default parameters. Next, quality of the sequencing reads was determined using FastQC (Andrews, 2010), which employs a "traffic light" system to indicate quality of the samples. Reference genomes were created using STAR (Dobin et al., 2013) (rat: Rnor_6.0.102; mouse: GRCm39.103), and trimmed reads mapped to them using the default parameters. Duplicates were marked using MarkDuplicates (Broad-Institute, 2019) and mapping quality examined using BamTools (Barnett et al., 2011). Finally, gene counts were produced using featureCounts (Liao et al., 2014).

2.4.2 Differential expression analysis

Differential expression analysis was undertaken in R (R Core Team, 2018) using limma/voom (Smyth, 2005;Law et al., 2014;Ritchie et al., 2015), as per the protocol described in Law et al., 2018 (Law et al., 2018). Details of the regression formulae used can be found in each appropriate chapter. The Benjamini-Hochberg False Discovery Rate (FDR) correction was applied to account for multiple comparisons, and genes were taken as significantly differentially expressed if the corrected p-value was below the threshold described in each chapter (0.1 in Chapter 3, 0.01 in Chapters 4 and 5).

2.4.2.1 Preparation of the FeatureCount files

FeatureCount files were read in and converted to a DGE-list object for differential expression using the "readDGE" function from the edgeR package (Robinson et al., 2009). It should be noted that the edgeR package was written and developed by the same research group as limma/voom (principal investigator: Gordon Smyth). Sample information, such as sample name, experimental condition, RIN score was added to the DGE list object for later inclusion in the differential expression model. Similarly, gene annotations such as entrez gene ID and external gene names were obtained from the biomaRt package and also added to the DGE list object (Smedley et al., 2009).

2.4.2.2 Data pre-processing

Raw counts were transformed into counts-per-million (CPM) and log counts-per-million (log-CPM) using the "cpm" function of edgeR. Transforming raw counts is important because it accounts for differences in library depth and size.

Next, genes that were either not expressed in any condition, or lowly expressed, were removed from the DGE list object. This is because genes that are not expressed in any condition will not be biologically relevant, as there is no expression across all samples, but will increase the number of multiple comparisons corrections that need to be made within the analysis. Low expression was determined automatically by the "filterByExpr" function in edgeR, whereby genes that have a count (CPM) of less than 10 in a minimum number of samples (the minimum group size) were removed. Again, genes that are not expressed at a meaningful level in a number of samples is likely to be due to erroneous variation (noise) and as such will increase the number of statistical tests whilst not adding biologically meaningful value. Similar filtering is recommended in other differential expression packages, such as DESeq2 (Love, 2022).

A second round of normalisation was conducted in order to ensure that expression distributions of each sample were similar across the entire experiment. In the sample preparation process, external factors such as pipetting error, sample processing order, laboratory temperature, can have an impact on the expression of individual samples. This normalisation step aims to reduce this, and was conducted using the trimmed mean of M-values (TMM) method. Similar normalisation methods are employed in DESeq2 (Love et al., 2014).

2.4.2.3 Differential expression analysis

A design matrix was created using the "model.matrix" function, specifying the design formula. The design formulae can be found in each experimental chapter. Next, a contrast matrix was specified using the "makeContrasts" function, specifying which experimental groups were to be compared against each other. Tables specifying the contrasts can be found in each experimental chapter.

Voom calculates precision weights which corrects for heteroscedascity in the data, without which RNA-seq data would often violate the linear regression assumption of homoscedasticity. RNA-seq data often violates this because the variance is not independent of the mean (Law et al., 2014).

Next, linear modelling was conducted using the "lmfit" and "contrasts.fit" function in limma. Empirical Bayes moderation was carried out using the "eBayes" function to obtain more precise

estimates of gene-wise variability. Again, empirical Bayes moderation is also carried out in DESeq2 (Love et al., 2014).

Finally, differentially expressed genes were extracted using topTable, which creates an R dataframe of all genes, log fold change, average expression, p-value and adjusted p-value, plus any additional gene information given earlier using biomaRt.

2.4.2.4 Other differential expression packages

As listed above, several of the normalisation steps carried out in the limma/voom pipeline are also employed by other differential expression algorithms, such as DESeq2. As such, the use of limma/voom does not introduce any erroneous steps which should be of concern. Comparisons between the differentially expressed genes identified using DESeq2 and limma have shown high concordance rates (over 90%), although DESeq2 has a tendency to identify more differentially expressed genes than the limma pipeline (Tong, 2021). Further, in a comparison of 6 differential expression algorithms, it has been shown that limma/voom and DESeq2 show comparable performance when differential expression from RNA-seq was compared to known changes using RT-qPCR (Rapaport et al., 2013). This research also found that limma/voom identified fewer false positives compared to DESeq2, potentially explaining the higher number of differentially expressed genes often found when using this method. Indeed, DESeq2 was found to find the highest number of false positives among the methods compared (Rapaport et al., 2013). Whilst DESeq2 is the most commonly used differential expression algorithm (Quinn et al., 2018), the Ritchie et al., 2015 publication on limma has over 19,000 citations to-date, indicating it is also a popular choice for this type of analysis.

2.4.3 Pathway analysis

Pathway analysis was undertaken in R using Fisher's Exact Test. Gene-pathway files were curated from the Gene Ontology (GO), or Mammalian Phenotype (MP) databases. The GO index was created using the GO.db package in R (Carlson, 2019). GO annotations were filtered to exclude the following evidence codes: NAS (non-traceable author statement), IEA (inferred from electronic annotation), and RCA (inferred from reviewed computational analysis). The MP phenotype-genotype index was downloaded from http://www.informatics.jax.org/downloads/reports/MGI_PhenoGenoMP.rpt in tab-delimited format. The names of the MP IDs were obtained from the ontologyIndex package in R (Greene et al., 2016).

Custom background gene sets were created using all expressed genes in the appropriate dataset to control for expression bias. Significant pathways were then subjected to a refinement procedure, in

order to determine the most specific pathways contributing to the enrichment. During refinement, pathway terms were re-tested for enrichment in the gene sets after the removal of genes from the term with the highest odds ratio in the original Fisher's Exact Test. For example, if the term with the highest odds ratio was "positive regulation of cell migration", the genes within that term in the gene set of interest would be removed in the refinement procedure and the pathway analysis re-run. Following the above example, any of the 1,711 mouse genes within this GO term would be removed from the gene set of interest, and the pathway analysis rerun. Terms that were no longer significant were dropped from the analysis. This is because their significant association is already covered in the term "positive regulation of cell migration", and thus is redundant. This process was repeated iteratively. For example, if the highest GO term after the first refinement process was "response to hormone" then all genes within this GO term that were also in the gene set of interest, plus those in the "positive regulation of cell migration" GO term which were already removed, would be removed, and the pathway analysis rerun. This process continued until only significant terms remained.

This refinement procedure avoids the production of lists of hundreds of significantly enriched pathways, many of which are parent/child terms of each other, which allows better exploration of biological pathways, and has been used in several peer-reviewed publications (Pocklington et al., 2015;Clifton et al., 2019;Clifton et al., 2021;Sanders et al., 2022).

2.5 Disease association analysis

2.5.1 Common Variation

2.5.1.1 SNP data

Genome-wide association study (GWAS) summary statistic data from patients with schizophrenia, bipolar disorder, autism spectrum disorder (ASD), Alzheimer's disease (AD), chronic kidney disease, and associated controls, were downloaded from the appropriate repositories.

Due to the positive correlation between common variation in bipolar disorder and schizophrenia (Brainstorm et al., 2018) and the evidence that bipolar type I symptomology overlaps with schizophrenia (Pearlson, 2015), the association between learning-associated gene sets and bipolar disorder was tested. Further, synaptic plasticity and learning related impairments have been found in animal models of autism and AD (Guang et al., 2018;Hansel, 2019;Piochon et al., 2014;Mango et al., 2019;Reiserer et al., 2007), and as such it was interesting to examine whether the association extended to other disorders with a synaptic plasticity component, or was restricted to schizophrenia. Chronic Kidney disease was chosen as a non-brain disorder control, with a case sample size similar to

that of the schizophrenia GWAS. In this way, any association found, if not found in the chronic kidney disease control, could not be attributed to disorder sample size.

The schizophrenia sample consisted of patients from the second wave of a UK study of patients taking clozapine (CLOZUK2) and the Psychiatric Genetics Consortium (PGC) (Pardinas et al., 2018), and the meta-analysis GWAS summary statistics were downloaded from the Walter's group data repository. The bipolar disorder sample consisted of patients from PGC3 (Mullins et al., 2021), and summary statistics from all cases, bipolar disorder subtype I and bipolar disorder subtype II were downloaded from the PGC data portal. The ASD sample consisted of patients from the iPSYCH cohort and PGC (Grove et al., 2019), and were downloaded from the PGC data portal. The AD sample consisted of patients with late-stage Alzheimer's disease from the PGC, the Alzheimer's disease sequencing project, and the International Genomics of Alzheimer's Project (Jansen et al., 2019). The chronic kidney disease sample consisted of patients from the Chronic Kidney Disease Genetics (CDKGen) consortium (Wuttke et al., 2019), and summary statistics were downloaded from the CDKGen consortium data portal. Although trans-ancestry data were collected as part of the CDKGen study, only patients of European ancestry were included here to maintain consistency with the other disorders. The number of cases and controls in each data set can be found in Table 2.8.

	n Cases	*n* Controls	*n* Total
Schizophrenia	40,675	64,643	105,318
Bipolar disorder (all)	41,917	371,549	413,466
Bipolar disorder Type I	25,060	449,978	475,038
Bipolar disorder Type II	6,781	364,075	370,856
ASD	18,382	27,969	46,351
AD	71,880	383,378	455,258
Chronic kidney disease	41,395	439,303	480,698

Table 2.8. *The number of cases, controls, and the total sample size for each of the data sets.*

2.5.1.2 Gene-set enrichment analysis

Gene-set enrichment analysis for common variation was undertaken using Multi-marker Analysis of GenoMic Annotation (MAGMA, version 1.08b) (de Leeuw et al., 2015). The GWAS summary statistic SNP files were filtered to remove SNP IDs that did not conform to the standard nomenclature, and therefore would not be mapped to a gene in the annotation step. In addition, these files were filtered to only include SNPs with an INFO score of greater than 0.8 (0.6 for bipolar disorder, as per the original study (Mullins et al., 2021)) and an allele frequency of greater than 1%, in order to exclude rare variants.

SNPs were mapped to genes using the NCBI build 37 gene location file as a reference, and gene analysis performed with linkage disequilibrium calculated using the 1000 genomes reference file (European ancestry). Both files were downloaded as auxiliary files from the MAGMA website (https://ctg.cncr.nl/software/magma). The resulting gene-based P-values were then used in the gene-set enrichment analyses using the SNP-wise mean model, with all genes expressed in the particular experiment included as a covariate within the model to account for the known association between brain expressed genes and psychiatric conditions. In addition to the p-value correction automatically applied by MAGMA, gene-set p-values were subjected to Bonferroni correction where appropriate to account for significant overlap between gene-sets.

2.5.2 Copy number variation (CNV) analysis

2.5.2.1 CNV data

CNV data from schizophrenia patients and healthy controls were collated from 4 large-scale studies: the International Schizophrenia Consortium (ISC) (ISC, 2008), the Molecular Genetics of

Schizophrenia study (MGS) (Levinson et al., 2011), CLOZUK (Rees et al., 2014) and CLOZUK2 (Rees et al., 2016).

2.5.2.2 CNV enrichment analysis

The CNV datasets were filtered to only include those larger than 100 kb in size and covered by more than 15 probes. The protein coding-genes overlapping each CNV in the 4 CNV datasets were then identified using the appropriate NCBI build (ISC: build 35, MGS: build 36, CLOZUK and CLOZUK2: build 37), and the number of genes overlapping each CNV was counted. The 4 CNV datasets were then collated to form the CNV dataset used in downstream analyses. This CNV dataset contained all CNVs present in the datasets from the original studies, in both cases and controls, leading to 19,301 CNVs being included that covered at least one protein-coding gene. This CNV dataset contained the following additional variables: case control status (coded as a binary variable where 0 = control and 1 = case), CNV size, the number of probes, the number of genes each CNV contained and the CNV study (ICS, MGS, CLOZUK, CLOZUK2). The number of genes overlapping each gene-set and each CNV was also calculated. Logistic regression analysis was used to investigate the relative enrichment of gene-sets in case CNVs compared to controls using the general linear model (glm, family = binomial) function in R.

The regression formula was as follows:

> *Case_control status ~ number of genes in CNV + CNV size + number of probes + CNV study + number of gene-set hits*

Following this, a permutation correction was applied in order to account for any background enrichment. The background gene-set was permutated 1000 times, producing 1000 gene sets of *n* length, where *n* represents the size of the gene-set being analysed. Each permutated gene-set underwent annotation and logistic regression analysis as above, producing a null distribution of p-values. An empirical p-value was then calculated for each gene-set as follows: the sum of the number of permutated gene sets with a p-value less than or equal to the original gene-set p-value divided by the number of permutations. P-values were adjusted with Bonferroni correction where appropriate.

Chapter 3: Gene expression profiles following retrieval of Contextual Fear Conditioning (CFC)

3.1 Introduction

The ability to encode, consolidate and retrieve information about the environment is key to the survival of nearly all living species. Whilst consolidation of contextual fear conditioning (CFC) will be discussed in Chapter 5, this chapter will focus on the retrieval of contextual fear conditioning, specifically downstream gene transcription events and the association with genetic risk for schizophrenia.

3.1.1 Associative learning

Associative learning is the process by which a neutral stimulus becomes associated with a response, such that presentation of the stimulus alone elicits a conditioned response. Initially documented by Pavlov in the 1920s, associative learning has been studied extensively over the past 50 years (Pavlov, 1927). CFC is one of the most widely used paradigms to study aversive associative learning, during which a rodent learns to associate a specific context (the conditioned stimulus) with an aversive foot shock (the unconditioned stimulus). When re-presented the context at a later time, rodents typically show a fear response similar to that elicited by the footshock itself (the conditioned response). This paradigm has been shown to lead to robust responses after just one conditioning trial, and as such has been widely used as a model to further understand the molecular correlates of learning (Wehner and Radcliffe, 2004).

3.1.2 Retrieval of conditioned fear

Memory retrieval is the process of recalling previously encoded information, and can elicit behaviour optimal for the previously learned conditions. In the case of CFC, re-exposure to the conditioned context leads to the retrieval of the footshock, and as such typically elicits a freezing response. It has

been proposed that re-exposure to the context can activate two processes: reconsolidation and extinction (Suzuki et al., 2004). During this re-exposure, previously consolidated memories become labile again, thus susceptible to disruption (reviewed in(Alberini and LeDoux, 2013)). In an early demonstration of this, Nader and colleagues (2000) administered anisomycin, a protein synthesis inhibitor, after an auditory conditioning retrieval trial (Nader et al., 2000). It was found that administering this immediately after memory retrieval, but not 6-hours later, led to impaired long term memory retrieval, indicating that the memory had moved to a labile state after retrieval and could be disrupted through interference with the synthesis of new proteins. Thus, reconsolidation if the process by which long-term memories are re-stabilised.

Brief re-exposure to the context has been suggested to lead to reconsolidation of the fear memory, and as such maintains the fear response in subsequent re-exposure trials (Blanchard, 1969). Longer re-exposure to the context initiates extinction processes, and leads to the diminishing of the fear response as the context no longer predicts foot shock. Rather than erasing the previous memory, it is widely accepted that extinction is a form of new learning, in which one forms an association between the context and no footshock (reviewed in (Dunsmoor et al., 2015)).

3.1.3 Molecular signatures underlying retrieval processes

Retrieval processes have been shown to require *de novo* gene expression, as demonstrated by work in which transcriptional processes or protein synthesis have been disrupted prior to reconsolidation (Nader et al., 2000;Mamiya et al., 2009;Gafford et al., 2011;Arguello et al., 2013). There is also evidence that retrieval processes may have dissociable molecular signatures, for example it has been shown that L-type voltage-gated calcium channels are required for extinction, but not reconsolidation (Cain et al., 2002;Suzuki et al., 2004). Similarly, it has been found that brain derived neurotrophic factor (BDNF) expression is reduced in rats that do not show extinction (Peters et al., 2010), but is not required for recall (Lee et al., 2004a).

Previously, our group has investigated gene expression profiles following retrieval of conditioned fear in the CA1 using microarray (Barnes et al., 2012;Scholz et al., 2016). Brain derived neurotrophic factor (BDNF) or zinc finger protein 268 (Zif268) antisense oligonucleotides were used to block the consolidation or reconsolidation, respectively, of the conditioned fear memory, which was used as the comparison condition for the consolidation and recall conditions. Extinction was investigated by returning the rats to the conditioned context for 10 minutes. It was found that genes up-regulated after recall were enriched for 10 functional clusters, mainly centred around immune associated terms such as inflammatory response, positive immune system regulation and immune-cell motion. Genes up-regulated after extinction were enriched for 12 functional clusters including cell-adhesion,

hormone response, signal transducer activity and regulation of developmental processes. Thus, these results suggest that genes associated with recall and extinction are enriched in different functional processes. Further, it has been found that different transcription factors are involved in recall and extinction. De la Fuente and colleagues (de la Fuente et al., 2011) showed that nuclear factor -κβ (NF-κβ) is required for recall but inhibited in extinction. In addition, they found that inhibiting the transcription factor nuclear factor of activated-T cells (*NFAT*) impaired extinction.

3.1.4 Associative learning and retrieval in psychiatric disorders

Whilst the retrieval of a previously adverse memory can be advantageous to survival, for example by leading to avoidance behaviour, aberrant associative learning has been associated with a variety of adverse outcomes, including anxiety disorders such as Post-Traumatic Stress Disorder (PTSD). It has been theorised that the persistence of delusional beliefs, such as those experienced by some patients with schizophrenia, may result from abnormally reinforced thoughts or associations (Jensen et al., 2008). Indeed, associative learning impairments have been documented in patients with schizophrenia. For example, patients with schizophrenia are impaired in their ability to learn object-location paired associations compared to healthy controls (Diwadkar et al., 2008) (Brambilla et al., 2011). In addition, it has been found that patients had impaired associative memory performance relative to first degree relatives and healthy controls, and had altered patterns of hippocampal activation during encoding and retrieval (Oertel et al., 2019). Patients have also been shown to have altered autonomic responses during fear conditioning, and an impaired ability to recall extinction memories (Holt et al., 2012). Given this, Clifton and colleagues combined human genetic studies, bioinformatics and experimental studies in rodents to further investigate the enrichment of schizophrenia associated CNVs in associative learning (Clifton et al., 2017b). Previously derived gene sets representing genes expressed following consolidation, recall or extinction of contextual fear (Scholz et al., 2016) were used to assess enrichment for patient CNVs. It was found that only extinction related genes were significantly enriched in schizophrenia associated CNVs, suggesting a selective impact of these CNVs on extinction learning processes.

3.1.5 Aims

The primary aim of this chapter was to characterise transcriptomic profiles related to associative learning, building the on previous methodology in 3 ways (Scholz et al., 2016;Clifton et al., 2017b). Firstly, the retrieval paradigm was simplified, such that rats were returned to the conditioning chamber 48 hours after conditioning for either 2 minutes (recall), 10 minutes (extinction), or remained in their home cages (no recall), without infusion of Zif268 antisense oligonucleotides. This

represents a more naturalistic version of the retrieval process. In addition, the behavioural paradigm and associated laboratory work were conducted at the same time by the same experimenter, serving to reduce variability compared to the combining of two data sources as previously. Thirdly, RNA sequencing was employed in order to get a hypothesis free insight into the genes expressed during consolidation, recall and extinction learning. Although microarrays are a useful way of examining gene expression, their use has been somewhat superseded by RNA sequencing, as the latter does not rely on pre-designed probes which can limit the range of differentially expressed genes that can be detected (Zhao et al., 2014a). Further to this, I aimed to test whether extinction learning related genes, as identified through RNA-seq, are enriched in common variants and CNVs associated with schizophrenia.

3.2 Methods

3.2.1 Animals

36 adult male Lister Hooded rats (weighing 250-275 grams on arrival) were used for each timepoint (72 total, see Figure 3.1), and housed as described in General Methods (2.1.1).

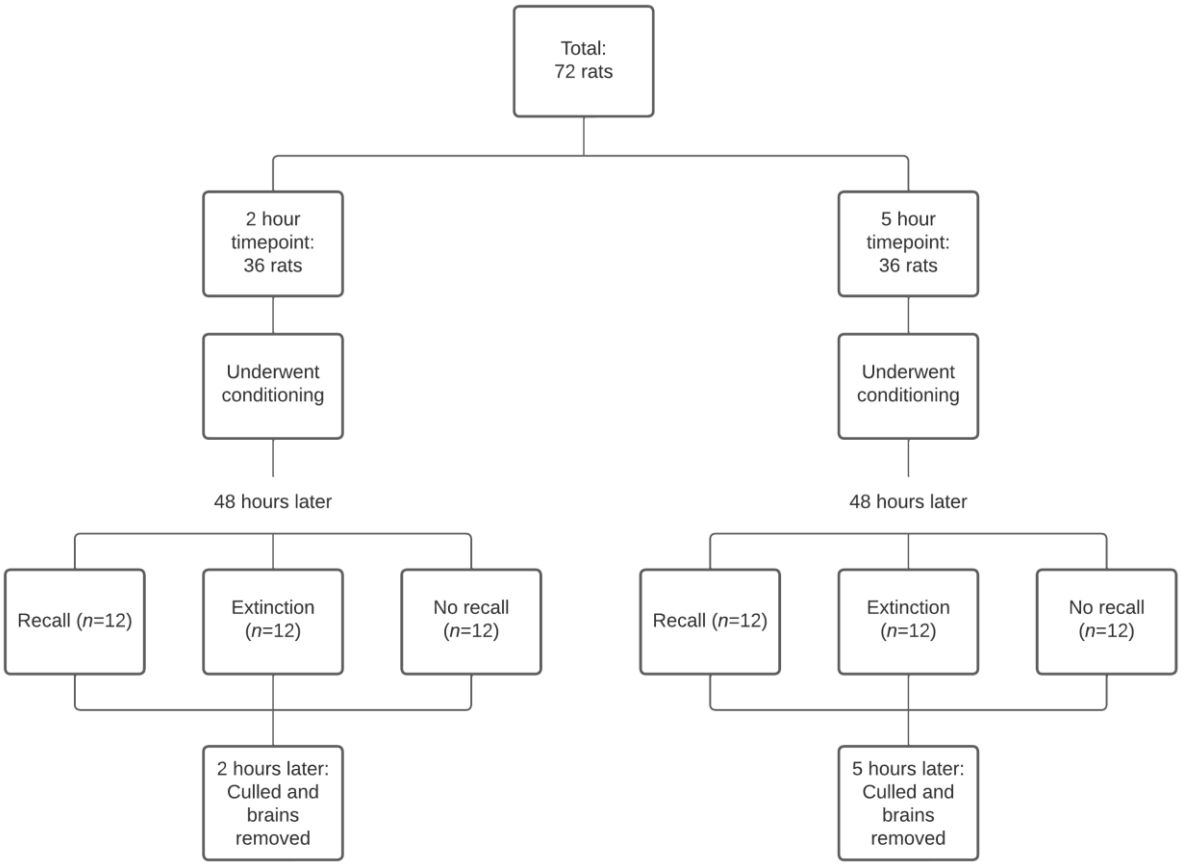


Figure 3.1. *Schematic diagram representing the flow of rats through the conditioning experiment, and numbers of rats in each conditioning group.*

3.2.2 Contextual Fear Conditioning (CFC)

Rats underwent CFC and retrieval, as described in General Methods (2.2.1). 2 or 5 hours after retrieval, rats were killed by CO_2 inhalation and whole brains removed and snap frozen on dry ice. Brains were stored at -75 °C until later microdissection (see General Methods: 2.3.1). Freezing behaviour was scored as described in General Methods section 2.2.3.

3.2.3 Nucleic acid isolation

3.2.3.1 2-hour timepoint

RNA was purified using the Qiagen AllPrep RNA/RNA micro kit (Qiagen [80284]). After homogenisation, samples were centrifuged for 3 minutes at full speed before the lysate was transferred to a DNA spin column and centrifuged for 30 seconds at 8,000 *x g*. The spin column was then placed in a 2 mL collection tube and stored at 4 ^{o}C for later DNA purification (DNA was purified, but no further analysis was conducted). The flow-through was combined with 350 µL 70 % ethanol diluted in nuclease-free water, and transferred to an RNA spin column before being centrifuged for 15 seconds at 8,000 *g*. Next, the membrane was washed by adding 350 µL buffer RW1 and centrifuging for 15 seconds at 8,000 *x g*. On column DNase digestion was performed by adding 10 µL DNase I stock solution to 70 µL buffer RDD (Qiagen [79254]) and incubating the membrane with this solution (80 µL total volume) for 15 minutes at room temperature. Following this, the membrane was washed again with 350 µL buffer RW1, and centrifuged for 15 seconds at 8,000 *x g*. In order to remove traces of salts from earlier buffers, 500 µL buffer RPE was added to the column and centrifuged for 15 seconds at 8,000 *x g*, before 500 µL 80 % ethanol was added and the column was centrifuged for 2 minutes at 8,000 *x g*. To prevent the carry-over of ethanol, the spin column was carefully removed from the collection tube and placed in a new 2 mL tube. In order to remove residual ethanol from the membrane, the spin column lid was opened and centrifuged for 5 minutes at full speed, before transferring the column to a 1.5 mL tube. To elute the RNA, 14 µL of RNase free water was added directly to the spin column membrane and centrifuged for 1 minute at 8,000 *x g*. The eluted RNA was stored at – 75 ^{o}C until library preparation.

3.2.3.2 5-hour timepoint

RNA was purified using the Qiagen RNeasy UCP micro kit (Qiagen, [74004]). Different kits were used between the time-points due to reagent availability in the COVID-19 pandemic. Tissue punches from dorsal CA1 were extracted via the micropunch method (Palkovits, 1973), as described in General Methods (2.3.1). Punches were expelled into a lysing matrix tube (MP Biomedicals [116913100]) containing buffer RLT with β-Mercaptoethanol added in a 100:1 ratio (Bio-Rad [1610710]). Punches were immediately homogenised for 12 seconds at 5000 rpm using a tissue homogeniser (Precellys [P00669-PR240-A]), and spun down for 15 seconds in a minifuge. The homogenate was transferred to a microcentrifuge tube and centrifuged for 3 minutes at full speed. After centrifugation, the supernatant was transferred to a new microcentrifuge tube, and stored at -75 °C until further use.

Prior to RNA isolation, homogenised lysates were removed from the freezer and defrosted on a heat block set to 25 °C for approximately 2 minutes. Once defrosted, 350 µL of 70 % ethanol (Fisher Scientific [E/0650DF/P17]) diluted in nuclease-free water (Ambion [AM9937]) was added to the lysate and then transferred to an RNA spin column. The column was then centrifuged for 15 seconds at 8,000 *x g*, and the flow through discarded. 350 µL of buffer RUWT was added to the column, and it was centrifuged for 15 seconds at 8,000 *x g*. Following this, on column DNase digestion was performed by adding 10 µL DNase I stock solution to 70 µL buffer RDD (Qiagen [79254]) and incubating the membrane for 15 minutes at room temperature. In order to wash the membrane, 350 µL of buffer RUWT was added and centrifuged for 15 seconds at 8,000 *x g*. The flow through was discarded and 500 µL of 80 % ethanol, diluted as stated previously, was added to the membrane. The column was centrifuged for 2 minutes at 8,000 *x g*, before the spin column lid was opened and it was centrifuged for a further 5 minutes at full speed. The spin column was placed in a new 1.5 mL collection tube, and 16 µL of ultra-clean water was added to the centre of the membrane. The column was centrifuged for 1 minute at full speed to elute the RNA. RNA was stored at – 75 ^{o}C until further use.

3.2.4 RNA Integrity Analysis

RNA integrity analysis was performed by Central Biotechnology Services (CBS, Cardiff University) using the Agilent 2100 bioanalyser system (Agilent [G2939BA]). Table 3.1 shows summary statistics for the observed RIN values. All RIN values were above 7, the cut-off below which samples are not of high enough quality for RNA-sequencing.

2-hour timepoint	Mean ± S.E.M	Range
No Recall	9.82 ± 0.13	8.4 - 10
Recall	9.78 ± 0.15	8.2 - 10
Extinction	9.7 ± 0.16	8.0 - 10
5- hour timepoint		
No Recall	9.9 ± 0.01	9.9 - 10
Recall	9.96 ± 0.02	9.8 - 10
Extinction	10 ± 0.0	10 - 10

Table 3.1*. RIN values summarised by experimental condition over the 2- and 5- hour retrieval datasets. RIN values represent the quality of the RNA, with 10 being the highest possible score. All RIN values exceeded 7, the minimum required standard for the KAPA mRNA HyperPrep library preparation kit.*

3.2.5 Sequencing

3.2.5.1 2-hour timepoint

Libraries were prepared following the protocol in General Methods (2.3.4), using 90 ng total RNA. Samples were pooled into 3 groups of 12, due to the availability of indexing adaptors at the time. Any samples with high concentrations were diluted in 10 mM Tris-HCL (pH 8.0 – 8.5), so that all samples to be pooled together were of a similar concentration. The range across all samples was 10.7 - 17.2 ng/ µL. Libraries were sequenced on the Illumina Hi-Seq 4000 platform, with an average mapped read depth of 45 million (min: 36.4 million, max: 53.7 million)

3.2.5.2 5-hour timepoint

Libraries were prepared following the protocol in General Methods (section 2.3.4), with the following adjustments. Firstly, as the RNA concentrations were higher, 240 ng RNA was used to prepare the libraries. Secondly, due to the increased RNA input, the number of PCR cycles used in the library amplification step was reduced from 14 to 12. Advances in technology meant that dual, rather than single, index adaptors were used (KAPA biosystems [KK8722]). This allowed all samples to be pooled together to be sequenced, rather than be pooled into multiple groups. Dual index adaptors were freshly prepared by diluting 1 µL of 15 µM adaptor stock in 10 µL dilution buffer, to a final concentration of 1.36 µM. In a second technological advancement, samples were sequenced on the Illumina NovaSeq 6000.

3.2.6 Analysis

3.2.6.1 Analysis of CFC

Statistical analyses were either performed in R (R Core Team, 2018) or SPSS (v25, IBM). Fear conditioning data were analysed using either a mixed model Analysis of Variance (ANOVA), or a one-way repeated measures ANOVA. Where Mauchly's test of Sphericity indicated that the assumption of Sphericity had been violated, the Greenhouse – Geisser correction was applied (Greenhouse and Geisser, 1959;Maxwell and Delaney, 2004).

3.2.6.2 Differential gene expression analysis

Raw sequencing data were processed as described in General Methods 2.4.2. In the 2-hour dataset, 3 samples were identified as outliers in an initial principal component analysis (PCA) plot (Figure 3.2). Notably, these 3 samples had the lowest initial RNA concentration, and as such were not able to be repeated when improvements to the RNA library preparation method were implemented, as with the other 33 samples. As such, these samples were excluded from differential expression analyses (see Figure 3.1). The new RIN summary statistics, after removal of the outliers, can be seen in Table 3.3. Differential expression analysis was undertaken with limma/voom using an FDR threshold of 0.1 using the following formulae:

2-hour timepoint:

$$\sim group + RIN + pool$$

5-hour timepoint:

$$\sim group + RIN$$

Contrast name	Groups
Recall	Recall – No recall
Extinction	Extinction – No recall

Table 3.2. *The contrasts used for differential expression analysis. Expression was experimental group minus control group, such that positive log fold changes represented higher expression in the experimental group, and negative log fold changes represented lower expression in the experimental group. The contrasts were identical for the 2- and 5- hour timepoints.*

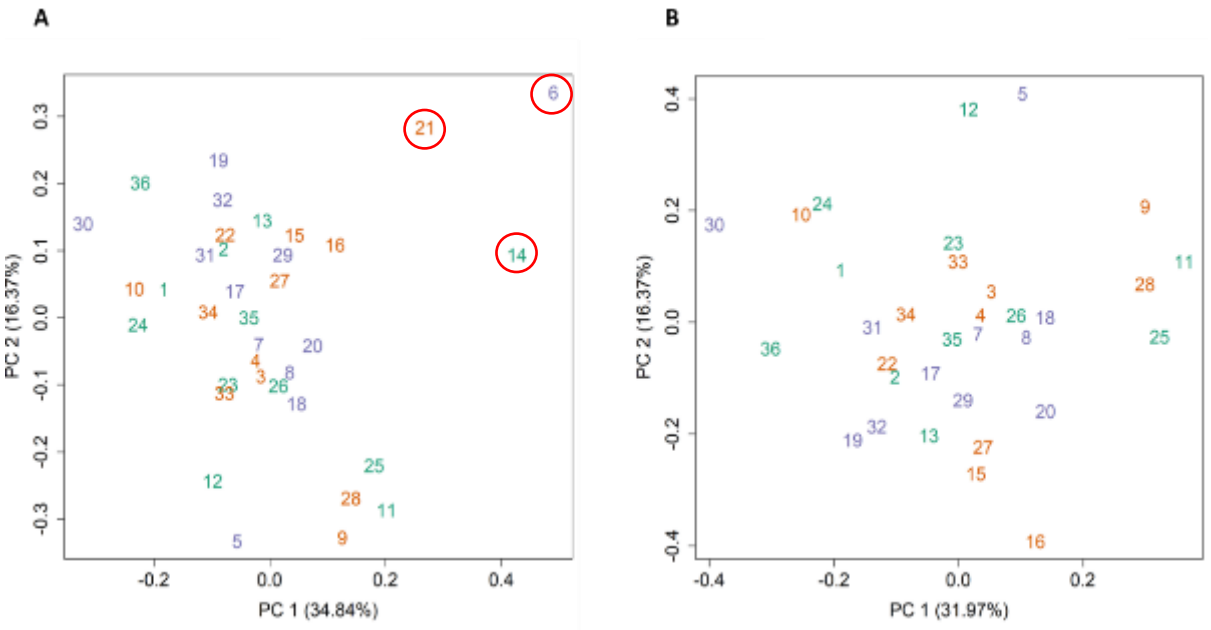


Figure 3.2 *PCA plots showing the distribution of normalised counts from each sample. The numbers correspond to the sample number and colours represent each of the conditions: Orange: Recall, Purple: Extinction, Green: Control (no retrieval).* ***A*** *PCA plot including 3 outlier samples (6, 14 and 21), each highlighted with a red circle.* ***B*** *PCA plot after removal of outlier samples.*

2-hour timepoint	Mean ± S.E.M	Range
No Recall	9.95 ± 0.04	9.6 - 10
Recall	9.78 ± 0.16	8.2 - 10
Extinction	9.85 ± 0.05	9.5 - 10

Table 3.3. *Summary of RIN values after the removal of 3 outlier samples in the 2- hour retrieval data. RIN values represent the quality of the RNA, with 10 being the highest possible score. All RIN values exceeded 7, the minimum required standard for the KAPA mRNA HyperPrep library preparation kit.*

3.2.6.3 Correlation analysis

Mean expression values for each gene from each dataset were calculated from the raw expression values, after filtering for low expression and applying TMM normalisation, on a group-wise basis. Pearson correlation analysis was performed in R using the "cor.test" function. Genes that were differentially expressed in *x* dataset were extracted from the expression analysis of *y* dataset. Gene expression values were then correlated. This was repeated, such that genes differentially expressed in *y* dataset were extracted from *x* dataset.

3.2.6.4 Pathway analysis

Pathway analysis was conducted using Fisher's exact test, with refinement, as described in General Methods section 2.4.3.

3.2.6.5 Transcription factor enrichment

In order to assess whether particular transcription factors were regulating the differentially expressed genes at recall and extinction, the DoRothEA package was used (Garcia-Alonso et al., 2019). This package provides a comprehensive resource of transcription factors and their interactions with genes (regulons), with a 5-level confidence assignment. Regulons with confidence scores of A, B or C were used for enrichment analysis (Figure 3.3).

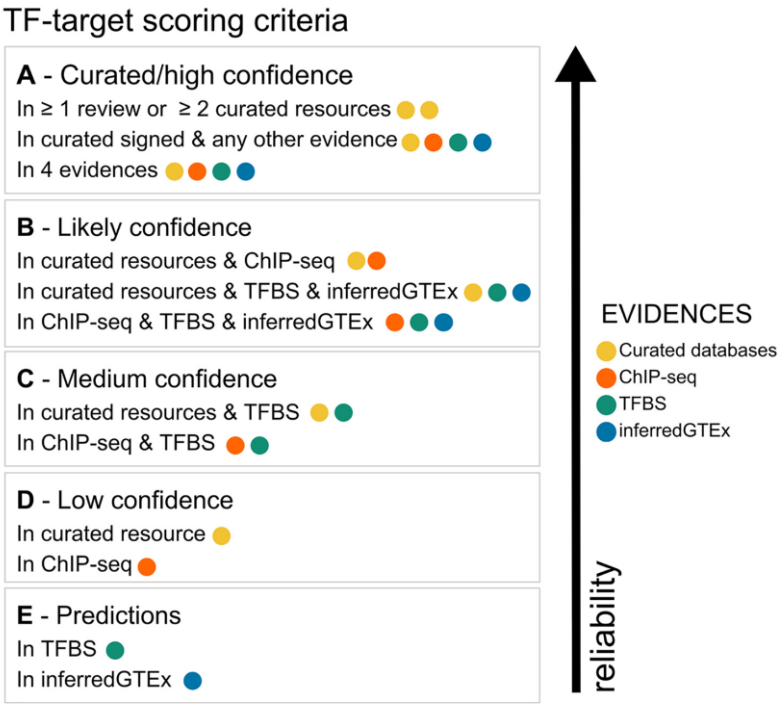


***Figure 3.3**. The scoring criteria for transcription factor-regulon relationships. This is copied with permission under a Creative Commons License (Attribution-NonCommercial 4.0 International) from Garcia-Alonso et al., 2019.*

Rat gene identifiers were converted to orthologous HGNC symbols using biomaRt (Durinck et al., 2009). Table 3.4 provides a summary of the genes which had a human ortholog. Fisher's exact test was used to calculate the enrichment of each transcription factor in the retrieval or extinction gene

sets compared to a background off all expressed genes. Bonferroni correction was applied to correct for multiple comparisons.

	Number of differentially expressed genes	Number of genes in background set
Extinction only	49 *(96%)*	13,626 *(86%)*
Retrieval	18 *(94%)*	

Table 3.4. *The number of genes in each dataset for the transcription factor analysis. Some genes from the differential expression set were not analysed as they do not have a human ortholog- the percentage of genes with a human equivalent are shown in brackets. The extinction only dataset contained genes that were differentially expressed in the extinction contrast only, and the retrieval dataset contained those genes that were differentially expressed in the recall and extinction conditions. As only 2 genes were differentially expressed only in the recall condition, these could not be analysed separately.*

3.2.6.6 Disease association

Disease association was conducted as described in General Methods section 2.5.

3.3 Results

In the following sections, when discussing gene expression, the term “recall” is used to mean those genes expressed in the recall condition, in which rats spent 2 minutes in the conditioned context during retrieval. The term “extinction” is used to mean those genes expressed in the extinction condition, in which rats spent 10 minutes in the conditioned context during retrieval. The term “retrieval” is used to mean those genes differentially expressed in both the recall and extinction conditions, that is, those genes that overlap. Tissue was collected at 2 timepoints, 2- or 5- hours after recall or extinction, and as such time qualifiers are added to the appropriate subsections.

3.3.1 Confirmation of successful contextual fear conditioning

3.3.1.1 2-hour timepoint

Figure 3.4 shows the mean percentage of time rats spent freezing across retrieval groups and timepoint in the 2-hour recall and extinction data. The timepoints were as follows: pre- unconditioned stimulus (US) was on the training day before the footshock, post- US was on the training day after the footshock, recall was on the retrieval day at the 2-minute timepoint, extinction was on the retrieval day at the 10 minute timepoint. There was a significant main effect of time point, but no group by time point interaction (Main effect: Time point ($F(1,30) = 157.625$, $p = 1.79 \times 10^{-13}$), Interaction: Group x Time point ($F(2,30) = 0.258$, $p = 0.774$)). This indicates that conditioning was successful, as the percentage of time spent freezing significantly increased between pre- and post-shock, and did not differ between experimental groups (No recall: pre-US mean = 3.78 ± 1.31, post- US mean = 49.99 ± 6.35; Recall pre-US mean = 3.66 ± 1.09, post-US mean = 46.96 ± 4.80; Extinction: pre-US mean = 3.78 ± 1.04, post-US mean = 43.93 ± 5.95). The percentage of time spent freezing at the 2 minute retrieval time point was not significantly different between groups ($t(19.66) = -0.44$, $p = 0.66$; Recall mean = 53.03 ± 7.37, Extinction mean = 57.95 ± 8.39). Time spent freezing significantly decreased between the 2- and 10- minute time point, indicating that the conditioned response underwent extinction ($t(10) = 5.24$, $p = 3.76 \times 10^{-4}$; 2-minute mean = 57.95 ± 8.39, 10-minute mean = 17.04 ± 3.99).

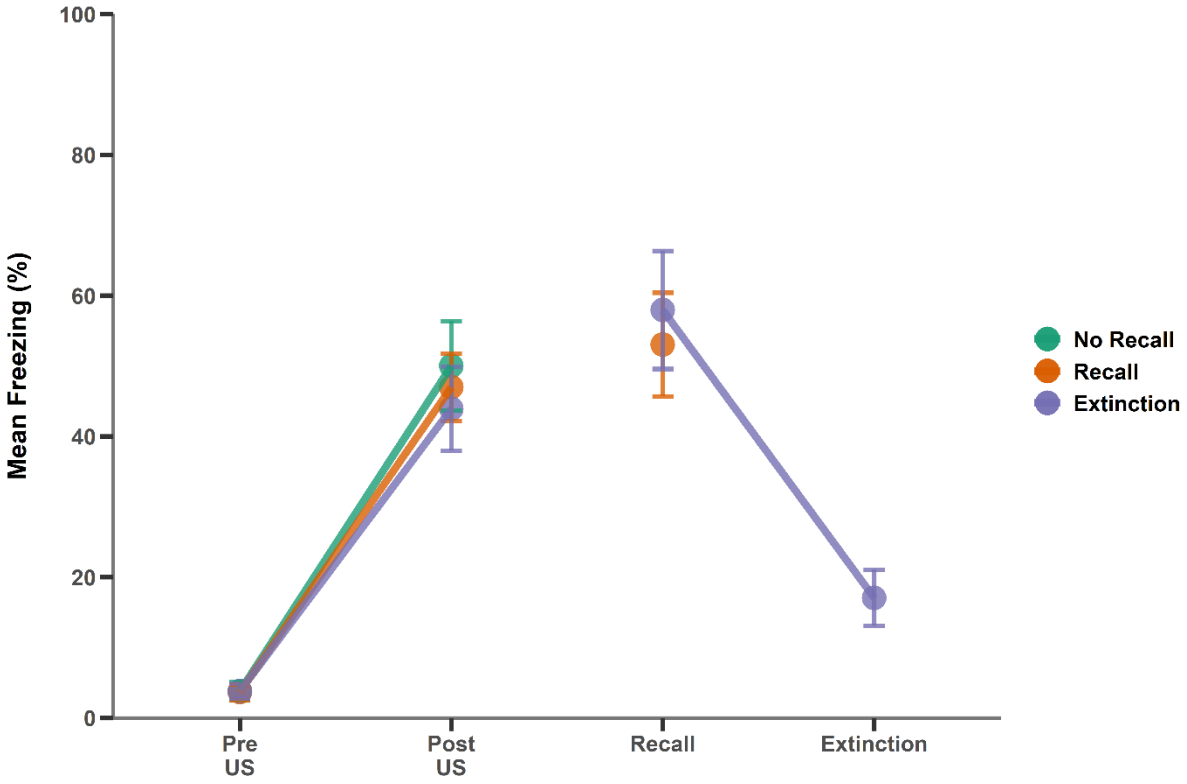


Figure 3.4. *Mean percentage of time that rats spent freezing across retrieval groups (No recall, Recall and Extinction) and timepoint (Pre US, Post US, recall (2 minutes) and extinction (10 minutes)) in the 2 hour dataset. These data excluded outlier samples 6, 14 and 21, as discussed in 3.2.6.2. Points represent mean freezing, error bars represent S.E.M.*

3.3.1.2 5-hour timepoint

Figure 3.5 shows the mean percentage of time rats spent freezing in the recall and extinction groups in the 5-hour dataset. Similar to the 2-hour timepoint, there was a significant main effect of time, but no group by time point interaction (Main effect: Time point ($F(1,33) = 302.63$, $p = 3.46 \times 10^{-18}$), Interaction: Group x Time point ($F(2,33) = 0.201$, $p = 0.819$); No recall: pre-US mean = 4.51 ± 1.08, post- US mean = 59.02 ± 5.75; Recall pre-US mean = 7.29 ± 1.92, post-US mean = 66.66 ± 5.52; Extinction: pre-US mean = 4.86 ± 1.00, post-US mean = 63.19 ± 6.83). The percentage of time spent freezing at the 2-minute retrieval time point was not significantly different between groups, although there was a tendency for rats in the recall group to freeze slightly more ($t(18.84) = 1.26$, $p = 0.22$; Recall mean = 59.50 ± 7.69, Extinction mean = 47.91 ± 4.98). Finally, time spent freezing significantly decreased between the 2- and 10- minute time point, indicating extinction of conditioned fear ($t(11) = 4.97$, $p = 4.19 \times 10^{-4}$); 2-minute mean = 47.91 ± 4.98, 10- minute mean = 16.31 ± 4.93). Taken together, these results suggest that contextual fear conditioning, and subsequent retrieval processes, where successfully activated by the experimental procedure.

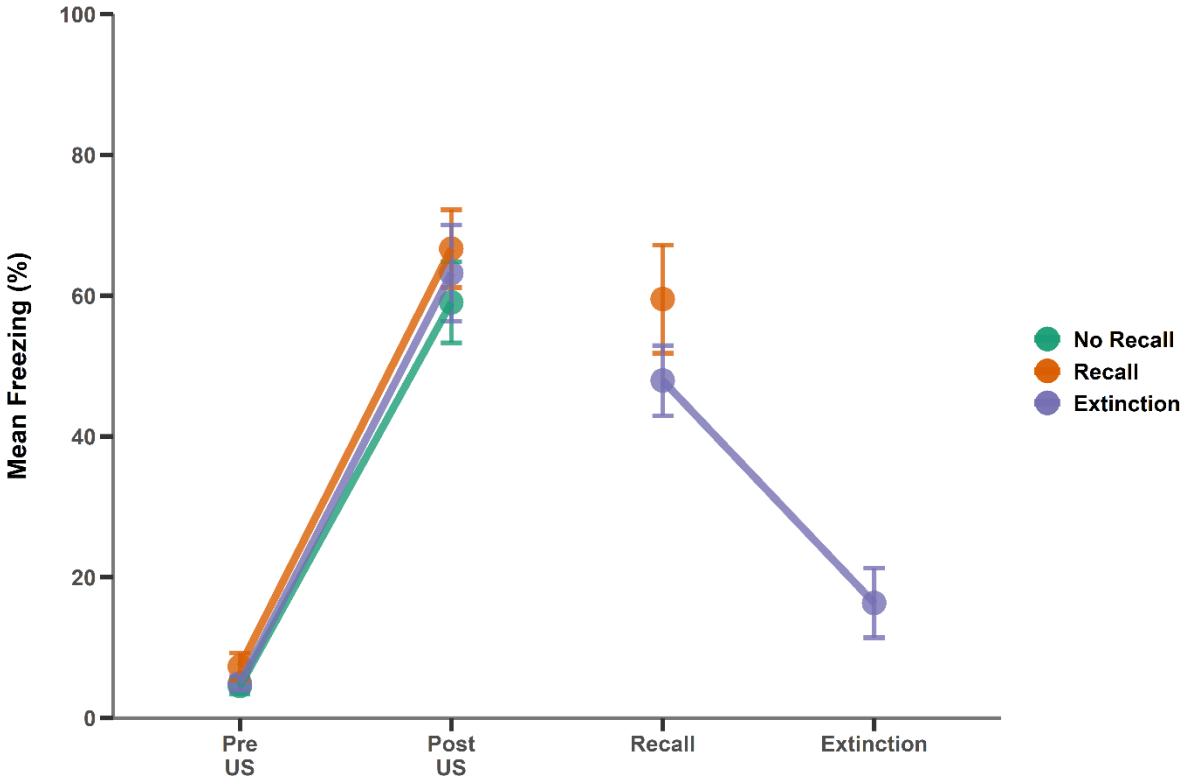


Figure 3.5. *Mean percentage of time that rats spent freezing across retrieval groups (No recall, Recall and Extinction) and timepoint (Pre US, Post US, 2 mins and 10 mins) in the 5-hour dataset. Points represent mean freezing, error bars represent S.E.M.*

3.3.2 Few genes were differentially expressed 2 hours after recall and extinction

The number of genes that were differentially expressed in the recall and extinction conditions, relative to the no recall condition (control), was examined 2- hours post-retrieval (Figure 3.6). In total, 21 genes were differentially expressed in the recall condition compared to control. The majority of these genes (90%) were also differentially expressed 2 hours after extinction. 70 genes were differentially expressed after extinction, with 27% shared with recall. Figures 3.7 and 3.8 show volcano plots of the expressed genes in each contrast, with -log10 FDR value plotted against log fold change. It can be seen that 4 of the top 5 differentially expressed genes in each contrast overlap: *FosB*, *Egr2, Egr4* and *Fam184b*. There were 2 genes that were uniquely differentially expressed in the recall condition: *Cry2* and *Egr3*. In both the recall and extinction conditions, the majority of differentially expressed genes were up-regulated, with only 3 down-regulated genes in the recall condition (14%) and 19 in the extinction condition (27%).

***Figure 3.6**. Venn diagram showing the overlap between differentially expressed genes in the recall vs control (orange), and extinction vs control (purple) conditions. The 19 genes that overlap between conditions are hereafter referred to as the retrieval dataset.*

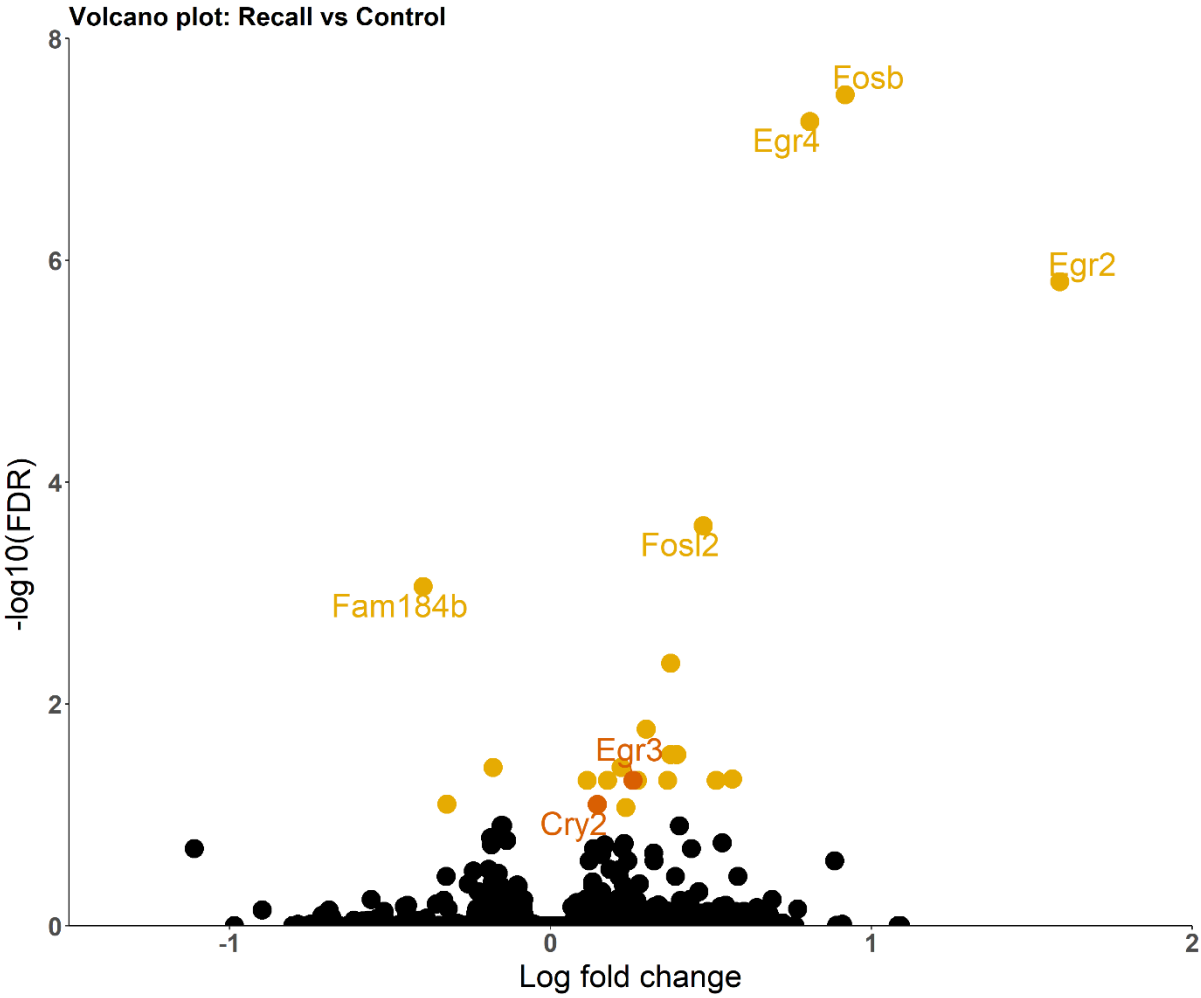


***Figure 3.7**. Volcano plot of log fold change against -log10 FDR values for each gene in the recall vs control contrast. Yellow represents those genes that are differentially expressed in both the recall and the extinction conditions and orange represents those genes differentially expressed in only the recall condition. The top 5 differentially expressed genes, ranked by FDR value, are labelled with gene names, as well as the 2 genes uniquely differentially expressed in the recall condition.*

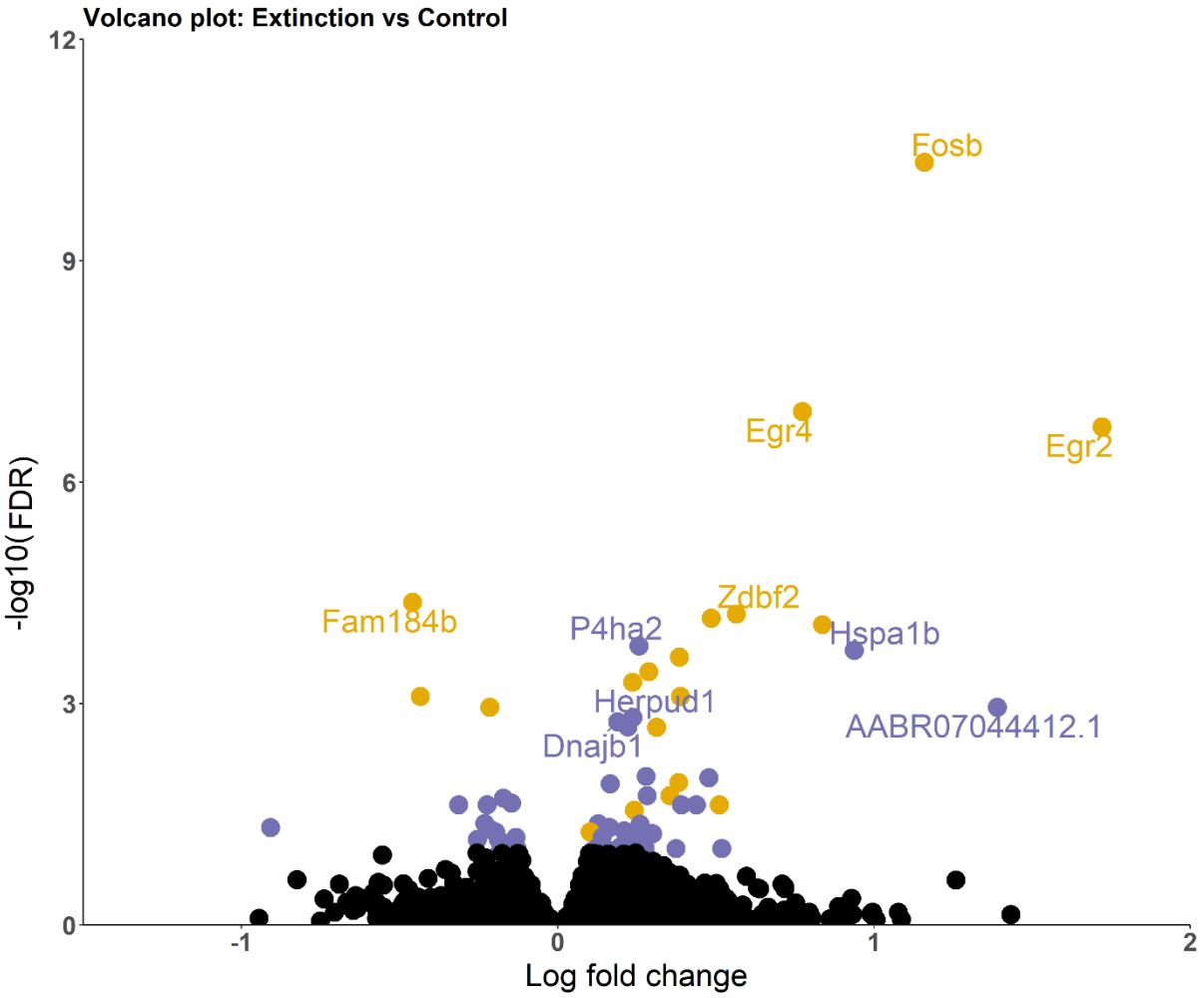


***Figure 3.8**. Volcano plot of log fold change against -log10 FDR values for each gene in the extinction vs control contrast. Yellow represents those genes that are differentially expressed in both the recall and the extinction conditions and purple represents those genes differentially expressed in only the extinction condition. The top 5 differentially expressed genes, ranked by FDR value, are labelled with gene names, as well as the top 5 genes uniquely differentially expressed in the extinction condition.*

3.3.3 Correlation of differentially expressed genes between the gene sets

In the previous section, I identified that there was significant overlap between the recall and extinction gene sets. Whilst some genes identified in one gene set were not significantly differentially expressed, it may be that their expression patterns are similar in the second gene set. To explore this, correlational analysis was performed.

Firstly, expression of genes that were significantly differentially expressed in the recall gene set were correlated with the expression of the same genes in the extinction dataset. It was found that there was a significant correlation between the mean expression of genes differentially expressed in the recall condition, and the mean expression of these genes in the extinction condition ($r(19) = 0.99$, $p = 2.2 \times 10^{-16}$; Figure 3.9).

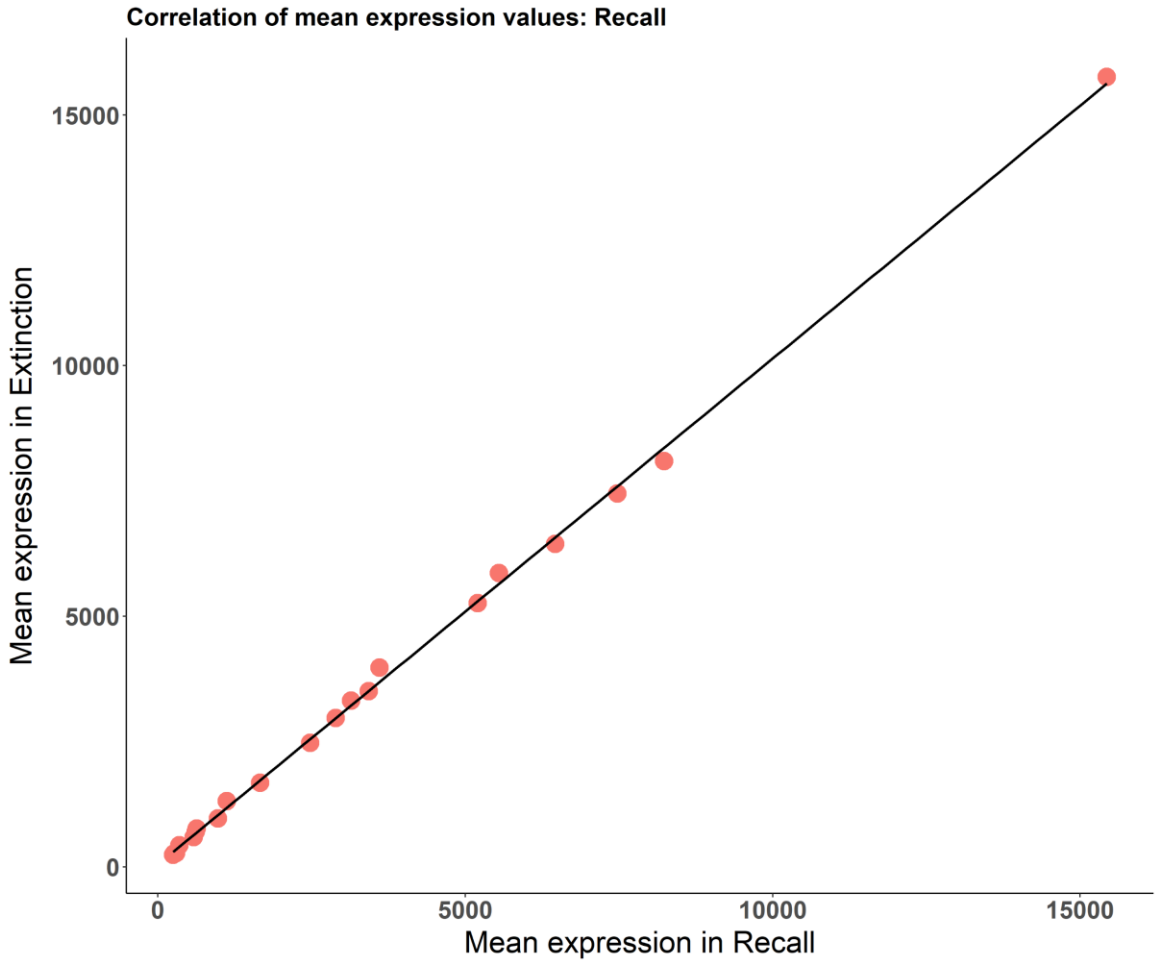


Figure 3.9. *Correlation of mean expression values for genes differentially expressed in recall, and expressed in the extinction condition. There is a strong correlation between the expression of genes differentially expressed in recall, and their expression in the extinction condition. Egr1 is the gene with the highest expression value in both datasets.*

Next, expression of genes that were significantly differentially expressed in the extinction gene set were correlated with the expression of the same genes in the recall dataset. It was found that there was a significant correlation between the mean expression of genes differentially expressed in the extinction condition, and the mean expression of these genes in the recall condition ($r(68) = 0.99$, $p = 2.2 \times 10^{-16}$; Figure 3.10).

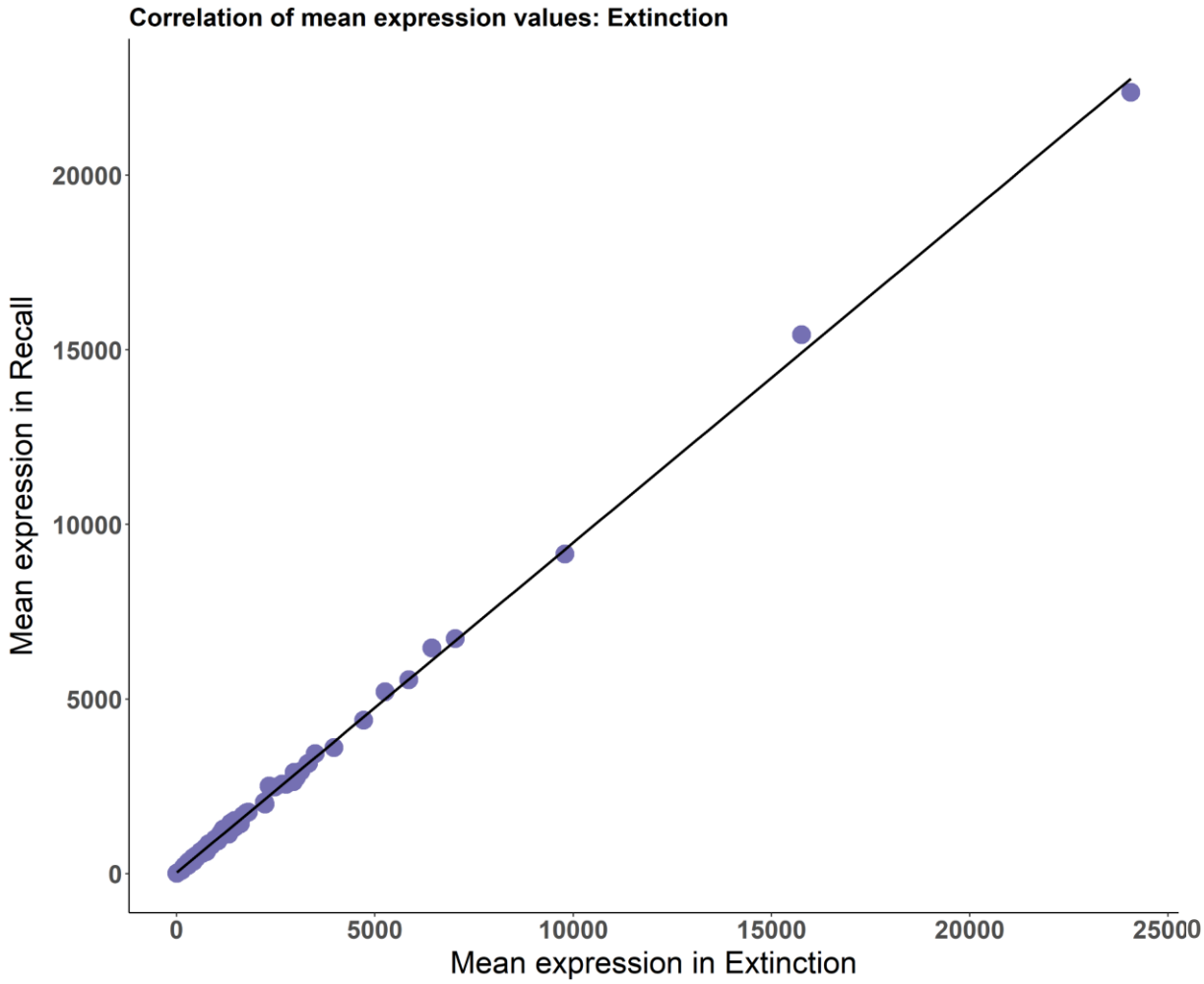


Figure 3.10. *Correlation of mean expression values for genes differentially expressed in extinction, and expressed in the recall condition. There is a strong correlation between the expression of genes differentially expressed in extinction, and their expression in the recall condition. Hspa5 is the gene with the highest expression value in both datasets.*

3.3.4 There was little overlap between recall and extinction gene sets and previous work

Next, I compared the overlap between differentially expressed genes in the present dataset (2-hours), and those in the microarray experiment (Scholz et al., 2016). There was little overlap between the datasets with no genes overlapping in the recall only condition, 2 overlapping in the extinction condition only, and 1 overlapping in retrieval (those shared between recall and extinction, see Table 3.5). *Hspb1* was present in the extinction condition only in our previous work, but was differentially expressed in both the recall and extinction conditions in the present work.

	Gene name
Recall	-
Extinction	*P4ha1*
	Tagln2
Retrieval	*Hspb1*

Table 3.5. *The overlap between the differentially expressed genes in each condition in the current data, and those presented in Scholz et al., 2016. The recall condition consists of genes differentially expressed only in the recall vs no recall contrast (2 genes). The extinction condition consists of genes differentially expressed in the extinction vs no recall contrast (51 genes). The retrieval condition consists of genes that were differentially expressed in both contrasts (19 genes). There was little overlap between the current data, and that found previously.*

3.3.5 Transcription-related pathways were enriched in recall and extinction gene sets

Pathway analysis was undertaken to explore the enrichment of the differentially expressed genes in biological processes, using the Gene Ontology database (GO) (Table 3.6). Genes that were only differentially expressed in the extinction condition were enriched in 3 biological pathways, all related to transcription. Indeed, these 3 enriched terms are part of the same biological process pathway within the GO network. Genes that were differentially expressed in both recall and extinction were enriched in 17 biological pathways, again largely relating to transcriptional processes. In addition, these genes were enriched in "response to peptide hormone" [GO:0043434]. As many of these terms were related to each other, refined pathway analysis was undertaken. This revealed 1 overarching term for extinction only genes: "Negative regulation of transcription from RNA polymerase II promotor in response to stress" [GO:0097201] (Odds ratio: 116, adjusted p-value: 0.0003), and 2 overarching terms for shared retrieval genes: "DNA binding transcription activator activity RNA polymerase II specific" [GO:0001228] and "response to peptide hormone" [GO:0043434] (Odds ratio: 23, adjusted p-value: 0.002; Odds ratio: 22, adjusted p-value: 4.9×10^{-4}

respectively). There were only 2 genes differentially expressed in recall only, and these were not significantly enriched in any GO terms.

Correlation analysis was undertaken to determine whether there was a relationship between the odds ratios of significantly enriched pathways in the extinction only and shared retrieval pathways. Taking pathways that were significantly enriched in the shared retrieval genes, it was found that there was no correlation between the odds ratios in the extinction pathway analysis ($r(15) = -0.09$, $p = 0.727$). When taking pathways that were significantly enriched in the extinction gene set, a strong negative correlation was found between the odds ratios in the shared retrieval pathway analysis ($r(1) = -1$, $p = 2.2 \times 10^{-16}$). This was likely driven by the GO term "Negative regulation of transcription from RNA polymerase II promoter in response to stress" [GO:0097201], which had an odds ratio of 116 in the extinction only gene set, and 0 in the shared retrieval gene set.

Gene set	Odds ratio	Adjusted p-value
Extinction only		
Negative regulation of transcription from RNA polymerase II promoter in response to stress [GO:0097201]	116	0.0037
Regulation of transcription from RNA polymerase II promoter in response to stress [GO:0043618]	31	0.017
Regulation of DNA templated transcription in response to stress [GO:0043620]	31	0.017
Retrieval (shared)		
Response to peptide hormone [GO:0043434]	22	4.9×10^{-4}
RNA polymerase II cis-regulatory region sequence-specific DNA binding [GO:0000978]	19	5.1×10^{-4}
Cis-regulatory region sequence-specific DNA binding [GO:0000987]	18	6.5×10^{-4}
Response to hormone [GO:0009725]	16	6.9×10^{-4}
Response to peptide [GO:1901652]	19	0.002
DNA binding transcription activator activity RNA polymerase II specific [GO:0001228]	23	0.002
DNA binding transcription activator activity [GO:0001216]	23	0.002
Response to organonitrogen compound [GO:0010243]	13	0.003
RNA polymerase II transcription regulatory region sequence-specific DNA binding [GO:0000977]	15	0.003
Response to nitrogen compound [GO:1901698]	13	0.006
Transcription regulatory region sequence-specific DNA binding [GO:0000976]	13	0.008
Regulatory region nucleic acid binding [GO:0001067]	13	0.009
Sequence-specific double-stranded DNA binding [GO:1990837]	13	0.012
Double-stranded DNA binding [GO:0003690]	12	0.021
Sequence-specific DNA binding [GO:0043565]	12	0.022
DNA binding transcription factor activity RNA polymerase II specific [GO:0000981]	13	0.023
DNA binding transcription factor activity [GO:0003700]	12	0.039

Table 3.6. *Enrichment of differentially expressed genes in GO pathways. Genes that were differentially expressed in the extinction only condition were enriched in 3 pathways. Genes that were differentially expressed in both retrieval conditions were enriched in 17 pathways. P-values for pathway analysis were Bonferroni corrected. All genes expressed in the tissue was used as a background dataset for the enrichment analyses.*

3.3.6 Transcription factor analysis

It was found that there was an enrichment of genes regulated by the transcription factor Heat Shock Factor Protein 1 (*HSF1*) in extinction, with 4 genes in this set being regulated by this transcription factor (Odds ratio: 199; Bonferroni adjusted p-value: 8.26×10^{-6}. In retrieval, there was an enrichment of genes regulated by cAMP responsive element binding protein 1 (*CREB1*) (Odds ratio: 60; Bonferroni adjusted p-value: 0.008).

3.3.7 No genes were differentially expressed 5-hours after recall or extinction

Pathway analysis 2 hours post-retrieval revealed that most of the differentially expressed genes related to transcriptional events. In order to explore the downstream pathways involved in retrieval processes relating to the consequence of these transcriptional changes, the experiment was repeated, with tissue taken 5-hours after recall or extinction. However, there were no differentially expressed genes observed at this time-point. Table 3.7 provides a summary of the top 5 genes for each condition, ranked by non-adjusted p-value.

Recall	Log fold-change	P-value	FDR
AABR07044301.1	-0.69	2.96×10^{-5}	0.23
Ubd	-0.84	2.32×10^{-5}	0.23
Nrn1	0.21	6.14×10^{-4}	0.99
Map6	-0.09	0.0016	0.99
Znhit1	0.14	0.0022	0.99
Extinction			
Hspa5	0.13	5.34×10^{-4}	0.99
Ifrd1	0.15	6.19×10^{-4}	0.99
Hsp90b1	0.09	7.05×10^{-4}	0.99
Egr1	-0.21	0.0011	0.99
Fgf12	0.10	0.0017	0.99

***Table 3.7**. Summary of the log fold-change, non-adjusted p-value and FDR p-value for the top 5 genes in the recall and extinction conditions, 5-hours after being returned to the context. No genes were significantly differentially expressed when adjusted using FDR.*

3.3.8 Enrichment in disease

3.3.8.1 Common variation

To see whether genes differentially expressed 2-hours after recall or extinction were enriched for common variants associated with schizophrenia, gene set enrichment analysis was undertaken with MAGMA (de Leeuw et al., 2015). The gene set analysis was conditioned on 'all tissue-expressed genes', to account for the background enrichment of brain expressed genes in schizophrenia datasets. No enrichment for schizophrenia association was found in either the extinction only dataset, or the shared retrieval dataset (Retrieval (shared) β value: 0.042 p-value: 0.439; Extinction β value: - 0.198, p-value: 0.865; Figure 3.9).

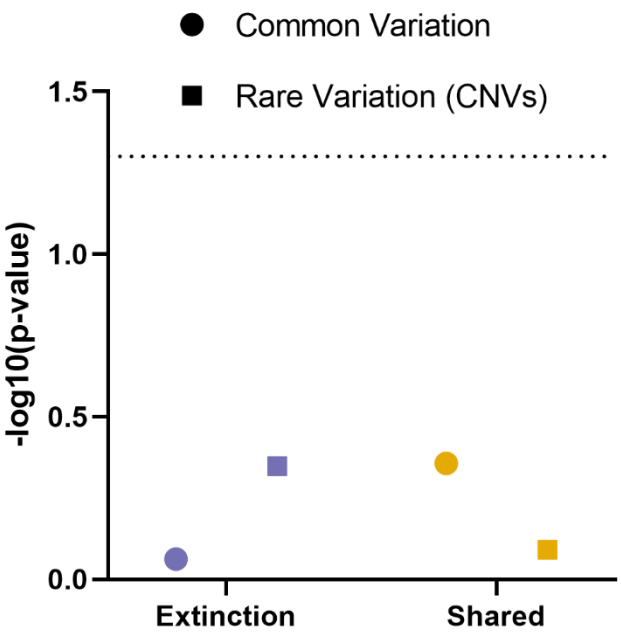


Figure 3.9. *There was no enrichment of extinction or retrieval gene sets in either common or rare structural variation for schizophrenia. The extinction gene set contained genes only differentially expressed in the extinction condition, whereas the shared gene set contained genes differentially expressed in both extinction and recall (retrieval). Dotted line represents significant association.*

3.3.8.2 CNV enrichment

In order to establish if there was an enrichment of recall or extinction genes in CNVs associated with schizophrenia, a binomial regression was undertaken. No enrichment of extinction-or retrieval-associated genes was found in the CNV gene set (Extinction: coefficient -0.136, p-value: 0.449; retrieval: coefficient 0.101, p-value: 0.811).

3.4 Discussion

The aim of this chapter was to derive recall- and extinction- related gene sets after contextual fear conditioning (CFC), implementing a more naturalistic paradigm and up-to-date methodology than previous work (Scholz et al., 2016). I found that recall and extinction were associated with an overlapping pattern of gene expression, and were enriched for transcription-related biological pathways. Further, I found no significantly differentially expressed genes 5 hours after retrieval, as determined by the current differential expression method used. Although previously Clifton and colleagues (2017b) found that extinction-related genes were enriched in rare variants associated with schizophrenia, this was not mirrored in the present data.

3.4.1 72 genes significantly differentially expressed across retrieval conditions.

In the present experiment, 19 genes were found to be differentially expressed in recall compared to the no recall condition, and 70 were found to be differentially expressed in the extinction condition. There was substantial overlap between conditions, with 17 of the genes identified as differentially expressed in recall also being differentially expressed in extinction, leaving only 2 uniquely differentially expressed genes in the recall condition. The genes uniquely differentially expressed in recall were Cryptochrome Circadian Regulator 2 (*Cry2)* and Early Growth Response (Egr) 3 (*Egr3)*. *Cry2* is a protein coding gene that forms a key part of the circadian core oscillator complex (GeneCards, 2022a). Whilst every effort was made to ensure that the recall and extinction conditions were counterbalanced across time-of-day, it cannot be ruled out that circadian rhythms may have had an impact on gene expression. *Egr3* is part of a family of transcription factors which have been linked to learning and memory (for review, see (Poirier et al., 2008)). Mice deficient in *Egr3* (Egr3 $^{-/-}$) have previously been shown to have impaired retrieval of contextual fear memories (Li et al., 2007). In addition, increased binding activity of *Egr3* after retrieval of an associative memory (inhibitory avoidance) and a trend towards increased protein expression has been found, providing further support for the present finding (Cheval et al., 2012).

Previously, our group has found that recall and extinction are associated with a distinct pattern of gene expression (Scholz et al., 2016). In further analysis of these gene sets, Clifton found that there was minimal overlap between the top 5% of genes, ranked by p-value, in consolidation, recall and extinction Clifton et al. (2017b). In contrast, the present experiment showed substantial overlap between the recall and extinction conditions. In addition, correlation analysis revealed strong positive correlations between the expression of genes differentially expressed in recall, and expression of these genes in the extinction dataset. Similarly, there was a strong positive correlation

between the expression of genes differentially expressed in extinction, and expression of those genes in the recall dataset. These results suggest that genes expressed after recall and extinction are similarly regulated, with similar mean expression values, but that some do not meet the threshold for significance in all datasets. The difference in overlap between the conditions is likely due to the differences in experimental paradigm. The previous work used BDNF and Zif268 antisense oligonucleotides to block the consolidation or reconsolidation, respectively, of the conditioned fear memory, which was used as the comparison condition. In this way, the resulting recall gene set was enriched for genes regulated by Zif268. Whilst this may give an insight into the core building blocks of retrieval memories, it ignores the interplay of neural processes, and as such the downstream genes are likely artificial. Although the extinction contrasts were almost identical in the two paradigms, by virtue of the Zif268 knock-down in the recall condition, the analysis of the overlap between the conditions would be unlikely to be similar. In undertaking the paradigm shown in this chapter, I have conducted the experiment in a more naturalistic way, which likely reflects the processes underlying recall and extinction memory formation. It should be noted that these results and conclusions are subject to the methodology used within each paradigm. As such, it cannot be confidently stated that the low numbers of genes found to be differentially expressed in the present experiment accurately reflect the physiological state-of-play in an awake animal, but that using the current technique and bioinformatic analysis, these were the results found.

Previously, our group has reported almost 1,000 genes to be differentially regulated following recall and extinction of conditioned fear (Scholz et al., 2016; Clifton et al., 2017b). One of the main reasons for the discrepancy in numbers is likely the application of multiple comparisons correction, FDR, to the current data, whereas the previous data was based on non-corrected p-values. Over the past decade, the field of genome-wide experiments has rapidly increased, and as such knowledge and analytical techniques to deal with such data has also rapidly evolved. As such, techniques and analytical practices that were once accepted have now been superseded. It is now widely accepted, and considered best practice, to use a multiple comparisons correction, such as FDR, when analysing large numbers of genes, as is the case in RNA-sequencing, to account for false positives. Of the genes found that were differentially expressed in the current data, only 3 overlapped with the previous experiment (Scholz et al., 2016). That there were so few differentially expressed genes that overlapped between the paradigms is not surprising, given their distinct methodologies on both an experimental and analytical level.

3.4.2 Pathway analysis showed enrichment of retrieval genes in transcriptional processes

In order to gain insight into the biological processes the differentially expressed genes may be involved in, pathway analysis was conducted. Genes that were differentially expressed only in extinction were enriched in biological processes related to the regulation of transcription, namely "Regulation of DNA templated transcription in response to stress" [GO:0043620], which was a parent term of the other 2 significantly enriched pathways. Similarly, genes differentially expressed in both recall and extinction (retrieval) were also enriched in transcriptional pathways, including "DNA binding transcription factor activator activity" [GO:0001216] and several other DNA binding GO terms. This is in line with other literature examining gene expression at this time point. For example, an enrichment of transcription associated genes was found when examining expression 2 hours after long-term potentiation (LTP) induction (Chen et al., 2017). Correlation analysis exploring the relationship between the odds ratios of pathways significantly enriched in recall or extinction genes (shared retrieval genes) and extinction only genes revealed no relationship. Whilst transcription related pathways were found to be significantly enriched in both recall and extinction (shared) and extinction only genes, these were different GO terms, likely explaining the lack of relationship found between the odds ratios for specific pathways. A strong negative correlation was found between the odds ratios of pathways that were significantly enriched in extinction only genes, but not significantly enriched in the recall and extinction (shared retrieval) genes. Again, this is likely due to the comparison of the odds ratios of specific GO terms.

3.4.3 Enrichment of *HSF1* and *CREB1* regulated genes in extinction and retrieval

Given that transcriptional processes were enriched in recall and extinction gene sets, and it has previously been shown that different transcription factors are involved in recall and extinction (de la Fuente et al., 2011), I investigated whether there was an enrichment of particular transcription factors regulating the differentially expressed genes. I found that there was an enrichment of genes regulated by *HSF1* in extinction, and an enrichment of genes regulated by *CREB1* in retrieval. Whilst *HSF1* has not previously been linked directly to learning, *HSP70*, which is synthesised following activation of *HSF1*, has been shown to be up-regulated following consolidation of contextual fear memories (Porto et al., 2018). It has previously been shown that NF-κβ is required for recall but not extinction (de la Fuente et al., 2011). However, neither of the genes differentially expressed at recall are regulated by this transcription factor. There was enrichment of genes differentially expressed in retrieval that were regulated by *CREB1*, a finding supported in the literature. Previously, retrieval of conditioned fear has been shown to induce *CREB* activation in the hippocampus (Huang et al., 2017) and amygdala (Hall et al., 2001). Similarly, there is a wealth of literature in which manipulation of

CREB1 has been shown to lead to memory deficits (Bourtchuladze et al., 1994;Mamiya et al., 2009;Matos et al., 2019).

Transcription factors and the genes they regulate, termed 'regulons', were accessed from the DoRothEA database (Garcia-Alonso et al., 2019). It should be noted that the DoRothEA database was originally developed for use with human data. It has recently been shown that it is also suitable for use with mouse data, due to the evolutionary conservation between humans and mice (Holland et al., 2020). However, it has not yet been validated with rat data, at least in published works, and as such should be regarded as exploratory. The conversion of rat gene identifiers to their human orthologs in order to run the analysis has precedent, as it is a similar method to that used in the mouse validation experiment where human identifiers were converted to their mouse orthologs (Holland et al., 2020).

3.4.4 No genes were significantly differentially expressed 5 hours after recall or extinction

Pathway analyses revealed an enrichment of transcription-associated biological processes in retrieval and extinction gene sets. Transcription is a relatively early molecular process, and so in an attempt to capture genes involved in more downstream processes, the experiment was repeated with tissue taken 5 hours after recall or extinction. In addition, studies into the time course of consolidation have shown that the second peak of gene transcription continues up to 6 hours after learning (Igaz et al., 2002), and as such 5-hours represented a sensible second time-point to investigate. However, no genes were found to be differentially expressed 5-hours after recall or extinction. One reason for this could be that the window of gene expression differs in retrieval compared to consolidation. Interestingly, the gene most likely to be differentially expressed after extinction learning at this time point, was *Hspa5* (alternatively known as *Hsp70*) which, as discussed above, has been linked to contextual fear memory. Similarly, the two of the genes most likely to be differentially expressed after recall, *NRN1* and *MAP6*, have been linked to synaptic maturation and plasticity respectively (reviewed in (Yap and Greenberg, 2018) and (Cuveillier et al., 2021) respectively). Given this, it is likely that gene expression continues into the 3-6 hour window previously established for consolidation, but that bulk RNA sequencing is unable to detect it after multiple comparisons correction. The CA1 contains a multitude of different cell-types, and due to the nature of bulk RNA-sequencing, genes that have differential expression in one cell-type but not in others are likely to be masked in this type of analysis. This may explain the low-to-no differential expression found at these time-points.

3.4.5 Methodological differences between the 2- and 5- hour timepoints

The 2- and 5- hour timepoints were conducted at different stages of the PhD, and as such methodological improvements were made in processing the 5-hour samples, which may have impacted the results. The first improvement was in the amount of total RNA used as input to the library preparation protocol, which was 90 ng at 2- hours and 240 ng at 5- hours. Total RNA contains all RNA species, including ribosomal RNA which makes up between 80-90 % of a total RNA sample (Invitrogen). As such, increasing the amount of total RNA as input to the library preparation would likely have improved the quality of the prepared libraries. Similarly, the lower amount of total RNA used in the library preparation method meant that a higher number of PCR cycles were required, which may have introduced artefacts (Wang et al., 2009a;Fang and Cui, 2011). However, the same library preparation kit and the same experimenter performed the RNA library preparation, with the same professionally calibrated pipettes, which may have reduced methodological artefacts between the two experiments. Another difference in the methodology was that single-index adapters were used for the 2-hour library preparations, and dual-index adapters were used for the 5-hour library preparations. Index adapters are short sequences which are ligated onto each DNA strand, to allow identification of which sample their originated from. Dual-index adapters are now the standard, and the single-index adapters were discontinued, preventing their use in the 5-hour experiment. The index adaptors were required to be used at different concentrations, which were piloted to ensure the lowest concentration of adapter primer-dimers. In the present experimenter's hands, it was found that more primer-dimers formed when using the single-index adaptors, and as such further bead cleaning was undertaken to remove them, which may have reduced the overall size of the prepared library. However, given that the 5-hour libraries were prepared using updated and superior methodologies, if this were to significantly impact the downstream genes found it would be expected that more differentially expressed genes would have been found at 5-hours compared to 2-hours. However, no differentially expressed genes were found at this time-point. Similarly, as presented in Chapter 5, when 2- and 5-hour libraries were prepared at the same time, using the same library preparation methods, no genes were differentially expressed at 5-hours. This indicates that it is likely that the results from the 5-hour dataset are not down to methodological differences. However, it may be that if these improvements had been available at the time of sequencing the 2-hour dataset, that more differentially expressed genes would have been found. As such, results have been caveated above that they reflect the methodologies used in the present experiments, and are not necessarily an accurate reflection of the state-of-play in a behaving animal system.

3.4.5 There was no enrichment of differentially expressed genes in schizophrenia risk datasets.

Contrary to previous work (Clifton et al., 2017b), I did not find an enrichment of the differentially expressed genes in retrieval or extinction in schizophrenia datasets, either common or rare variation. The previous work made use of the recall and extinction gene sets reported in Scholz et al., (2016), taking forward the top 5% of genes for disease enrichment analysis. As discussed above, the gene sets reported in the previous microarray work were not corrected for multiple comparisons which, in addition to the technology used, represents a major difference between the works.

3.4.6 Summary

In conclusion, I found far fewer differentially expressed genes across the recall and extinction conditions than previously reported, likely due to the methodological and statistical differences between the works. No genes were found to be differentially expressed at 5 hours, and there was no enrichment of recall or extinction genes in common or rare variation associated with schizophrenia, contrary to previous work. So far, the current work has focused on nuanced retrieval conditions, which although have previously been shown to have distinct molecular signatures, may be too subtle to detect in bulk RNA sequencing. As such, I next used gene sets derived from an LTP experiment to investigate association with schizophrenia. These gene sets had the advantage of being derived after induction of LTP, which is likely to elicit a much stronger cellular response than a behavioural experiment, which in turn may alter detectable gene expression to a greater degree. In addition, expression data were also derived using TRAP technology, which allows investigation of specific cell-types, ameliorating some of the detection issues found in bulk RNA-sequencing.

Chapter 4: The enrichment of LTP associated genes in genetic variation for schizophrenia

4.1 Introduction

In the previous chapter, the transcriptomic profiles underlying retrieval processes were explored. I found 70 genes significantly differentially expressed after extinction, and 21 significantly differentially genes expressed after recall. These gene sets were significantly enriched for transcriptional processes, with no evidence of enrichment for common or rare variation associated with schizophrenia. The aim of the current chapter was to further explore gene expression in synaptic plasticity processes, specifically Long-Term Potentiation (LTP), and the enrichment of LTP-associated genes in schizophrenia risk.

4.1.1 LTP

LTP is defined as the persistent increase in synaptic strength following repeated stimulation of a synapse, and was first discovered in 1973 (Bliss and Lomo, 1973). In the hippocampus, one of the best characterised forms of LTP occurs in CA3-CA1 Schaffer collateral synapses and is N-methyl-D-aspartate receptor (NMDAr) dependent (Collingridge et al., 1983;Harris et al., 1984). Upon release from the presynaptic cell, glutamate binds to α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors on the post-synaptic membrane, which triggers an influx of sodium ions. This causes the post-synaptic cell to depolarise, therefore removing the magnesium block from the NMDA receptor and allowing an influx of calcium ions into the cell. Calcium ions bind to calmodulin to form a complex, which in turn activates several enzymes, including calcium/calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC), which in turn phosphorylate several proteins including AMPA receptors, increasing their conductance (Derkach et al., 1999). In addition, influx of calcium ions into the post synaptic cell mediates the trafficking of AMPA receptors to the cell membrane (Shi et al., 1999). This early-phase LTP (E-LTP) is protein-synthesis independent, and is not sustained beyond a few hours (Frey et al., 1993). In contrast, late-phase LTP (L-LTP) is protein

synthesis dependent, requiring gene expression to modulate long-term structural synaptic changes such as dendritic spine enlargement (Stanton and Sarvey, 1984;Harris et al., 2003;Bosch et al., 2014). Large influxes of calcium ions trigger multiple downstream signalling cascades, leading to the phosphorylation of cAMP-response element-binding protein (CREB). Subsequently, CREB binds to the cAMP-response element of the promotor of its target genes, leading to the initiation of gene transcription (Impey et al., 1996). A summary of mechanisms of LTP is shown in Figure 4.1.

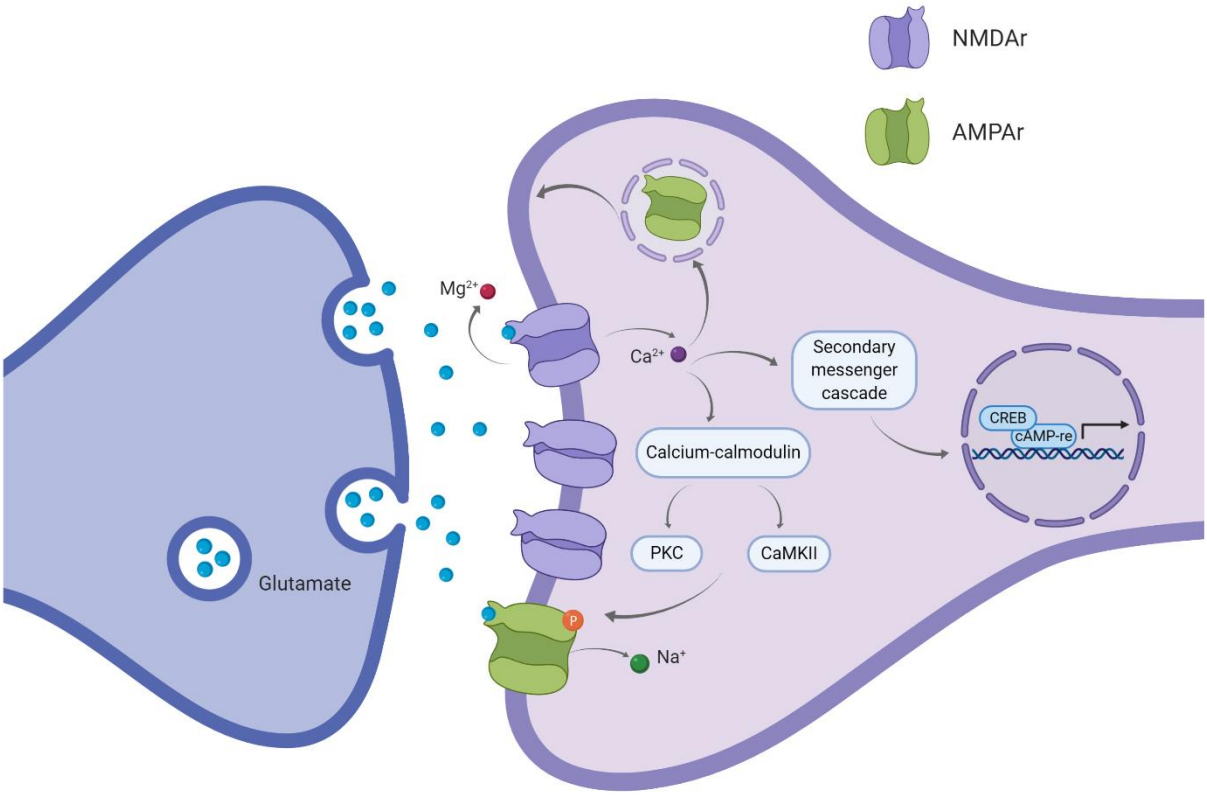


***Figure 4.1**. A simplified summary of mechanisms of NMDA receptor-dependent LTP. Glutamate, released from the presynaptic cell, binds to AMPA receptors triggering an influx of sodium. The subsequent depolarisation removes the magnesium block from NMDA receptors, allowing an influx of calcium ions into the post-synaptic cell. Calcium binds to the calmodulin complex, activating several enzymes which phosphorylate AMPA receptors and increasing their conductance. Calcium ions also mediate the trafficking of AMPA receptors to the cell membrane. Several secondary messenger cascades are activated upon influx of calcium into the post-synaptic cell, leading to gene transcription via CREB activation.*

Several studies have investigated hippocampal gene expression following induction of LTP. Early studies focused on candidate gene expression, finding that immediate early genes such as zif268 (Cole et al., 1989), c-jun and jun-b (Demmer et al., 1993) were expressed following LTP. In addition, the mRNA of proteins such as BDNF (Patterson et al., 1992) and transcription factors such as NF-kappa B (Meberg et al., 1996) were also identified after LTP induction. As technology progressed, microarray studies began to identify more extensive lists of genes expressed following LTP, including those involved in transmitter transport, growth factors, ion channels, arc interactors and regulation

of the cytoskeleton, amongst others (Thompson et al., 2003;Wibrand et al., 2006;Park et al., 2006;Ryan et al., 2012).

4.1.2 Gene expression following LTP induction

The advancement of profiling technologies, and subsequent reduction in cost, has allowed detailed investigation of the hippocampal transcriptome (reviewed in (Valor and Barco, 2012)). In particular, the advent of Translating Ribosome Affinity Purification (TRAP) has allowed the transcriptome of individual populations of cells to be characterised (Heiman et al., 2008;Doyle et al., 2008). Briefly, this methodology involves genetically tagging a ribosomal protein under the control of a tissue-specific promotor, to drive expression in the cell-type of interest. Ribosome-associated mRNAs can then be isolated by immunoprecipitation and be profiled with microarray or RNA-sequencing technologies. Chen and colleagues (2017) utilised this technique to profile the genes expressed 30-, 60- and 120- minutes after chemically inducing LTP in hippocampal slices using forskolin (see 4.2.1 for further methodology). RiboTag mice (Sanz et al., 2009) were bred with mice in which cre-expression was driven by the αCaMKII promotor (Tsien et al., 1996), in order to profile LTP gene expression specifically in CA1 excitatory pyramidal neurons. The authors found that TRAP-sequencing identified hundreds more differentially expressed genes compared to bulk RNA-sequencing (TRAP-seq: 1035 genes; bulk RNA-seq: 399 genes, across the 3 timepoints), indicating the advantages of cell-type specificity in identifying activity-regulated transcripts. Subsequent pathway analyses revealed that genes identified through TRAP-seq were enriched for cell adhesion and cytoskeletal genes, whilst those identified through bulk RNA-seq were enriched for cytokine signalling pathways. Genes identified through both TRAP- and bulk RNA-seq were enriched for transcription related pathways (Chen et al., 2017). Further, the authors used cluster analysis to examine the temporal profile of the differentially expressed genes identified in the study. They found 3 clusters of genes, those which were generally upregulated over time (cluster 1), those which were generally downregulated between 30- and 60- minutes and then stayed downregulated at 120-minutes (cluster 2), and those which became generally down regulated over time (cluster 3). GO pathway analysis again revealed that cluster 1, those genes that were generally upregulated over time, were enriched in biological processes relating to transcription. Genes in clusters 2 and 3 were not significantly enriched in any biological pathway.

4.1.3 Synaptic plasticity and schizophrenia

Aberrant synaptic plasticity mechanisms have been described in several brain disorders, including Alzheimer's disease (AD) (Koch et al., 2012) and epilepsy (Lenck-Santini and Scott, 2015). In addition,

there is increasing evidence for the role of synaptic plasticity mechanisms in the aetiology psychiatric disorders, including schizophrenia. Over the past decade, genomic studies have been at the forefront of the schizophrenia research landscape, and several genetic variations that increase schizophrenia risk have been identified. Genetic risk variants for schizophrenia typically fall into one of two categories: common variants with individually low effect sizes, for example single nucleotide polymorphisms (SNPs) identified through genome-wide association studies (GWAS), and rare variants with larger effect sizes, for example copy number variants (CNVs). In a landmark GWAS of almost 37,000 cases and over 113,000 controls, 128 associations spanning 108 loci were found to be associated with schizophrenia (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014). In PGC2, 93 of these loci were replicated another 52 implicated, taking the total to 145 (Pardinas et al., 2018). Similarly, rare CNVs at several loci have been found to associate with schizophrenia, including 1q21.1, 3q29, 16p11.2 and 22q11.2 (Stefansson et al., 2008;Rees et al., 2014;Marshall et al., 2016;Green et al., 2009). Ultra-rare de novo mutations, which deleteriously impact proteins, have also been identified through exome sequencing of schizophrenia cases (Rees et al., 2019;Rees et al., 2021;Singh et al., 2022).

Several of the genetic variants identified through common variation and CNV analyses converge on synaptic processes. In 2012, Kirov and colleagues combined CNV and proteomics datasets to conduct a systematic analysis of synaptic protein complexes (Kirov et al., 2012). They found an enrichment of post synaptic density (PSD) genes within schizophrenia associated CNVs, and when investigated further, found a particular enrichment of activity regulated cytoskeleton-associated protein (ARC) interactors and NMDA receptor genes. Pocklington and colleagues investigated the impact of schizophrenia associated CNVs on biological processes using gene-sets derived from the Mammalian Genome Index (MGI)(Pocklington et al., 2015). In addition to confirming the association between patient CNVs and NMDAr and ARC associated genes, they also found an enrichment of genes associated with abnormal LTP and abnormal synaptic transmission. Further, recent evidence from the Schizophrenia Exome Meta-Analysis Consortium (SCHEMA) found protein-damaging mutations in genes encoding NMDA and AMPA receptor subunits, key in the LTP cellular mechanism cascade (Singh et al., 2022).

4.1.3 Chapter aim

The aim of this chapter was to quantify patterns of gene expression following long term potentiation (LTP), with particular focus on CA1 excitatory neurons, and test for association with genetic variants from patients with schizophrenia, schizophrenia-related disorders, and appropriate control disorders. The data used was the publicly available gene expression data from Chen et al., (2017), in

which chemical LTP was induced in hippocampal slices and gene expression measured 30-, 60- and 120- minutes after LTP. Previously, the link between plasticity and schizophrenia has been demonstrated using curated databases such as Gene Ontology (GO) or MGI, rather than explored using gene-sets derived from a single, functional LTP experiment. In particular, the availability of data obtained via TRAP-seq methods enabled the explicit exploration of the association between genes expressed in CA1 hippocampal excitatory neurons during LTP, and schizophrenia risk, across several timepoints.

4.2 Methodology

4.2.1 LTP induction by Chen and colleagues (2017)

LTP RNA-sequencing data were generated by Chen and colleagues, and described in detail in the original paper (Chen et al., 2017). RiboTag mice, which express a floxed HA-tagged ribosomal protein L22 in Cre recombinase expressing cells (The Jackson Laboratory: 011029), were crossed with CaMKIIα-cre mice (The Jackson Laboratory: 005359), to direct expression to the CA1 pyramidal cell layer of the hippocampus. At 8-12 weeks old, a chemical LTP (cLTP) induction protocol was applied to *ex-vivo* hippocampal slices, with the dentate gyrus (DG) removed, as shown in Figure 4.2. Immediately after culling, 400 µm hippocampal slices were prepared and allowed to recover in oxygenated artificial cerebral spinal fluid (ACSF) for 2 hours. Pilot work conducted by Chen found that 2 hours recovery time, compared to 30- or 60-minutes, allowed activation of ERK1/2 (part of the MAPK pathway involved in cell survival and gene expression) to return to a baseline level (Chen, 2016). After recovery, experimental slices (those which underwent LTP) were submerged in 50 µM forskolin for 10 minutes, before being perfused with ASCF for 30-, 60- or 120- minutes. Control slices, those that did not undergo LTP, were perfused with ASCF with 0.2 % DSMO for those 10 minutes, and then perfused with ASCF for either 30-, 60- or 120- minutes. The addition of DSMO for the control slices mirrors the DSMO that was in the forskolin solution.

Forskolin has been shown to induce NMDA receptor-dependent late-phase LTP in the hippocampus (Otmakhov et al., 2004;Gobert et al., 2008). Forskolin is a naturally occurring organic chemical, most commonly extracted from the root of the Coleus forskohlii plant, medicinally used to treat hypertension, respiratory disorders and heart failure, with anti-inflammatory properties (Ju et al., 2021). It's mechanism of action for LTP induction is by increasing cyclic AMP (cAMP) production. Previously it has been shown that at concentrations greater than 50 µM, forskolin stimulated a 100-fold increase in cAMP content in the exposed cells (Barovsky et al., 1984). As described in the General Introduction (section 1.4.1), cAMP is a crucial second messenger, activating a range of kinases such as PKA and ERK which in turn phosphorylate transcription factors required for gene expression and maintenance of LTP processes (Frödin and Gammeltoft, 1999;Anjum and Blenis, 2008). In addition, forskolin has been shown to mediate AMPA receptor trafficking to the postsynaptic membrane, another key component of LTP expression and maintenance (Gomes et al., 2004;Oh et al., 2006). Clinically, off-target effects of forskolin have not been reported, although research in this area is limited. Recently, a single 100 µM dose of forskolin administered for 24 hours to Mesenchymal stem cells (MSCs) was found to have limited cytotoxicity (Awale et al., 2022),

potentially indicating that the 10 minute 50 µM dose administered in the Chen et al., 2017 study would have been unlikely to induce cell toxicity.

Frozen slices were homogenised in immunoprecipitation (IP) buffer, and an aliquot of the homogenate was taken for subsequent RNA isolation and RNA-sequencing. This was the 'bulk RNA-sequencing' condition. The remainder of the homogenate underwent IP and then subsequent RNA purification to isolate ribosome-associated mRNAs. RNA was then sequenced to form the 'TRAP-seq' condition. This design meant that the bulk- and TRAP-seq conditions were obtained from the same biological replicate, reducing between-animal variability between the bulk- and TRAP-seq conditions. For each biological replicate, 4 mice were used, and in turn there were 3 biological replicates per condition.

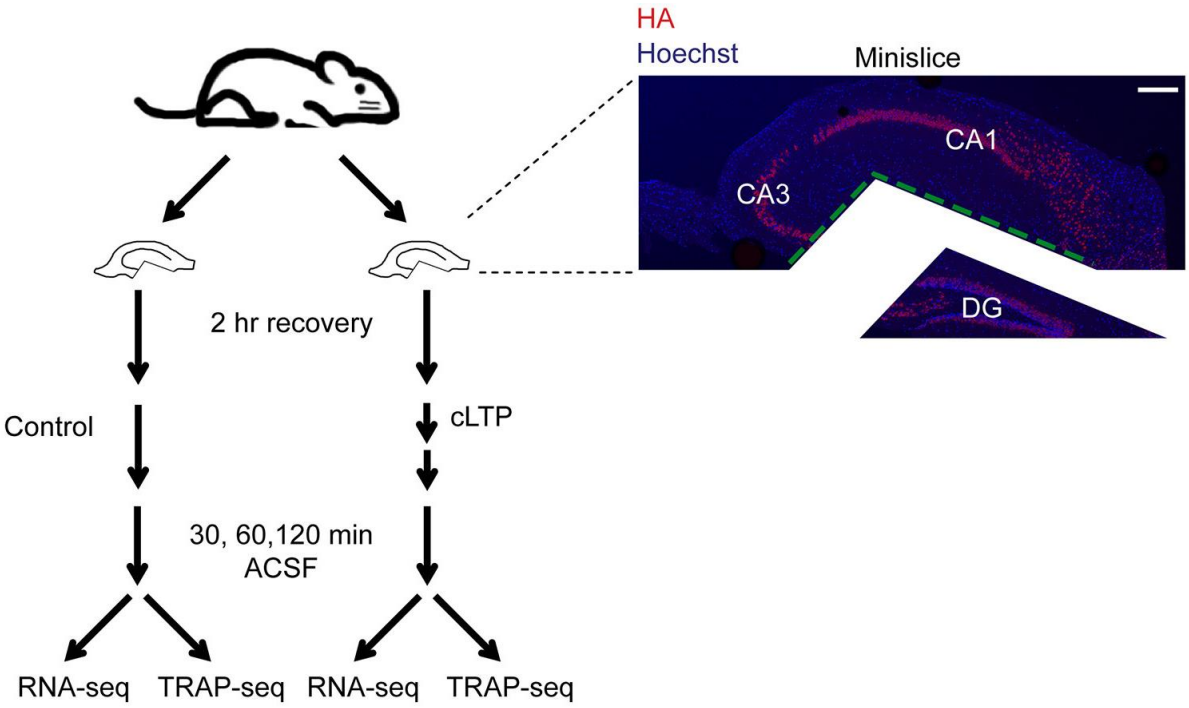


***Figure 4.2**. The experimental design of the cLTP experiment. Ex-vivo hippocampal slices recovered for 2 hours in artificial cerebral spinal fluid before cLTP was induced. Slices were then perfused with artificial cerebral spinal fluid for 30-, 60- or 120 minutes before being frozen for later processing. Control slices were exposed to ACSF with 0.2 % DSMO for the 10 minutes that the experimental slices were exposed to forskolin. Figure reproduced from the original paper under the Creative Commons Attribution License (Chen et al., 2017).*

4.2.2. LTP data

Following RNA-sequencing, reads were mapped to the GRCm38 mouse genome (NCBI) using the STAR aligner (Dobin et al., 2013). The edgeR algorithm (Robinson et al., 2009), including TMM normalisation (TMM described in section 2.4.2.2.) was used to examine differential expression (Chen et al., 2017). The contrasts used in the present work are detailed in Table 4.1.

Contrast name	Groups
30-minute TRAP-seq	TRAP-seq experimental – TRAP-seq control
30-minute Bulk RNA-seq	Bulk-seq experimental – Bulk-seq control
60-minute TRAP-seq	TRAP-seq experimental – TRAP-seq control
60-minute Bulk RNA-seq	Bulk-seq experimental – Bulk-seq control
120-minute TRAP-seq	TRAP-seq experimental – TRAP-seq control
120-minute Bulk RNA-seq	Bulk-seq experimental – Bulk-seq control

Table 4.1. *The contrasts from Chen et al., 2017 that were downloaded for the present work. The control slices were exposed to ACSF with 0.2 % DSMO, the experimental slices were exposed to 50 µM forskolin, both for 10 minutes.*

Processed RNA sequencing datasets were downloaded from the NCBI Gene Expression Omnibus (GEO), accession number GSE79790. These datasets contained the log fold change (logFC) and false discovery rate (FDR) for each gene, for each contrast of interest. These contrasts were: "TRAP-LTP vs. TRAP- basal" and "Total-LTP vs Total-basal", for each of the three time points (30, 60 and 120 minutes). The gene symbol, Entrez ID, logFC and FDR values for each contrast were extracted to form the background dataset. These datasets were further filtered to include only those genes with an FDR < 0.01 to form the gene sets of interest, hereafter referred to as: 30-minute TRAP-seq, 30-minute Bulk RNA-seq, 60-minute TRAP-seq, 60-minute Bulk RNA-seq, 120-minute TRAP-seq, 120-minute Bulk RNA-seq. In order to facilitate comparisons with human data, mouse Entrez IDs were converted to their human homologs. Genes that did not have a human homolog were excluded from further analyses.

4.2.2 Association with disease

Enrichment for common and rare variation was undertaken as described in General Methods (Section 2.5).

Where a gene set was significantly associated with common variation for a disorder, the MAGMA programme outputs a separate gene list file with the individual p-values (file suffix ".gsa.sets.genes.out.txt", gene lists can be found in the Appendix). Significant genes in this file ($p < 0.05$) were taken forward for additional pathway analyses.

4.2.2.1 Ultra-rare coding variants

Ultra-rare coding variants from 3444 schizophrenia trios (Rees et al., 2021) were obtained. Protein truncating variants (PTVs) and missense variants with a "Missense badness, Polyphen-2, constraint" (MPC) score of 2 or greater were taken forward for analysis. MPC is a measure of pathogenicity, with

a score greater than 2 having been shown to confer enrichment in neurodevelopmental disorder cases (Samocha et al., 2017).

4.3 Results

Throughout the rest of this chapter, I refer to 'bulk' and 'TRAP' RNA-sequencing. The bulk RNA-seq gene sets contain genes expressed from all cell types, derived from the tissue homogenate prior to immunoprecipitation. The TRAP RNA-seq gene sets contain genes expressed specifically from excitatory neurons, derived after immunoprecipitation selection for this specific cell-type.

4.3.1 Pattern of differential expression

Figure 4.3 shows the number of differentially expressed genes in the TRAP- and bulk- RNA seq conditions at each time point. It can be seen that at all timepoints, the number of differentially expressed genes was highest in the TRAP-seq gene-sets, that is, from excitatory neurons, compared to the bulk RNA-seq gene-sets containing all cell types (Figure 4.3A). In the TRAP-seq gene-sets, the highest number of differentially expressed genes was found at 60 minutes, with almost 1500 genes. This was followed by the 120-minute timepoint with just over 1000 genes. There were relatively few differentially expressed genes at 30 minutes. In the bulk RNA-seq condition, most differentially expressed genes were identified at 120 minutes, with an almost 4-fold increase compared to the 30- and 60- minute timepoints combined.

Across conditions, it can be seen that the majority of genes differentially expressed at the 30-minute timepoint are also differentially expressed at the two other timepoints, with only 10% of genes uniquely differentially expressed in TRAP-seq and 14% in bulk-seq at 30-minutes. In the TRAP-seq gene-sets, around one third of genes expressed at 60 minutes were shared with the other timepoints, and just under half of genes differentially expressed at 120 minutes were shared between timepoints (66% and 53% uniquely differentially expressed respectively). In the bulk RNA-seq gene-sets, three quarters of genes at the 60-minute timepoint were shared across timepoints, which reduced to 15% at the 120-minute timepoint. Thus, whilst more genes were differentially expressed in the TRAP-seq gene-set at 120-minutes, there was a more distinct pattern of gene expression in the bulk-seq at this time.

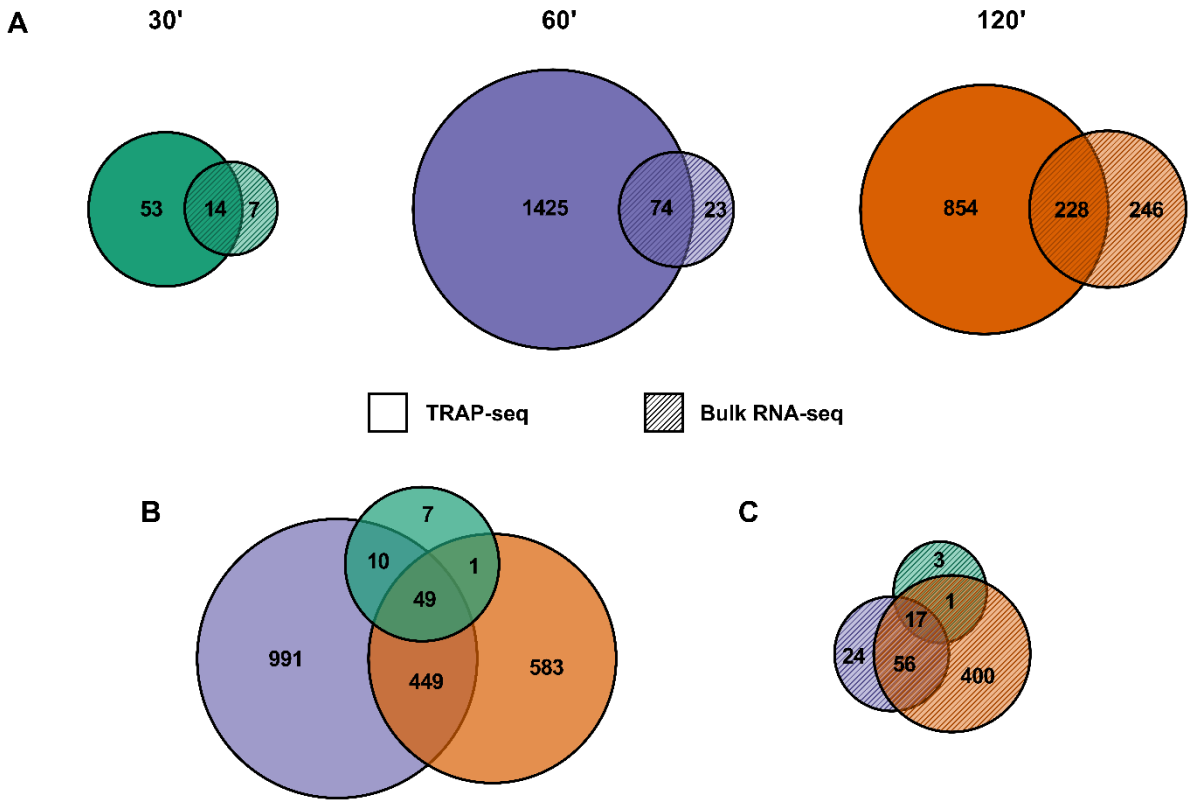


Figure 4.3. *The intersection between genes differentially expressed (FDR < 0.01) in the TRAP and Bulk sequencing gene-sets.* ***A.*** *The number of genes differentially expressed at 30 minutes (green), 60 minutes (purple) and 120 minutes (orange).* ***B.*** *The overlap between genes differentially expressed in the TRAP-seq condition across time points.* ***C.*** *The overlap between genes differentially expressed in the Bulk RNA- seq condition across time points. Solid fill represents the TRAP-seq condition, patterned fill represents the Bulk RNA-seq condition.*

4.3.2 Association with common variation

In order to assess whether there was an enrichment of LTP-related genes in common variation for psychiatric disorder, gene set enrichment analyses were undertaken using MAGMA (de Leeuw et al., 2015), conditioned on all expressed genes to account for known enrichments of brain expressed genes in psychiatric disease. Bonferroni multiple comparison adjustments were applied to account for the gene overlap between timepoints. Single genes that were significant in each association, as outputted by the MAGMA programme, are listed in the Appendix.

There was a significant enrichment of genes identified through TRAP-seq at 60- and 120- minutes and schizophrenia risk, but not 30-minutes, as conferred through common variation (Figure 4.4 and 4.5, 30 minutes: p = 0.638; 60 minutes: p = 0.002, 120 minutes: p = 0.002). The enrichment of bulk RNA-seq gene sets and schizophrenia risk were not significant at any time point (30 minutes: p = 0.635, 60 minutes: p = 0.37, 120 minutes: p = 0.18).

Similarly, there was a significant enrichment of genes identified through TRAP-seq and bipolar risk at 60- and 120- minutes post LTP induction (TRAP-seq: 30 minutes: p = 1.0, 60 minutes: p = 2.2 x 10^{-5}, 120 minutes: p = 0.0073; Bulk RNA-seq: 30 minutes: p= 0.983, 60 minutes: p= 1.0, 120 minutes: p = 1.0). Further, when the two bipolar subtypes were analysed separately, it was found that this enrichment was restricted to bipolar subtype I (Table 4.2).

	Bipolar Disorder subtype	β value (SE)	Adjusted p-value
120- minute TRAP-seq	**Bipolar I**	**0.105 (0.024)**	**0.004**
	Bipolar II	0.007 (0.002)	1.0
120- minute bulk RNA-seq	Bipolar I	0.005 (7.91 x 10 -4)	1.0
	Bipolar II	-0.03404 (- 0.005)	1.0
60- minute TRAP-seq	**Bipolar I**	**0.1394 (0.037511)**	**2.69 x 10 -6**
	Bipolar II	0.0157 (0.004)	1.0
60- minute bulk RNA-seq	Bipolar I	0.0775 (0.0056)	0.898
	Bipolar II	- 0.079 (- 0.005)	1.0
30- minute TRAP-seq	Bipolar I	-0.085 (- 0.005)	1.0
	Bipolar II	- 0.001 (-8.42 x 10 -5)	1.0
30- minute bulk RNA-seq	Bipolar I	0.0476 (0.0016)	1.0
	Bipolar II	0.069 (0.002)	1.0

Table 4.2*. Enrichment for common variation for bipolar disorder, disaggregated by bipolar disorder subtypes (I and II). There was a significant enrichment of genes expressed in CA1 excitatory neurons 120- and 60- minutes after LTP induction in bipolar disorder type I. Bold text indicated statistical significance. P-values were adjusted to account for the significant overlap in genes between the timepoints. SE = Standard error.*

There was no significant enrichment of any gene sets in common variation for autism spectrum disorder (ASD) (TRAP-seq: 30 minutes: p = 0.635, 60 minutes: p = 0.33, 120 minutes: p = 0.445; Bulk RNA-seq: 30 minutes: 0.286, 60 minutes: 0.638, 120 minutes: 0.665) or Alzheimer's disease (AD), indicating potential disorder specificity (TRAP-seq: 30 minutes: p = 1.0 , 60 minutes: p = 1.0, 120 minutes: p = 0.18; Bulk RNA-seq: 30 minutes: 1.0, 60 minutes: 1.0, 120 minutes: 1.0). In addition, there was no significant enrichment of any gene sets in common variation for chronic kidney disease, suggesting that the enrichment was not simply a product of large gene set sizes (TRAP-seq: 30 minutes: p= 0.848, 60 minutes: p = 0.848, 120 minutes: p = 0.635; bulk RNA-seq: 30 minutes: p = 0.878, 60 minutes: 0.635, 120 minutes: 0.665).

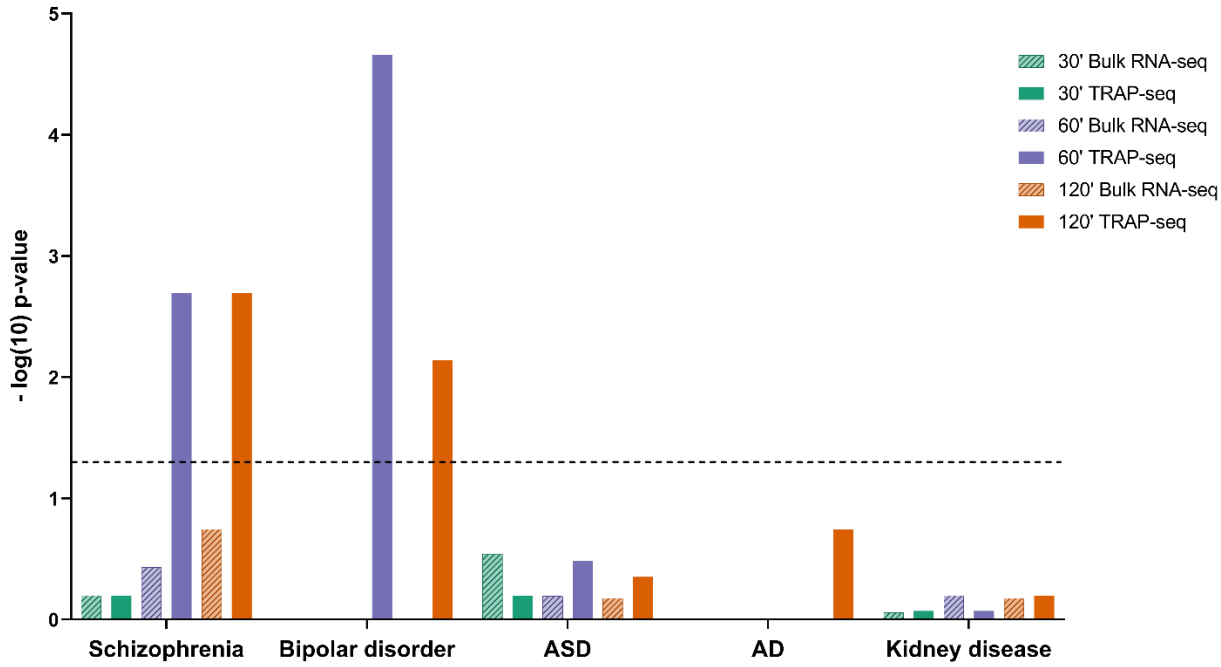


***Figure 4.4**. Gene set enrichment analysis of LTP datasets and common variation for Schizophrenia, Bipolar disorder, ASD, AD and kidney disease. P-values have been log[10] transformed for visualisation purposes, such that -log[10] p-values close to 0 represent untransformed p-values of close to 1. For AD, the majority of gene set enrichment analyses had adjusted p-values of 1.0, and therefore are not visible on the graph. It can be seen that there was significant enrichment of schizophrenia and bipolar gene sets in the 60- and 120- minute TRAP-sequencing conditions, consisting of excitatory neurons. All other conditions were not significant. Dotted line represents p = 0.05 threshold for significance (log transformed).*

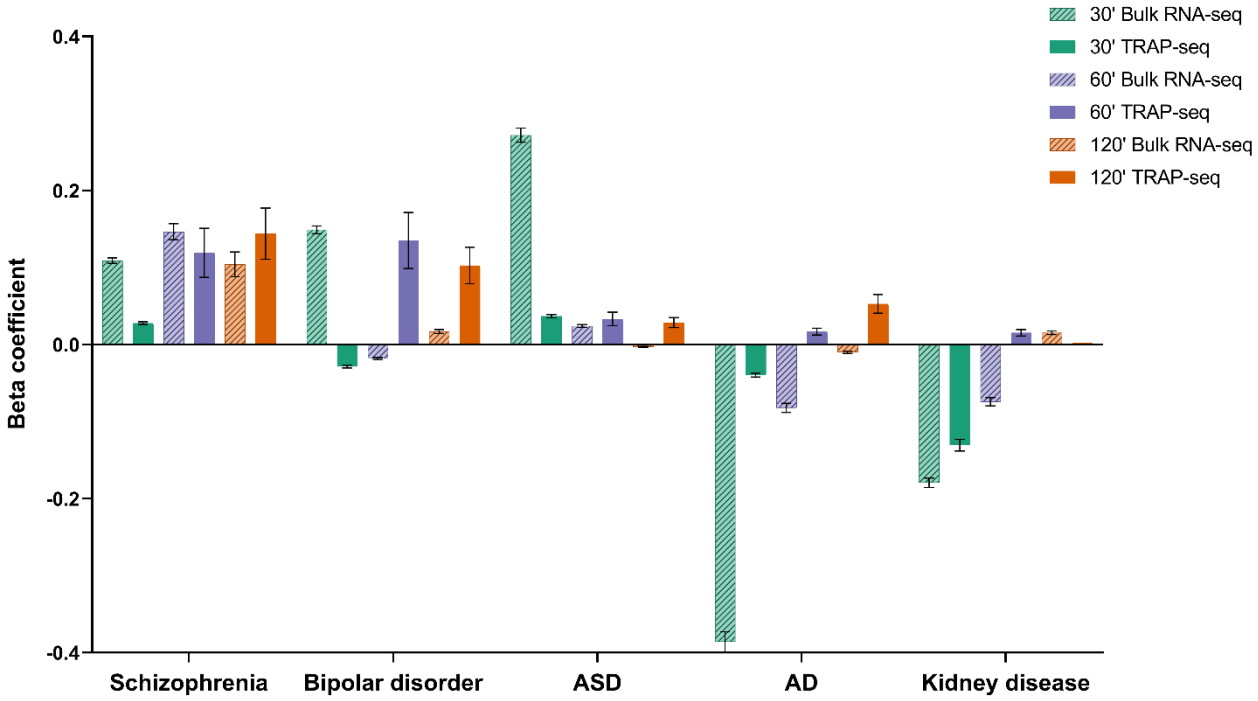


***Figure 4.5**. Effect sizes (β coefficient values) for gene set enrichment analysis of LTP datasets and common variation for Schizophrenia, Bipolar disorder, ASD, AD and kidney disease.*

4.3.2.1 Pathway analysis of significantly associated genes

To understand if these genes potentially driving the enrichment with common variation for schizophrenia and bipolar disorder were enriched in a particular biological pathway, pathway analysis was undertaken. Genes that had significant single-gene p-values in each gene set were taken forward for analysis.

Genes driving the enrichment in schizophrenia in the 120-minute TRAP-seq gene set were enriched in 126 biological GO pathways, 18 of which remained significant after refinement (Figure 4.6). The pathway with the highest odds ratio was “protein localisation to axon” [GO: 0099612], followed by “axon initial segment” [GO:0043194]. General plasticity related pathways, such as “regulation of neuronal synaptic plasticity” [GO:0048168] and “synapse organisation” [GO:0050808], were also significantly enriched. Interestingly, “learning” [GO: 0007612] was also a significantly enriched pathway.

In the 60-minute TRAP-seq gene set, 120 GO pathways were significantly enriched, 21 of which remained significant after refinement. Whilst the genes driving association with schizophrenia at 120 minutes were enriched in more generalise synaptic plasticity processes, those driving association at 60 minutes were enriched in more specific processes such as “neuronal ion channel clustering” [GO:0045161] and “regulation of postsynaptic membrane neurotransmitter receptor levels” [GO:0099072]. Further, “inhibitory synapse” [GO: 0060077] and “GABAergic synapse” [GO:0098982] were also significantly enriched terms.

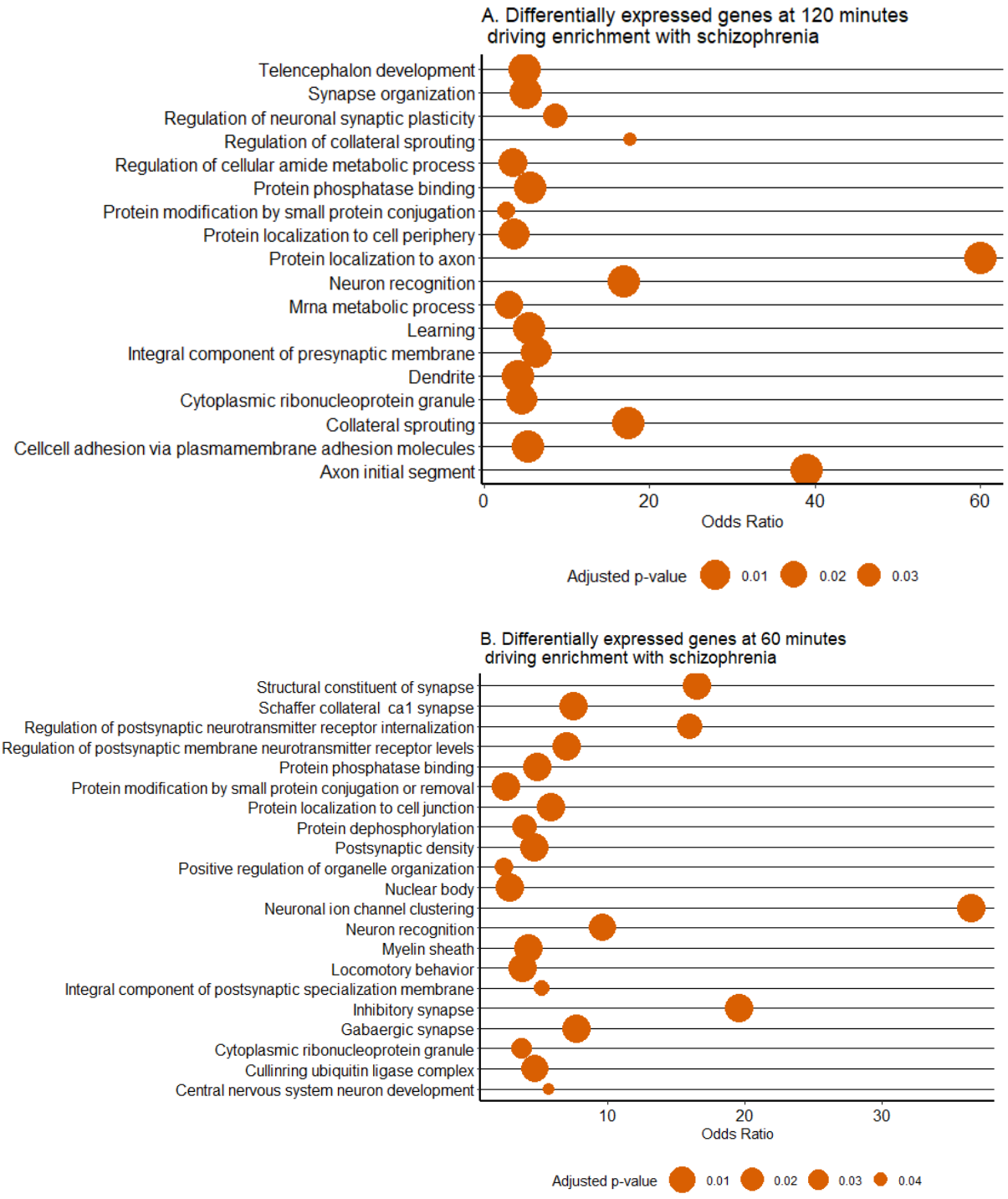


Figure 4.6. *Enrichment of gene ontology (GO) pathways in genes driving enrichment between LTP-TRAP gene sets and common variation for schizophrenia.* ***A.*** *GO pathways enriched in genes driving enrichment at 120-minutes.* ***B****. GO pathways enriched in genes driving enrichment at 60-minutes. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

Genes driving enrichment with bipolar disorder in the 120-minute TRAP-seq gene set were enriched in 87 biological GO pathways, 13 of which survived the refinement procedure (Figure 4.7). 3 learning related pathways were significantly enriched: “learning” [GO:0007612], “memory” [GO:0007613] and “adult behaviour” [GO:0030534]. In addition, 2 transcription related pathways were significantly enriched “RNA polymerase II specific DNA binding transcription factor binding” [GO:0061629] and “chromatin binding” [GO:0003682]. Examining genes driving the enrichment with bipolar disorder type I, 99 pathways were significantly enriched, corresponding to 15 major pathways (Figure 4.8). Again, “learning” [GO:0007612] was significantly enriched, as was “chromatin binding” [GO:0003682].

Genes driving enrichment with bipolar disorder in the 60-minute TRAP-seq gene set were enriched in 87 biological GO pathways, 15 of which survived the refinement procedure. These were similar to those pathways enriched at 120-minutes, including learning and transcription-related pathways. When examining genes driving the enrichment for bipolar disorder type I, 120 pathways were significantly enriched, corresponding to 13 pathways after refinement. Again, many of these overlapped with the enrichment with both bipolar disorder subtypes, including “inhibitory synapse” [GO:0060077], “post synapse” [GO:0098794] and “dendrite” [GO:0030425].

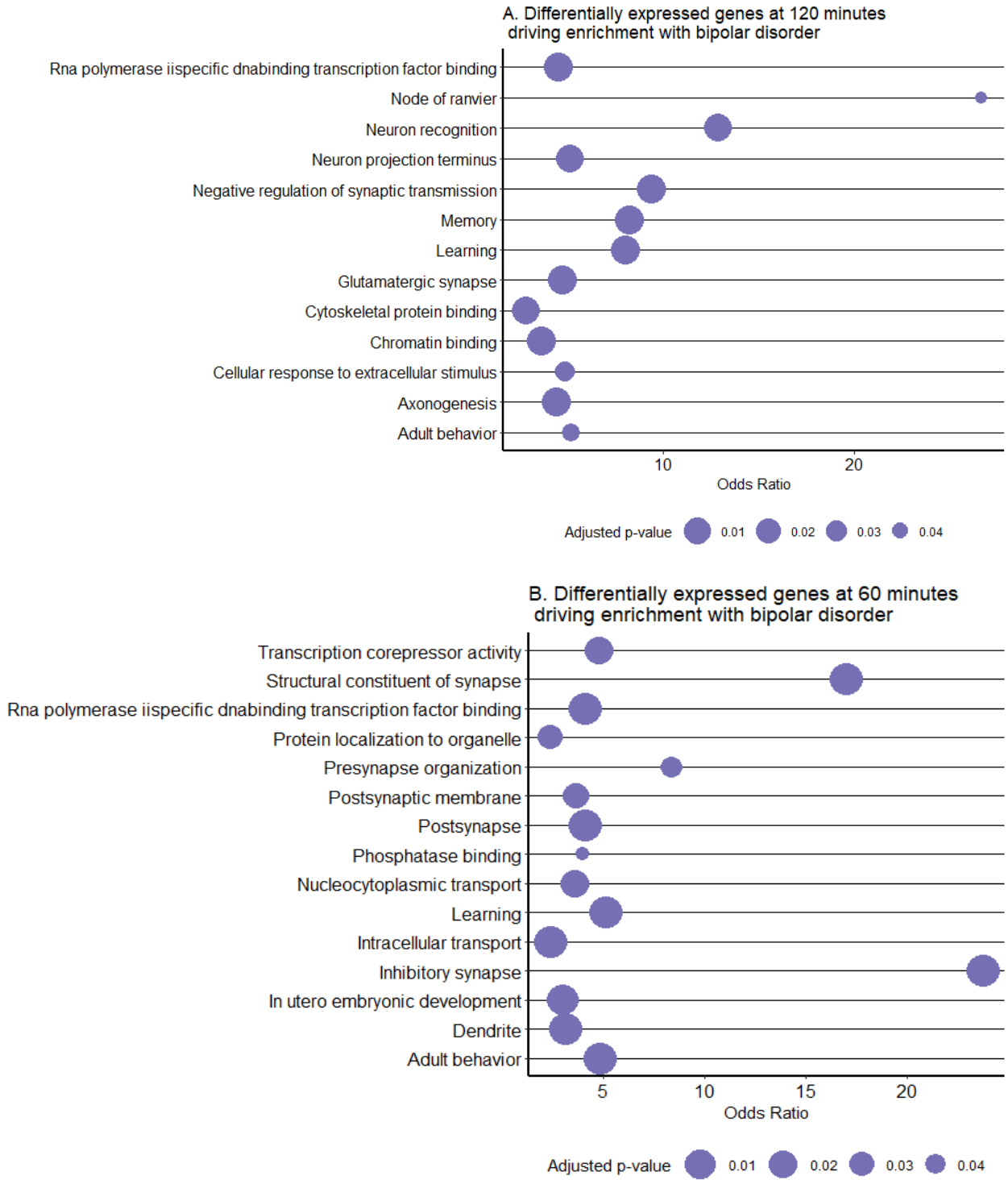


Figure 4.7. *Enrichment of gene ontology (GO) pathways in genes driving enrichment between LTP-TRAP gene sets and common variation for bipolar disorder.* ***A.*** *GO pathways enriched in genes driving enrichment at 120- minutes.* ***B.*** *GO pathways enriched in genes driving enrichment at 60-minutes. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

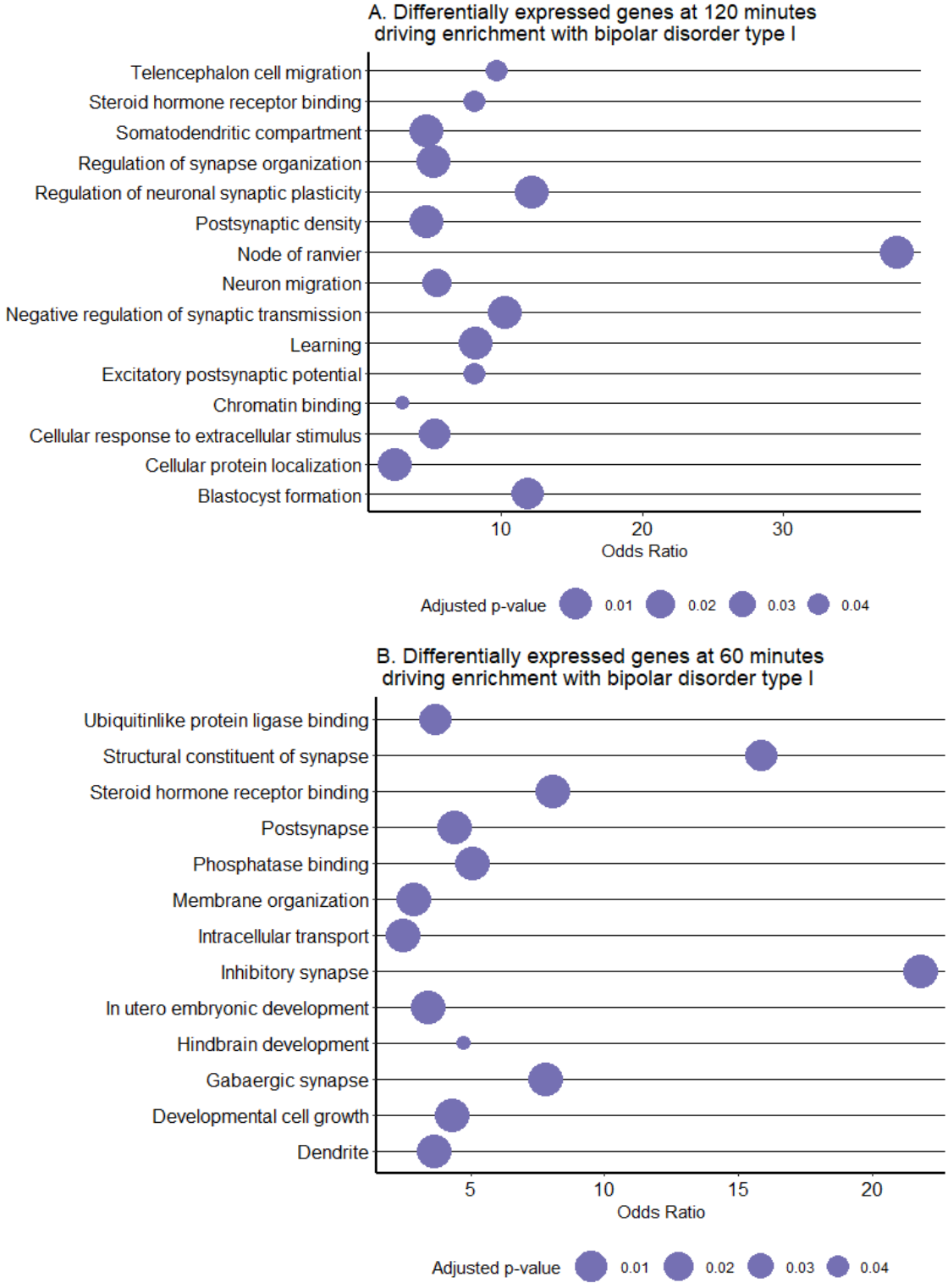


Figure 4.8. *Enrichment of gene ontology (GO) pathways in genes driving enrichment between LTP-TRAP gene sets and common variation for bipolar disorder type I.* ***A.*** *GO pathways enriched in genes driving enrichment at 120- minutes.* ***B.*** *GO pathways enriched in genes driving enrichment at 60-minutes. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

4.3.3 CNV enrichment

In order to investigate the enrichment of LTP gene-sets in schizophrenia-associated CNVs, CNV enrichment analysis was undertaken for all schizophrenia-associated CNVs and for deletions and duplications separately (Figure 4.9). Similar to common variation enrichment, genes expressed 60-minutes post-LTP in CA1 excitatory neurons were found to be enriched in schizophrenia associated CNVs (60-minute TRAP-seq: p= 0.024). However, no further LTP gene-sets were enriched in the combined CNV analysis, including those identified at 120 minutes which were significantly enriched for common variation (30 minute bulk RNA-seq: p = 0.228; 30 minute TRAP-seq: p = 1.0; 60-minute bulk RNA-seq: p = 0.072; 120 minute bulk RNA-seq: p = 0.678; 120 minute TRAP-seq: p = 1.0).

Deletion and duplication CNVs were then analysed separately to investigate whether these enrichments were driven by a particular subset of CNVs. There was no significant enrichment of any LTP gene-sets in deletion CNVs (30-minute bulk RNA-seq: p = 0.174; 30-minute TRAP-seq: p = 1.0; 60-minute bulk RNA-seq: p = 1.0; 60-minute TRAP-seq: p = 0.972; 120-minute bulk RNA-seq: p = 0.852; 120-minute TRAP-seq: p = 1.0). For duplication CNVs, there was a selective enrichment for LTP genes expressed in all cell-types at 60- minutes (60-minute bulk RNA-seq: p = 0.024), and this approached significance at 30-minutes (30-minute bulk RNA-seq: p = 0.057). There was no enrichment of duplication CNVs in any other gene-set (30-minute TRAP-seq: p = 1.0; 60-minute TRAP-seq: p = 0.588; 120-minute bulk RNA-seq: p = 1.0; 120-minute TRAP-seq: p = 1.0).

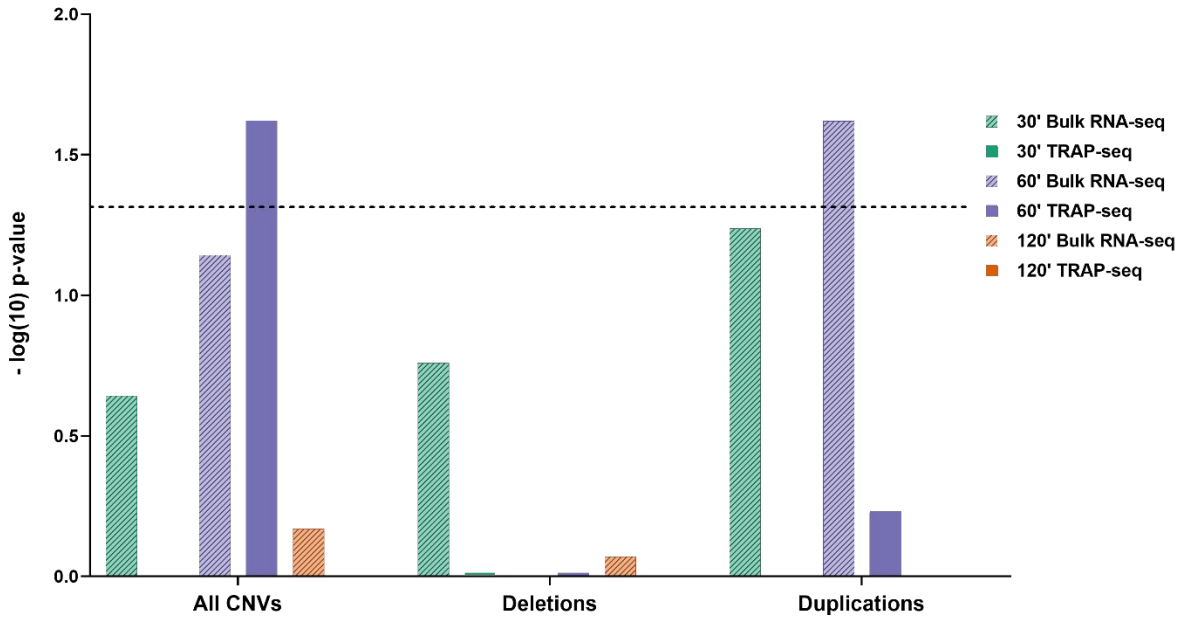


***Figure 4.9**. Enrichment of LTP gene-sets in schizophrenia-associated CNVs. P-values have been log[10] transformed for ease of visualisation, -log[10] p-values close to 0 represent untransformed p-values close to 1. P-values shown here have undergone permutation correction to account for potential background enrichment, and post-hoc Bonferroni correction to adjust for overlap within the gene-sets. When deletions and duplicates were analysed together (all CNVs), there was a significant enrichment in the 60-minute TRAP-seq gene set. When CNV types were analysed separately, there was an enrichment in the 60-minute bulk RNA-seq gene set only. Adjusted p-values = 1.0 for several gene sets, and thus are not visible on the graph. Dotted line represents p = 0.05.*

4.3.4 Ultra-rare coding variant enrichment

Finally, enrichment of ultra-rare coding mutations in the LTP gene sets was examined. Loss-of-function protein truncating variants (PTVs) and missense mutations with an MPC score of greater than 2 were tested. There was no significant enrichment of these variants of either mutation class in the TRAP- or bulk-seq conditions, when corrected for multiple comparisons (Table 4.3).

LTP gene set	Schizophrenia ultra-rare coding variant enrichment			
	Mutation class	**Observed/expected**	**Adjusted p-value**	**Rate ratio (95% confidence intervals)**
30-minute bulk	PTV	1/0.175	0.788	4.56 (0.115 - 25.6)
	Missense	1/0.324	0.834	3.09 (0.0776 - 17.5)
30-minute TRAP	PTV	2/1.75	1	0.912 (0.11 - 3.32)
	Missense	2/1.58	1	1.27 (0.152 - 4.66)
60-minute bulk	PTV	5/1.93	0.39	2.08 (0.672 - 4.9)
	Missense	2/1.25	0.56	1.61 (0.193 - 5.9)
60-minute TRAP	PTV	50/32.2	0.39	1.27 (0.925 - 1.72)
	Missense	31/25.9	0.56	1.24 (0.808 - 1.86)
120-minute bulk	PTV	13/6.51	0.318	1.61 (0.85 - 2.79)
	Missense	4/7.38	0.398	0.527 (0.142 - 1.38)
120-minute TRAP	PTV	52/29.9	0.062	1.45 (1.06 - 1.95)
	Missense	27/21.4	0.398	1.31 (0.831 - 2.01)

Table 4.3. *LTP gene sets were tested for enrichment of protein truncating variants (PTVs) and missense variants with an MPC score of greater than 2 from schizophrenia trios. There was no significant enrichment when corrected for multiple comparisons (Bonferroni adjustment).*

4.4 Discussion

The aim of the current chapter was to quantify patterns of gene expression following long term potentiation (LTP), and test for association with genetic variants from patients with schizophrenia, schizophrenia-related disorders, and appropriate control disorders. Gene-sets were derived from both bulk RNA-sequencing, comprising of gene expression profiles from all cell types, and TRAP-sequencing, comprising of expression profiles specifically from CA1 excitatory pyramidal neurons. The combination of this, and multiple experimental timepoints, allowed for an in-depth investigation into the manner in which schizophrenia risk variants impact on LTP genes.

4.4.1 Over 2,000 differentially expressed genes identified using TRAP-seq after induction of LTP

Candidate gene and micro-array studies have identified a number of genes involved in LTP, including zif268 (Cole et al., 1989) and BDNF (Patterson et al., 1992), and have identified pathways including transmitter transport, arc interactors and regulation of the cytoskeleton as being involved (Thompson et al., 2003). Here, using an FDR threshold of 0.01, 501 differentially expressed genes were identified through bulk RNA-seq, and over 2,000 through TRAP-seq, summed across timepoints. It should be noted that the differentially expressed genes described below are subject to the differential expression method used, and as such may not represent the "true" state of play. The increase in differentially expressed genes identified through TRAP-seq was noted by the original authors, who highlighted that there is a dilution effect at play in bulk RNA-seq, which can diminish the ability to detect relevant differentially expressed transcripts (Chen et al., 2017).

Taking each time-point individually, the highest number of differentially expressed genes was identified at 60 minutes indicating that, at this time-point, gene expression is particularly active. Almost 94 % of differentially expressed genes at this time-point were identified through TRAP-seq, indicating that excitatory neurons were driving this increase in gene expression. In the original study, which used a different differential expression criteria (logFC > 0.4, FDR < 0.1), the number of differentially expressed genes was highest 120-minutes post LTP-induction. The addition of a logFC criteria limits the identification of differentially expressed transcripts, and thus may have removed some genes that contribute to the nuanced expression of LTP. In addition, higher logFC is not necessarily indicative of being more biologically relevant. Although in some circumstances it can be useful to limit the analysis in this way, for example when trying to identify specific transcriptional markers of certain cell populations, for the current purpose this extra criterion was deemed unnecessary. The finding that gene expression decreased at 120- minutes compared to 60-minutes is

broadly consistent with previous literature which suggests that gene expression occurs in a relatively early time window (Fonseca et al., 2006). However, although comparatively fewer genes were differentially expressed compared to 60-minutes, it was still in excess of 1,300 genes. Fewer than 100 genes were found to be differentially expressed 30-minutes post-LTP induction. Given that 30-minutes likely represents an early phase of L-LTP, and that in preliminary experiments this time-point was the earliest in which the immediate early genes *arc* and *c-Fos* were detected (Chen, 2016), this is not unexpected.

4.4.2 Significant enrichment of LTP gene-sets in common variation for schizophrenia and bipolar disorder

Previously, pathway analysis of common variants in schizophrenia found an association between schizophrenia risk and genes encoding calcium channels, dopamine receptors and those involved in glutamatergic neurotransmission (Schizophrenia Working Group of the Psychiatric Genomics et al., 2014). Here, using LTP gene-sets derived from a functional experiment, I confirm and extend these findings. Firstly, it was found that there was an enrichment of LTP associated genes and schizophrenia risk, as conferred through common variation, at 60- and 120- minutes post LTP-induction. This enrichment was only identified via cell-type specific TRAP-seq, suggesting that common variation for schizophrenia has a selective impact on plasticity associated genes in CA1 excitatory neurons. This selective enrichment again highlights the advantages of using cell-specific methods, as bulk RNA-sequencing of all cell types does not show this enrichment. These results are consistent with previous literature suggesting that CA1 pyramidal cells are impacted by schizophrenia variants. For example, a recent study examining schizophrenia-associated regulatory SNPs found that CA1 pyramidal cells had the highest number of genes targeted by such SNPs (Huo et al., 2019). However, it cannot be ruled out that other cell types may also be impacted, and further work investigating this with other TRAP-seq drivers would be informative.

In order to further investigate the pattern of LTP gene-set enrichment, gene-set analyses were conducted for other psychiatric disorders. Enrichment was also found for genes expressed in excitatory hippocampal neurons 120- and 60- minutes following LTP induction and bipolar disorder. Further, when the subtypes were analysed separately, it was found that this enrichment was restricted to bipolar I. Bipolar type I is characterised by periods of mania, that is, abnormally and persistently elevated mood and abnormal goal-directed behaviour, with or without depressive episodes (DSM-V (APA, 2013)). Clinically, bipolar disorder type I is more strongly associated with schizophrenia, whereas bipolar disorder II is more associated with major depressive disorder (Stahl et al., 2019;Mullins et al., 2021). As such, that enrichment of LTP genes in CA1 excitatory neurons is

found in schizophrenia and bipolar type I, but not type II, may have been expected. Recently, visual cortex plasticity has been examined using visually evoked potentials (VEP) in patients with bipolar disorder (Valstad et al., 2021). It was found that patients had reduced modulation of the N1b component of the VEP, which has previously been implicated as a candidate for NMDA receptor-dependent LTP-like plasticity in the visual cortex. Further, common variants associated with bipolar disorder have recently been found to be enriched in genes expressed in the hippocampus, including hippocampal pyramidal neurons, and in GO terms relating to synaptic signalling (Mullins et al., 2021). Thus, the current finding adds to the growing literature implicating synaptic processes in bipolar disorder aetiology.

It has previously been suggested that synaptic processes may be implicated in ASD, however I found no evidence of an enrichment of LTP genes in ASD risk, as conferred through common variation. Previously, the link has been investigated through the genetic mutation of synaptic genes, such as *SHANK3* and *FMR1*, and the subsequent finding that animals with altered gene dosage have social deficits and/or impaired LTP (Wang et al., 2011;Lauterborn et al., 2007). It could be that LTP genes are enriched in rare variation, for example in CNVs in which altered gene dosage is more characteristic, however the limited availability of large-scale data makes this difficult to establish at the present time. Finally, there was no association between LTP gene-sets and common variation for chronic kidney disease, suggesting that the previous enrichments were not simply an artefact of large gene-set sizes. The fact that the number of cases for chronic kidney disease were similar to that of schizophrenia and bipolar cases, means that they should be similarly matched in power to detect an association, if present (chronic kidney disease *n* cases: 41,395; schizophrenia *n* cases: 40,675; bipolar disorder *n* cases: 41,917).

Whilst effect sizes, as illustrated by β coefficients from the MAGMA output, were not particularly large, this does not detract from the present results. Given that common variants tend to have small effect, and are thought to exert their effect through multiplicative action of multiple variants, large effect sizes would not necessarily be expected.

4.4.3 Pathway analysis of genes driving enrichment with disease revealed role in synaptic plasticity processes

Next, in order to understand whether the genes in each gene-set driving the association were enriched in biological pathways, pathway analysis was undertaken using those genes in each LTP set with significant single gene p-values in the disease enrichment analysis. These were the genes driving enrichment between: LTP-120 and schizophrenia, LTP-60 and schizophrenia, LTP-120 and

bipolar disorder, LTP-60 and bipolar disorder, LTP-120 and bipolar disorder type I, LTP-60 and bipolar disorder type I. Whilst the exact pathways enriched in each of these gene sets was different, the majority were either related to synaptic plasticity or related to synaptic structures, such as "dendrite", "structural constituent of the synapse", "node of Ranvier" and "post-synapse", both 60- and 120- minutes after LTP induction. This is unsurprising, given that the genes were derived from a functional LTP experiment, and that LTP has been shown to induce structural changes at the synapse such as AMPA receptor trafficking, increased spine density and dendritic spine enlargement (Watson et al., 2016;Harris, 2020). Further, synaptic alterations such as reduced dendritic spine density and reduced dendritic arborisation have been identified in post-mortem brains from patients with schizophrenia (Glantz and Lewis, 2000;Broadbelt et al., 2002;Sweet et al., 2009), and reduced dendrite length has been reported in patients with bipolar disorder (Konopaske et al., 2014). As such, it is likely that the gene expression changes seen at 60- and 120- minutes post LTP induction have relevance to schizophrenia and bipolar disorder. Learning and memory associated GO terms were also enriched in gene sets associated with both schizophrenia and bipolar disorder, further supporting the notion that these processes are impacted by schizophrenia and bipolar disorder risk.

GO terms relating to inhibitory synapses were also found to be significantly enriched in these gene sets. Whilst this protocol specifically enriches for transcripts from excitatory neurons, it does not mean that these genes are always exclusively expressed in this neuronal cell type. For example, it has been found that genes regulated by MEF2 are expressed in both excitatory and inhibitory neurons throughout the lifespan (Lyons et al., 2012;Shalizi and Bonni, 2005). As such, it would not be expected that genes expressed during LTP in excitatory hippocampal neurons would be exclusively expressed in this cell type. Of further interest is that inhibitory neurons have been linked to schizophrenia pathophysiology. Decreased inhibitory drive from interneurons onto post-synaptic pyramidal cells has been postulated to be a core feature of schizophrenia (Rotaru et al., 2012), and reduced GABAergic neurotransmission has been found in a rat model of schizophrenia (Selten et al., 2016). Thus, gene expression of inhibitory neurons and enrichment in psychiatric disorders may be an interesting avenue to explore in future work.

4.4.4 Significant enrichment of LTP gene-sets in rare copy number variation

In order to investigate the enrichment of LTP gene-sets in schizophrenia risk further, I examined enrichment in rare CNVs. Previously, schizophrenia-associated CNVs have been found to be enriched for genes involved in synaptic plasticity, including Arc-interactors, NMDA receptor genes, and those associated with abnormal synaptic transmission (Kirov et al., 2012;Pocklington et al., 2015). In combined analyses of both deletion and duplication CNVs, an enrichment of LTP genes expressed at

60 minutes was found in TRAP-seq, containing excitatory neurons. This mirrors what was found in the common variation analysis. In separate analyses of CNV subtypes, there was no enrichment of any gene-set in deletion CNVs. In duplication CNVs, there was an enrichment of LTP genes expressed in all cell-types, but no specific excitatory neuron enrichment, at 60-minutes. The finding that genes expressed at 60-minutes in CA1 excitatory neurons were enriched in combined analyses, but not in sub-type specific analyses, indicates that the enrichment is not driven by a particular sub-type of CNV in this instance.

Taken together, these results suggest that genetic risk for schizophrenia, as conferred through CNVs, impacts a different subset of plasticity-associated genes compared to common variation. Firstly, the results suggest that the genes particularly enriched are those expressed at 60-minutes, rather than 120-minutes. Whilst this was also the largest gene-set, permutation analyses generate random gene-sets of the same size and then test for enrichment. Correspondingly, if gene-set size was the driving factor, you would expect a corrected p-value greater than the significance threshold, which was not the case here. Secondly, there was a particular enrichment of genes identified in bulk RNA-seq in duplication CNVs. Bulk RNA-seq contains genes expressed by all cell-types and so this indicates that schizophrenia-associated duplication CNVs may particularly impact on plasticity-related genes expressed by cell-types other than CA1 excitatory neurons.

4.4.5 No enrichment of ultra-rare coding variants

Recently, exome sequencing studies of patients with schizophrenia have identified ultra-rare protein damaging variants (Rees et al., 2019;Rees et al., 2021;Singh et al., 2022). I investigated enrichment of LTP gene-sets in exome sequencing data from just over 3,000 schizophrenia trios (Rees et al., 2021), and found no significant enrichment after correcting for multiple comparisons. The enrichment of genes expressed in excitatory neurons 120- minutes after LTP induction approached significance (corrected p-value 0.062; uncorrected 0.0155), mirroring the results found for common variation. This is consistent with previous results which found an enrichment of ultra-rare protein coding mutations in protein coding genes impacted by common variation, as identified by GWAS (Singh et al., 2022).

4.4.6. Consideration of the effect of forskolin administration

In the present chapter, LTP was chemically induced using forskolin, a cAMP activator. Whilst exposing cells to a 100 µM dose of forskolin for 24 hours has previously been shown not to impact on cell viability (Awale et al., 2022), there is limited clinical literature about the off-target effects of forskolin. However, administering octopamine, another compound which increases cAMP activation,

to hippocampal neurons has been shown to ameliorate sleep deprivation associated memory deficits in mice (Havekes et al., 2014), which may indicate an enhancement, rather than a detriment, of increased cAMP. However, it cannot be completely ruled out that off-target effects are driving the enrichment with schizophrenia and bipolar disorder, rather than the LTP processes themselves. In order to attempt to disentangle the effects of forskolin from the effects of LTP induction, the experiment could be repeated with a different compound which induces LTP, such as exogenous L-glutamate, which has been shown to induce LTP in hippocampal neuronal cultures (Molnár, 2011). If the enrichment with schizophrenia and bipolar risk variants is still found, it could be concluded the association is driven by LTP processes, rather than off-target effects of forskolin administration. Alternatively, a dose-dependent study of forskolin could be undertaken, whereby slices were exposed to a concentration of forskolin which does not induce LTP, or induces LTP to a lesser extent. If the association with disease risk was still evidence, it could be concluded that off-target effects of forskolin administration was driving this enrichment.

It has previously been found that clozapine, an atypical antipsychotic used for treatment-resistant schizophrenia, acts to reduce cAMP activation in post-mortem hippocampus from patients with schizophrenia, the opposite effect of forskolin (Marazziti et al., 2014). The present study examines the genes significantly differentially expressed following induction of LTP with forskolin, containing both up- and down-regulated genes. Direction of fold-change was not taken into consideration as part of the disease association analysis. Thus, it may be that genes whose expression is induced by forskolin administration are impacted by clozapine in the opposite direction, and thus SNPs impacting those genes would contribute to the association between LTP induced genes and disease risk.

4.4.7 Summary

In conclusion, I found an enrichment of common variation for schizophrenia and bipolar disorder in gene sets expressed in CA1 excitatory neurons 60- and 120- minutes post LTP induction. Further, I found an enrichment of rare variation in genes expressed in CA1 excitatory neurons 60 minutes post LTP induction, and an enrichment of genes impacted by duplication CNVs 60 minutes post LTP induction in bulk RNA-sequencing data. Thus far, I have demonstrated that few genes are significantly differentially expressed at retrieval when analysed through bulk RNA-seq. Further, in the current chapter I have demonstrated that analysing cell-type specific gene expression patterns not only increases the number of differentially expressed genes found, but also shows divergent patterns of enrichment for disease risk. In the next chapter, I combine and build upon these findings

by applying cell-type specific TRAP-sequencing to examine gene expression following acquisition of contextual fear conditioning.

Chapter 5: Gene expression profiles in hippocampal excitatory neurons during consolidation of contextual fear conditioning (CFC)

5.1 Introduction

In the previous chapter, it was shown that genes associated with schizophrenia and bipolar disorder were enriched in gene sets obtained from excitatory neurons 2 hours after chemical LTP induction. Given that LTP is thought to underlie consolidation processes, and the increased specificity shown with TRAP-seq compared to bulk-seq, in this chapter I used TRAP-seq to examine the gene expression profile of hippocampal excitatory neurons 2- and 5- hours after acquisition of CFC, during the consolidation window.

5.1.2 Molecular processes underlying consolidation

Consolidation is the process of memory stabilisation, and occurs in the minutes-to-hours following acquisition of a learning event. After acquisition, initial short-term memories (STM) are subject to disruption, and as such consolidation is vital to supporting the formation of long-term memories (LTM).

The process of consolidation requires *de novo* protein synthesis and gene transcription (reviewed in (McGaugh, 2000), (Kandel, 2001)). For example, it has been shown that inhibiting protein synthesis prior to CFC impaired long-term, but not short-term, memory for the context-shock association (Schafe et al., 1999). In addition, inhibiting mammalian target of rapamycin (mTOR), which regulates protein synthesis, immediately after CFC training led to a reduction in the expression of fear behaviour (Gafford et al., 2011). Similarly, inhibiting *de novo* gene expression with RNA polymerase inhibitors also prevents the consolidation of associative learning paradigms. Using a one-trial inhibitory avoidance paradigm, in which rodents received a footshock when they stepped down off a platform, Igaz and colleagues demonstrated that intrahippocampal infusion of an RNA polymerase II

inhibitor immediately after conditioning led to a reduced latency to step down to the platform in a LTM trial 24 hours later. Further, they demonstrated that 2 periods of gene expression were required for LTM consolidation, one immediately following training and one 3-6 hours later (Igaz et al., 2002).

5.1.3 Consolidation and the hippocampus

The hippocampus was first associated with memory consolidation in the 1950s when patient H.M., whose medial temporal lobe resection surgery included the hippocampal formation, demonstrated memory consolidation impairments (Scoville and Milner, 1957;Corkin, 1984). Since then, the involvement of the hippocampus in consolidation has been well documented (reviewed in (Squire et al., 2015)), including the consolidation of contextual fear memories. Bilateral hippocampal lesions made 1 day after acquisition of CFC have been shown to diminish expression of conditioned fear, suggesting interruption to the consolidation process (Kim and Fanselow, 1992). This was further demonstrated in research by Phillips and LeDoux, in which dorsal hippocampal lesions impaired contextual fear conditioning, but not cued fear conditioning (Phillips and LeDoux, 1992a).

In addition to lesion studies, the involvement of the hippocampus in CFC has been demonstrated through expression of immediate early genes (IEG). IEGs, such as *c-Fos*, *Arc* and *Egr1* (*Zif268*), are rapidly upregulated after induction of neuronal activity, either through electrophysiological stimulation or after learning (Guzowski et al., 1999;Guzowski et al., 2006);reviewed in (Minatohara et al., 2016). *Arc* mRNA has been shown to be increased in the dorsal and ventral hippocampus after CFC (Czerniawski et al., 2011). Similar results have been found for other IEGs, including *Egr1* (Schreiber et al., 2014) and *c-Fos* (Huff et al., 2006).

5.1.4 Consolidation and Long-term potentiation (LTP)

One of the most studied forms of long-term potentiation (LTP) in the hippocampus is that which occurs in the CA3 to CA1 Schaffer collateral pathway, which is dependent on NMDA receptors (Collingridge et al., 1983). Upon depolarisation of the post-synaptic neuron via AMPA receptors, the magnesium block is released from NMDA receptors, leading to influx of calcium ions (Seeburg et al., 1995). This triggers multiple downstream signalling cascades, resulting in cellular and structural changes which support LTP.

Consolidation of fear memories and LTP have been shown to share similar molecular mechanisms (reviewed in (Martin and Morris, 2002)). For example, inhibiting NMDA receptors with APV immediately following inhibitory avoidance (IA) significantly decreased the latency to step down to the shock-associated platform, indicating impairment in consolidation of the fear association

(Cammarota et al., 2000). Similarly, intrahippocampal infusion of APV prior to CFC training resulted in a lack of fear-related freezing behaviour upon re-exposure to the context (Athos et al., 2002). Further, staining revealed that APV blocked the cre-mediated gene expression seen in control animals, suggesting NMDA receptors are required both for the expression of contextual fear and cre-mediated gene transcription. In a series of experiments, using an IA task as the learning paradigm, Whitlock and colleagues demonstrated that fear learning induced LTP in CA1 (Whitlock et al., 2006). Firstly, it was demonstrated that IA training induced increased phosphorylation of AMPA receptor subunit GluR1 at Serine^{831}, a marker of LTP (Lee et al., 2000). Secondly, IA led to an increase of AMPA receptors at synapses, measured through immunoblotting of AMPA receptor subunits GluR1 and GluR2 in synaptoneurosome fractions. Finally, it was found that IA, but not presentation of the apparatus or shock alone, produced enhanced field excitatory post-synaptic potentials in activated-CA1 Schaffer collateral neurons. Taken together, these results provide evidence that consolidation of fear memories and LTP share underlying molecular mechanisms.

5.1.5 Chapter aims

The aim of this chapter was to explore the gene expression profile induced during the consolidation window following acquisition of CFC in excitatory hippocampal neurons using TRAP sequencing (TRAP-seq). Previously, I found few differentially expressed genes after retrieval of conditioned fear using bulk RNA-sequencing (bulk RNA-seq) (Chapter 3) and demonstrated that cell-type specific TRAP-seq allows the identification of an increased number of differentially expressed genes compared to bulk RNA-seq (Chapter 4). Thus, in the present chapter I used TRAP-seq methodology to examine gene expression profiles in excitatory hippocampal neurons. Given that I previously found an enrichment of LTP gene sets in common and rare variants identified from patients with schizophrenia, and that LTP processes are thought to underlie consolidation, I also aimed to test for the enrichment of consolidation gene sets in schizophrenia.

5.2 Methods

5.2.1 Contextual fear conditioning

Contextual fear conditioning was conducted as described in the General Methods, section 2.2.2.2.

5.2.2 Dissection

2 or 5 hours after training, mice were culled by inhalation of carbon dioxide, as described in General Methods, section 2.2.4. After death was confirmed, mice were decapitated and a vertical cut was made in the skull. The skull was removed with curved forceps, taking care to sever blood vessels to ensure the brain was not damaged. Once exposed, brains were removed and placed on a thermal block on wet ice. Prefrontal cortex was removed using a razor blade and snap frozen on dry ice. The cerebellum was removed to allow access to the posterior portion of the cortex. Using closed curved forceps, the brain was cut down the midline a few millimetres to allow the cortex to be rolled. Using 2 pairs of closed curved forceps (one a the posterior and anterior end or the brain), the cortex was slowly rolled away from the rest of the tissue. The hippocampus can be distinguished in texture and colour from the cortex and, using a gentle rolling motion, comes away from the cortical tissue. The hippocampus was then removed and snap frozen on dry ice. This process was repeated for the other hemisphere of the brain. In this way, the whole hippocampus was removed, including dorsal and ventral portions (Figure 5.1). Once frozen, brain regions were stored at -75 °C until further use.

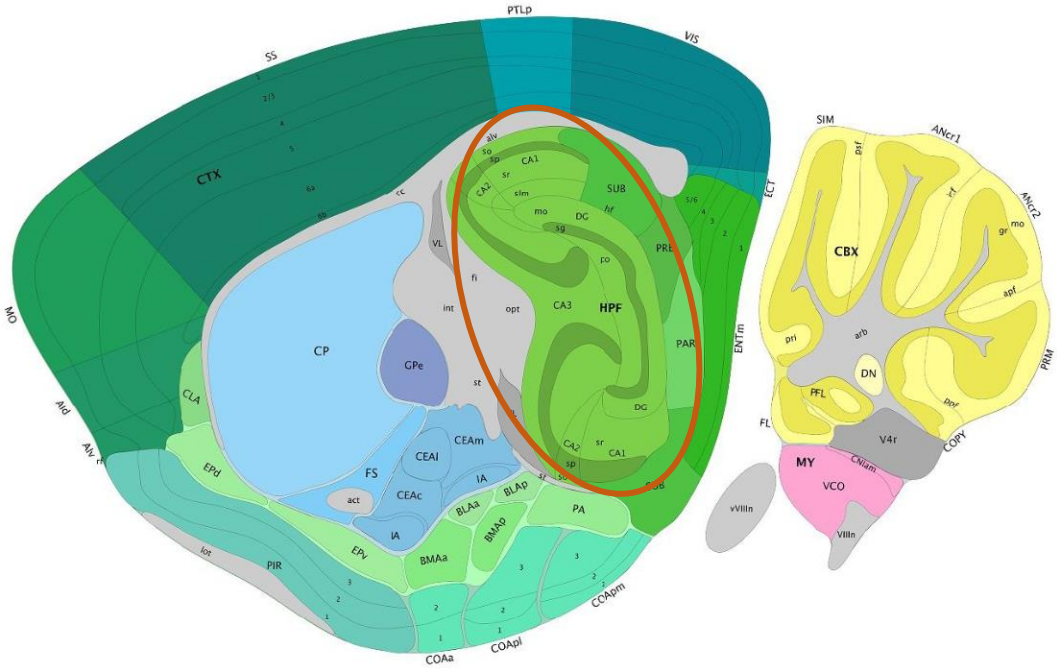


Figure 5.1. *Sagittal plane of mouse brain from (Allen Reference Atlas). Hippocampal dissection included removal of the hippocampal formation, displayed here in pale green and schematically indicated by an oval.*

5.2.3 Immunoprecipitation (IP)

In order to look at cell-type specific gene expression, immunoprecipitation was performed on the hippocampi of RiboTag x CaMKIIα-cre mice. The protocol was followed as directed in Sanz et al., 2019, as follows. Homogenisation buffer (see Table 5.1) was prepared fresh and chilled on ice at 4 °C. Two hippocampi (one per animal, both hemispheres), were homogenised in 1 mL of ice-cold homogenisation buffer using a Dounce homogeniser (Sigma [cat number], ~35 strokes pestle A followed by ~35 strokes pestle B). Homogenate was transferred to a clean microcentrifuge tube, and centrifuged at 10,000 x *g* for 10 minutes at 4 °C. 65 µL of homogenate was removed for input analysis (Total RNA) and stored at -75 °C until further use. One sample from the 2-hour timepoint did not yield enough homogenate to take a total RNA sample.

Homogenisation buffer	
Chemical	*Supplier [catalogue number]*
50 mM Tris-cl, pH 7.4	SLS [LZ51237]
100 mM KCl	Merck [60142-100ML-F]
12 mM $MgCl_2$	Merck [63069-100ML]
1% Nonidet P-40 substitute	Merck [11332473001]
1 mM DTT	Merck [43816-10ML]
200 units/mL RNasin	Promega [N2111]
1 mg/mL Heparin	Merck [H3393-50KU]
100 µg/mL Cycloheximide	Merck [C7698-1G]
1 x Protease inhibitor mixture	Merck [P8340-1ML]

High-salt buffer	
Chemical	*Supplier*
50 mM Tris, pH 7.4	SLS [LZ51237]
300 mM KCl	Merck [60142-100ML-F]
12 mM $MgCl_2$	Merck [63069-100ML]
1% Nonidet P-40 substitute	Merck [11332473001]
0.5 mM DTT	Merck [43816-10ML]
100 µg/mL Cycloheximide	Merck [C7698-1G]

Table 5.1. *Solutions for homogenisation- and high-salt buffers. These solutions were prepared by adding defined volumes of chemicals to nuclease-free water.*

800 µL of the remaining homogenate was transferred to a pre-chilled microcentrifuge tube, and anti-HA antibody (BioLegend UK [901516]) was added in 1:150 ratio. The homogenate was incubated with the antibody for 4 hours at 4 °C on a microtube rotator (end-over-end mixing).

Immediately prior to use, the A/G magnetic protein beads (ThermoFisher Scientific [88803]) were resuspended by gently vortexing, and 200 µL transferred to a clean microcentrifuge tube (one tube per sample). The tube was placed on a magnetic stand (BioRad Laboratories [1614916]) on ice to collect the beads, and the storage buffer removed and discarded. 400 µL of ice-cold homogenisation buffer was added to the beads, and gently pipetted to resuspend. Beads were placed on an end-over-end rotator and washed for 5 minutes at 4 °C. Tubes were briefly spun and the beads were then collected using the magnetic stand, and homogenisation buffer removed and discarded. The

lysate-antibody solution was added to the beads, resuspended by gentle pipetting, and rotated at 4 °C overnight (approximately 15 hours).

The next day, high-salt buffer was prepared according to the recipe in Table 5.1 and chilled on ice at 4 °C. The tubes containing the lysate-antibody solution were placed on the magnetic stand on ice, and the supernatant removed and stored at -75 °C. 800 µL high-salt buffer was added and beads were briefly and gently resuspended. Tubes were placed on and end-over-end rotator for 5 minutes to wash the beads, before being placed on the magnetic stand and the solution removed. This washing process was repeated 2 additional times, so that the beads had been washed 3 times in total. After the 3rd wash, beads were resuspended in 800 µL fresh high-salt buffer, mixed on a rotator for 5 minutes before being transferred to a clean microcentrifuge tube. Tubes were placed on a magnetic stand and the high-salt buffer was carefully removed. 350 µL RLT buffer (Qiagen, from RNeasy micro kit [74004]), supplemented with β-Mercaptoethanol, was added to the beads and vortexed for 30 seconds at room temperature. Tubes were placed on a magnetic stand (at room temperature) and RLT buffer removed and transferred to a clean microcentrifuge tube. RNA isolation was performed immediately.

5.2.4 RNA-sequencing

5.2.4.1 RNA isolation

350 µL 70 % ethanol (freshly prepared in nuclease-free water) was added to the RLT buffer, and centrifuged at 8,000 x *g* for 15 seconds. 350 µL RW1 buffer was added to the column, and centrifuged at 8,000 x *g* for 15 seconds. On-column DNase digestion was performed by adding 10 µL DNase I stock solution to 70 µL buffer RDD, gently inverting to mix, and then transferring to the spin column membrane. This was incubated for 15 minutes at room temperature. 350 µL RW1 buffer was added to the spin column, and centrifuged at 8,000 x *g* for 15 seconds. Next, 500 µL buffer RPE was added to the spin column, and it was centrifuged at 8,000 x *g* for 15 seconds. 500 µL 80 % ethanol (freshly prepared in nuclease-free water) was added to the spin column, and centrifuged for 2 minutes at 8,000 x *g* to wash the membrane. Following this, the lid of the spin column was opened, and centrifuged at full speed for 5 minutes to dry the membrane. Finally, 16 µL nuclease-free water was added to the spin column membrane and centrifuged for 1 minute at full speed to elute the RNA. RNA was stored at -75 °C until further processing.

To extract RNA from the input sample, samples were thawed on wet ice. 50 µL was removed and added to supplemented RLT buffer, and the protocol continued from the addition of 70% ethanol, as described above.

5.2.4.2 RNA-sequencing

Quality checks, library preparation and sequencing were performed by GeneWiz Ltd. RNA was quantified using the Qubit Fluorometer and RNA integrity was checked using the Agilent Fragment Analyzer. One bulk RNA-seq sample (2-hour time point) was removed at this point due to low RNA quantity. The Agilent Fragment Analyzer outputs an RNA quality number (RQN), an alternative to RIN, whereby 1 represents the most degraded RNA and 10 represents the least degraded RNA. RQN summary statistics are presented in Table 5.2.

Condition	***n***	**RQN (range)**
Bulk 2 hour	6	8.8 (6.9 – 9.3)
IP 2 hour	9	8.9 (8.5 – 9.0)
Bulk 5 hour	8	9.1 (8.2 – 9.6)
IP 5 hour	8	8.8 (7.4 – 10.0)
Home-cage bulk	10	9.2 (8.9 – 9.4)
Home-cage IP	9	9.0 (8.4 – 9.9)

Table 5.2. *RQN summary statistics for the RNA quality in each condition.*

Libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina following the manufacturers instructions. After libraries had been generated, they were quality assured on the Fragment Analyzer and quantified, before being loaded onto an Illumina flow cell for sequencing on the NovaSeq 6000.

5.2.5 Bioinformatic analysis

Raw sequencing reads were processed to produce counts per gene, as described in General Methods section 2.4.1. The average library depth was 55.8 million.

5.2.5.1 Differential gene expression

Differential expression analysis was undertaken with limma/voom using an FDR threshold of 0.01 and the following formula:

$$\sim group + RIN$$

The contrasts can be found in Table 5.3.

Contrast name	Groups
Bulk 2 hour	2 hour bulk – home cage bulk
IP 2 hour	2 hour IP – home cage IP
Bulk 5 hour	5 hour bulk – home cage bulk
IP 5 hour	5 hour IP- home cage IP

Table 5.3. *The contrasts used for differential expression analysis. Expression was experimental group minus control group, such that positive log fold changes represented higher expression in the experimental group, and negative log fold changes represented lower expression in the experimental group.*

5.2.5.2 Pathway analysis

Pathway analysis was conducted using Fisher's exact test, with refinement, as described in General Methods section 2.4.3.

5.2.5.3 Temporal pattern of differential gene expression (DGE)

Genes that were significantly differentially expressed at each time point were categorised according to whether they were not differentially expressed, up-regulated, or down regulated at the other time point. For example, *Fosb* was significantly upregulated 2-hours post-CFC, but not significantly differentially expressed 5-hours post-CFC. Therefore, it was categorised into the up-regulated not differentially expressed category (Table 5.4). The frequency of genes in each category was calculated. It should be noted that this is not an unsupervised clustering analysis, but counts of genes which fall into each possible category.

Temporal category	Differential expression status at 2-hours	Differential expression status at 5- hours
Down-regulated, down-regulated	Down-regulated	Down-regulated
Down-regulated, not differentially expressed	Down-regulated	Not differentially expressed
Not differentially expressed, down-regulated	Not differentially expressed	Down-regulated
Not differentially expressed, up-regulated	Not differentially expressed	Up-regulated
Up-regulated, not differentially expressed	Up-regulated	Not differentially expressed

Table 5.4. *The categories for the temporal pattern of differential gene expression analysis.*

5.2.5.4 Disease association

Disease association was conducted as described in General Methods section 2.5.

5.2.6 Quantitative PCR

5.2.6.1 cDNA synthesis

500 ng total RNA was converted to cDNA using the SuperScript IV VILO Master Mix (ThermoFisher Scientific [11766050]). Immediately prior to reverse transcription, total RNA concentration was measured using the Qubit, as described in General Methods section 2.3.4.1. On ice, 4 µL of SuperScript IV VILO Master Mix and 500 ng template RNA were added to a PCR tube. An appropriate volume of nuclease-free water was added, such that the total reaction volume was 20 µL. The reaction was set up as in Table 5.4. Following this, cDNA was diluted 1:5 in nuclease free water and stored at -75 °C until use.

Step	Temperature	Time
Anneal primers	25 °C	10 minutes
Reverse transcription	50 °C	10 minutes
Inactive enzyme	85 °C	5 minutes

Table 5.4. *Thermocycler set up for reverse transcription.*

5.2.6.2 Quantitative PCR

qPCR was conducted using TaqMan technology. TaqMan Fast Advanced Master Mix (ThermoFisher Scientific [4444557]), TaqMan assays (ThermoFisher Scientific [4331182], see Table 5.5) and cDNA samples were thawed on ice. The qPCR reaction was assembled for each probe (see Table 5.6) and vortexed briefly to mix, before 18 µL was pipetted into each well of a PCR plate (ThermoFisher Scientific [4346906]). 2 µL cDNA (or nuclease-free water for no template control) was added to each well, and each sample was plated in triplicate for each probe. The plate was sealed with an optical PCR film (ThermoFisher Scientific [4306311]), and centrifuged briefly.

Gene probe	Neuron type	Assay ID
Arc	Excitatory	Mm00437967_m1
CaMKIIα	Excitatory	Mm01204954_g1
Gad1	Inhibitory	Mm04207432_g1
GFAP	Astrocyte	Mm01253033_m1
CNpase	Oligodendrocyte	Mm01306640_m1
SDHA	Housekeeping	Mm01352366_m1
UBC	Housekeeping	Mm02525934_g1

Table 5.5*. Gene probes and associated TaqMan Assay IDs. All assays had the FAM-MGB quencher.*

Component	Volume per reaction	Volume in triplicate (+10%)
TaqMan Fast Advanced Master Mix (2X)	10 µL	33 µL
TaqMan Assay (20X)	1 µL	3.3 µL
Nuclease-free water	8 µL	23.1 µL
Total volume per reaction	**18 µL**	**59.4 µL**

Table 5.6*. Master Mix reaction set-up.*

The StepOne plus RT-PCR system was used for the qPCR reaction, using the Fast setting. The experiment was set up as shown in Table 5.7.

Step	Temperature	Time
UNG incubation	50 °C	2 minutes
Polymerase activation	95 °C	2 minutes
PCR (40 cycles)	95 °C	1 second
	60 °C	20 seconds

***Table 5.7**. Conditions for qPCR.*

5.2.6.3 qPCR analysis

Cycle threshold (Ct) values ere outputted from the qPCR, representing the number of cycles it took for a signal to be detected above that of the background fluorescence. Threshold levels were automatically generated by the StepOne plus system. Quantification of the qPCR data was undertaken using the comparative $2^{-\Delta\Delta Ct}$ method, as described in 'Guide to performing relative quantitation of gene expression using real-time quantitative PCR' by AppliedBiosystems (Applied Biosystems, 2008). As two housekeeping genes were used, the average and standard deviation (SD) were calculated for the two genes and used in the calculations.

Error for $2^{-\Delta\Delta Ct}$ values was expressed as a range (as per the Applied Biosystems documentation) and was calculated as follows:

$$2^{-\Delta\Delta Ct} + \text{SD of IP sample}$$

$$2^{-\Delta\Delta Ct} - \text{SD of IP sample}$$

One-sided paired-sample t-tests were conducted (the total and IP were paired for each sample) to assess the differences in gene expression. One-sided tests were used because we knew the expected direction of the results. T-tests were conducted on the delta Ct values.

5.2.7 Immunohistochemistry (IHC)

5.2.7.1 Perfusion

Mice were sacrificed via intraperitoneal injection of Euthatal (100 mg/mL) and remained in a holding cage until cessation of heartbeat. Mice were rapidly dissected and a perfusion needle was inserted into the left ventricle and clamped into place. The right atrium was cut to allow the flow of blood out of the body. Ice cold 0.1 M phosphate buffered saline (PBS) was released at a rate of 13-20 mL/minute into the heart to flush out the blood. Following this, the solution was changed to ice-cold

4 % paraformaldehyde (PFA), which was released at a flow rate of 20 mL/minute for approximately 15 minutes. The brain was then rapidly removed and put into fresh 4 % PFA. Brains remained in the PFA solution for 24 hours at 4 °C. Once fixed, brains were stored in 30 % sucrose at 4 °C to cryoprotect the tissue.

5.2.7.2 Hippocampal sectioning

Perfused brains were removed from the sucrose solution and embedded in Optimal Cutting Temperature compound (OCT, Agar Scientific [AGR1180]) until set. Embedded brains were attached to a cryostat chuck using OCT, and left at – 20 °C until securely attached. 40 µm coronal sections were collected using a cryostat (Leica Microsystems CM1860UV), and placed into a 12-well plate containing 0.1M PBS.

5.2.7.3 Staining

Sections were blocked in 10 % Donkey serum (Generon [0030-01]), 1 % triton (from 10 x stock) in 0.1 M PBS for 2 hours. Primary antibodies (Table 5.8) were diluted in blocking solution and left overnight on gentle rotation at 4 °C.

The next day, the primary antibody was removed and sections were washed 3 times for 5 minutes each with blocking solution. Appropriate Alexa secondary antibodies, diluted in blocking solution, were added to the sections and incubated in the dark at room temperature for 2 hours. The secondary antibody solution was removed, and sections were washed for 5 minutes in 0.1M PBS. Sections were then incubated with DAPI stain (1:1000) in 0.1 M PBS for 5 minutes. Finally, sections were washed in 0.1 M PBS. Sections were mounted on standard microscopy slides using Mowiol aqueous mounting solution and covered with standard cover slips. Sections were stored at 4 °C in the dark until use.

Target (species)	Antibody manufacturer	Dilution
Map2 (rabbit)	Abcam [ab32454]	1:1000
HA.11 (mouse)	Biolegend [MMS-101R]	1:1000

Table 5.8. *Primary antibodies used for IHC.*

5.2.7.4 Microscopy

Sections were imaged using an upright microscope (Leica DM6000B Upright Timelapse System) at 5 x or 20 x magnification.

5.3 Results

5.3.1 Confirmation of successful CFC in mice

To check that the CFC parameters generated a fear memory, five male mice of the same genotype and age as the experimental animals were used for a pilot. The mice underwent CFC as described in 2.2.2.2. 24 hours later, mice were placed back into the conditioning chamber for 3 minutes to test recall of the fear memory. Percentage of time spent freezing was taken as the behavioural output of fear memory. Figure 5.2 shows that the mice froze around 40% of the time, indicating that mice learned the association between the context and the shock.

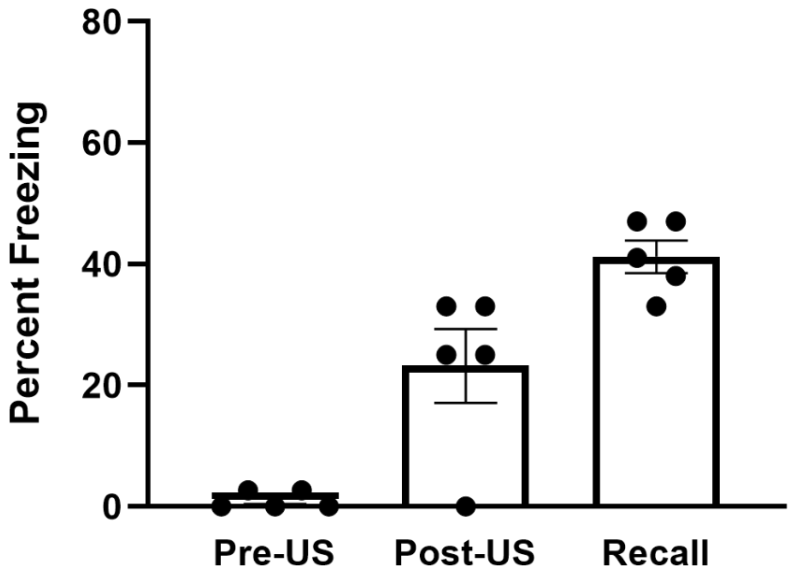


Figure 5.2. *Percentage of time mice exhibited freezing behaviour before the foot shock (Pre-US), in the one minute after footshock (Post-US) and in the 3 minute recall trial 24 hours later. Mice exhibited minimal freezing behaviour before the foot shock (Mean = 1.08; S.E.M. = 0.66), but exhibited freezing behaviour both Post-US (mean = 23.2; S.E.M= 6.06) and at recall 24 hours later (Mean = 41.2; S.E.M. = 2.69). One-way repeated measures ANOVA indicated that were significant differences between timepoints (F(2, 8) = 24.03, p = 0.0058). Dunnett's multiple comparisons tests indicated that there was a significant difference between pre-US freezing and Post-US freezing, and Pre-US freezing and recall freezing (adjusted p- values of 0.0295 and 0.0003 respectively). n = 5 per group.*

Further, freezing behaviour of the experimental animals prior to the footshock, and in the 1 minute afterwards, was quantified. Figure 5.3 shows that the mice in both the 2-hour and 5-hour condition froze around 40% of the time after the shock, indicating that mice learned the association between the context and the shock.

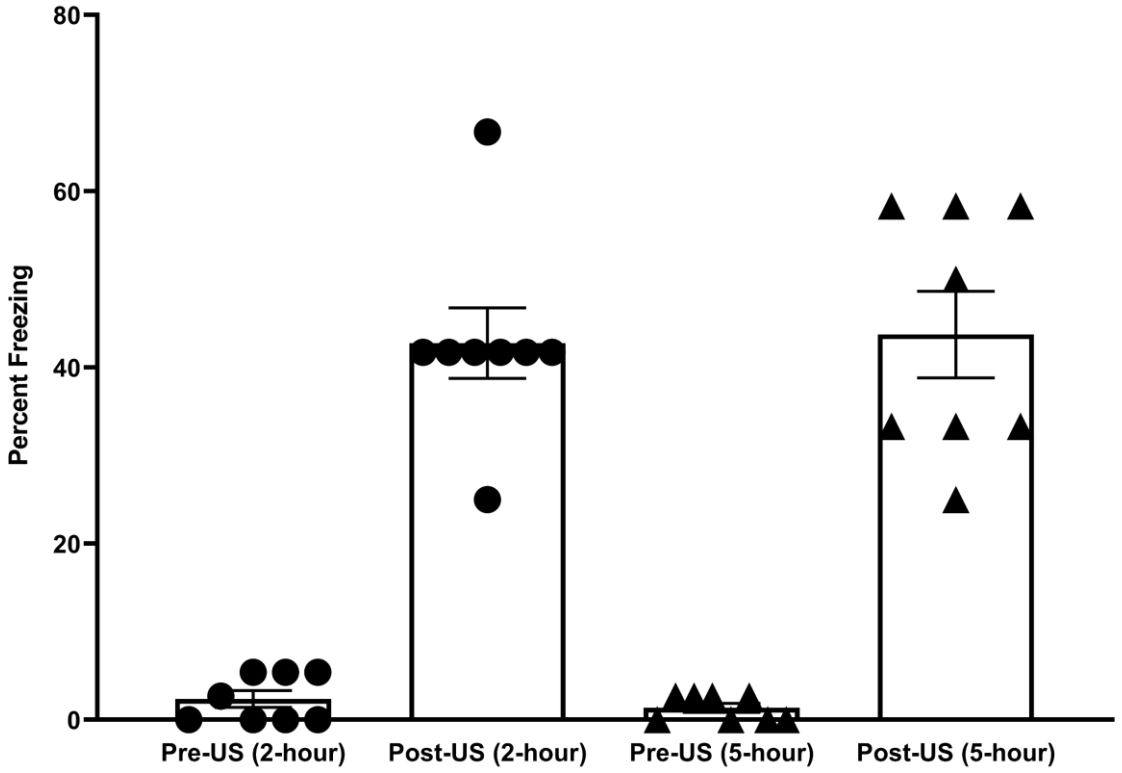


Figure 5.3. *Percentage of time mice exhibited freezing behaviour before the foot shock (Pre-US) and in the one minute after footshock (Post-US). Mice exhibited minimal freezing behaviour before the foot shock (2- hour mean = 2.363, S.E.M. = 0.946; 5-hour mean = 1.350, S.E.M. = 0.510), but exhibited freezing behaviour Post-US (2-hour mean = 42.74, S.E.M= 3.99; 5-hour mean = 43.73, S.E.M. = 4.9). One-way repeated measures ANOVA indicated that were significant differences between timepoints (F(3, 7) = 63.10, p < 0.0001). Dunnett's multiple comparisons tests indicated that there was a significant difference between pre-US freezing and Post-US freezing between groups (adjusted p-values: 2-hour group: < 0.0001; 5-hour group: 0.0003). Points represent individual mouse freezing values, circles represent those mice in the 2-hour group, triangles represent those mice in the 5-hour group.*

5.3.2 qPCR shows de-enrichment non-excitatory targets

Real-time quantitative PCR (RT-qPCR) was undertaken to confirm the de-enrichment of non-excitatory neuron targets lysate that had undergone IP. IP RNA is the ribosome enriched fraction, equivalent to the RNA used in the TRAP-sequencing condition. Total RNA contains transcripts from all cell types, equivalent to the RNA used in the Bulk-sequencing condition. Expression of *GAD1* (inhibitory neurons), *GFAP* (astrocytes) and *CNPase* (oligodendrocytes), as well as the excitatory neuronal markers *ARC* and *CaMKIIα*, were measured in the IP samples compared to the total RNA sample. It can be seen in Figure 5.4 that *GAD1*, *GFAP* and *CNPase* were de-enriched in IP compared to total RNA. Excitatory neuronal markers *ARC* and *CaMKIIα* were present in the IP RNA, as expected. There was also an enrichment of *ARC* in IP vs Total RNA.

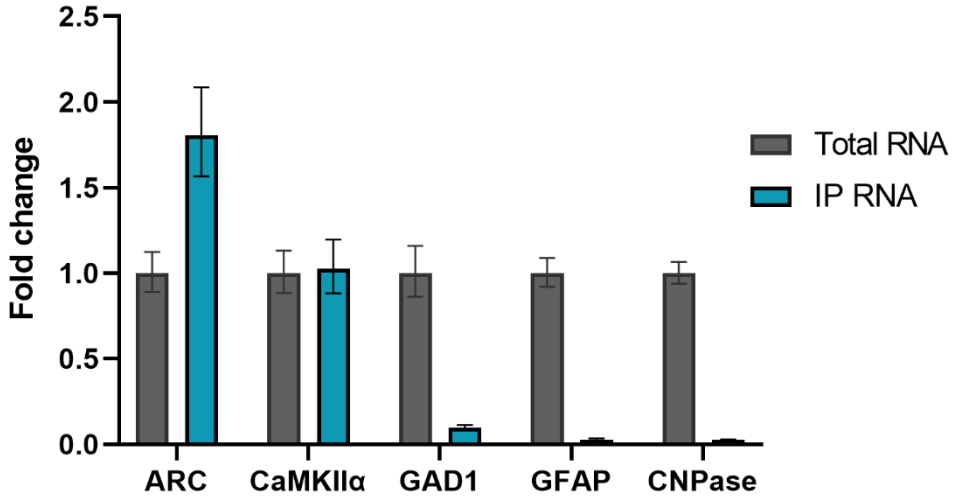


Figure 5.4. *Expression of Arc, CaMKIIα, GAD1, GFAP and CNPase expressed as the fold change between total (grey) and IP (blue) RNA. Error bars represent the upper and lower fold-change error limits. n = 3 per group.*

Paired t-tests confirmed that the delta Ct values were significantly different between IP and total RNA for ARC ($t(2)= 6.502$, $p = 0.022$), GAD1 ($t(2)= -185.55$, $p = 2.9 \times 10^{-5}$), GFAP ($t(2)= -33.93$, $p = 8.6 \times 10^{-4}$) and CNPase ($t(2)= -141.4$, $p = 5 \times 10^{-5}$). Delta Ct values for CaMKIIα were not significantly different ($t(2)= 0.21$, $p = 0.85$).

5.3.3 Immunohistochemistry shows expression of RiboTag sequence in excitatory hippocampal neurons

Immunohistochemistry (IHC) was undertaken to examine the spatial location of the RiboTag protein, using an antibody against the HA tag. It can be seen that the RiboTag protein was present in

hippocampal sub-regions, particularly the CA1 and dentate gyrus (DG) (Figure 5.5). Further, co-localisation with an antibody against MAP2 confirmed it was present in excitatory neurons (Figure 5.6).

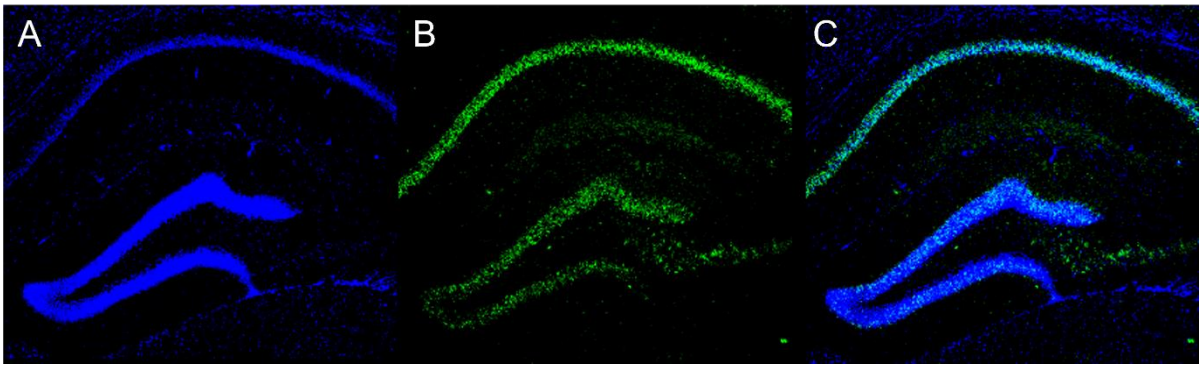


Figure 5.5. *Immunohistochemistry (IHC) showing the spatial expression of the RiboTag protein. A. DAPI expression. B. HA-tag expression. C. Overlay of DAPI and HA-tag. Images are at 5x magnification.*

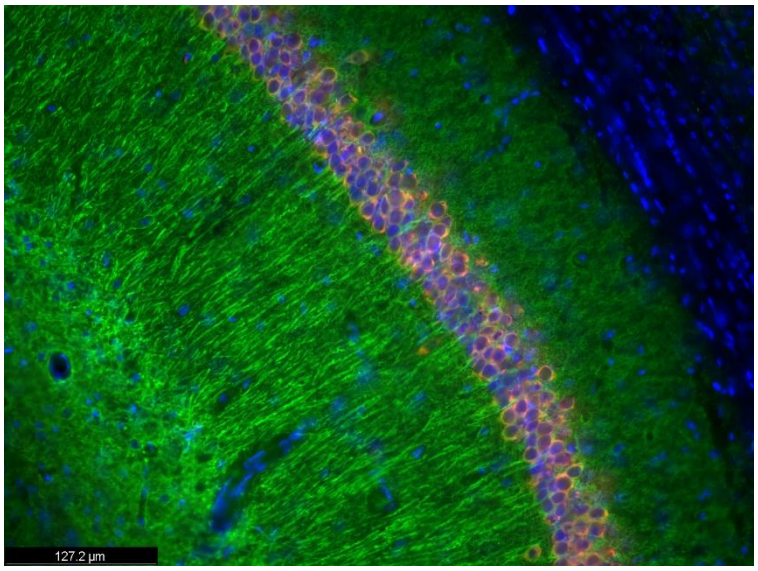


Figure 5.6. *IHC showing the expression of the RiboTag protein using an antibody against the HA tag (red), neuronal marker MAP2 (green) and DAPI (blue). Yellow indicates co-localisation. 20 x magnification.*

5.3.4 Differential gene expression 2 and 5 hours post CFC acquisition

In order to examine transcriptomic expression profiles induced by contextual fear conditioning, hippocampal tissue was extracted 2 or 5 hours after training. Tissue from each subject underwent bulk RNA sequencing, to examine gene expression from all cell types, and TRAP sequencing to examine the gene expression profile specifically from hippocampal excitatory neurons. The number of genes that were differentially expressed in each condition is shown in Figure 5.7. 846 genes were differentially expressed in excitatory neurons 2 hours after acquisition of CFC, and 341 differentially expressed in this cell type 5 hours after CFC. Only 7 genes were differentially expressed in all cell types 2 hours after CFC, all of these were also differentially expressed in the excitatory population. No genes were differentially expressed 5 hours after CFC when all cell types were sequenced.

Further, this was maintained even when the FDR cut-off was increased to FDR < 0.1. This mirrors the temporal pattern of results found in bulk sequencing after retrieval (Chapter 3). 710 were uniquely differentially expressed in the 2-hour TRAP condition, just over 83%. 212 genes, just over 62%, were uniquely differentially expressed in the 5-hour TRAP condition. This overlap was statistically significant ($p = 2.2 \times 10^{-16}$, Fisher's Exact Test).

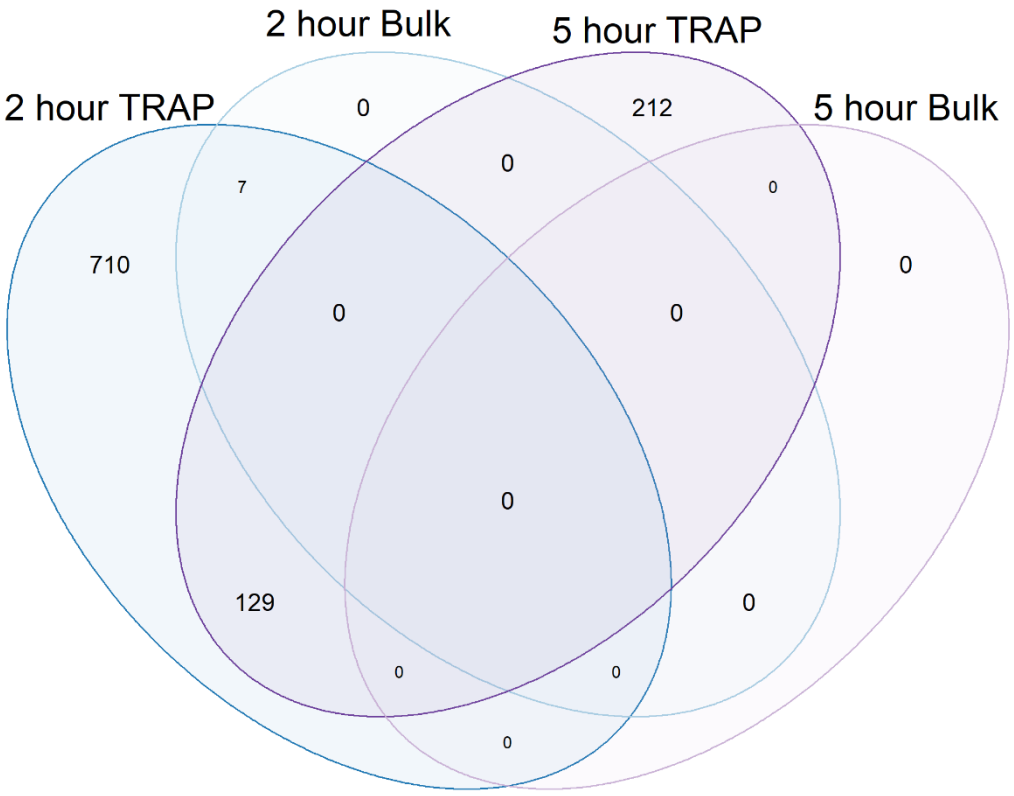


Figure 5.7. *The number of genes that were differentially expressed in each condition following acquisition of CFC. The 2 hour TRAP condition had the highest number of differentially expressed genes, followed by the 5 hour TRAP condition. There were very few differentially expressed genes in the 2 hour bulk condition, and none in the 5 hour bulk condition.*

Table 5.9 summarises the pattern of differential expression in each condition. In excitatory neurons, 2 hours after acquisition of CFC there was a roughly equal split between up- and down- regulated

genes. However, 5 hours after acquisition, this had shifted such that most of the differentially expressed genes were down-regulated. When expression in all cell types was examined, all differentially expressed genes were upregulated.

	Direction of expression	Number of genes
2-hour TRAP	Up-regulated	432
	Down-regulated	414
2- hour bulk	Up-regulated	7
	Down-regulated	0
5-hour TRAP	Up-regulated	24
	Down-regulated	317
5- hour bulk	Up-regulated	0
	Down-regulated	0

Table 5.9*. Pattern of differential expression in each condition. The 2-hour TRAP condition had almost equal numbers of genes up- and down- regulated. In contrast, by 5 hours most differentially expressed genes were down regulated.*

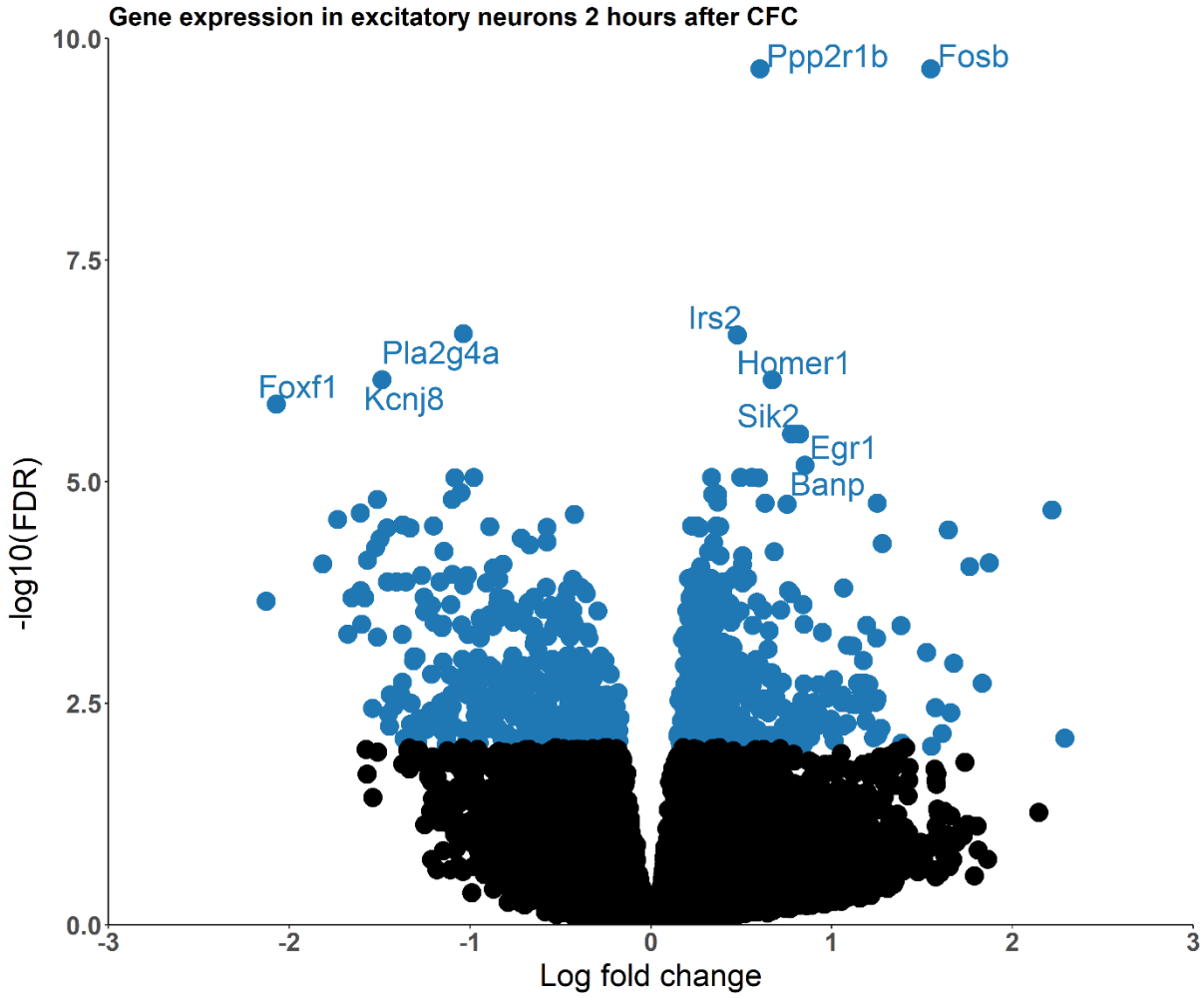


Figure 5.8. *Volcano plot of the log fold change against -log10 FDR values for genes expressed 2 hours after CFC in excitatory neurons. The top 10 most differentially expressed genes, as measured by FDR, are labelled with their gene symbol. Blue genes are those that were significantly differentially expressed at FDR < 0.01.*

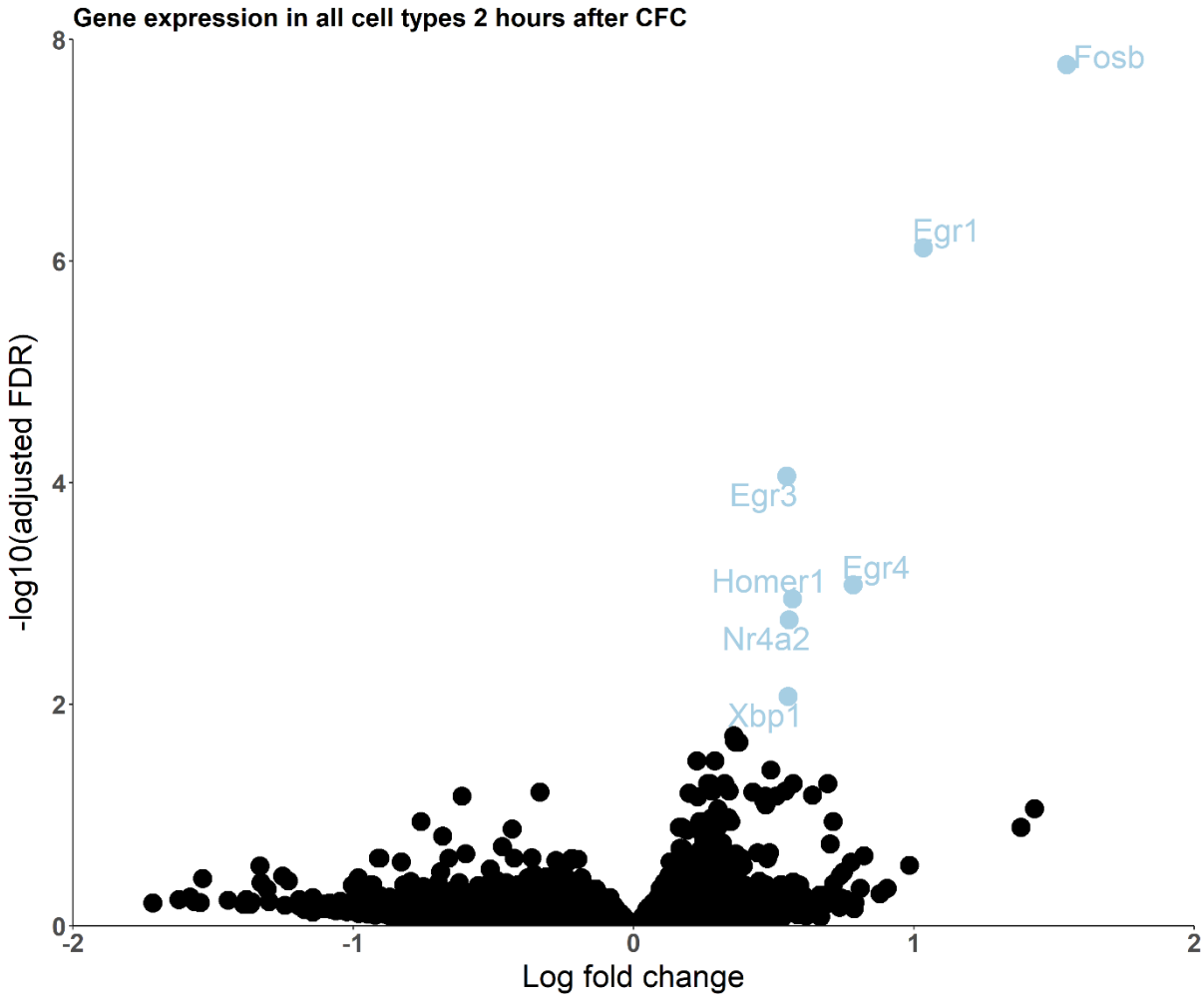


Figure 5.9. *Volcano plot of the log fold change against -log10 FDR values for genes expressed 2 hours after CFC in all cell types. The 7 genes that were significantly differentially expressed are labelled with their gene symbol and highlighted in light blue. FDR < 0.01*

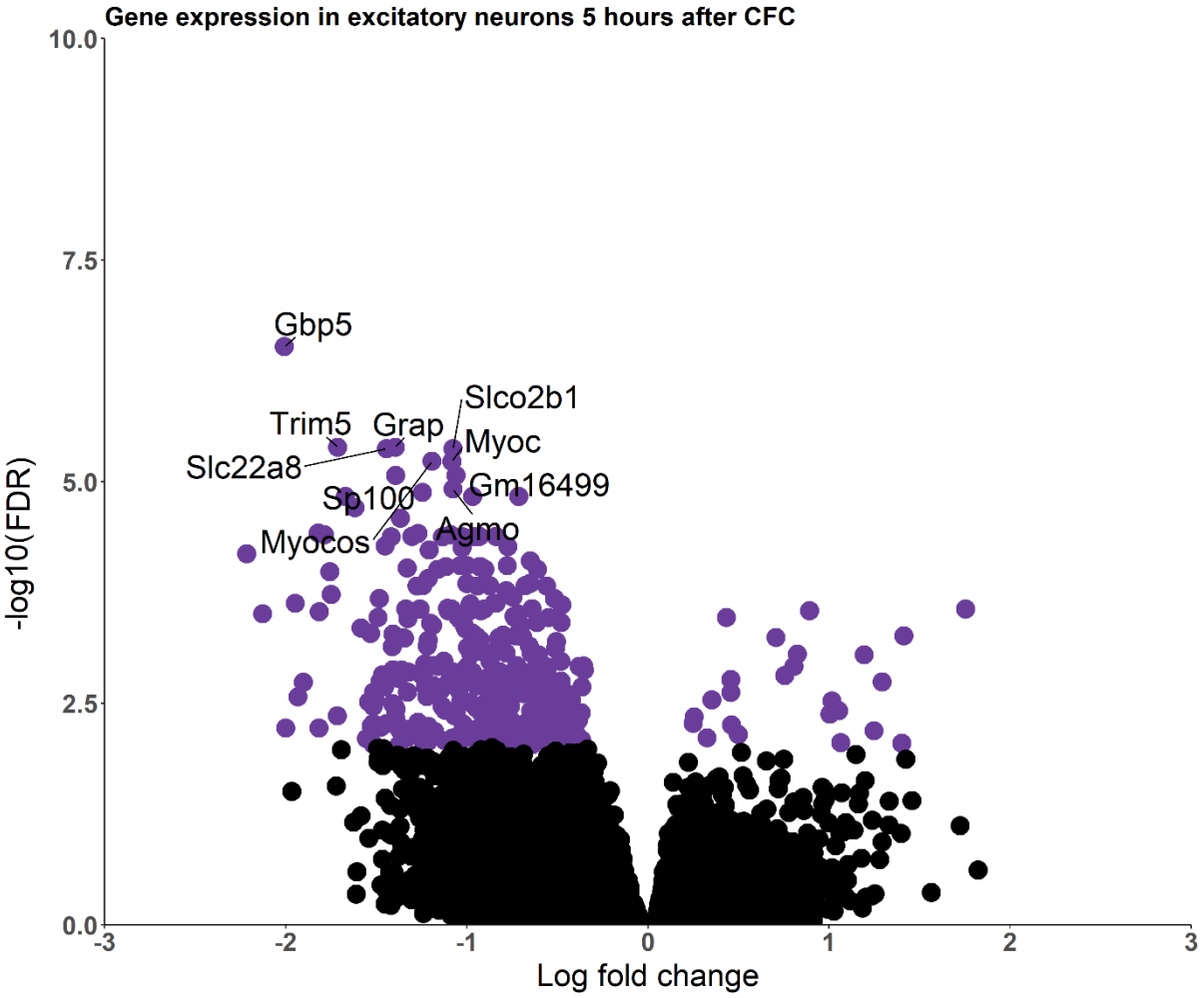


Figure 5.10. *Volcano plot of log fold change against -log 10 FDR value for each gene expressed 5 hours after CFC in excitatory neurons. The top 10 most differentially expressed genes, as measured by FDR, are labelled with their gene symbol. Purple genes are those that were significantly differentially expressed at FDR < 0.01.*

5.3.5 Pathway analysis

5.3.5.1 Gene ontology enrichment

Gene ontology (GO) analysis revealed 33 biological pathways enriched in the 2-hour TRAP seq gene set, corresponding to 7 major pathways after refinement. These included pathways such as “positive regulation of cell migration” [GO:0030335] and “regulation of catalytic activity” [GO:0050790] (Figure 5.9). When limited to only those differentially expressed genes (DEGs) that were upregulated, there were 42 significantly enriched pathways, corresponding to 6 major pathways. These major pathways were mainly related to neuronal development, including “positive regulation of dendrite development” [GO:1900006] and “neurogenesis” [GO:0022008]. Similarly, when limited to only those DEGs that were downregulated, there were 37 significantly enriched pathways, corresponding to 8 major pathways. These pathways were mainly related to blood vessel development.

Further, GO analysis revealed 134 biological pathways enriched in the 5-hour TRAP seq gene set, a 4-fold increase in the number of enriched pathways compared to the 2-hour gene set. These 134 pathways corresponded to 16 major pathways after enrichment, 6 of which were related to immune pathways including “Regulation of cytokine production involved in inflammatory response” [GO:1900015] and “Response to interferongamma” [GO:0034341] (Figure 5.11). Further, when limited to only those DEGs that were downregulated, there were 142 significantly enriched pathways, corresponding to 17 major pathways after refinement. Again, several of these were related to immune responses, including both aforementioned biological pathways: “Regulation of cytokine production involved in inflammatory response” [GO: 1900015] and “Response to interferongamma” [GO:0034341]. This is perhaps unsurprising, given that the majority of DEGs at 5 hours were downregulated, and highlights that genes involved in immune responses are significantly de-enriched in excitatory neurons 5- hours after contextual fear conditioning. There was no biological pathway enrichment of up-regulated DEGs.

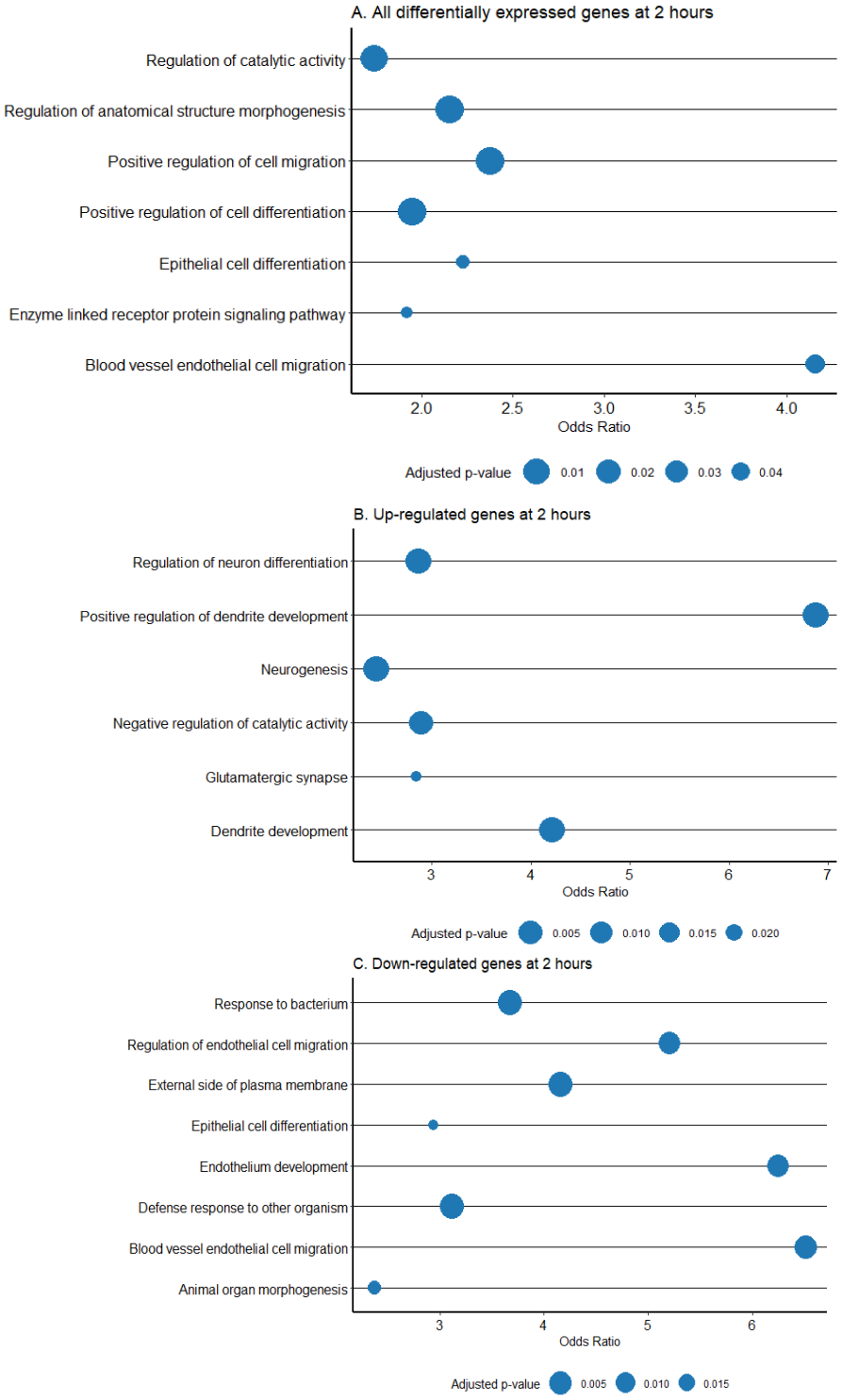


Figure 5.11. *Enrichment of gene ontology (GO) pathways in gene sets that were significantly differentially expressed (DEGs) in excitatory neurons 2 hours after contextual fear conditioning (CFC).* ***A.*** *GO pathways enriched in both up- and down-regulated DEG gene sets.* ***B.*** *GO pathways enriched in up-regulated DEGs.* ***C.*** *GO pathways enriched in down-regulated DEGs. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

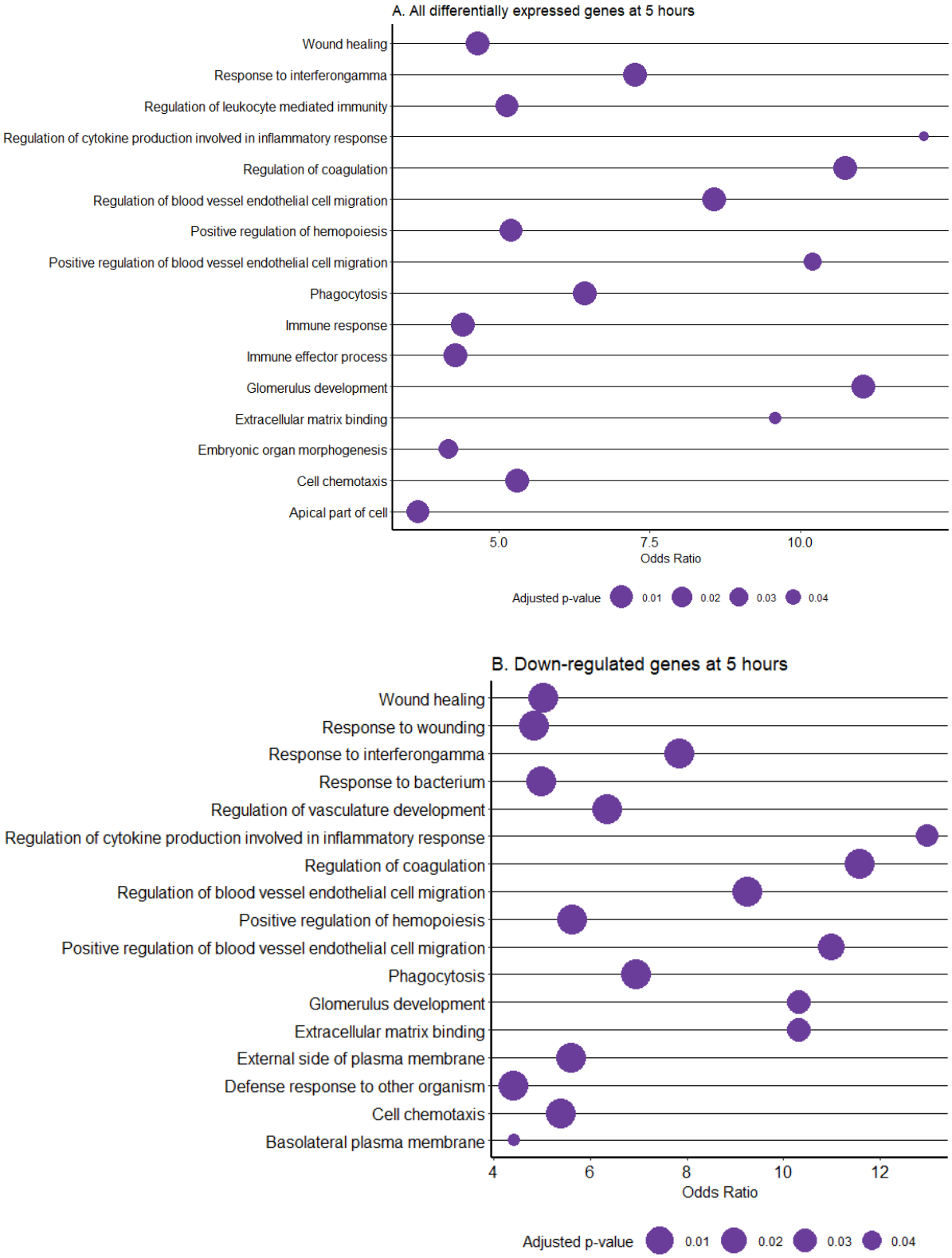


Figure 5.12. *Enrichment of gene ontology (GO) pathways in gene sets that were significantly differentially expressed (DEGs) in excitatory neurons 5 hours after contextual fear conditioning (CFC).* ***A.*** *GO pathways enriched in both up- and down- regulated DEG gene sets.* ***B.*** *GO pathways enriched in down-regulated DEGs. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value. There were no GO pathways enriched when analyses were restricted to only those genes which were up-regulated at 5 hours.*

5.3.5.2 Mammalian phenotype enrichment

Mammalian phenotype (MP) analysis revealed 5 phenotypes that were enriched in the 2-hour TRAP seq gene-set, all of which were independent terms after refinement (Figure 5.13). 2 terms were related to spleen morphology, 2 to post-natal lethality, and the other vascular permeability. When limited to only those genes that were upregulated, the 2 post-natal lethality pathways remained significantly enriched. When analysing only those genes that were downregulated at 2-hours in the TRAP seq gene-set, 7 phenotypes were significantly enriched. After refinement, 5 phenotypes remained enriched, most of which were vascular.

Further, MP enrichment analyses revealed 24 phenotypes that were enriched in the 5-hour TRAP seq gene-set, 14 of which remained significant after refinement (Figure 5.14). Similar to the GO enrichment analyses, abnormal phenotype terms were related to immune pathways, including "abnormal immunoglobulin levels" [MP:0002490], "abnormal cytokine level" [MP:0008713] and "abnormal chemokine secretion" [MP:0008722]. When limited to genes that were downregulated, there were 24 significantly enriched phenotypes, and 14 after further refinement. Similar to previous findings, several of these phenotypes were related to the immune response, including "abnormal adaptive immunity" [MP:0002420], "abnormal cytokine level" [MP:0008713] and "abnormal chemokine secretion" [MP:0008722]. No phenotypes were enriched in genes that were up-regulated in the 5-hour TRAP seq gene-set.

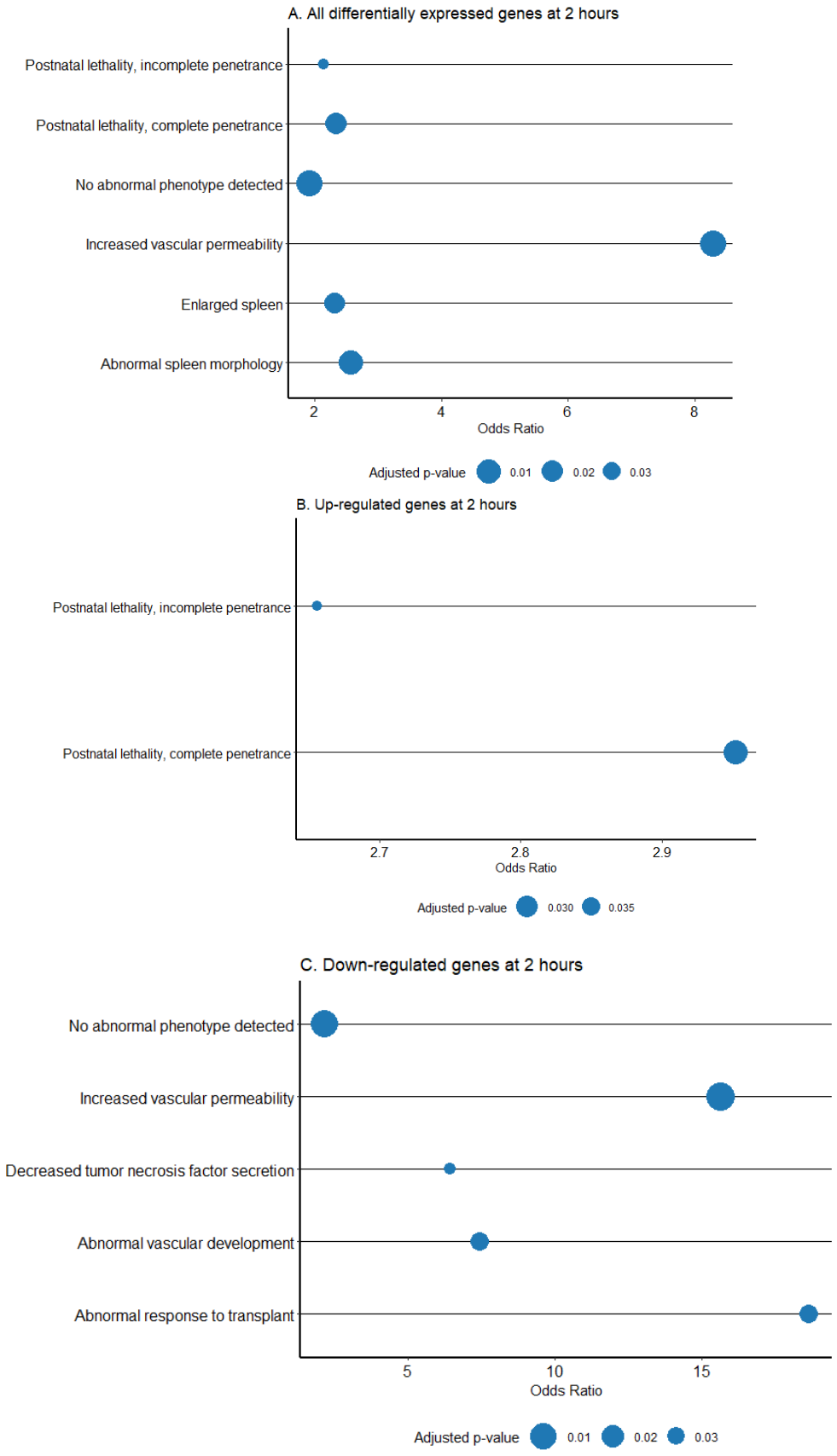


Figure 5.13. *Enrichment of Mammalian Phenotypes (MP) in gene sets that were significantly differentially expressed (DEGs) in excitatory neurons 2 hours after contextual fear conditioning (CFC).* ***A.*** *MPs enriched in both up- and down- regulated DEG gene sets.* ***B.*** *MPs enriched in up-regulated DEGs.* ***C.*** *MPs enriched in down-regulated DEGs, "Abnormal vein development" not shown as the odds ratio was infinity, p-value= 0.046. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

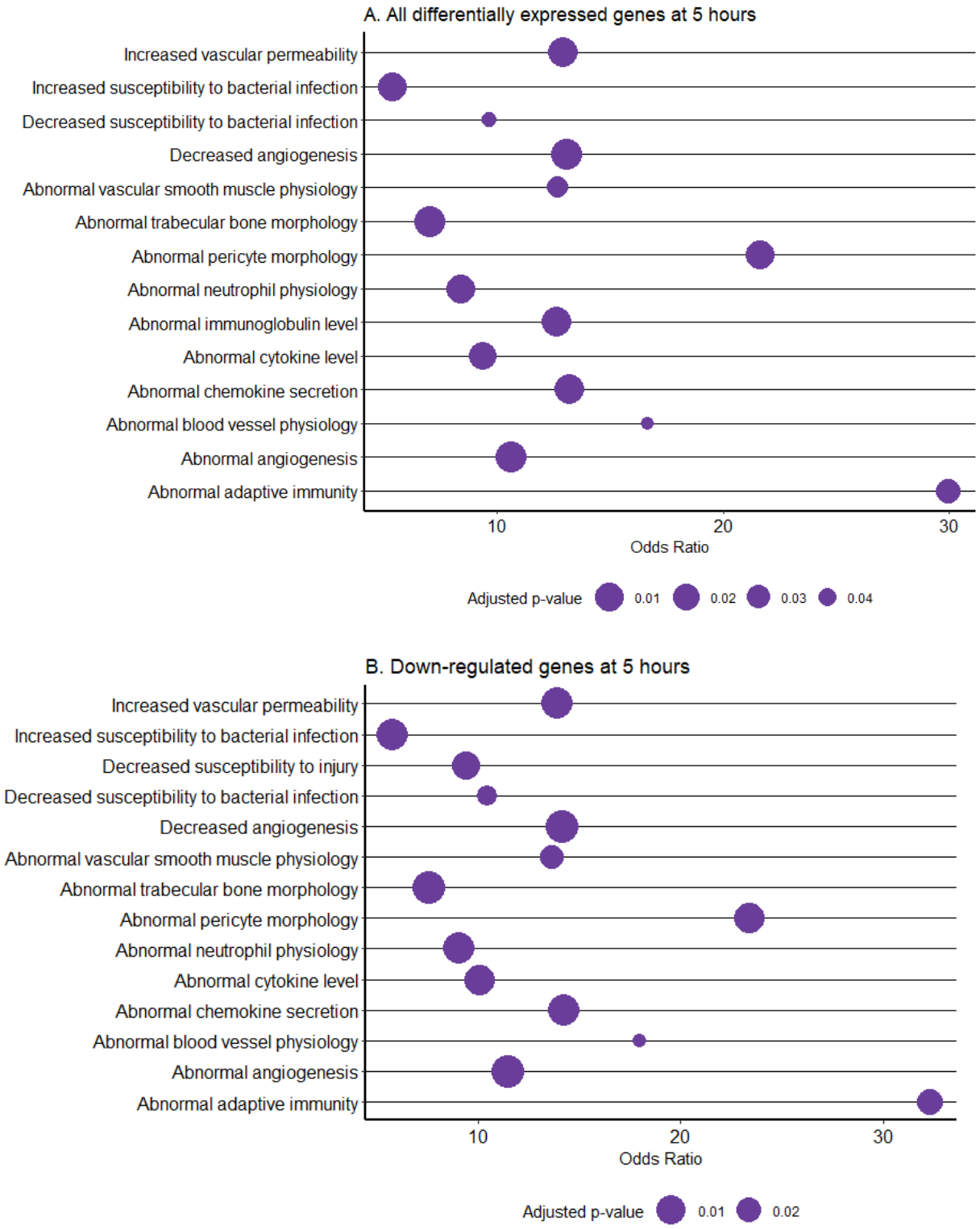


Figure 5.14. *Enrichment of Mammalian Phenotypes (MP) in gene sets that were significantly differentially expressed (DEGs) in excitatory neurons 5 hours after contextual fear conditioning (CFC).* ***A.*** *MPs enriched in both up- and down- regulated DEG gene sets.* ***B.*** *MPs enriched in down-regulated DEGs. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

5.3.6 Pattern of differential gene expression over time

Genes that were significantly differentially expressed in hippocampal excitatory neurons (TRAP-seq) at either 2 hours or 5 hours after acquisition of CFC were categorised according to whether they were not differentially expressed, up-regulated, or down regulated at each time point. The most common pattern of gene expression between the timepoints was to be up-regulated and 2 hours, and then not differentially expressed at 5-hours (Figure 5.15). Examples of genes in this category include *Fosb*, *Homer1* and *Egr1*. Similarly, the second most common pattern was to be down-regulated at 2 hours and then not differentially expressed at 5 hours, likely reflecting the increased numbers of differentially expressed genes at 2 hours compared to 5 hours. At 5 hours, the most common pattern of expression was not differentially expressed at 2 hours, and then down-regulated at 5 hours, followed by being down-regulated at both timepoints. Only 7 genes were significantly up-regulated at both 2- and 5- hours.

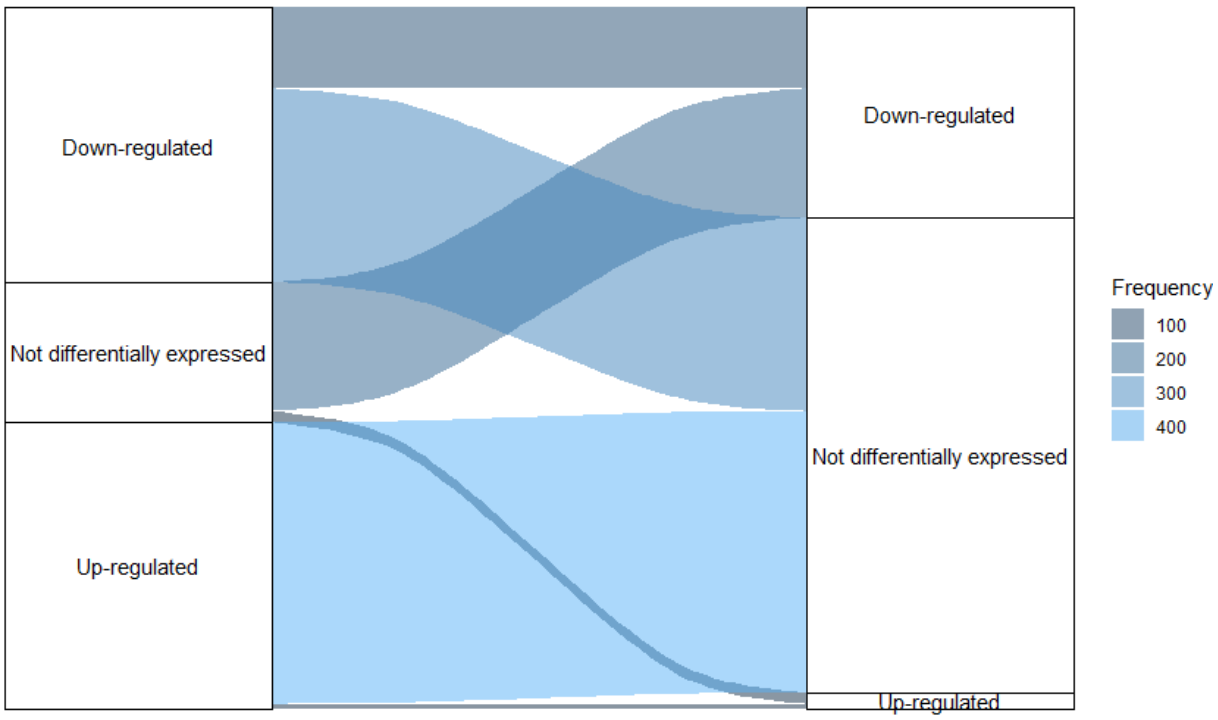
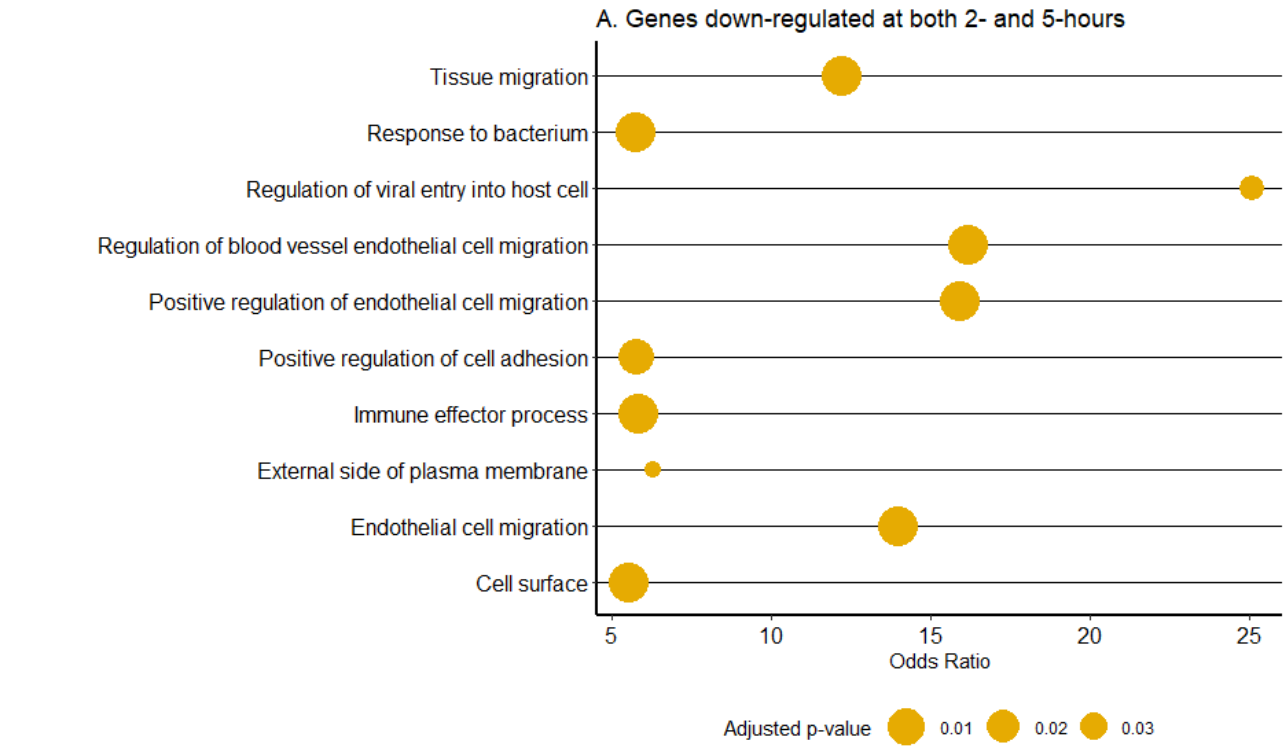


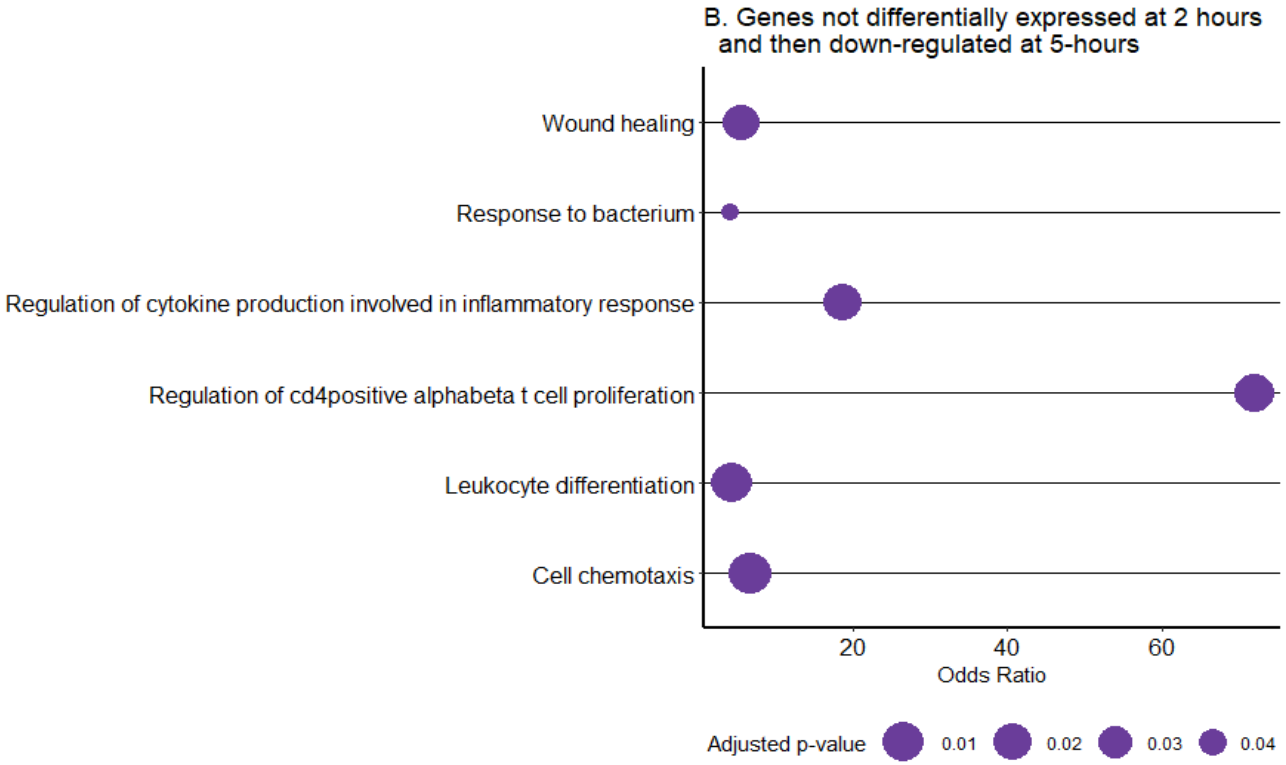
Figure 5.15. *The temporal dynamics of differentially expressed genes at 2- and 5- hours post CFC, in hippocampal excitatory neurons (TRAP-seq). The left-hand side is gene expression status at 2 hours, the right-hand side is gene expression status at 5 hours. The width and colour of the bar represents the frequency, with higher frequencies represented by thicker bars and lighter colour. The most common pattern of gene expression between the time points was to be up-regulated at 2 hours, and then not differentially expressed at 5 hours. At 5 hours, the most common pattern was to not have been differentially expressed at 2 hours, and then down-regulated at 5 hours.*

5.3.6.1 GO enrichment

Gene Ontology (GO) enrichment analyses were undertaken to establish whether patterns of differential expression over time were associated with particular biological pathways. Of the 6 patterns of differential expression, 3 were significantly enriched for genes found in at least one GO pathway: genes down-regulated at both time points, genes not differentially expressed at 2 hours and then down-regulated at 5 hours, and genes up-regulated at 2 hours and not differentially expressed at 5 hours (Figure 5.16).

Firstly, 66 GO terms were enriched in the gene set containing those which were down-regulated at both 2- and 5- hours post CFC, 10 of which remained significant after refinement. 4 of these terms were related to cell- or tissue migration. Those genes which were not differentially expressed at 2 hours and then down-regulated at 5 hours were enriched for 46 GO terms, 6 of which survived the refinement procedure to remain statistically significant. These 6 GO terms were all broadly related to immune processes, including “regulation of cytokine production involved in inflammatory response” [GO:1900015]. Finally, those genes up-regulated at 2 hours and not differentially expressed at 5 hours had 44 enriched GO terms, 6 of which were significant after refinement. These were related to neuronal and synaptic development.


A. Genes down-regulated at both 2- and 5-hours
Tissue migration
Response to bacterium
Regulation of viral entry into host cell
Regulation of blood vessel endothelial cell migration
Positive regulation of endothelial cell migration
Positive regulation of cell adhesion
Immune effector process
External side of plasma membrane
Endothelial cell migration
Cell surface
5
10
15
20
25
Odds Ratio
Adjusted p-value
0.01
0.02
0.03


B. Genes not differentially expressed at 2 hours and then down-regulated at 5-hours
Wound healing
Response to bacterium
Regulation of cytokine production involved in inflammatory response
Regulation of cd4positive alphabeta t cell proliferation
Leukocyte differentiation
Cell chemotaxis
20
40
60
Odds Ratio
Adjusted p-value
0.01
0.02
0.03
0.04

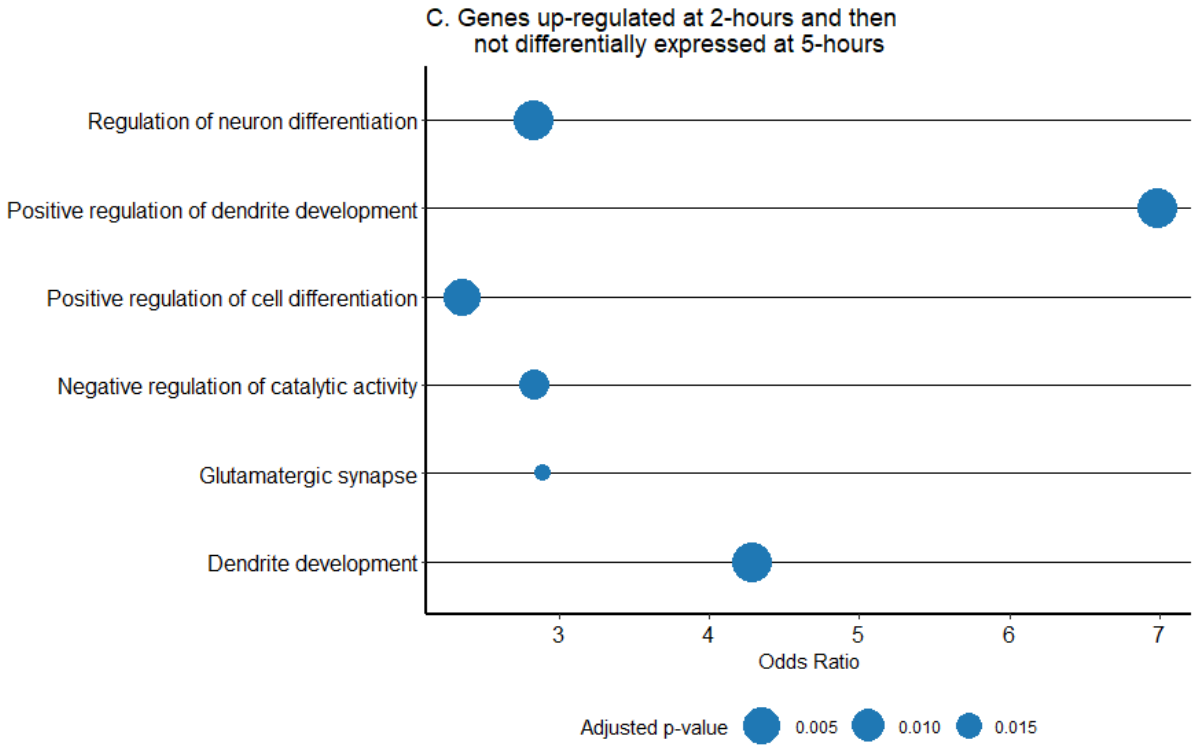


Figure 5.16. *Enrichment of gene ontology (GO) pathways in differentially expressed genes that were either:* ***A.*** *Significantly down-regulated at both 2- and 5-hours post CFC in hippocampal excitatory neurons* ***B.*** *Not differentially expressed at 2-hours and then significantly down-regulated 5-hours post CFC in hippocampal excitatory neurons.* ***C.*** *Significantly up-regulated at 2-hours and then not differentially expressed 5-hours post CFC in hippocampal excitatory neurons. The size of the point represents the Bonferroni adjusted p-value, whereby a larger point is a lower (more significant) p-value.*

5.3.7 Enrichment in disease risk

5.3.7.1 Common variation

To see whether genes differentially expressed in hippocampal excitatory neurons after acquisition of CFC were enriched for common variation for disease, gene set enrichment analysis was undertaken with MAGMA (de Leeuw et al., 2015). The gene set analysis was conditioned on 'all expressed genes' to account for the enrichment of brain expressed genes in psychiatric disease risk sets. No enrichment was found for any of the diseases tested (Table 5.10). Exploratory analysis was undertaken using genes differentially expressed at FDR < 0.05 and schizophrenia, but no enrichment was found (2-hour TRAP: p = 0.73, 5-hour TRAP p = 0.99, p-values adjusted for multiple comparisons).

	Psychiatric disorder	β value (SE)	Adjusted p- value
2- hour TRAP-seq	Schizophrenia	0.001 (0.0002)	0.974
	Bipolar disorder	-0.017 (-0.003)	1
	Autism Spectrum Disorder	0.016 (0.003)	0.664
5- hour TRAP-seq	Schizophrenia	-0.173 (-0.02)	0.993
	Bipolar disorder	-0.127 (-0.015)	1
	Autism Spectrum Disorder	-0.076 (-0.009)	0.919

***Table 5.10**. Enrichment for common variation for schizophrenia, bipolar disorder and autism spectrum disorder in TRAP-seq data. There was no significant enrichment in any gene set. P-values were adjusted to account for the significant overlap in genes between the 2- and 5- hour TRAP datasets. SE = standard error.*

5.3.7.2 CNV enrichment

Further, binomial regression was undertaken to see if there was an enrichment of CFC acquisition-related genes in CNVs associated with schizophrenia. No enrichment was found for either dataset (2-hour TRAP-seq: co efficient = 0.06, p-value = 0.342, 5-hour TRAP-seq: co-efficient = -0.03, p-value = 1, p-values adjusted for multiple comparisons).

5.3.8 Comparison to 2-hour LTP TRAP results

Given that LTP processes are thought to underlie acquisition of CFC, I examined the correlation between genes that were differentially expressed 2 hours after CFC in the TRAP-seq dataset, and the expression of these genes in the 2-hour TRAP LTP dataset from Chapter 4. A strong positive

correlation between the mean expression of genes significantly differentially expressed 2-hours after CFC, and expression of these genes in the LTP TRAP dataset ($r(698) = 0.8$, $p = 2.2 \times 10^{-16}$; Figure 5.17). However, whilst the correlation between the expression of the genes was strong, the number of genes that were significantly differentially expressed in both conditions was low, only 60 genes (4.8 %).

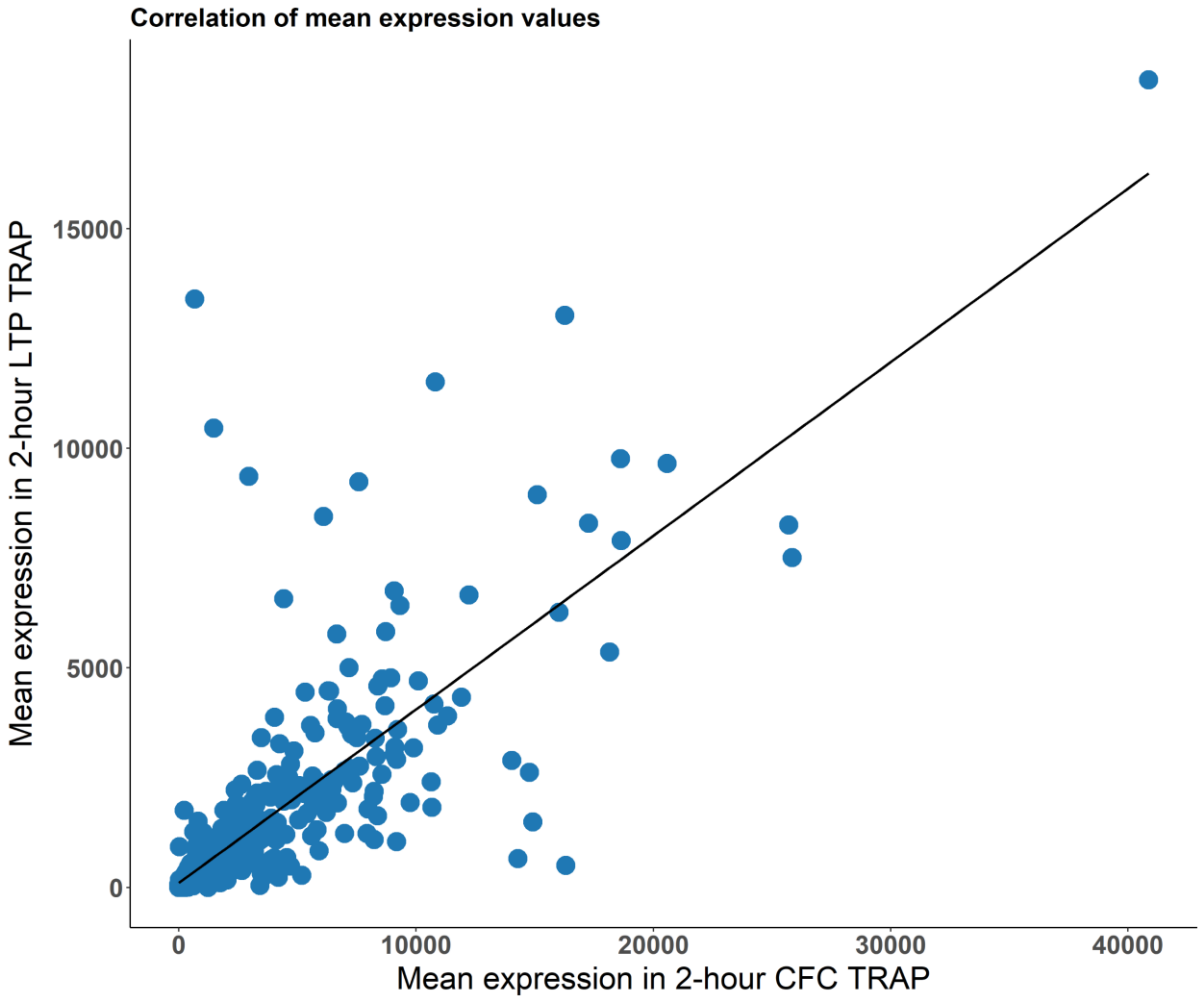


Figure 5.17. *Correlation of mean expression values for genes differentially expressed in 2-hour CFC TRAP and 2-hour LTP TRAP from Chapter 4. There is a strong correlation between the expression of genes differentially expressed in 2-hour CFC TRAP and their expression in the 2-hour LTP TRAP dataset. Map4 is the gene with the highest expression value in both datasets.*

5.4 Discussion

The aim of this chapter was to explore the gene expression profile induced by the consolidation of CFC in excitatory hippocampal neurons, and to test for association with psychiatric risk variants. Further, bulk RNA-sequencing (bulk RNAseq) was conducted to explore gene expression in all cell types, mirroring the gene profiling conducted in Chapter 3. I found that over 1,100 genes were differentially expressed in excitatory hippocampal neurons across the 2 time-points following acquisition of CFC, as determined by the current differential expression method used. In contrast, there was little evidence of differential gene expression in bulk RNAseq, consisting of all cell types, at either time-point. In addition, there was no evidence that genes differentially expressed after acquisition of CFC were enriched for schizophrenia-associated variation.

5.4.1 TRAP-seq methodology successfully de-enriches non-excitatory neuronal targets

In the present chapter, cre-mediated expression of the RiboTag construct, driven by the CaMKIIα promotor, allowed the isolation, purification and sequencing of ribosome-associated RNA transcripts from hippocampal excitatory neurons. Real-time qPCR (RT-qPCR) confirmed the de-enrichment of *GAD1* (inhibitory neurons), *GFAP* (astrocytes) and *CNPase* (oligodendrocytes) in the immunoprecipitate (IP). This confirms previous results using this mouse line (Chen et al., 2017). Further, immunohistochemistry (IHC) confirmed localisation of the HA tag to the hippocampus, particularly the CA1 and dentate gyrus (DG) subfields, and co-localisation with MAP2 protein in excitatory neurons. This is in contrast to the original laboratory report, that the cre driver line (*B6.Cg-Tg(Camk2a-cre)T29-1Stl/J*) drove expression predominantly in the CA1 (Tsien et al., 1996). However, data from the Genetic Resource Science group (Mouse-Genome-Database) has shown expression in several hippocampal subfields, which is consistent with the present results. In light of the IHC results, data was referred to within this chapter as from “excitatory hippocampal neurons” rather than CA1 specifically.

5.4.2 Hundreds of differentially expressed genes identified through TRAP-seq 2- and 5- hours after acquisition of contextual fear conditioning (CFC)

A total of 846 significantly differentially expressed genes were identified from excitatory hippocampal neurons 2-hours after acquisition of CFC, and 341 significantly differentially expressed genes were identified 5-hours after acquisition of CFC. Only 7 genes were found to be differentially expressed in the bulk RNA-seq, containing all cell-types, all of which were identified 2-hours after CFC, and were also significantly differentially expressed in the excitatory cell type fraction. The top 4,

in terms of significant p-values, were *Fosb*, *Egr1*, *Egr3* and *Egr4*, all of which were also significantly differentially expressed 2-hours after retrieval and/or extinction of CFC (Chapter 3). These 4 genes are all involved in transcription, either as transcription factors or transcriptional regulators (GeneCards, 2022b), suggesting that bulk RNAseq, containing all cell-types, is capturing genes that underpin basic cellular mechanisms.

In excitatory hippocampal neurons, 2-hours after CFC training, there was a roughly equal split of up- and down- regulated differentially expressed genes. Previous TRAP-seq studies examining expression of excitatory transcripts after LTP identified slightly more up-regulated genes 2-hours post LTP than down-regulated (354 up-regulated and 238 down-regulated) (Chen et al., 2017). However, given the differences in paradigm, chemical induction of LTP compared to acquisition of CFC in an intake animal, these slight differences would be expected. Marco and colleagues (2020) identified similar numbers of up- and down-regulated genes 1.5 to 2 hours following CFC (41 up-regulated, 47 down-regulated) (Marco et al., 2020). Whilst this study examined mRNA levels from all cell-types, the authors specifically looked at those neurons which were activated by the CFC task, and thus might explain why more differentially expressed genes were found compared to the bulk sequencing presented here. Given that LTP processes are thought to underlie acquisition of CFC, the correlation between the expression of genes that were significantly differentially expressed 2-hours after CFC in the TRAP-seq dataset and expression of these genes in the 2-hour LTP dataset from Chapter 4 (Chen et al., 2017), was examined. A strong positive correlation was found between these genes, indicating that despite little overall gene overlap between the gene sets, genes that were significantly differentially expressed in the current study showed similar expression patterns in data derived from an LTP experiment. Different differential expression algorithms were used in each study (DESeq2 in the LTP experiment and Limma/voom in the present experiment) which may explain why despite a strong expression correlation, there was little gene overlap. In this way, this correlation analysis should also be regarded as experimental, as in order to accurately compare the expression between 2 gene expression studies the same algorithm should be used.

5-hours after training, in the same cell-type, the majority of differentially expressed genes (over 90%) were down-regulated. This pattern is similar to that reported by Cho and colleagues, who found that 4 hours after CFC, the majority of genes differentially expressed in the hippocampus were downregulated (Cho et al., 2015). Using ribosomal profiling, Cho and colleagues reported that transcript level downregulation at 4-hours post CFC was largely driven by regulation by estrogen receptor 1 (ESR1), and that half of the differentially expressed genes at this timepoint were downstream of ESR1. In the present study, none of the ESR1 downregulated genes listed in this 2015

study were significantly differentially expressed. However, the previous authors did not do cell-type specific ribosomal profiling, and so this may account for differences in the genes reported.

5.4.3 Pathway analysis revealed downregulation of immune pathways 5-hours after acquisition of CFC

Gene ontology (GO) and mammalian phenotype (MP) pathway enrichment analyses were undertaken on the 2- and 5- hour excitatory neuron TRAP gene sets. 2-hours after CFC training, pathway analyses revealed that 7 biological pathways (GO), after refinement analysis, were enriched in genes that were significantly up- or down-regulated, mainly relating to "basic" cellular functions such as regulation of catalytic activity, cell differentiation and protein signalling pathways. Further, when analysing up- and down- regulated genes separately, up-regulated genes were enriched in pathways related to neuronal differentiation and development, whilst down-regulated genes were enriched in terms related to cellular function, such as endothelial cell migration and differentiation. That upregulated genes were enriched in terms related to neuronal differentiation is expected, given that consolidation processes likely involve structural and functional synaptic changes to support the new learning. MP enrichment did not reveal any particular phenotypes of interest to the current work, indeed one of the significantly enriched pathways was "no abnormal phenotype detected" [MP:0002169]. When analysing up-regulated genes separately, postnatal lethality (both complete and incomplete penetrance) was significantly enriched. Given that GO pathway analysis implicated basic cellular and blood vessel terms, it is not surprising that phenotypes associated with these genes would result in lethality. The MP index does include terms related to learning and memory, including contextual fear conditioning and associative learning, and as such it is perhaps surprising that these phenotypes are not significantly enriched in the 2-hour consolidation dataset. However, it could be that the significantly differentially expressed genes, such as *Fosb*, *Egr1* and *Homer1*, underpin such a range of cellular and molecular functions that the signal is not specific enough to significantly associate with learning and memory phenotypes.

5-hours after CFC training, genes that were significantly differentially expressed in excitatory hippocampal neurons were enriched in GO terms relating to immune processes, such as "regulation of cytokine production involved in inflammatory response" [GO:1900015] and "immune response" [GO:0006955]. This enrichment was further supported by MP pathway analysis, in which phenotype terms relating to dysregulation of the immune system or immune cells, such as "abnormal cytokine level" [MP:0008713], "increased susceptibility to bacterial infection" [MP:0002412] and "abnormal neutrophil physiology" [MP:0002463] were significantly enriched. That similar pathways were enriched in both GO and MP analyses, suggests that this is a robust finding. Given that the majority of differentially expressed genes were downregulated at 5-hours in excitatory hippocampal neurons

(90%), these results suggest that immune processes are downregulated in animals consolidating a recently learned memory compared to controls. Involvement of the immune response in consolidation of fear conditioning has previously been demonstrated. For example, in a mouse model of sepsis, which produces a robust immune response, it has been shown that mice freeze significantly less than controls to a context previously associated with shock, indicating dysregulated consolidation of associative memory (Huerta et al., 2016). Similarly, Toxoplasma infection reduced the freezing response of mice after CFC (Ihara et al., 2016;Ihara et al., 2019). In addition, gene expression studies following contextual fear conditioning have found enrichment of immune pathways (Barnes et al., 2012;Scholz et al., 2016), although given previous discussions (Chapter 3, section 3.4), these should be interpreted with caution. The GO term with the highest odds ratio in the genes significantly downregulated 5-hours after CFC acquisition was "regulation of cytokine production involved in inflammatory response" [GO:1900015]. Depino and colleagues investigated the role of the interleukin-1 (IL-1) cytokine family in a hippocampal-dependent inhibitory avoidance task, and found that inhibiting IL-1α and IL-1β in the dorsal hippocampus facilitated both short- and long-term memory, resulting in increased latencies to step down to a shock-associated platform (Depino et al., 2004). Thus, it could be that down-regulating cytokines during the consolidation process improves memory, potentially an evolutionary advantage. Whilst IL-1 family cytokines themselves weren't significantly downregulated in the present study, Sigirr an IL-1 receptor, was. It has also been demonstrated that increased levels of IL-1β impairs contextual, but not auditory, fear conditioning, resulting in a reduction of freezing behaviour (Pugh et al., 1999). Interestingly, increased levels of cytokines, particularly those in the IL-1 family, have been shown to disrupt expression of LTP in the CA1, CA3 and DG (Bellinger et al., 1993;Katsuki et al., 1990;O'Connor and Coogan, 1999) (Ross et al., 2003), potentially indicating the cellular mechanism through which over expression of IL-1 family cytokines impairs memory consolidation. Taken together, these results support the present finding that immune responses are downregulated following fear-dependent associative learning, and highlight the potential evolutionary advantages of doing so in relation to consolidating memories.

Further, pathway analysis revealed that genes that were significantly downregulated 2- or 5- hours after CFC were enriched in vascular related pathways. For example, at 2 hours post-CFC, GO terms such as "Regulation of endothelial cell migration" [GO:0010594], "Endothelium development" [GO:0003158] and "Blood vessel endothelial cell migration" [GO:0043534] were all enriched. Similarly, MP pathway analysis found enrichment in phenotypes including "Increased vascular permeability" [MP:0003070] and "Abnormal vascular development" [MP:0000259]. 5-hours post-CFC, enriched GO terms included "Regulation of vasculature development" [GO:1901342],

“Regulation of blood vessel endothelial cell migration” [GO:0043535] and “Positive regulation of hemopoiesis” [GO:1903708]. MP terms included “Increased vascular permeability” [MP:0003070] and “Abnormal blood vessel physiology” [MP:0000249]. These results indicate that genes downregulated at 2- or 5- hours post-CFC play a role in vascular development.

In the literature, changes to vasculature and learning and memory processes have mainly been studied in relation to vascular dementia, whereby vascular pathology such as cerebral infarcts and white matter lesions lead to difficulties with executive function, attention and memory (Nyenhuis et al., 2004) (O'Brien and Thomas, 2015;Gorelick et al., 2016). Within schizophrenia research, a recent study found that mRNAs important in maintaining blood brain barrier (BBB) physiology, such as intercellular adhesion molecule-1 (ICAM1) and VE-cadherin were upregulated in frontal cortex of patients with schizophrenia who also had high inflammatory markers (Cai et al., 2020). Further, decreases in cerebral blood volume (CBV) have been found in patients with schizophrenia (Brambilla et al., 2007) and reductions in frontal cerebral blood flow has been associated with psychotic symptoms (Katsel et al., 2017). However, vascular processes and endothelial physiology have not been comprehensively studied in relation to associative learning. Thus, whilst a link between vascular pathology and disease processes has been established, it is unclear the extent to which they are important in this particular paradigm.

5.4.4. Most genes were up-regulated 2-hours after CFC and then not differentially expressed at the 5-hour timepoint

Temporal patterns of gene expression in excitatory hippocampal neurons (TRAP-seq) 2- and 5- hours after CFC training were examined. It was found that the most common temporal dynamic was upregulation at 2-hours, and then no significant differential expression at the 5-hour timepoint. No genes were upregulated at 2-hours and then significantly downregulated at 5-hours, suggesting that 3 hours may be too short a time-frame to examine these significant changes.

Pathway analysis revealed that genes that were upregulated at 2-hours and then not significantly differentially expressed at 5-hours were enriched for neuronal and synaptic terms, including “regulation of neuronal differentiation” [GO:0045664] and “glutamatergic synapse” [GO:0098978]. “Positive regulation of dendrite development” [GO:1900006] was the GO term with the highest odds ratio. This suggests that transcripts encoding proteins involved in synaptic and dendritic changes after learning are only up-regulated for a short period of time. Following the trend of previous results, genes that were not differentially expressed at 2 hours but then significantly downregulated 5-hours after CFC training were enriched for GO terms involving the immune and inflammatory

response. Those down-regulated at both time-points were enriched for endothelial cell-related terms.

5.4.5 There was no association between genes expressed during the consolidation window and schizophrenia risk variants

Given the previously found enrichment of genes expressed after LTP in common and rare schizophrenia risk variants (Chapter 4), and that LTP is thought to underpin consolidation, I sought to investigate whether genes expressed 2- or 5-hours after CFC were also enriched in psychiatric disease risk variants. However, there was no evidence of enrichment of schizophrenia, bipolar or autism spectrum disorder common risk variants in either consolidation gene set. Further, there was no evidence of enrichment in rare variation for schizophrenia, as determined by CNV enrichment. Although no association between consolidation gene sets and rare schizophrenia risk variants had been found previously (Clifton et al., 2017b), given the mechanistic similarity of LTP and consolidation it could be hypothesised that this null association was an artefact of the methodology. However, given the present null results, it suggests that whilst the cellular mechanisms are similar, genes expressed after LTP and during the consolidation window do not share the same enrichment with schizophrenia risk. Comparison of the pathway analysis undertaken of the genes driving disease enrichment in Chapter 4, and the pathway analysis undertaken in the present chapter, further highlights differences in the genes expressed in between the LTP and the CFC experiment. Genes that drive the disease enrichment in the LTP experiment were enriched in pathways largely related to synaptic plasticity and neuronal structures, whilst genes expressed 2 hours after CFC were mainly enriched in vascular processes. Given that synaptic plasticity related genes have previously been found in genetic risk for schizophrenia, whilst vascular related genes have not, it is perhaps unsurprising that genes expressed 2-hours after CFC were not enriched in schizophrenia risk. There are also methodological reasons why these differences might occur, despite similar cellular properties. In the previous chapter, LTP was induced chemically with forskolin, which will lead to widespread neural activation, perhaps unlike that which occurs in response to learning *in vivo*. Thus, this exaggerated neural activation increases the power to detect significant enrichment. One way of overcoming this would be to sequence only those neurons which are activated during consolidation or retrieval of CFC, which can be achieved through the use of activity reporter mice such as Arc-dVenus (Eguchi and Yamaguchi, 2009). However, it cannot be ruled out that, despite LTP underpinning consolidation processes, there is no enrichment of consolidation-related genes in common and rare genetic variation for schizophrenia.

5.4.6 Summary

In conclusion, when using TRAP-seq methodology which allows the selective sequencing of ribosomal-associated genes specifically in excitatory hippocampal neurons, I found over 1,100 genes significantly differentially expressed 2- and 5- hours after CFC training. Only 7 genes were found to be differentially expressed in all cell types, derived from bulk sequencing, further confirming the advantages of cell-type specific sequencing for behavioural experiments. Whilst no association with genetic risk for schizophrenia was found, as might have been expected given that LTP is thought to underpin consolidation processes, it may be that this reflects the difference between artificial *ex-vivo* induction of LTP and *in-vivo* learning.

6: General discussion

6.1 Overview

The ability to learn and recall information is key for the survival of all living species. There are several stages underlying long term memory (LTM), including acquisition, consolidation and retrieval. Both consolidation and retrieval are underpinned by molecular and cellular changes, and have been demonstrated to require new gene expression and protein synthesis (McGaugh, 2000), (Kandel, 2001) (Debiec et al., 2002;Gafford et al., 2011)**.** Associative learning, the process through which one learns the relationship between two or more stimuli, has many adaptive applications, for example to avoid food that previously led to illness, and has been extensively researched over the past 50 years. Impairments in associative learning have been reported in several psychiatric disorders, including post-traumatic stress disorder (PTSD), anxiety, and schizophrenia (reviewed in (Lambert and McLaughlin, 2019;Pittig et al., 2018;Byrom, 2013)). Contextual fear conditioning (CFC) is an aversive fear conditioning paradigm which involves learning the association between a particular context and a footshock, resulting in the expression of fear behaviour (freezing) in response to presentation of the context alone (Blanchard and Blanchard, 1969). CFC is a discrete learning task that produces robust fear behaviour after a single conditioning trial, and has been shown to be hippocampal dependent (Phillips and LeDoux, 1992b;Daumas et al., 2005). Hippocampal abnormalities have been reported in patients with schizophrenia, including reduced pyramidal cell size (Benes et al., 1991;Arnold et al., 1995;Zaidel et al., 1997), reduced hippocampal volume (Velakoulis et al., 1999;Shenton et al., 2001;Szeszko et al., 2003) and increased hippocampal activity at rest (Tregellas et al., 2014). Given this, CFC represents a good paradigm with which to investigate learning and memory processes and schizophrenia.

Long term potentiation (LTP) is defined as the persistent increase in synaptic strength following synaptic activity. One of the most commonly studied forms of hippocampal LTP is between the CA3-CA1 Schaffer collaterals and is NDMA receptor dependent (Collingridge et al., 1983). LTP results in CREB-mediated transcription of new gene transcripts underpinning the structural changes needed to support LTP (Impey et al., 1996). Cellular and molecular processes underlying LTP have been shown to be important in the consolidation of fear memories (Rogan et al., 1997;McKernan and Shinnick-Gallagher, 1997;Martin et al., 2000). For example, NMDA receptors are crucial for the consolidation of inhibitory avoidance (Cammarota et al., 2000) and CFC (Athos et al., 2002). LTP processes have also been implicated in schizophrenia aetiology through studies of genetic variants in patients with schizophrenia. It has been found that single nucleotide polymorphisms (SNPs) found in patients with

schizophrenia are enriched for genes involved in synaptic plasticity, dopaminergic synapses and LTP (Schijven et al., 2018). Further, studies of rare copy number variants (CNVs) found in patients has uncovered associations with the NMDA receptor family, as well as learning and memory processes such as contextual conditioning and associative learning (Kirov et al., 2012;Pocklington et al., 2015). Finally, it has previously been reported that genes expressed 2- hours after extinction of CFC, but not recall, were enriched in rare genetic variants from patients with schizophrenia, suggesting a selective impact on extinction learning processes (Clifton et al., 2017b).

Previously, the link between plasticity and schizophrenia was demonstrated using curated databases such as Gene Ontology (GO) or Mammalian Phenotype (MP), rather than explored using gene-sets derived from a single, functional LTP experiment. Further, research conducted by Clifton and colleagues (2017) involved infusion of Zif268 antisense oligonucleotides to block reconsolidation processes in the control group, which may have introduced artefacts into the analysis. Therefore, the aims of this thesis were as follows:

- To test and extend previous findings that genes expressed during extinction of CFC are enriched for schizophrenia risk variants (Chapter 3).
- Quantify patterns of gene expression following long term potentiation (LTP), with particular focus on CA1 excitatory neurons, and test for association with genetic variants from patients with schizophrenia, schizophrenia-related disorders, and appropriate control disorders (Chapter 4).
- Explore the gene expression profile induced by the consolidation of CFC in excitatory hippocampal neurons, and test for psychiatric genetic association (Chapter 5).

The work described in this thesis has been independently discussed in each experimental chapter, and thus this chapter serves as a general overview of the main findings, implications, limitations, and future directions of the presented work.

6.2 Main findings

The main findings from each chapter of the thesis are presented in Table 6.1

Chapter	Key Findings
Chapter 3: Gene expression profiles following retrieval of Contextual Fear Conditioning (CFC) Species: Rat	• 72 unique genes were significantly differentially expressed in CA1 following recall or extinction of CFC • Gene ontology (GO) pathway analysis showed that these were mainly enriched in transcriptional processes • No genes were significantly differentially expressed 5-hours after recall or extinction of CFC • There was no enrichment of genes expressed after recall or extinction of CFC in either common or rare variation for schizophrenia
Chapter 4: The enrichment of LTP associated genes in genetic variation for schizophrenia Species: Mouse	• Cell-type specific sequencing (TRAP-seq) revealed over 2,000 genes were significantly differentially expressed following LTP induction in excitatory hippocampal neurons across 3 time points (30-, 60-, and 120- minutes after LTP induction) • This was over 3 times the number identified through bulk-seq of all cell types (501 genes) • An enrichment of genes expressed by excitatory neurons 60- and 120- minutes after LTP induction was found in common variation for schizophrenia and bipolar disorder; there was no enrichment for genes found through bulk RNA-seq • Genes expressed 60-minutes after induction of LTP in excitatory hippocampal neurons were enriched in CNVs from patients with schizophrenia when looking at deletions and duplications combined • When analysed separately, there was an enrichment of genes expressed in all cell-types 60 minutes after LTP in duplication CNVs from schizophrenia patients • There was no enrichment in ultra-rare coding variants from patients with schizophrenia

Chapter 5: Gene expression profiles in hippocampal excitatory neurons during consolidation of contextual fear conditioning (CFC) Species: Mouse	• Over 1,000 genes were found to be significantly differentially expressed in excitatory hippocampal neurons 2- and 5- hours after acquisition of CFC (during the consolidation window) • Only 7 differentially expressed genes were identified in the bulk RNA-seq condition containing transcripts from all cell-types • Genes that were significantly differentially expressed 5-hours after CFC were mainly down-regulated, and were enriched in immune processes and phenotypes • There was no association between genes expressed during the consolidation window in excitatory hippocampal neurons and genetic risk for schizophrenia, through either common or rare variation

Table 6.1*. Summary of the key findings of each experimental chapter.*

6.3 Implications

6.3.1 Implications for schizophrenia

The overarching aim of this thesis was to explore the enrichment of plasticity-related genes and schizophrenia risk, to better understand the processes upon which such risk variants act. In this section, I will discuss the results in terms of the implications for our knowledge of schizophrenia.

One of the first aims of this thesis was to test and replicate the finding that extinction-associated genes were enriched in rare variation for schizophrenia, as previously found in our group (Clifton et al., 2017b), using a simplified CFC protocol and updated RNA-seq methodology. However, as presented in Chapter 3, I found no evidence that genes expressed following extinction of CFC memory were enriched in rare variation as identified through CNVs from patients with schizophrenia. Similarly, I found no enrichment with common variation from SNPs identified in patients with schizophrenia, although this replicates the original study. Prior to the present work, previous work from our laboratory indicated that there was an association between associative learning genes and rare variation in schizophrenia, and as such the current result casts doubt on this. In addition, the results of Chapter 5 indicated that there was no association between genes expressed in hippocampal excitatory neurons following acquisition of CFC and schizophrenia.

Numerous studies of associative learning in patients with schizophrenia have found that patients display deficits in associative learning and retrieval of such learning, and the results of the present work do not call this into question. Rather, what has been discovered as a result of this work is that risk variants for schizophrenia potentially do not impact on genes involved in the acquisition or expression of negatively valenced associative learning. Fear is often associated with schizophrenia, mainly through the often-persecutory content of delusions and hallucinations reported by patients, and following publication of Clifton et al., (2017b) it was suggested that fear processing may be fundamental to schizophrenia pathogenesis (Sullivan, 2017). Further, it had been suggested that the association between adult hippocampal learning and risk variants opened new research pathways into the development of treatments for schizophrenia (Hall and Bray, 2022). However, the present results suggest that this may not be the case, and it would be prudent to conduct further basic neuroscientific research before investing in research aimed at investigating potential treatment pathways focused on the association between adult learning and schizophrenia. Whilst it may be disappointing to find negative results, especially ones which are contradictory to previous work carried out by one's research group, such results are important and, if upheld, add a valuable contribution to the field of schizophrenia research by preventing research funding being spent based on erroneous associations.

In the present work, fear conditioning was used to assess learning and explore how the subsequently expressed genes were associated with schizophrenia risk variants. Patients with schizophrenia have been shown to have impairments in the recall of extinction fear memories, as measured by a human equivalent of the present contextual fear conditioning paradigm (Holt et al., 2012). Therefore, this task was appropriate to recapitulate the negative valenced associative learning deficits found in patients with schizophrenia. In relation to the present results, this means that the lack of association between genes expressed after associative learning and schizophrenia risk variants can only be interpreted in terms of negatively valenced associations, and within the methodological parameters used (see 6.4). These results cannot be extended to interpret that there is no association between positive or neutral valenced associations, for which the present work does not provide evidence for.

In a further exploration of the overarching thesis aim, Chapter 4 explored whether genes expressed after long term potentiation (LTP), particularly in CA1 excitatory neurons, were enriched in genetic variants from patients with schizophrenia, schizophrenia-related disorders, and appropriate control disorders. Previously, association between schizophrenia and LTP has been shown using gene set enrichment analyses (Pocklington et al., 2015) which, whilst informative, may be subject to artefacts during the curation process. Here, for the first time, I demonstrated enrichment of LTP-associated genes from a functional experiment in common variation for schizophrenia, and rare CNV variation.

These results confirm previous findings in the literature that common and rare variants found in patients with schizophrenia impact on genes involved in synaptic plasticity processes (Kirov et al., 2012;Pocklington et al., 2015;Singh et al., 2022). Further, these results support the notion that risk variants particularly impact on CA1 excitatory neurons, as the association was specific to ribosome-associated transcripts in excitatory hippocampal neurons (Pocklington et al., 2015;Trubetskoy et al., 2022). That the association was specific to ribosome-associated mRNAs identified through TRAP-seq indicates that risk variants act upon genes that are actively being translated in excitatory hippocampal neurons, though it cannot tell us whether these were those newly transcribed following LTP or whether they were translating already in-situ mRNAs. Thus, the results of Chapter 4 have added to our knowledge of schizophrenia risk variant pathogenesis by demonstrating that they are enriched in genes that are being actively translated following LTP induction, a step up from our previous knowledge that there was an association based on curated databases.

In addition, I demonstrated, again for the first time using a functional LTP experiment, that genes expressed 60- and 120- minutes after LTP induction were enriched in common variation for bipolar disorder, and that this enrichment was specific to bipolar disorder type I. Bipolar disorder type I is more strongly associated with schizophrenia than bipolar disorder type II (Stahl et al., 2019;Mullins et al., 2021). The results presented here add to this literature and suggest a functional pathway (LTP processes) that may link genetic risk variants between the two disorders.

Whilst the work presented here did partially achieve the overall aim of this thesis, to explore the enrichment of plasticity-related genes and schizophrenia risk, it was not completely fulfilled. Having contradicted the previous work it was set to replicate; uncertainty has been left as to the extent to which learning-associated genes are enriched in schizophrenia risk variants. Further, only small facets of learning and schizophrenia were explored here, and as such broader knowledge into the enrichment of other learning paradigms, brain regions, and schizophrenia risk variants are still unknown. Future work that may help to elucidate this is presented in section 6.5.

6.3.2 Implications for learning and memory

Whilst not the overarching aim of the present work, the protocols used to explore associative learning and schizophrenia also gave the opportunity to add to the literature of gene expression following learning and memory. In Chapter 5, the gene expression profile of consolidation of CFC in excitatory hippocampal neurons was examined. The majority of genes significantly differentially expressed 5-hours after acquisition of CFC were significantly down-regulated, and were enriched in immune pathways and phenotypes, such as “regulation of cytokine production involved in

inflammatory response" [GO: 1900015] and "abnormal cytokine level" [MP:0008713]. Immune pathways have previously been implicated in LTM consolidation, particularly in rodent models where the immune system has been augmented, for example through over expression of cytokines or their receptors (Pugh et al., 1999;O'Connor and Coogan, 1999) (Ross et al., 2003), or through deliberate infection (Huerta et al., 2016;Ihara et al., 2016;Ihara et al., 2019). Further, reducing levels of IL-1 family cytokines has been found to facilitate long-term inhibitory avoidance memory (Depino et al., 2004). The results presented in this thesis further support this body of literature by showing a down-regulation of immune pathways during the consolidation of CFC memory. Specifically, the present results demonstrate that immune pathways are down-regulated following a natural demonstration of consolidation of fear, without altering the immune system, which may have had off-target effects. Further, as TRAP-seq enriches for genes that are ribosome-associated, the present findings indicate that genes in particular immune pathways are not being actively translated, an additional step beyond current knowledge that reducing levels of specific immune components impacts fear memories.

6.3.3 Considerations of TRAP-seq

The results presented in this thesis have shown that cell-type specific TRAP- seq has demonstrable advantages over the more commonly used bulk RNA-seq, particularly for examining gene expression following behavioural experiments. This is likely because behavioural experiments elicit more subtle gene expression patterns, the nuances of which are lost in bulk RNA-seq which contains transcripts expressed from cell-types. Even following chemical LTP, a targeted *ex vivo* manipulation that elicited widespread neuronal activation, TRAP-seq identified nearly 3 times as many differentially expressed genes compared to bulk RNA-seq. This mirrors previous findings in the literature, where cell-type specific sequencing has identified an increased number of biologically relevant differentially expressed genes than bulk RNA-seq alone in a range of contexts (Chen et al., 2017) (Allison et al., 2015;Haimon et al., 2018).

Whilst TRAP-seq has several advantages over bulk RNA-seq, there are practical considerations to consider for implementation. Firstly, TRAP-seq requires the extra step of immunoprecipitation (IP) prior to RNA extraction, which entails extra reagent and time costs. Similarly, IP may not be set up in many laboratories which focus on RNA-seq, as it was not in the current workspace before this thesis. A detailed protocol for IP using RiboTag mice is available in Sanz et al., 2019, which was used in the present work to set up and conduct IP experiments. Whilst the protocol would need to be piloted in each laboratory prior to beginning experimental work, this protocol is easy to use and required no adaptations in the present work. The protocol requires many reagents commonly found in existing

laboratories, such as Tris, KCl, M_gCl_2, DTT and NP-40, however other less common reagents such as cycloheximide, heparin and RNasin would need to be bought in. Further, the protocol requires antibodies and magnetic protein A/G beads, which can be costly. One of the most expensive elements of the present work was the use of RiboTag and CaMKIIα-cre mice, which were not present at Cardiff University and therefore had to be imported from The Jackson Laboratory in America. With prior planning, a few mice could be imported, reducing the costs, and then the experimental cohort bred-up internally. Once the experimental cohort were bred, no extra mice were required for the TRAP-seq element over more traditional bulk RNA-seq, as TRAP- and bulk- RNA were extracted from the same hippocampus of experimental animals as part of the IP process. However, the production of both TRAP- and bulk-seq samples meant extra samples were required to be sequenced, increasing sequencing costs compared to standard bulk RNA-seq. In terms of the bioinformatic analysis, no additional steps were required beyond the standard RNA-seq analysis pipeline, thus there is no additional barrier to entry for TRAP-seq compared to standard RNA-seq.

Potentially one of the main disadvantages of TRAP-seq is the need to know which cre driver line to use to drive expression of the RiboTag transcript. Whilst many behavioural paradigms have been extensively validated and the underlying neurobiology established, this is not the case for all applications of RNA-seq, or in new behavioural protocols. Thus, further work prior to TRAP-seq would need to be performed in order to establish the cell types involved. One way in which the cell-type of interest could be established is by using spatial gene expression analyses such as RNAScope, which is an in-situ hybridisation protocol which allows one to visualise single RNA molecules (Wang et al., 2012). Conducting an RNAScope analysis on a gene which one knows to be regulated following the experimental manipulation of interest, and then cross referencing it with available spatial brain atlases, would allow one to determine which cell type driver to use.

6.4 Limitations

6.4.1 Methodological

When interpreting the results presented in this thesis, there are several methodological limitations to be considered. Firstly, whilst it has been shown that the contextual fear conditioning paradigm is a contextual configuration task (Rudy and O'Reilly, 1999;Fanselow, 2000;Rudy and O'Reilly, 2001), this was not explicitly tested. Therefore, it cannot be stated for certain that the genes identified in this thesis were a result of contextual fear conditioning specifically, or fear conditioning more generally, as the context in itself may have acted as a cue. In order to test this, future experiments

could test the context specificity by exposing rodents to a second, neutral context (not previously associated with shock), and measure freezing responses. It would be expected that freezing responses would be reduced in the neutral context compared to the conditioned context. In addition, the behavioural output of the CFC (freezing behaviour) was only scored by a single experimenter. To confirm robustness of the freezing behaviour and indicate quality of the initial scoring, behaviour is ideally scored by at least 2 independent raters. As such, whilst a standard CFC paradigm was used which has been previously shown to induce fear responses (Lee et al., 2004a;Clifton et al., 2017a), it cannot be stated for certain that appropriate levels of freezing responses were generated from the current experiments.

Further, a limited number of timepoints were used in the present study, owing to the practical and cost considerations of conducting RNA-seq and TRAP-seq experiments, within the context of the global pandemic. The 2-hour time point was chosen as it replicated the previous work done in our laboratory, and it represents the start of the second peak of gene transcription following learning (Barnes et al., 2012;Scholz et al., 2016;Clifton et al., 2017b). Following the results of the first experiment conducted (2-hour Chapter 3), it was decided that a second later timepoint would be done to capture genes that might be involved in more downstream processes, such as immune functioning, as pathway analysis at 2-hours showed enrichments in transcription-related processes. 5-hours was chosen as a later point in the second transcription peak window, which was replicated in Chapter 5 for consistency between the chapters. However, with hindsight it may have been better to undertake the later timepoint at 4-hours, for consistency with other papers such as Cho et al., (2015). Further, given that significant association was found between genes expressed 60-minutes after LTP and schizophrenia and bipolar risk variants, in future work (see section 6.5.1.) I would undertake gene expression analyses at 1 hour, 2 hours and 4 hours.

It has been demonstrated that patients with schizophrenia may show differential associative learning impairments in neutral compared to aversive stimuli, in that deficits in acquisition of associative learning are found for neutral stimuli, but aberrant extinction is shown for aversive conditioning (Diwadkar et al., 2008;Brambilla et al., 2011;Holt et al., 2012). Aversive conditioning was used in the current work due to extensive prior research regarding the time points, neurobiology and circuitry underlying fear conditioning. Further, the protocol for aversive contextual fear conditioning is similar in rodents and humans, in that both involve exposure to a context (virtually for humans) and aversive shock to extremities (feet in rodents, hands in humans), which may increase the translational value of such studies. Whilst neutral pair associations can be readily applied to protocols in humans, for example names-faces, these are less able to be translated to neutral conditioning in rodents. As such, using an aversive associative learning paradigm was optimal

in the present study. However, it would be interesting to examine whether a positively valenced associative learning paradigm, for example operating a foot lever for a food pellet, results in similar gene expression profiles and potential association with schizophrenia risk genes.

Further, the results described in this thesis are subject to the analytical methods used, such as the FDR threshold, and differential expression analysis pipeline used. As such, when genes are described as "significantly differentially expressed" or "not significantly differentially expressed" this is within the context of the current paradigm and subject to the experimental methods. Whilst well documented differential expression analysis pipelines were used, it may be that the results would differ upon usage of other packages such as DESeq2 or EdgeR. In order to validate the bioinformatic methods used here, the analysis could have been repeated with a second differential expression package and the overlap quantified. Given that previous research has found a high concordance rate between DESeq2 and Limma (Tong, 2021), I would not expect large differences in differential expression to be found, or for this to change the significance of the present results. Nonetheless, it would be an important step in validating the present results.

6.4.2 Updated schizophrenia GWAS

Since conducting the present research, a new wave of the Psychiatric Genetic Consortium (PGC) schizophrenia GWAS has been released (PGC3). This release contains data from over 76,000 patients with schizophrenia, greatly extending PGC2 sample (Trubetskoy et al., 2022). Given the p-values of the current null results were not close to being significant, and that the β values were close to 0, I would not expect these results to change (Table 6.2). However, given the increased sample size, it would be interesting to examine whether the effect sizes or p-values increased in the 120- minute TRAP-seq gene set of excitatory hippocampal genes.

	Gene-set	β value	p-value
Chapter 3	Retrieval (shared)	0.042	0.439
	Extinction	- 0.198	0.865
Chapter 5	Excitatory neurons 2-hours post CFC acquisition	0.001	0.974
	Excitatory neurons 5-hours post CFC acquisition	-0.173	0.993

Table 6.2. *β values and p-values of null results from Chapters 3 and 5: enrichment of gene sets in common variation for schizophrenia.*

6.4.3 CaMKIIα promotor targeting other cell types

Recently, a pre-print paper found that CaMKIIα promotor manipulations target both excitatory and inhibitory neurons (Veres et al., 2022). This was established through visualisation of adeno-

associated viral vectors (AAV) containing the CaMKIIα-channel-rhodopsin 2-mCherry construct, which showed the mCherry construct in a variety of interneuron types. Whilst this may cause doubt on the appropriateness of CaMKIIα promotor construct for this type of work, this model has been extensively validated, both by previous authors and in the present work (Benson et al., 1991;Jones et al., 1994;Sík et al., 1998;Liu and Murray, 2012). In the present work, de-enrichment of *GAD1*, expressed in inhibitory neurons, was found, indicating that the RiboTag construct effectively targeted excitatory neurons. However, methodological issues with the perfusion of test mice meant that immunohistochemical co-localisation of the HA tag (as part of the RiboTag construct) and inhibitory neuronal protein markers could not be conducted in the present work. Therefore, whilst it is unlikely to have impacted the present work, this cannot be ruled out.

6.5 Future directions

6.5.1 TRAP-seq following retrieval of CFC

The overall aim of this thesis was to further explore the enrichment of plasticity-associated genes in schizophrenia risk. Whilst this was achieved in regard to LTP, the association with extinction-related genes is still unknown. No association between extinction-related genes and genes impacted by common or rare variation in schizophrenia was found, but due to the low numbers of genes identified through bulk sequencing it is not known whether this is an artefact of the methodology. Given the demonstration in this thesis that cell-type specific TRAP-seq both identifies more differentially expressed genes, and potentially with a greater relevance to both the biological pathways and association with disease risk, the next experiment I would do is conduct cell-type specific sequencing following the retrieval of conditioned fear, as presented in Chapter 3. Given the present results, it is likely that several hundred differentially expressed genes would be identified following recall and extinction of conditioned fear, which should allow adequate power to detect an association with variation in schizophrenia if one exists. If such an association was found, it would demonstrate experimentally that associative learning processes are impacted by schizophrenia risk variants, which would have implications for the aetiology of such impairments in patients with schizophrenia. If no association was found, there would again be implications for the aetiology of associative learning impairments, namely that these are potentially not driven by genetic risk factors, but potentially more social or environmental outcomes. If this were the case, then research could be done within a clinical setting to examine if psycho-social interventions reduced associative learning impairments found in patients with schizophrenia.

Whilst conducting TRAP-seq after retrieval was considered for the current work, the global pandemic restricted the time available to conduct such experiments. Previous pilot work from our lab found that, following footshock, mice did not readily extinguish fear memories, displaying consistent freezing behaviour of around 40 % across 7 extinction sessions, and 1 month later (Trent, 2018). Whilst extinction behaviour has been demonstrated in mice (reviewed in (Curzon et al., 2011)**)** given the time restrictions imposed by the pandemic it was not possible to troubleshoot and produce a working protocol in our lab. However, successful implementation of this protocol would permit gene expression profiling of excitatory hippocampal neurons following retrieval of CFC. This is of interest both in itself, to further understand the translatomic landscape of retrieval processes, and to establish whether it was methodological confounds which led to an association (or lack of) with schizophrenia risk variants, or whether this result is robust.

6.5.2 Other cognitive tasks

The focus of this thesis was the enrichment of genes expressed after consolidation and retrieval of CFC in genetic variation for schizophrenia. However, a range of cognitive impairments have been identified in patients with schizophrenia, including attention, working memory and problem solving (Kelly et al., 2000;Eryilmaz et al., 2016;Zhang et al., 2017). Thus, there may be enrichments of other learning and memory related gene sets in common and rare variation for schizophrenia. Whilst CFC is methodologically ideal for discerning such associations, due to the discrete learning point which can be linked to gene expression and the extensive knowledge about the underlying brain circuitry, there are other paradigms that also offer this opportunity. Working memory impairments are a core feature of schizophrenia, with impairments reported across several domains including spatial-, auditory- and object- working memory (for example (Badcock et al., 2008;Gooding and Tallent, 2004;Liu et al., 2021b); reviewed in (Park and Gooding, 2014;Lett et al., 2014)). In rodents, one of the most commonly used working memory tasks is the radial arm maze, in which the outstretched arms are baited, and rodents must visit each in turn to receive reward, whilst remembering which arms have been visited so as not to commit a working memory error (Olton and Samuelson, 1976). Whilst the discrete learning point may not be as easily determined compared to CFC, previous studies have identified upregulation of a number of genes, including Arc and Homer-1α (Nikbakht et al., 2012). Further, the radial arm maze has been shown to be hippocampal dependent (Olton et al., 1979;Floresco et al., 1997;Kawabe et al., 1998), thus it may be of interest to examine gene expression patterns following the radial arm maze, and investigate enrichment in schizophrenia risk.

In addition, a further task that has ecological validity is the intra- extra-dimensional set-shift task, which is an attentional set-shifting and reversal learning task which mimics the intra-extra

dimensional (IED) set shift task in the Cambridge Neuropsychological Test Automated Battery (CANTAB) human cognitive function test battery. In the human version of the task, pink shape and white line configurations are shown in two of 4 positions on the screen, and participants must select the correct configuration using the feedback provided (Cambridge Cognition). The rule changes after 6 correct responses. In the rodent version of the task, rodents are presented with stimuli which differ in odour, sight and tactile perceptual dimensions, and food reward is administered upon selection of the correct configuration of stimuli (Scheggia and Papaleo, 2016). After 8 consecutive correct responses, the rule is changed. Performance in these tasks are used as a measure of executive functioning, similar to the WCST, and patients with schizophrenia have been shown to have impaired performance in the task (Jazbec et al., 2007;Ceaser et al., 2008). Executive functioning is key to allow an individual to adapt to new situations, and has been suggested to be necessary for formulating an objective, planning and implementing actions to achieve such tasks (Lezak et al., 2004). Thus, executive dysfunction in patients with schizophrenia may go some way to explain their poorer social outcomes, such as lower employment rates, lower rates of independent living and poor self-care (Orellana and Slachevsky, 2013;Strassnig et al., 2018). Therefore, given both the ecological validity of the task, and that executive functioning impairments likely represent large symptom burden and impact social functioning, examining the association between genes expressed after the IED task and schizophrenia would go further in answering the overarching aim of this thesis. Tasks of executive function have been shown to be dependent on the dorsolateral prefrontal cortex, a brain region which has also been found to be structurally changed in patients with schizophrenia (Harrison and Owen, 2002;Rüsch et al., 2007). The cre-driver line used in the present work has been shown to have forebrain expression, and has been used previously in studies of prefrontal cortex (Pinto and Dan, 2015). As such, it could also be used in experiments of executive functioning which are dependent on the prefrontal cortex. Following the administration of the IED task in RiboTag x CaMKIIα-cre mice, animals could be culled and prefrontal cortex dissected and snap frozen to undergo IP and RNA sequencing as described in the present work. This would allow gene expression profiles following an executive function task to be explored, and association with schizophrenia to be established. Similar to TRAP-seq following retrieval of CFC, a significant association would suggest that executive functioning, as tested through a set-shifting task, leads to the expression of genes implicated in schizophrenia risk. Further pathway analysis could then be used to determine pathways impacted by these genes, potentially opening paths to further experimental work or association with known drug targets for future therapeutic use. Alternatively, no association may suggest that these impairments in patients with schizophrenia may be an

interaction between environmental, social and genetic factors, and thus lead the path to more clinical psychosocial interventions being tested.

6.5.3 Association of LTP genes and Major depressive disorder (MDD)

There is evidence that glutamatergic dysfunction in the hippocampus contributes to the aetiology of MDD (Pittenger and Duman, 2008). For example, it has been found that patients with treatment-resistant and remitted-recurrent depression had significantly lower levels of glutamate, particularly in the right hippocampus, compared to controls, and that levels were correlated with longer illness duration (de Diego-Adeliño et al., 2013). Further, reduced metabotropic glutamate receptor 5 (mGluR5) binding has been found in the hippocampi of patients with MDD, and severity of depressive symptoms was found to negatively correlate with mGluR5 binding in this brain region (Deschwanden et al., 2011). Given that LTP requires glutamate signalling, it would be interesting to examine whether genes expressed after LTP were also associated with risk for MDD. A recent GWAS meta-analysis of patients with MDD identified 102 loci associated with the disorder, pathway analysis of which revealed enrichment in synaptic structures and neurotransmission (Howard et al., 2019), making it an interesting avenue of research. Currently, in order to use a large proportion of the data within this GWAS, a data transfer agreement is required with 23andme, which takes a considerable amount of time and was not in place for the current work. However, it could be requested in the future to test the association between LTP related genes and genes associated with MDD.

6.6 Concluding remarks

Advances in the understanding of the genetic architecture of schizophrenia, and in gene expression profiling techniques, allows the opportunity to investigate enrichment of functional gene sets in genetic risk variants identified in schizophrenia patients. Using the CFC paradigm, which is relevant to schizophrenia due to identified associative learning deficits in patients and the dependency on the hippocampus, this thesis has identified genes expressed during consolidation and retrieval of long-term fear memories, although no association with schizophrenia risk variants were found. Enrichment of LTP-associated gene sets, specifically in excitatory hippocampal neurons, were found to be enriched in schizophrenia- and bipolar- associated risk variants. The next step in progressing this research would be to undertake cell-type specific TRAP-seq following retrieval of conditioned fear, potentially combined with an activity-reporter mechanism to ensure sequencing of only those

cells activated by retrieval processes, in order to further elucidate the enrichment of memory-related mechanisms in schizophrenia risk.

7: Bibliography

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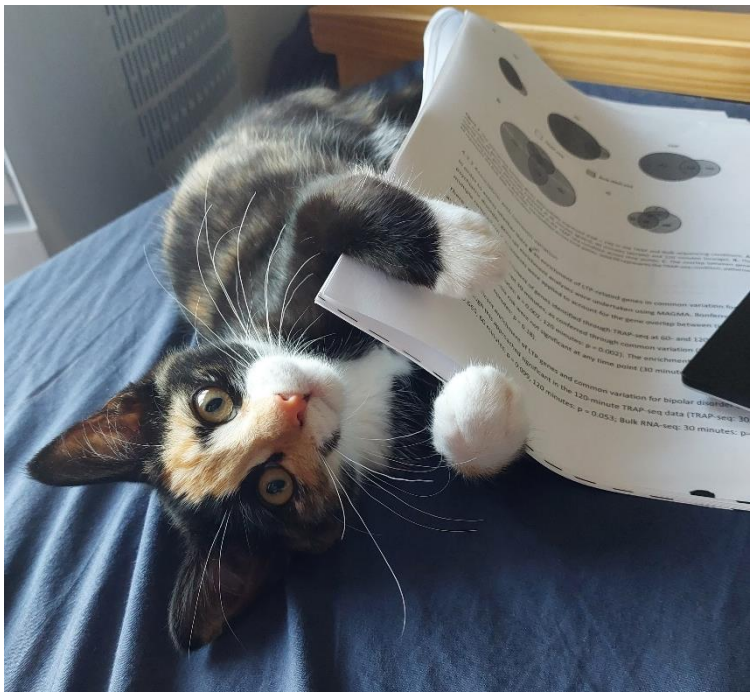
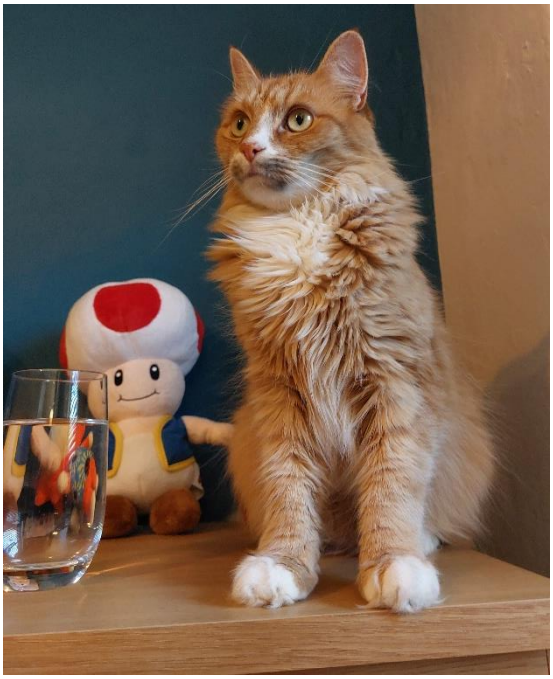
Acknowledgements Part II

I wasn't sure that cats were appropriate for the main acknowledgements section, so I have included them as an appendix at the end. With special thanks to Bonnie, who made writing this thesis cuter, if a little harder by stomping on my keyboard (any continuous spells of nonsense numbers and letters left in this thesis are her input!). And Arnie, who brings joy to my life and whose fluffy love cheered me up after bad days in the lab. Sorry I brought Bonnie home and made you a grump!





8: Appendix

8.1: Table of the genes driving the enrichment with common variation in Chapter 4

Gene set	Entrez ID	Symbol	P-value
120 TRAP schizophrenia	57449	PLEKHG5	9.23E-04
120 TRAP schizophrenia	473	RERE	2.88E-07
120 TRAP schizophrenia	23095	KIF1B	0.024668
120 TRAP schizophrenia	8672	EIF4G3	1.92E-04
120 TRAP schizophrenia	8289	ARID1A	8.10E-04
120 TRAP schizophrenia	84065	TMEM222	0.016373
120 TRAP schizophrenia	27245	AHDC1	0.0056835
120 TRAP schizophrenia	6421	SFPQ	9.34E-04
120 TRAP schizophrenia	9202	ZMYM4	0.014296
120 TRAP schizophrenia	149175	MANEAL	7.39E-04
120 TRAP schizophrenia	643314	NA	0.001684
120 TRAP schizophrenia	284716	RIMKLA	2.08E-04
120 TRAP schizophrenia	79033	ERI3	0.030574
120 TRAP schizophrenia	55225	RAVER2	0.028984
120 TRAP schizophrenia	57554	LRRC7	0.014788
120 TRAP schizophrenia	84146	ZNF644	2.32E-04
120 TRAP schizophrenia	64783	RBM15	0.0010696
120 TRAP schizophrenia	51592	TRIM33	0.026401
120 TRAP schizophrenia	51177	PLEKHO1	3.65E-09
120 TRAP schizophrenia	23248	RPRD2	2.35E-06
120 TRAP schizophrenia	80222	TARS2	2.41E-06
120 TRAP schizophrenia	4170	MCL1	0.03176
120 TRAP schizophrenia	57459	GATAD2B	6.98E-06
120 TRAP schizophrenia	103	ADAR	0.02147
120 TRAP schizophrenia	23623	RUSC1	0.0063138
120 TRAP schizophrenia	55870	ASH1L	0.0018853
120 TRAP schizophrenia	54856	GON4L	0.04523
120 TRAP schizophrenia	4000	LMNA	0.003005
120 TRAP schizophrenia	93183	PIGM	0.028268
120 TRAP schizophrenia	1314	COPA	2.39E-06
120 TRAP schizophrenia	23385	NCSTN	1.21E-06
120 TRAP schizophrenia	22920	KIFAP3	0.029892
120 TRAP schizophrenia	7143	TNR	9.82E-06
120 TRAP schizophrenia	460	ASTN1	6.04E-04
120 TRAP schizophrenia	9462	RASAL2	0.025488
120 TRAP schizophrenia	777	CACNA1E	0.0030366
120 TRAP schizophrenia	1660	DHX9	0.0042446
120 TRAP schizophrenia	5997	RGS2	0.047416
120 TRAP schizophrenia	23528	ZNF281	2.85E-05

120 TRAP schizophrenia	23114	NFASC	1.83E-04
120 TRAP schizophrenia	6900	CNTN2	1.35E-06
120 TRAP schizophrenia	5362	PLXNA2	1.25E-05
120 TRAP schizophrenia	56776	FMN2	2.57E-04
120 TRAP schizophrenia	9859	CEP170	5.04E-07
120 TRAP schizophrenia	50618	ITSN2	0.0146
120 TRAP schizophrenia	2355	FOSL2	0.0039659
120 TRAP schizophrenia	6654	SOS1	0.03889
120 TRAP schizophrenia	6546	SLC8A1	2.50E-08
120 TRAP schizophrenia	57142	RTN4	0.0057382

gene_set	entrez_ID	Symbol	P-value
120 TRAP schizophrenia	55704	CCDC88A	0.018339
120 TRAP schizophrenia	53335	BCL11A	2.76E-08
120 TRAP schizophrenia	27332	ZNF638	0.00822
120 TRAP schizophrenia	1961	EGR4	0.00124
120 TRAP schizophrenia	23020	SNRNP200	7.73E-04
120 TRAP schizophrenia	26504	CNNM4	0.0013541
120 TRAP schizophrenia	9392	TGFBRAP1	0.0024192
120 TRAP schizophrenia	53353	LRP1B	6.15E-06
120 TRAP schizophrenia	9839	ZEB2	1.24E-07
120 TRAP schizophrenia	55183	RIF1	0.039854
120 TRAP schizophrenia	4929	NR4A2	0.0076207
120 TRAP schizophrenia	57282	SLC4A10	0.045887
120 TRAP schizophrenia	6328	SCN3A	0.0018671
120 TRAP schizophrenia	6326	SCN2A	0.031619
120 TRAP schizophrenia	6323	SCN1A	2.03E-04
120 TRAP schizophrenia	151556	GPR155	0.0058017
120 TRAP schizophrenia	23451	SF3B1	1.95E-11
120 TRAP schizophrenia	23314	SATB2	3.85E-10
120 TRAP schizophrenia	205327	C2orf69	1.81E-12
120 TRAP schizophrenia	659	BMPR2	0.001808
120 TRAP schizophrenia	57683	ZDBF2	0.0044465
120 TRAP schizophrenia	80309	SPHKAP	2.34E-04
120 TRAP schizophrenia	92737	DNER	0.016157
120 TRAP schizophrenia	4691	NCL	0.0069447
120 TRAP schizophrenia	26058	GIGYF2	1.21E-12
120 TRAP schizophrenia	152330	CNTN4	4.20E-14
120 TRAP schizophrenia	201595	STT3B	0.021995
120 TRAP schizophrenia	10015	PDCD6IP	0.029627
120 TRAP schizophrenia	353274	ZNF445	0.010386
120 TRAP schizophrenia	10869	USP19	0.0088763
120 TRAP schizophrenia	1605	DAG1	0.012703
120 TRAP schizophrenia	8927	BSN	0.005303
120 TRAP schizophrenia	10181	RBM5	8.40E-04
120 TRAP schizophrenia	1849	DUSP7	0.019788
120 TRAP schizophrenia	80335	WDR82	1.36E-04
120 TRAP schizophrenia	55193	PBRM1	7.38E-08
120 TRAP schizophrenia	200845	KCTD6	0.017376
120 TRAP schizophrenia	5793	PTPRG	9.15E-04
120 TRAP schizophrenia	55079	FEZF2	0.045742
120 TRAP schizophrenia	166336	PRICKLE2	1.04E-06
120 TRAP schizophrenia	6092	ROBO2	0.018209
120 TRAP schizophrenia	6091	ROBO1	0.017112
120 TRAP schizophrenia	200894	ARL13B	0.015674
120 TRAP schizophrenia	214	ALCAM	0.0062886
120 TRAP schizophrenia	26137	ZBTB20	0.019246
120 TRAP schizophrenia	50512	PODXL2	0.031437
120 TRAP schizophrenia	80012	PHC3	0.006662
120 TRAP schizophrenia	57709	SLC7A14	0.028857
120 TRAP schizophrenia	55689	YEATS2	0.034326

gene_set	entrez_ID	Symbol	P-value
120 TRAP schizophrenia	285382	C3orf70	0.0015126
120 TRAP schizophrenia	165918	RNF168	0.0049357
120 TRAP schizophrenia	152	ADRA2C	0.023833
120 TRAP schizophrenia	23216	TBC1D1	0.0041545
120 TRAP schizophrenia	65997	RASL11B	0.035439
120 TRAP schizophrenia	23284	ADGRL3	2.06E-04
120 TRAP schizophrenia	2044	EPHA5	4.34E-05
120 TRAP schizophrenia	55236	UBA6	0.011409
120 TRAP schizophrenia	56978	PRDM8	0.030988
120 TRAP schizophrenia	9348	NDST3	1.15E-04
120 TRAP schizophrenia	84162	KIAA1109	0.04394
120 TRAP schizophrenia	10424	PGRMC2	0.042585
120 TRAP schizophrenia	11275	KLHL2	8.02E-04
120 TRAP schizophrenia	4750	NEK1	6.44E-05
120 TRAP schizophrenia	108	ADCY2	0.025979
120 TRAP schizophrenia	1008	CDH10	0.026316
120 TRAP schizophrenia	2668	GDNF	0.0013679
120 TRAP schizophrenia	3350	HTR1A	0.011756
120 TRAP schizophrenia	3156	HMGCR	0.0014572
120 TRAP schizophrenia	10087	CERT1	2.25E-04
120 TRAP schizophrenia	133746	JMY	0.0071531
120 TRAP schizophrenia	9456	HOMER1	0.0014541
120 TRAP schizophrenia	57561	ARRDC3	0.0084327
120 TRAP schizophrenia	5122	PCSK1	0.0046031
120 TRAP schizophrenia	3295	HSD17B4	0.017707
120 TRAP schizophrenia	1958	EGR1	1.02E-08
120 TRAP schizophrenia	3313	HSPA9	1.75E-09
120 TRAP schizophrenia	54882	ANKHD1	1.02E-05
120 TRAP schizophrenia	10307	APBB3	0.0031454
120 TRAP schizophrenia	929	CD14	2.67E-06
120 TRAP schizophrenia	56124	PCDHB12	6.08E-04
120 TRAP schizophrenia	56113	PCDHGA2	0.0026248
120 TRAP schizophrenia	56112	PCDHGA3	0.0027228
120 TRAP schizophrenia	56107	PCDHGA9	0.0019898
120 TRAP schizophrenia	56105	PCDHGA11	0.0020479
120 TRAP schizophrenia	56097	PCDHGC5	0.0022945
120 TRAP schizophrenia	1729	DIAPH1	0.049875
120 TRAP schizophrenia	5097	PCDH1	0.01854
120 TRAP schizophrenia	81848	SPRY4	0.020291
120 TRAP schizophrenia	133522	PPARGC1B	0.0051356
120 TRAP schizophrenia	79646	PANK3	0.0074496
120 TRAP schizophrenia	6586	SLIT3	0.038874
120 TRAP schizophrenia	1627	DBN1	0.045468
120 TRAP schizophrenia	3187	HNRNPH1	0.0027687
120 TRAP schizophrenia	8878	SQSTM1	9.11E-04
120 TRAP schizophrenia	9856	KIAA0319	0.014498
120 TRAP schizophrenia	8968	H3C7	9.67E-06
120 TRAP schizophrenia	79692	ZNF322	1.04E-13
120 TRAP schizophrenia	80317	ZKSCAN3	1.74E-26

gene_set	entrez_ID	Symbol	P-value
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120 TRAP schizophrenia	23469	PHF3	1.22E-06
120 TRAP schizophrenia	577	ADGRB3	2.78E-07
120 TRAP schizophrenia	10492	SYNCRIP	4.71E-05
120 TRAP schizophrenia	23036	ZNF292	0.049418
120 TRAP schizophrenia	2045	EPHA7	0.018492
120 TRAP schizophrenia	81491	GPR63	0.016047
120 TRAP schizophrenia	2309	FOXO3	1.66E-06
120 TRAP schizophrenia	5570	PKIB	0.02554
120 TRAP schizophrenia	23345	SYNE1	1.37E-05
120 TRAP schizophrenia	55975	KLHL7	0.044187
120 TRAP schizophrenia	8887	TAX1BP1	0.0013714
120 TRAP schizophrenia	63974	NEUROD6	0.0034004
120 TRAP schizophrenia	3624	INHBA	0.0020037
120 TRAP schizophrenia	9031	BAZ1B	0.0098611
120 TRAP schizophrenia	2969	GTF2I	0.012896
120 TRAP schizophrenia	27445	PCLO	4.23E-09
120 TRAP schizophrenia	1595	CYP51A1	0.012873
120 TRAP schizophrenia	4885	NPTX2	0.048419
120 TRAP schizophrenia	64599	GIGYF1	1.15E-06
120 TRAP schizophrenia	7425	VGF	0.011422
120 TRAP schizophrenia	4897	NRCAM	0.0018201
120 TRAP schizophrenia	4189	DNAJB9	1.05E-05
120 TRAP schizophrenia	9732	DOCK4	7.69E-04
120 TRAP schizophrenia	3475	IFRD1	0.042223
120 TRAP schizophrenia	83992	CTTNBP2	0.0026514
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120 TRAP schizophrenia	79269	DCAF10	0.014786
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120 TRAP schizophrenia	29979	UBQLN1	0.001206
120 TRAP schizophrenia	9568	GABBR2	7.27E-09

gene_set	entrez_ID	Symbol	P-value
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120 TRAP schizophrenia	221079	ARL5B	1.74E-04
120 TRAP schizophrenia	220213	OTUD1	0.01019
120 TRAP schizophrenia	57512	GPR158	0.038278
120 TRAP schizophrenia	94134	ARHGAP12	4.21E-04
120 TRAP schizophrenia	3799	KIF5B	3.88E-04
120 TRAP schizophrenia	8829	NRP1	0.0018146
120 TRAP schizophrenia	23283	CSTF2T	9.82E-05
120 TRAP schizophrenia	288	ANK3	7.57E-07
120 TRAP schizophrenia	84159	ARID5B	3.61E-06
120 TRAP schizophrenia	1959	EGR2	0.017451
120 TRAP schizophrenia	221037	JMJD1C	0.012395
120 TRAP schizophrenia	10580	SORBS1	0.030197
120 TRAP schizophrenia	55719	SLF2	0.0018443
120 TRAP schizophrenia	8945	BTRC	8.44E-04
120 TRAP schizophrenia	23082	PPRC1	0.011714
120 TRAP schizophrenia	9118	INA	3.41E-07
120 TRAP schizophrenia	9748	SLK	0.0079647
120 TRAP schizophrenia	85450	ITPRIP	0.0014436
120 TRAP schizophrenia	22986	SORCS3	1.08E-07
120 TRAP schizophrenia	1847	DUSP5	0.013097
120 TRAP schizophrenia	10579	TACC2	0.045006
120 TRAP schizophrenia	1571	CYP2E1	0.044802
120 TRAP schizophrenia	1200	TPP1	0.0062739
120 TRAP schizophrenia	10418	SPON1	0.034691
120 TRAP schizophrenia	144110	TMEM86A	0.034554
120 TRAP schizophrenia	338645	LUZP2	5.43E-04
120 TRAP schizophrenia	627	BDNF	5.45E-04
120 TRAP schizophrenia	4076	CAPRIN1	5.00E-04
120 TRAP schizophrenia	1132	CHRM4	2.72E-12
120 TRAP schizophrenia	9793	CKAP5	1.29E-07
120 TRAP schizophrenia	79096	CSTPP1	4.08E-06
120 TRAP schizophrenia	85456	TNKS1BP1	0.0056644
120 TRAP schizophrenia	1500	CTNND1	2.85E-09
120 TRAP schizophrenia	1642	DDB1	0.033002
120 TRAP schizophrenia	79026	AHNAK	0.035119
120 TRAP schizophrenia	56834	GPR137	0.016095
120 TRAP schizophrenia	11007	CCDC85B	0.0069229
120 TRAP schizophrenia	9158	FIBP	0.0060475
120 TRAP schizophrenia	10992	SF3B2	0.0024908
120 TRAP schizophrenia	55690	PACS1	0.036879
120 TRAP schizophrenia	266743	NPAS4	0.028978
120 TRAP schizophrenia	5091	PC	0.0072916
120 TRAP schizophrenia	5499	PPP1CA	0.029433
120 TRAP schizophrenia	56946	EMSY	0.041885

gene_set	entrez_ID	Symbol	P-value
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120 TRAP schizophrenia	4684	NCAM1	4.20E-05
120 TRAP schizophrenia	57453	DSCAML1	1.30E-04
120 TRAP schizophrenia	6230	RPS25	0.0037109
120 TRAP schizophrenia	57476	GRAMD1B	5.28E-04
120 TRAP schizophrenia	3703	STT3A	0.0033394
120 TRAP schizophrenia	29118	DDX25	0.030784
120 TRAP schizophrenia	399979	SNX19	3.78E-10
120 TRAP schizophrenia	22997	IGSF9B	4.24E-17
120 TRAP schizophrenia	6515	SLC2A3	2.66E-04
120 TRAP schizophrenia	2904	GRIN2B	0.048032
120 TRAP schizophrenia	55605	KIF21A	1.76E-07
120 TRAP schizophrenia	144165	PRICKLE1	0.029808
120 TRAP schizophrenia	6334	SCN8A	0.016366
120 TRAP schizophrenia	84975	MFSD5	0.0064818
120 TRAP schizophrenia	4035	LRP1	5.70E-08
120 TRAP schizophrenia	55832	CAND1	0.014874
120 TRAP schizophrenia	7184	HSP90B1	0.02714
120 TRAP schizophrenia	255394	TCP11L2	6.04E-04
120 TRAP schizophrenia	326625	MMAB	1.67E-04
120 TRAP schizophrenia	88455	ANKRD13A	8.67E-05
120 TRAP schizophrenia	121665	SPPL3	5.94E-06
120 TRAP schizophrenia	51433	ANAPC5	0.0025472
120 TRAP schizophrenia	605	BCL7A	6.19E-05
120 TRAP schizophrenia	6249	CLIP1	1.01E-07
120 TRAP schizophrenia	9612	NCOR2	0.0047549
120 TRAP schizophrenia	23504	RIMBP2	0.023974
120 TRAP schizophrenia	23141	ANKLE2	0.0022222
120 TRAP schizophrenia	2802	GOLGA3	0.017919
120 TRAP schizophrenia	11215	AKAP11	0.030333
120 TRAP schizophrenia	5100	PCDH8	1.43E-04
120 TRAP schizophrenia	10464	PIBF1	5.96E-04
120 TRAP schizophrenia	64062	RBM26	2.88E-07
120 TRAP schizophrenia	259232	NALCN	4.41E-05
120 TRAP schizophrenia	7174	TPP2	0.0037603
120 TRAP schizophrenia	8660	IRS2	0.039738
120 TRAP schizophrenia	161253	REM2	0.0023345
120 TRAP schizophrenia	22985	ACIN1	0.0042163
120 TRAP schizophrenia	2290	FOXG1	0.019657
120 TRAP schizophrenia	25831	HECTD1	3.78E-04
120 TRAP schizophrenia	9472	AKAP6	7.77E-05
120 TRAP schizophrenia	254170	FBXO33	0.016536
120 TRAP schizophrenia	161357	MDGA2	1.58E-06
120 TRAP schizophrenia	51199	NIN	0.0013148
120 TRAP schizophrenia	5926	ARID4A	0.0019092
120 TRAP schizophrenia	9495	AKAP5	0.0069235
120 TRAP schizophrenia	10243	GPHN	0.0072248
120 TRAP schizophrenia	56252	YLPM1	0.0058731
120 TRAP schizophrenia	145567	TTC7B	5.20E-05

gene_set	entrez_ID	Symbol	P-value
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120 TRAP schizophrenia	81693	AMN	1.79E-05
120 TRAP schizophrenia	342184	FMN1	0.0035224
120 TRAP schizophrenia	6263	RYR3	0.0034774
120 TRAP schizophrenia	85455	DISP2	0.01092
120 TRAP schizophrenia	22893	BAHD1	0.020303
120 TRAP schizophrenia	7158	TP53BP1	5.38E-06
120 TRAP schizophrenia	9728	SECISBP2L	0.0010636
120 TRAP schizophrenia	23312	DMXL2	0.0010353
120 TRAP schizophrenia	54778	RNF111	3.48E-05
120 TRAP schizophrenia	388125	C2CD4B	0.020662
120 TRAP schizophrenia	83660	TLN2	2.29E-05
120 TRAP schizophrenia	83464	APH1B	0.0031588
120 TRAP schizophrenia	26035	GLCE	6.89E-04
120 TRAP schizophrenia	3658	IREB2	1.24E-08
120 TRAP schizophrenia	8120	AP3B2	1.10E-09
120 TRAP schizophrenia	53339	BTBD1	0.013945
120 TRAP schizophrenia	1106	CHD2	2.57E-07
120 TRAP schizophrenia	83886	PRSS27	2.36E-05
120 TRAP schizophrenia	115	ADCY9	1.43E-05
120 TRAP schizophrenia	29855	UBN1	0.032225
120 TRAP schizophrenia	2903	GRIN2A	1.75E-05
120 TRAP schizophrenia	162073	ITPRIPL2	0.038962
120 TRAP schizophrenia	123876	ACSM2A	0.048718
120 TRAP schizophrenia	123879	DCUN1D3	0.014762
120 TRAP schizophrenia	730094	MOSMO	8.81E-07
120 TRAP schizophrenia	11273	ATXN2L	0.0025401
120 TRAP schizophrenia	26470	SEZ6L2	1.09E-09
120 TRAP schizophrenia	9344	TAOK2	5.47E-12
120 TRAP schizophrenia	79724	ZNF768	0.0015664
120 TRAP schizophrenia	23019	CNOT1	5.79E-10
120 TRAP schizophrenia	1006	CDH8	0.048426
120 TRAP schizophrenia	1009	CDH11	8.13E-04
120 TRAP schizophrenia	7084	TK2	0.017111
120 TRAP schizophrenia	1014	CDH16	0.0018178
120 TRAP schizophrenia	79567	RIPOR1	1.53E-04
120 TRAP schizophrenia	23644	EDC4	7.77E-06
120 TRAP schizophrenia	10725	NFAT5	0.039848
120 TRAP schizophrenia	16	AARS1	0.017893
120 TRAP schizophrenia	164	AP1G1	0.01107
120 TRAP schizophrenia	2734	GLG1	2.58E-07
120 TRAP schizophrenia	124641	OVCA2	4.31E-05
120 TRAP schizophrenia	23140	ZZEF1	7.08E-05
120 TRAP schizophrenia	5430	POLR2A	0.047088
120 TRAP schizophrenia	23135	KDM6B	0.019309
120 TRAP schizophrenia	124925	SEZ6	0.0022932
120 TRAP schizophrenia	85464	SSH2	0.036688
120 TRAP schizophrenia	1362	CPD	2.21E-05
120 TRAP schizophrenia	5469	MED1	8.48E-06

gene_set	entrez_ID	Symbol	P-value
120 TRAP schizophrenia	94103	ORMDL3	0.019271
120 TRAP schizophrenia	4077	NBR1	0.0055974
120 TRAP schizophrenia	10642	IGF2BP1	3.01E-04
120 TRAP schizophrenia	8913	CACNA1G	3.12E-06
120 TRAP schizophrenia	9110	MTMR4	1.95E-04
120 TRAP schizophrenia	22843	PPM1E	2.05E-04
120 TRAP schizophrenia	10351	ABCA8	0.049663
120 TRAP schizophrenia	79902	NUP85	0.0014776
120 TRAP schizophrenia	23163	GGA3	0.018265
120 TRAP schizophrenia	63893	UBE2O	0.018168
120 TRAP schizophrenia	71	ACTG1	0.0039532
120 TRAP schizophrenia	2194	FASN	0.029595
120 TRAP schizophrenia	23136	EPB41L3	0.0042423
120 TRAP schizophrenia	23253	ANKRD12	0.0034547
120 TRAP schizophrenia	3998	LMAN1	0.040441
120 TRAP schizophrenia	5725	PTBP1	0.019926
120 TRAP schizophrenia	2879	GPX4	0.016657
120 TRAP schizophrenia	90007	MIDN	0.0062817
120 TRAP schizophrenia	1455	CSNK1G2	4.07E-04
120 TRAP schizophrenia	23030	KDM4B	0.021813
120 TRAP schizophrenia	3726	JUNB	0.02779
120 TRAP schizophrenia	9592	IER2	0.0088133
120 TRAP schizophrenia	773	CACNA1A	6.61E-05
120 TRAP schizophrenia	3337	DNAJB1	6.47E-05
120 TRAP schizophrenia	10331	B3GNT3	0.003649
120 TRAP schizophrenia	1463	NCAN	1.06E-04
120 TRAP schizophrenia	57130	ATP13A1	1.21E-06
120 TRAP schizophrenia	7538	ZFP36	0.012963
120 TRAP schizophrenia	4858	NOVA2	0.026453
120 TRAP schizophrenia	27113	BBC3	0.028121
120 TRAP schizophrenia	6625	SNRNP70	0.002086
120 TRAP schizophrenia	6203	RPS9	0.030434
120 TRAP schizophrenia	10155	TRIM28	0.0024191
120 TRAP schizophrenia	5126	PCSK2	0.019202
120 TRAP schizophrenia	22803	XRN2	8.06E-04
120 TRAP schizophrenia	83737	ITCH	0.0013046
120 TRAP schizophrenia	7150	TOP1	1.03E-05
120 TRAP schizophrenia	23051	ZHX3	0.0027049
120 TRAP schizophrenia	84181	CHD6	0.041615
120 TRAP schizophrenia	57468	SLC12A5	0.0039624
120 TRAP schizophrenia	1434	CSE1L	9.80E-05
120 TRAP schizophrenia	5105	PCK1	0.015195
120 TRAP schizophrenia	6874	TAF4	0.003755
120 TRAP schizophrenia	8204	NRIP1	5.58E-07
120 TRAP schizophrenia	1525	CXADR	0.0033986
120 TRAP schizophrenia	2618	GART	0.0042822
120 TRAP schizophrenia	6651	SON	0.0012846
120 TRAP schizophrenia	23544	SEZ6L	0.00502
120 TRAP schizophrenia	162	AP1B1	0.0014017

gene_set	entrez_ID	Symbol	P-value
120 TRAP schizophrenia	10291	SF3A1	2.52E-05
120 TRAP schizophrenia	6948	TCN2	0.016967
120 TRAP schizophrenia	22880	MORC2	0.013047
120 TRAP schizophrenia	1454	CSNK1E	0.029303
120 TRAP schizophrenia	4248	MGAT3	1.48E-07
120 TRAP schizophrenia	91582	RPS19BP1	1.03E-08
120 TRAP schizophrenia	23112	TNRC6B	0.0031234
120 TRAP schizophrenia	57591	MRTFA	2.34E-05
120 TRAP schizophrenia	5905	RANGAP1	2.84E-07
120 TRAP schizophrenia	10766	TOB2	0.013039
120 TRAP schizophrenia	22866	CNKSR2	4.32E-05
120 TRAP schizophrenia	6853	SYN1	0.049967
120 TRAP schizophrenia	51132	RLIM	0.045648
120 TRAP schizophrenia	79589	RNF128	0.0086697
60 TRAP schizophrenia	148479	PHF13	3.96E-07
60 TRAP schizophrenia	9903	KLHL21	5.83E-07
60 TRAP schizophrenia	8672	EIF4G3	1.92E-04
60 TRAP schizophrenia	84196	USP48	0.0188
60 TRAP schizophrenia	23038	WDTC1	0.02264
60 TRAP schizophrenia	27245	AHDC1	0.0056835
60 TRAP schizophrenia	6883	TAF12	4.48E-04
60 TRAP schizophrenia	6421	SFPQ	9.34E-04
60 TRAP schizophrenia	149175	MANEAL	7.39E-04
60 TRAP schizophrenia	643314	NA	0.001684
60 TRAP schizophrenia	4802	NFYC	0.0025438
60 TRAP schizophrenia	284716	RIMKLA	2.08E-04
60 TRAP schizophrenia	26135	SERBP1	0.010763
60 TRAP schizophrenia	84146	ZNF644	2.32E-04
60 TRAP schizophrenia	128338	DRAM2	0.043515
60 TRAP schizophrenia	54879	ST7L	6.56E-04
60 TRAP schizophrenia	51592	TRIM33	0.026401
60 TRAP schizophrenia	333932	H3C15	0.020636
60 TRAP schizophrenia	9900	SV2A	0.03014
60 TRAP schizophrenia	51177	PLEKHO1	3.65E-09
60 TRAP schizophrenia	9129	PRPF3	4.02E-05
60 TRAP schizophrenia	4170	MCL1	0.03176
60 TRAP schizophrenia	405	ARNT	0.031196
60 TRAP schizophrenia	10500	SEMA6C	2.74E-06
60 TRAP schizophrenia	9909	DENND4B	2.08E-05
60 TRAP schizophrenia	10899	JTB	1.14E-05
60 TRAP schizophrenia	57198	ATP8B2	0.005792
60 TRAP schizophrenia	4580	MTX1	3.31E-04
60 TRAP schizophrenia	10712	FAM189B	0.016484
60 TRAP schizophrenia	23623	RUSC1	0.0063138
60 TRAP schizophrenia	23381	SMG5	0.03371
60 TRAP schizophrenia	477	ATP1A2	0.045031
60 TRAP schizophrenia	93185	IGSF8	0.029346
60 TRAP schizophrenia	8682	PEA15	0.0086291
60 TRAP schizophrenia	7143	TNR	9.82E-06

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	64326	COP1	0.006181
60 TRAP schizophrenia	460	ASTN1	6.04E-04
60 TRAP schizophrenia	777	CACNA1E	0.0030366
60 TRAP schizophrenia	1660	DHX9	0.0042446
60 TRAP schizophrenia	81563	C1orf21	0.0068539
60 TRAP schizophrenia	5997	RGS2	0.047416
60 TRAP schizophrenia	8707	B3GALT2	6.27E-04
60 TRAP schizophrenia	163486	DENND1B	2.28E-04
60 TRAP schizophrenia	23528	ZNF281	2.85E-05
60 TRAP schizophrenia	2848	GPR25	0.0093648
60 TRAP schizophrenia	55705	IPO9	0.0014237
60 TRAP schizophrenia	6900	CNTN2	1.35E-06
60 TRAP schizophrenia	5929	RBBP5	8.76E-06
60 TRAP schizophrenia	25778	DSTYK	7.05E-06
60 TRAP schizophrenia	9911	TMCC2	3.58E-04
60 TRAP schizophrenia	55699	IARS2	0.01137
60 TRAP schizophrenia	149111	CNIH3	0.0019679
60 TRAP schizophrenia	1131	CHRM3	1.35E-04
60 TRAP schizophrenia	56776	FMN2	2.57E-04
60 TRAP schizophrenia	9859	CEP170	5.04E-07
60 TRAP schizophrenia	23369	PUM2	0.041513
60 TRAP schizophrenia	3777	KCNK3	5.47E-04
60 TRAP schizophrenia	29959	NRBP1	7.73E-04
60 TRAP schizophrenia	64838	FNDC4	2.43E-04
60 TRAP schizophrenia	2355	FOSL2	0.0039659
60 TRAP schizophrenia	6546	SLC8A1	2.50E-08
60 TRAP schizophrenia	9378	NRXN1	3.55E-07
60 TRAP schizophrenia	57142	RTN4	0.0057382
60 TRAP schizophrenia	55704	CCDC88A	0.018339
60 TRAP schizophrenia	114800	CCDC85A	0.0036489
60 TRAP schizophrenia	53335	BCL11A	2.76E-08
60 TRAP schizophrenia	5534	PPP3R1	0.021893
60 TRAP schizophrenia	119	ADD2	0.030259
60 TRAP schizophrenia	27332	ZNF638	0.00822
60 TRAP schizophrenia	10322	SMYD5	3.57E-04
60 TRAP schizophrenia	1961	EGR4	0.00124
60 TRAP schizophrenia	1844	DUSP2	0.0045114
60 TRAP schizophrenia	56910	STARD7	2.55E-04
60 TRAP schizophrenia	10865	ARID5A	0.018232
60 TRAP schizophrenia	6549	SLC9A2	0.0013762
60 TRAP schizophrenia	9392	TGFBRAP1	0.0024192
60 TRAP schizophrenia	64682	ANAPC1	3.62E-04
60 TRAP schizophrenia	9839	ZEB2	1.24E-07
60 TRAP schizophrenia	55183	RIF1	0.039854
60 TRAP schizophrenia	4929	NR4A2	0.0076207
60 TRAP schizophrenia	64844	MARCHF7	1.10E-04
60 TRAP schizophrenia	57282	SLC4A10	0.045887
60 TRAP schizophrenia	100131390	SP9	6.38E-05
60 TRAP schizophrenia	151556	GPR155	0.0058017

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	151126	ZNF385B	4.83E-04
60 TRAP schizophrenia	23451	SF3B1	1.95E-11
60 TRAP schizophrenia	23314	SATB2	3.85E-10
60 TRAP schizophrenia	205327	C2orf69	1.81E-12
60 TRAP schizophrenia	659	BMPR2	0.001808
60 TRAP schizophrenia	57683	ZDBF2	0.0044465
60 TRAP schizophrenia	55022	PID1	0.0097061
60 TRAP schizophrenia	4691	NCL	0.0069447
60 TRAP schizophrenia	26058	GIGYF2	1.21E-12
60 TRAP schizophrenia	9922	IQSEC1	0.0026803
60 TRAP schizophrenia	23228	PLCL2	1.30E-05
60 TRAP schizophrenia	201595	STT3B	0.021995
60 TRAP schizophrenia	23171	GPD1L	0.014079
60 TRAP schizophrenia	9881	TRANK1	2.88E-09
60 TRAP schizophrenia	9852	EPM2AIP1	0.0040991
60 TRAP schizophrenia	51188	SS18L2	0.047482
60 TRAP schizophrenia	10425	ARIH2	0.014032
60 TRAP schizophrenia	10869	USP19	0.0088763
60 TRAP schizophrenia	7375	USP4	0.0025587
60 TRAP schizophrenia	8927	BSN	0.005303
60 TRAP schizophrenia	9807	IP6K1	0.013448
60 TRAP schizophrenia	79012	CAMKV	9.66E-04
60 TRAP schizophrenia	10181	RBM5	8.40E-04
60 TRAP schizophrenia	1849	DUSP7	0.019788
60 TRAP schizophrenia	80335	WDR82	1.36E-04
60 TRAP schizophrenia	55193	PBRM1	7.38E-08
60 TRAP schizophrenia	5580	PRKCD	0.001907
60 TRAP schizophrenia	50650	ARHGEF3	0.044162
60 TRAP schizophrenia	54899	PXK	0.0044195
60 TRAP schizophrenia	5793	PTPRG	9.15E-04
60 TRAP schizophrenia	55079	FEZF2	0.045742
60 TRAP schizophrenia	166336	PRICKLE2	1.04E-06
60 TRAP schizophrenia	6092	ROBO2	0.018209
60 TRAP schizophrenia	6091	ROBO1	0.017112
60 TRAP schizophrenia	214	ALCAM	0.0062886
60 TRAP schizophrenia	50512	PODXL2	0.031437
60 TRAP schizophrenia	8971	H1-10	0.0065575
60 TRAP schizophrenia	6578	SLCO2A1	0.0083882
60 TRAP schizophrenia	7545	ZIC1	1.51E-04
60 TRAP schizophrenia	23007	PLCH1	0.04391
60 TRAP schizophrenia	165918	RNF168	0.0049357
60 TRAP schizophrenia	10815	CPLX1	0.011125
60 TRAP schizophrenia	1487	CTBP1	0.0071436
60 TRAP schizophrenia	152	ADRA2C	0.023833
60 TRAP schizophrenia	132884	EVC2	0.0012528
60 TRAP schizophrenia	9948	WDR1	0.023173
60 TRAP schizophrenia	57620	STIM2	0.012453
60 TRAP schizophrenia	5099	PCDH7	2.58E-08
60 TRAP schizophrenia	55276	PGM2	0.0033858

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	285527	FRYL	0.0041471
60 TRAP schizophrenia	65997	RASL11B	0.035439
60 TRAP schizophrenia	56978	PRDM8	0.030988
60 TRAP schizophrenia	23001	WDFY3	0.043864
60 TRAP schizophrenia	5530	PPP3CA	0.0023802
60 TRAP schizophrenia	152503	SH3D19	0.032611
60 TRAP schizophrenia	11275	KLHL2	8.02E-04
60 TRAP schizophrenia	83891	SNX25	0.0024481
60 TRAP schizophrenia	54888	NSUN2	0.023578
60 TRAP schizophrenia	11044	TENT4A	4.61E-04
60 TRAP schizophrenia	108	ADCY2	0.025979
60 TRAP schizophrenia	10409	BASP1	0.0028107
60 TRAP schizophrenia	1008	CDH10	0.026316
60 TRAP schizophrenia	10884	MRPS30	3.80E-07
60 TRAP schizophrenia	3350	HTR1A	0.011756
60 TRAP schizophrenia	5295	PIK3R1	0.002181
60 TRAP schizophrenia	3156	HMGCR	0.0014572
60 TRAP schizophrenia	133746	JMY	0.0071531
60 TRAP schizophrenia	9456	HOMER1	0.0014541
60 TRAP schizophrenia	10085	EDIL3	0.0086425
60 TRAP schizophrenia	57561	ARRDC3	0.0084327
60 TRAP schizophrenia	5122	PCSK1	0.0046031
60 TRAP schizophrenia	64839	FBXL17	2.05E-04
60 TRAP schizophrenia	6695	SPOCK1	0.002826
60 TRAP schizophrenia	1958	EGR1	1.02E-08
60 TRAP schizophrenia	3313	HSPA9	1.75E-09
60 TRAP schizophrenia	54882	ANKHD1	1.02E-05
60 TRAP schizophrenia	3550	IK	1.54E-06
60 TRAP schizophrenia	8641	PCDHGB4	0.0019876
60 TRAP schizophrenia	56097	PCDHGC5	0.0022945
60 TRAP schizophrenia	1729	DIAPH1	0.049875
60 TRAP schizophrenia	5097	PCDH1	0.01854
60 TRAP schizophrenia	662	BNIP1	0.0024613
60 TRAP schizophrenia	1627	DBN1	0.045468
60 TRAP schizophrenia	8878	SQSTM1	9.11E-04
60 TRAP schizophrenia	11282	MGAT4B	0.0010811
60 TRAP schizophrenia	10667	FARS2	0.0034361
60 TRAP schizophrenia	9856	KIAA0319	0.014498
60 TRAP schizophrenia	81688	C6orf62	0.018243
60 TRAP schizophrenia	8359	H4C1	1.37E-14
60 TRAP schizophrenia	3006	H1-2	8.76E-12
60 TRAP schizophrenia	8360	H4C4	0.014817
60 TRAP schizophrenia	8365	H4C8	1.16E-09
60 TRAP schizophrenia	79692	ZNF322	1.04E-13
60 TRAP schizophrenia	8294	H4C9	2.06E-14
60 TRAP schizophrenia	79897	RPP21	5.19E-16
60 TRAP schizophrenia	5514	PPP1R10	2.40E-16
60 TRAP schizophrenia	221545	C6orf136	1.05E-14
60 TRAP schizophrenia	57176	VARS2	6.81E-25

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	534	ATP6V1G2	1.71E-07
60 TRAP schizophrenia	80736	SLC44A4	1.05E-19
60 TRAP schizophrenia	6048	RNF5	7.64E-28
60 TRAP schizophrenia	5089	PBX2	2.36E-27
60 TRAP schizophrenia	51596	CUTA	7.79E-12
60 TRAP schizophrenia	29993	PACSIN1	0.0077064
60 TRAP schizophrenia	5190	PEX6	0.038329
60 TRAP schizophrenia	116138	KLHDC3	0.006199
60 TRAP schizophrenia	25844	YIPF3	2.57E-04
60 TRAP schizophrenia	2729	GCLC	0.011162
60 TRAP schizophrenia	23469	PHF3	1.22E-06
60 TRAP schizophrenia	22999	RIMS1	0.0024029
60 TRAP schizophrenia	9892	SNAP91	7.52E-13
60 TRAP schizophrenia	23036	ZNF292	0.049418
60 TRAP schizophrenia	114792	KLHL32	0.0010912
60 TRAP schizophrenia	23097	CDK19	0.03985
60 TRAP schizophrenia	57480	PLEKHG1	0.047987
60 TRAP schizophrenia	23345	SYNE1	1.37E-05
60 TRAP schizophrenia	57492	ARID1B	5.39E-05
60 TRAP schizophrenia	6950	TCP1	0.020183
60 TRAP schizophrenia	84498	FAM120B	2.35E-04
60 TRAP schizophrenia	28514	DLL1	1.92E-04
60 TRAP schizophrenia	80028	FBXL18	9.03E-04
60 TRAP schizophrenia	9265	CYTH3	0.001119
60 TRAP schizophrenia	54468	MIOS	0.048612
60 TRAP schizophrenia	2115	ETV1	0.015704
60 TRAP schizophrenia	6671	SP4	1.61E-06
60 TRAP schizophrenia	63974	NEUROD6	0.0034004
60 TRAP schizophrenia	23080	AVL9	0.0040666
60 TRAP schizophrenia	27072	VPS41	1.96E-04
60 TRAP schizophrenia	3624	INHBA	0.0020037
60 TRAP schizophrenia	816	CAMK2B	0.025848
60 TRAP schizophrenia	107	ADCY1	0.01736
60 TRAP schizophrenia	23242	COBL	0.012846
60 TRAP schizophrenia	9031	BAZ1B	0.0098611
60 TRAP schizophrenia	2969	GTF2I	0.012896
60 TRAP schizophrenia	27445	PCLO	4.23E-09
60 TRAP schizophrenia	154661	RUNDC3B	0.011863
60 TRAP schizophrenia	440	ASNS	0.047492
60 TRAP schizophrenia	4885	NPTX2	0.048419
60 TRAP schizophrenia	7425	VGF	0.011422
60 TRAP schizophrenia	80228	ORAI2	5.13E-04
60 TRAP schizophrenia	4897	NRCAM	0.0018201
60 TRAP schizophrenia	830	CAPZA2	0.035587
60 TRAP schizophrenia	83992	CTTNBP2	0.0026514
60 TRAP schizophrenia	51530	ZC3HC1	0.0010156
60 TRAP schizophrenia	23008	KLHDC10	1.29E-04
60 TRAP schizophrenia	26047	CNTNAP2	0.0049988
60 TRAP schizophrenia	9258	MFHAS1	0.010616

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	9108	MTMR7	1.76E-05
60 TRAP schizophrenia	5533	PPP3CC	3.63E-05
60 TRAP schizophrenia	5520	PPP2R2A	1.36E-04
60 TRAP schizophrenia	148	ADRA1A	0.027875
60 TRAP schizophrenia	7976	FZD3	7.23E-04
60 TRAP schizophrenia	9530	BAG4	4.59E-08
60 TRAP schizophrenia	115294	PCMTD1	6.54E-05
60 TRAP schizophrenia	5862	RAB2A	2.60E-05
60 TRAP schizophrenia	254778	VXN	0.02944
60 TRAP schizophrenia	734	OSGIN2	0.0062069
60 TRAP schizophrenia	64168	NECAB1	0.0050615
60 TRAP schizophrenia	7071	KLF10	8.00E-05
60 TRAP schizophrenia	9699	RIMS2	0.031089
60 TRAP schizophrenia	3646	EIF3E	0.045387
60 TRAP schizophrenia	9897	WASHC5	0.031506
60 TRAP schizophrenia	23237	ARC	5.27E-07
60 TRAP schizophrenia	84948	TIGD5	0.024733
60 TRAP schizophrenia	57589	RIC1	0.019774
60 TRAP schizophrenia	158358	KIAA2026	0.0094479
60 TRAP schizophrenia	1993	ELAVL2	0.0037691
60 TRAP schizophrenia	9373	PLAA	2.30E-04
60 TRAP schizophrenia	80173	IFT74	1.76E-04
60 TRAP schizophrenia	55234	SMU1	9.36E-04
60 TRAP schizophrenia	1271	CNTFR	0.006494
60 TRAP schizophrenia	2592	GALT	0.020826
60 TRAP schizophrenia	7415	VCP	0.0016892
60 TRAP schizophrenia	26267	FBXO10	0.011508
60 TRAP schizophrenia	79269	DCAF10	0.014786
60 TRAP schizophrenia	8395	PIP5K1B	0.00022135
60 TRAP schizophrenia	9615	GDA	0.010582
60 TRAP schizophrenia	23287	AGTPBP1	0.0178
60 TRAP schizophrenia	10927	SPIN1	0.012411
60 TRAP schizophrenia	10558	SPTLC1	0.0013811
60 TRAP schizophrenia	23196	FAM120A	5.30E-07
60 TRAP schizophrenia	5253	PHF2	2.36E-06
60 TRAP schizophrenia	138639	PTPDC1	0.0018727
60 TRAP schizophrenia	9568	GABBR2	7.27E-09
60 TRAP schizophrenia	58499	ZNF462	2.75E-04
60 TRAP schizophrenia	6136	RPL12	0.001285
60 TRAP schizophrenia	25792	CIZ1	0.0012856
60 TRAP schizophrenia	10444	ZER1	1.75E-04
60 TRAP schizophrenia	220213	OTUD1	0.01019
60 TRAP schizophrenia	5599	MAPK8	0.016853
60 TRAP schizophrenia	23283	CSTF2T	9.82E-05
60 TRAP schizophrenia	288	ANK3	7.57E-07
60 TRAP schizophrenia	84890	ADO	0.02343
60 TRAP schizophrenia	1959	EGR2	0.017451
60 TRAP schizophrenia	221035	REEP3	0.010259
60 TRAP schizophrenia	55749	CCAR1	0.002265

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	2894	GRID1	2.01E-04
60 TRAP schizophrenia	10580	SORBS1	0.030197
60 TRAP schizophrenia	56889	TM9SF3	2.64E-04
60 TRAP schizophrenia	8945	BTRC	8.44E-04
60 TRAP schizophrenia	30819	KCNIP2	1.27E-04
60 TRAP schizophrenia	8861	LDB1	0.011109
60 TRAP schizophrenia	5662	PSD	0.0043619
60 TRAP schizophrenia	81603	TRIM8	1.48E-07
60 TRAP schizophrenia	9118	INA	3.41E-07
60 TRAP schizophrenia	9748	SLK	0.0079647
60 TRAP schizophrenia	85450	ITPRIP	0.0014436
60 TRAP schizophrenia	22986	SORCS3	1.08E-07
60 TRAP schizophrenia	1847	DUSP5	0.013097
60 TRAP schizophrenia	57700	FHIP2A	0.0069835
60 TRAP schizophrenia	10579	TACC2	0.045006
60 TRAP schizophrenia	9024	BRSK2	0.0035197
60 TRAP schizophrenia	406	ARNTL	0.011447
60 TRAP schizophrenia	10418	SPON1	0.034691
60 TRAP schizophrenia	5286	PIK3C2A	2.15E-05
60 TRAP schizophrenia	55327	LIN7C	0.023383
60 TRAP schizophrenia	4076	CAPRIN1	5.00E-04
60 TRAP schizophrenia	8534	CHST1	0.027145
60 TRAP schizophrenia	8525	DGKZ	1.78E-12
60 TRAP schizophrenia	9793	CKAP5	1.29E-07
60 TRAP schizophrenia	85456	TNKS1BP1	0.0056644
60 TRAP schizophrenia	10313	RTN3	0.040267
60 TRAP schizophrenia	10963	STIP1	0.032621
60 TRAP schizophrenia	56834	GPR137	0.016095
60 TRAP schizophrenia	9379	NRXN2	7.72E-04
60 TRAP schizophrenia	10992	SF3B2	0.0024908
60 TRAP schizophrenia	266743	NPAS4	0.028978
60 TRAP schizophrenia	78999	LRFN4	0.0036313
60 TRAP schizophrenia	9828	ARHGEF17	0.010786
60 TRAP schizophrenia	5612	THAP12	0.0055491
60 TRAP schizophrenia	283219	KCTD21	0.024145
60 TRAP schizophrenia	2977	GUCY1A2	0.0035307
60 TRAP schizophrenia	4684	NCAM1	4.20E-05
60 TRAP schizophrenia	51092	SIDT2	0.04872
60 TRAP schizophrenia	57453	DSCAML1	1.30E-04
60 TRAP schizophrenia	6230	RPS25	0.0037109
60 TRAP schizophrenia	29118	DDX25	0.030784
60 TRAP schizophrenia	334	APLP2	0.008774
60 TRAP schizophrenia	4978	OPCML	6.20E-10
60 TRAP schizophrenia	50865	HEBP1	0.040523
60 TRAP schizophrenia	121504	H4-16	6.30E-04
60 TRAP schizophrenia	51729	WBP11	3.47E-04
60 TRAP schizophrenia	6660	SOX5	1.52E-07
60 TRAP schizophrenia	83857	TMTC1	0.0011845
60 TRAP schizophrenia	10526	IPO8	0.023768

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	1272	CNTN1	0.0027674
60 TRAP schizophrenia	23416	KCNH3	9.29E-04
60 TRAP schizophrenia	25840	METTL7A	0.016384
60 TRAP schizophrenia	5502	PPP1R1A	0.002526
60 TRAP schizophrenia	55832	CAND1	0.014874
60 TRAP schizophrenia	8835	SOCS2	0.026285
60 TRAP schizophrenia	23074	UHRF1BP1L	9.44E-04
60 TRAP schizophrenia	51559	NT5DC3	9.07E-04
60 TRAP schizophrenia	7184	HSP90B1	0.02714
60 TRAP schizophrenia	29915	HCFC2	0.04853
60 TRAP schizophrenia	89910	UBE3B	5.20E-06
60 TRAP schizophrenia	51347	TAOK3	0.024762
60 TRAP schizophrenia	121665	SPPL3	5.94E-06
60 TRAP schizophrenia	51433	ANAPC5	0.0025472
60 TRAP schizophrenia	65082	VPS33A	0.0025865
60 TRAP schizophrenia	6249	CLIP1	1.01E-07
60 TRAP schizophrenia	57605	PITPNM2	9.65E-13
60 TRAP schizophrenia	80212	CCDC92	2.70E-07
60 TRAP schizophrenia	144348	ZNF664	1.55E-07
60 TRAP schizophrenia	9612	NCOR2	0.0047549
60 TRAP schizophrenia	23141	ANKLE2	0.0022222
60 TRAP schizophrenia	2802	GOLGA3	0.017919
60 TRAP schizophrenia	55743	CHFR	0.028568
60 TRAP schizophrenia	5100	PCDH8	1.43E-04
60 TRAP schizophrenia	64062	RBM26	2.88E-07
60 TRAP schizophrenia	22873	DZIP1	0.0063658
60 TRAP schizophrenia	7546	ZIC2	0.030527
60 TRAP schizophrenia	259232	NALCN	4.41E-05
60 TRAP schizophrenia	7174	TPP2	0.0037603
60 TRAP schizophrenia	1948	EFNB2	0.018876
60 TRAP schizophrenia	84945	ABHD13	0.030504
60 TRAP schizophrenia	8660	IRS2	0.039738
60 TRAP schizophrenia	22821	RASA3	1.10E-06
60 TRAP schizophrenia	8881	CDC16	0.036
60 TRAP schizophrenia	22985	ACIN1	0.0042163
60 TRAP schizophrenia	2290	FOXG1	0.019657
60 TRAP schizophrenia	9472	AKAP6	7.77E-05
60 TRAP schizophrenia	161357	MDGA2	1.58E-06
60 TRAP schizophrenia	122773	KLHDC1	0.030178
60 TRAP schizophrenia	8814	CDKL1	0.012265
60 TRAP schizophrenia	51062	ATL1	0.0066998
60 TRAP schizophrenia	55860	ACTR10	6.50E-05
60 TRAP schizophrenia	5926	ARID4A	0.0019092
60 TRAP schizophrenia	83851	SYT16	3.53E-04
60 TRAP schizophrenia	9495	AKAP5	0.0069235
60 TRAP schizophrenia	10243	GPHN	0.0072248
60 TRAP schizophrenia	26037	SIPA1L1	3.01E-05
60 TRAP schizophrenia	56252	YLPM1	0.0058731
60 TRAP schizophrenia	145567	TTC7B	5.20E-05

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	5265	SERPINA1	0.024879
60 TRAP schizophrenia	7187	TRAF3	9.77E-04
60 TRAP schizophrenia	123606	NIPA1	0.046702
60 TRAP schizophrenia	2562	GABRB3	0.010252
60 TRAP schizophrenia	2558	GABRA5	2.63E-05
60 TRAP schizophrenia	7082	TJP1	0.033059
60 TRAP schizophrenia	342184	FMN1	0.0035224
60 TRAP schizophrenia	10125	RASGRP1	1.98E-05
60 TRAP schizophrenia	440275	EIF2AK4	0.011072
60 TRAP schizophrenia	85455	DISP2	0.01092
60 TRAP schizophrenia	23339	VPS39	4.71E-05
60 TRAP schizophrenia	9836	LCMT2	1.66E-05
60 TRAP schizophrenia	7158	TP53BP1	5.38E-06
60 TRAP schizophrenia	9728	SECISBP2L	0.0010636
60 TRAP schizophrenia	79811	SLTM	5.41E-04
60 TRAP schizophrenia	54778	RNF111	3.48E-05
60 TRAP schizophrenia	388125	C2CD4B	0.020662
60 TRAP schizophrenia	23060	ZNF609	0.0035912
60 TRAP schizophrenia	4088	SMAD3	0.0087098
60 TRAP schizophrenia	26035	GLCE	6.89E-04
60 TRAP schizophrenia	9493	KIF23	0.021316
60 TRAP schizophrenia	1445	CSK	0.026437
60 TRAP schizophrenia	4123	MAN2C1	0.036856
60 TRAP schizophrenia	8120	AP3B2	1.10E-09
60 TRAP schizophrenia	9455	HOMER2	0.0082213
60 TRAP schizophrenia	53339	BTBD1	0.013945
60 TRAP schizophrenia	4122	MAN2A2	2.94E-09
60 TRAP schizophrenia	1106	CHD2	2.57E-07
60 TRAP schizophrenia	79641	ROGDI	0.032485
60 TRAP schizophrenia	112479	ERI2	0.027837
60 TRAP schizophrenia	123879	DCUN1D3	0.014762
60 TRAP schizophrenia	7385	UQCRC2	7.64E-06
60 TRAP schizophrenia	730094	MOSMO	8.81E-07
60 TRAP schizophrenia	57478	USP31	5.77E-04
60 TRAP schizophrenia	5579	PRKCB	3.72E-06
60 TRAP schizophrenia	10368	CACNG3	3.11E-04
60 TRAP schizophrenia	5930	RBBP6	0.042604
60 TRAP schizophrenia	11273	ATXN2L	0.0025401
60 TRAP schizophrenia	25970	SH2B1	0.0032533
60 TRAP schizophrenia	26470	SEZ6L2	1.09E-09
60 TRAP schizophrenia	8479	HIRIP3	8.63E-11
60 TRAP schizophrenia	83723	TLCD3B	8.80E-09
60 TRAP schizophrenia	226	ALDOA	1.42E-08
60 TRAP schizophrenia	64400	AKTIP	0.018053
60 TRAP schizophrenia	2775	GNAO1	0.032865
60 TRAP schizophrenia	55239	OGFOD1	0.039994
60 TRAP schizophrenia	23019	CNOT1	5.79E-10
60 TRAP schizophrenia	8883	NAE1	0.004618
60 TRAP schizophrenia	9114	ATP6V0D1	1.02E-04

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	146206	CARMIL2	5.73E-04
60 TRAP schizophrenia	65057	ACD	7.12E-05
60 TRAP schizophrenia	55512	SMPD3	2.20E-04
60 TRAP schizophrenia	84916	UTP4	1.49E-05
60 TRAP schizophrenia	10725	NFAT5	0.039848
60 TRAP schizophrenia	28987	NOB1	0.044592
60 TRAP schizophrenia	16	AARS1	0.017893
60 TRAP schizophrenia	7571	ZNF23	0.0014947
60 TRAP schizophrenia	79791	FBXO31	0.041057
60 TRAP schizophrenia	23174	ZCCHC14	0.011174
60 TRAP schizophrenia	7531	YWHAE	3.35E-07
60 TRAP schizophrenia	1398	CRK	7.72E-04
60 TRAP schizophrenia	124641	OVCA2	4.31E-05
60 TRAP schizophrenia	9905	SGSM2	4.15E-04
60 TRAP schizophrenia	5048	PAFAH1B1	4.74E-04
60 TRAP schizophrenia	23108	RAP1GAP2	0.0044242
60 TRAP schizophrenia	56919	DHX33	0.0057403
60 TRAP schizophrenia	1856	DVL2	3.84E-04
60 TRAP schizophrenia	9196	KCNAB3	8.85E-05
60 TRAP schizophrenia	58485	TRAPPC1	2.03E-04
60 TRAP schizophrenia	3996	LLGL1	0.038333
60 TRAP schizophrenia	124925	SEZ6	0.0022932
60 TRAP schizophrenia	399687	MYO18A	0.0054744
60 TRAP schizophrenia	7756	ZNF207	6.85E-06
60 TRAP schizophrenia	84152	PPP1R1B	4.62E-06
60 TRAP schizophrenia	94103	ORMDL3	0.019271
60 TRAP schizophrenia	5709	PSMD3	0.0077626
60 TRAP schizophrenia	4077	NBR1	0.0055974
60 TRAP schizophrenia	4836	NMT1	0.0041997
60 TRAP schizophrenia	113026	PLCD3	9.52E-05
60 TRAP schizophrenia	4137	MAPT	1.67E-04
60 TRAP schizophrenia	4905	NSF	3.10E-04
60 TRAP schizophrenia	65264	UBE2Z	5.78E-05
60 TRAP schizophrenia	8913	CACNA1G	3.12E-06
60 TRAP schizophrenia	4591	TRIM37	1.57E-05
60 TRAP schizophrenia	10238	DCAF7	0.0091606
60 TRAP schizophrenia	10672	GNA13	7.40E-05
60 TRAP schizophrenia	85302	FBF1	0.030756
60 TRAP schizophrenia	63893	UBE2O	0.018168
60 TRAP schizophrenia	71	ACTG1	0.0039532
60 TRAP schizophrenia	23136	EPB41L3	0.0042423
60 TRAP schizophrenia	23253	ANKRD12	0.0034547
60 TRAP schizophrenia	494470	RNF165	0.026661
60 TRAP schizophrenia	3998	LMAN1	0.040441
60 TRAP schizophrenia	80148	SLC66A2	3.05E-07
60 TRAP schizophrenia	513	ATP5F1D	0.0024828
60 TRAP schizophrenia	90007	MIDN	0.0062817
60 TRAP schizophrenia	399664	MEX3D	0.033856
60 TRAP schizophrenia	1455	CSNK1G2	4.07E-04

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	148252	DIRAS1	7.66E-07
60 TRAP schizophrenia	29985	SLC39A3	1.37E-04
60 TRAP schizophrenia	23030	KDM4B	0.021813
60 TRAP schizophrenia	4298	MLLT1	0.0083659
60 TRAP schizophrenia	4670	HNRNPM	0.031025
60 TRAP schizophrenia	3726	JUNB	0.02779
60 TRAP schizophrenia	4784	NFIX	0.017849
60 TRAP schizophrenia	9592	IER2	0.0088133
60 TRAP schizophrenia	3337	DNAJB1	6.47E-05
60 TRAP schizophrenia	342865	VSTM2B	6.19E-05
60 TRAP schizophrenia	7538	ZFP36	0.012963
60 TRAP schizophrenia	593	BCKDHA	0.0058699
60 TRAP schizophrenia	1762	DMWD	0.030935
60 TRAP schizophrenia	4858	NOVA2	0.026453
60 TRAP schizophrenia	27113	BBC3	0.028121
60 TRAP schizophrenia	6625	SNRNP70	0.002086
60 TRAP schizophrenia	23521	RPL13A	4.42E-04
60 TRAP schizophrenia	57479	PRR12	4.31E-08
60 TRAP schizophrenia	126129	CPT1C	2.13E-07
60 TRAP schizophrenia	7376	NR1H2	0.022026
60 TRAP schizophrenia	126119	JOSD2	1.69E-04
60 TRAP schizophrenia	51157	ZNF580	0.038326
60 TRAP schizophrenia	63934	ZNF667	0.028946
60 TRAP schizophrenia	6193	RPS5	0.0093993
60 TRAP schizophrenia	27111	SDCBP2	0.0018429
60 TRAP schizophrenia	140885	SIRPA	2.19E-06
60 TRAP schizophrenia	54453	RIN2	0.019266
60 TRAP schizophrenia	58476	TP53INP2	0.0099012
60 TRAP schizophrenia	26133	TRPC4AP	0.048199
60 TRAP schizophrenia	6714	SRC	0.025212
60 TRAP schizophrenia	140679	SLC32A1	4.52E-11
60 TRAP schizophrenia	26051	PPP1R16B	4.64E-19
60 TRAP schizophrenia	7150	TOP1	1.03E-05
60 TRAP schizophrenia	5335	PLCG1	2.21E-05
60 TRAP schizophrenia	1434	CSE1L	9.80E-05
60 TRAP schizophrenia	57169	ZNFX1	0.041535
60 TRAP schizophrenia	128553	TSHZ2	0.030713
60 TRAP schizophrenia	9885	OSBPL2	7.98E-04
60 TRAP schizophrenia	3785	KCNQ2	0.0024333
60 TRAP schizophrenia	30811	HUNK	0.0025108
60 TRAP schizophrenia	6651	SON	0.0012846
60 TRAP schizophrenia	5594	MAPK1	0.0018768
60 TRAP schizophrenia	23544	SEZ6L	0.00502
60 TRAP schizophrenia	4744	NEFH	0.0071436
60 TRAP schizophrenia	4771	NF2	0.00754
60 TRAP schizophrenia	8897	MTMR3	0.0073926
60 TRAP schizophrenia	10291	SF3A1	2.52E-05
60 TRAP schizophrenia	150290	DUSP18	0.019402
60 TRAP schizophrenia	25828	TXN2	0.04209

gene_set	entrez_ID	Symbol	P-value
60 TRAP schizophrenia	1454	CSNK1E	0.029303
60 TRAP schizophrenia	3761	KCNJ4	0.0019973
60 TRAP schizophrenia	6122	RPL3	0.038781
60 TRAP schizophrenia	9145	SYNGR1	0.0025598
60 TRAP schizophrenia	468	ATF4	1.55E-04
60 TRAP schizophrenia	57591	MRTFA	2.34E-05
60 TRAP schizophrenia	83746	L3MBTL2	1.99E-05
60 TRAP schizophrenia	150356	CHADL	3.40E-06
60 TRAP schizophrenia	5905	RANGAP1	2.84E-07
60 TRAP schizophrenia	10766	TOB2	0.013039
60 TRAP schizophrenia	25813	SAMM50	0.0054223
60 TRAP schizophrenia	415116	PIM3	1.09E-04
60 TRAP schizophrenia	6305	SBF1	0.0017302
60 TRAP schizophrenia	23542	MAPK8IP2	0.012274
60 TRAP schizophrenia	5931	RBBP7	0.0015473
60 TRAP schizophrenia	9282	MED14	0.0019097
60 TRAP schizophrenia	6853	SYN1	0.049967
60 TRAP schizophrenia	56850	GRIPAP1	0.0067589
60 TRAP schizophrenia	23708	GSPT2	0.022621
60 TRAP schizophrenia	9500	MAGED1	0.013182
60 TRAP schizophrenia	51132	RLIM	0.045648
60 TRAP schizophrenia	8933	RTL8C	0.046652
60 TRAP schizophrenia	3423	IDS	0.017656
120 TRAP bipolar	9696	CROCC	0.0022841
120 TRAP bipolar	149420	PDIK1L	0.0026844
120 TRAP bipolar	9698	PUM1	0.0066428
120 TRAP bipolar	8565	YARS1	3.32E-04
120 TRAP bipolar	6421	SFPQ	4.98E-04
120 TRAP bipolar	9202	ZMYM4	0.026984
120 TRAP bipolar	149175	MANEAL	0.011631
120 TRAP bipolar	643314	NA	0.026222
120 TRAP bipolar	284716	RIMKLA	9.34E-04
120 TRAP bipolar	3725	JUN	0.011754
120 TRAP bipolar	84251	SGIP1	0.003107
120 TRAP bipolar	84146	ZNF644	0.0056845
120 TRAP bipolar	284613	CYB561D1	0.0023238
120 TRAP bipolar	57463	AMIGO1	0.038711
120 TRAP bipolar	51177	PLEKHO1	6.12E-07
120 TRAP bipolar	23248	RPRD2	1.36E-05
120 TRAP bipolar	80222	TARS2	7.79E-06
120 TRAP bipolar	4170	MCL1	0.0016341
120 TRAP bipolar	23623	RUSC1	0.037686
120 TRAP bipolar	4720	NDUFS2	1.91E-04
120 TRAP bipolar	5999	RGS4	0.010114
120 TRAP bipolar	22920	KIFAP3	0.033589
120 TRAP bipolar	7143	TNR	1.19E-04
120 TRAP bipolar	777	CACNA1E	0.032627
120 TRAP bipolar	1660	DHX9	0.0080168
120 TRAP bipolar	23114	NFASC	0.01978

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	5362	PLXNA2	0.032938
120 TRAP bipolar	142	PARP1	0.0045376
120 TRAP bipolar	84886	C1orf198	0.034923
120 TRAP bipolar	56776	FMN2	0.020705
120 TRAP bipolar	9859	CEP170	0.015801
120 TRAP bipolar	6664	SOX11	0.049368
120 TRAP bipolar	57498	KIDINS220	0.01923
120 TRAP bipolar	50618	ITSN2	0.026124
120 TRAP bipolar	6546	SLC8A1	0.020454
120 TRAP bipolar	57142	RTN4	3.81E-04
120 TRAP bipolar	53335	BCL11A	0.001006
120 TRAP bipolar	1961	EGR4	0.0068944
120 TRAP bipolar	23020	SNRNP200	1.69E-04
120 TRAP bipolar	26504	CNNM4	3.41E-04
120 TRAP bipolar	23505	TMEM131	3.12E-04
120 TRAP bipolar	11320	MGAT4A	0.017154
120 TRAP bipolar	9669	EIF5B	0.0011727
120 TRAP bipolar	9392	TGFBRAP1	0.039335
120 TRAP bipolar	3625	INHBB	0.039594
120 TRAP bipolar	4929	NR4A2	0.0012562
120 TRAP bipolar	10716	TBR1	2.76E-05
120 TRAP bipolar	57282	SLC4A10	3.14E-04
120 TRAP bipolar	6328	SCN3A	0.0056145
120 TRAP bipolar	6326	SCN2A	0.0043526
120 TRAP bipolar	9360	PPIG	0.00398
120 TRAP bipolar	151230	KLHL23	3.72E-04
120 TRAP bipolar	151556	GPR155	0.0096594
120 TRAP bipolar	3676	ITGA4	0.017123
120 TRAP bipolar	57181	SLC39A10	0.010789
120 TRAP bipolar	23451	SF3B1	2.82E-05
120 TRAP bipolar	23314	SATB2	0.0099068
120 TRAP bipolar	205327	C2orf69	7.52E-04
120 TRAP bipolar	2043	EPHA4	0.04227
120 TRAP bipolar	80309	SPHKAP	0.0065441
120 TRAP bipolar	26058	GIGYF2	0.011441
120 TRAP bipolar	8864	PER2	0.019105
120 TRAP bipolar	4705	NDUFA10	0.0027297
120 TRAP bipolar	547	KIF1A	3.06E-05
120 TRAP bipolar	152330	CNTN4	0.020663
120 TRAP bipolar	2803	GOLGA4	0.0061435
120 TRAP bipolar	353274	ZNF445	4.91E-07
120 TRAP bipolar	29072	SETD2	0.028273
120 TRAP bipolar	1605	DAG1	0.013841
120 TRAP bipolar	8927	BSN	0.0053692
120 TRAP bipolar	10181	RBM5	0.0037869
120 TRAP bipolar	2771	GNAI2	8.51E-04
120 TRAP bipolar	11186	RASSF1	0.0058985
120 TRAP bipolar	1795	DOCK3	0.028773
120 TRAP bipolar	80335	WDR82	6.14E-05

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	55193	PBRM1	7.39E-11
120 TRAP bipolar	200845	KCTD6	0.047109
120 TRAP bipolar	166336	PRICKLE2	0.011629
120 TRAP bipolar	253559	CADM2	4.00E-07
120 TRAP bipolar	214	ALCAM	0.0010726
120 TRAP bipolar	26137	ZBTB20	0.042917
120 TRAP bipolar	8930	MBD4	0.027691
120 TRAP bipolar	9819	TSC22D2	0.028913
120 TRAP bipolar	9197	SLC33A1	0.0029094
120 TRAP bipolar	8833	GMPS	0.0030881
120 TRAP bipolar	80012	PHC3	0.038892
120 TRAP bipolar	1981	EIF4G1	2.17E-05
120 TRAP bipolar	23527	ACAP2	0.033976
120 TRAP bipolar	23284	ADGRL3	6.44E-04
120 TRAP bipolar	9348	NDST3	2.42E-04
120 TRAP bipolar	84162	KIAA1109	3.02E-04
120 TRAP bipolar	11275	KLHL2	0.034489
120 TRAP bipolar	108	ADCY2	2.55E-07
120 TRAP bipolar	3156	HMGCR	0.020521
120 TRAP bipolar	10087	CERT1	0.028419
120 TRAP bipolar	9456	HOMER1	9.78E-05
120 TRAP bipolar	116068	LYSMD3	4.75E-05
120 TRAP bipolar	1958	EGR1	3.39E-06
120 TRAP bipolar	3313	HSPA9	2.69E-05
120 TRAP bipolar	54882	ANKHD1	0.0026414
120 TRAP bipolar	10307	APBB3	0.015536
120 TRAP bipolar	929	CD14	1.67E-04
120 TRAP bipolar	56124	PCDHB12	6.90E-05
120 TRAP bipolar	56113	PCDHGA2	0.01888
120 TRAP bipolar	6879	TAF7	0.032564
120 TRAP bipolar	56112	PCDHGA3	0.016731
120 TRAP bipolar	56107	PCDHGA9	0.018407
120 TRAP bipolar	56105	PCDHGA11	0.017853
120 TRAP bipolar	56097	PCDHGC5	0.032394
120 TRAP bipolar	81848	SPRY4	0.033882
120 TRAP bipolar	51520	LARS1	4.19E-04
120 TRAP bipolar	10915	TCERG1	0.0015679
120 TRAP bipolar	133522	PPARGC1B	4.43E-04
120 TRAP bipolar	6586	SLIT3	7.30E-04
120 TRAP bipolar	57472	CNOT6	0.049791
120 TRAP bipolar	221692	PHACTR1	1.46E-04
120 TRAP bipolar	29116	MYLIP	0.038309
120 TRAP bipolar	9856	KIAA0319	0.0021154
120 TRAP bipolar	8968	H3C7	6.67E-04
120 TRAP bipolar	79692	ZNF322	1.79E-04
120 TRAP bipolar	80317	ZKSCAN3	1.96E-06
120 TRAP bipolar	5514	PPP1R10	0.04796
120 TRAP bipolar	23787	MTCH1	0.031498
120 TRAP bipolar	114781	BTBD9	0.011044

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	6722	SRF	4.40E-05
120 TRAP bipolar	24149	ZNF318	1.31E-05
120 TRAP bipolar	23469	PHF3	0.00292
120 TRAP bipolar	577	ADGRB3	0.0041903
120 TRAP bipolar	3351	HTR1B	6.89E-05
120 TRAP bipolar	55023	PHIP	0.035763
120 TRAP bipolar	55603	TENT5A	7.17E-04
120 TRAP bipolar	8936	WASF1	0.037578
120 TRAP bipolar	10370	CITED2	0.0373
120 TRAP bipolar	23345	SYNE1	2.59E-07
120 TRAP bipolar	84629	TNRC18	0.028474
120 TRAP bipolar	8887	TAX1BP1	6.17E-06
120 TRAP bipolar	154807	VKORC1L1	0.0046404
120 TRAP bipolar	27445	PCLO	3.98E-06
120 TRAP bipolar	1595	CYP51A1	0.021169
120 TRAP bipolar	79027	ZNF655	0.0026826
120 TRAP bipolar	7589	ZSCAN21	0.034076
120 TRAP bipolar	64599	GIGYF1	0.0074529
120 TRAP bipolar	4897	NRCAM	8.41E-04
120 TRAP bipolar	4189	DNAJB9	1.91E-05
120 TRAP bipolar	83992	CTTNBP2	8.55E-04
120 TRAP bipolar	168850	ZNF800	0.041558
120 TRAP bipolar	27044	SND1	0.020828
120 TRAP bipolar	154790	CLEC2L	0.038888
120 TRAP bipolar	26047	CNTNAP2	0.037968
120 TRAP bipolar	9601	PDIA4	0.016348
120 TRAP bipolar	64478	CSMD1	0.0027339
120 TRAP bipolar	8658	TNKS	0.0015319
120 TRAP bipolar	65986	ZBTB10	0.047854
120 TRAP bipolar	862	RUNX1T1	7.68E-04
120 TRAP bipolar	3788	KCNS2	0.042985
120 TRAP bipolar	7071	KLF10	0.033243
120 TRAP bipolar	11236	RNF139	0.036662
120 TRAP bipolar	4715	NDUFB9	0.036967
120 TRAP bipolar	51059	FAM135B	4.54E-06
120 TRAP bipolar	65268	WNK2	0.0026575
120 TRAP bipolar	9568	GABBR2	0.0046665
120 TRAP bipolar	23245	ASTN2	0.0043303
120 TRAP bipolar	54461	FBXW5	0.016726
120 TRAP bipolar	774	CACNA1B	0.0014804
120 TRAP bipolar	57512	GPR158	0.0078659
120 TRAP bipolar	23283	CSTF2T	0.0043465
120 TRAP bipolar	288	ANK3	3.73E-06
120 TRAP bipolar	1959	EGR2	1.65E-04
120 TRAP bipolar	7414	VCL	0.012864
120 TRAP bipolar	23223	RRP12	0.033434
120 TRAP bipolar	8945	BTRC	0.024582
120 TRAP bipolar	23082	PPRC1	0.025674
120 TRAP bipolar	22986	SORCS3	2.74E-04

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	57698	SHTN1	0.0022259
120 TRAP bipolar	627	BDNF	0.032421
120 TRAP bipolar	1132	CHRM4	3.17E-04
120 TRAP bipolar	9793	CKAP5	0.012209
120 TRAP bipolar	79096	CSTPP1	0.017251
120 TRAP bipolar	8567	MADD	0.026844
120 TRAP bipolar	1500	CTNND1	0.0043328
120 TRAP bipolar	56834	GPR137	0.0021984
120 TRAP bipolar	10992	SF3B2	7.60E-07
120 TRAP bipolar	55690	PACS1	1.03E-08
120 TRAP bipolar	266743	NPAS4	1.56E-05
120 TRAP bipolar	10432	RBM14	1.97E-04
120 TRAP bipolar	5091	PC	1.62E-06
120 TRAP bipolar	22941	SHANK2	2.52E-06
120 TRAP bipolar	2915	GRM5	5.74E-04
120 TRAP bipolar	85459	CEP295	0.0013174
120 TRAP bipolar	91893	FDXACB1	0.027906
120 TRAP bipolar	4684	NCAM1	2.56E-06
120 TRAP bipolar	53826	FXYD6	0.02124
120 TRAP bipolar	6230	RPS25	0.030931
120 TRAP bipolar	399979	SNX19	1.35E-04
120 TRAP bipolar	5927	KDM5A	0.049704
120 TRAP bipolar	25900	IFFO1	0.0060793
120 TRAP bipolar	8078	USP5	1.21E-04
120 TRAP bipolar	6515	SLC2A3	0.027474
120 TRAP bipolar	9052	GPRC5A	0.0034001
120 TRAP bipolar	2904	GRIN2B	0.02318
120 TRAP bipolar	55729	ATF7IP	0.017735
120 TRAP bipolar	636	BICD1	0.032337
120 TRAP bipolar	55605	KIF21A	0.0040506
120 TRAP bipolar	3164	NR4A1	0.01002
120 TRAP bipolar	4141	MARS1	0.0027341
120 TRAP bipolar	8089	YEATS4	0.0081573
120 TRAP bipolar	8411	EEA1	0.0052873
120 TRAP bipolar	7184	HSP90B1	0.045703
120 TRAP bipolar	10985	GCN1	0.01582
120 TRAP bipolar	121665	SPPL3	7.28E-04
120 TRAP bipolar	6249	CLIP1	0.0063249
120 TRAP bipolar	9612	NCOR2	5.39E-04
120 TRAP bipolar	10808	HSPH1	0.014497
120 TRAP bipolar	11215	AKAP11	0.047229
120 TRAP bipolar	26512	INTS6	0.027062
120 TRAP bipolar	27253	PCDH17	0.0050952
120 TRAP bipolar	23077	MYCBP2	0.043605
120 TRAP bipolar	79596	OBI1	0.011654
120 TRAP bipolar	64062	RBM26	1.51E-04
120 TRAP bipolar	3843	IPO5	0.013052
120 TRAP bipolar	7174	TPP2	3.07E-05
120 TRAP bipolar	57680	CHD8	0.037688

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	9878	TOX4	0.043524
120 TRAP bipolar	2290	FOXG1	1.14E-04
120 TRAP bipolar	25831	HECTD1	0.02303
120 TRAP bipolar	9472	AKAP6	0.048021
120 TRAP bipolar	253959	RALGAPA1	0.0011096
120 TRAP bipolar	23116	TOGARAM1	0.010737
120 TRAP bipolar	161357	MDGA2	3.07E-04
120 TRAP bipolar	10243	GPHN	0.033228
120 TRAP bipolar	2353	FOS	0.019489
120 TRAP bipolar	145567	TTC7B	0.021762
120 TRAP bipolar	440193	CCDC88C	0.002645
120 TRAP bipolar	64919	BCL11B	3.32E-09
120 TRAP bipolar	81693	AMN	0.0017439
120 TRAP bipolar	388021	TMEM179	0.0065711
120 TRAP bipolar	23359	FAM189A1	5.64E-04
120 TRAP bipolar	22893	BAHD1	0.0138
120 TRAP bipolar	7158	TP53BP1	0.019908
120 TRAP bipolar	23312	DMXL2	0.021144
120 TRAP bipolar	54778	RNF111	0.031662
120 TRAP bipolar	54832	VPS13C	0.0021461
120 TRAP bipolar	388125	C2CD4B	0.01476
120 TRAP bipolar	83660	TLN2	0.036932
120 TRAP bipolar	8925	HERC1	0.023617
120 TRAP bipolar	3658	IREB2	0.0067611
120 TRAP bipolar	8120	AP3B2	1.96E-04
120 TRAP bipolar	53339	BTBD1	5.15E-06
120 TRAP bipolar	1106	CHD2	0.010436
120 TRAP bipolar	57585	CRAMP1	0.0064745
120 TRAP bipolar	23162	MAPK8IP3	0.035118
120 TRAP bipolar	29855	UBN1	0.0043214
120 TRAP bipolar	29035	C16orf72	1.38E-07
120 TRAP bipolar	2903	GRIN2A	5.23E-08
120 TRAP bipolar	26470	SEZ6L2	1.55E-05
120 TRAP bipolar	9344	TAOK2	3.37E-06
120 TRAP bipolar	6376	CX3CL1	0.0026889
120 TRAP bipolar	3801	KIFC3	0.034373
120 TRAP bipolar	23019	CNOT1	7.19E-04
120 TRAP bipolar	1006	CDH8	2.36E-05
120 TRAP bipolar	1009	CDH11	0.033408
120 TRAP bipolar	79567	RIPOR1	3.44E-04
120 TRAP bipolar	23644	EDC4	2.91E-05
120 TRAP bipolar	10725	NFAT5	0.033617
120 TRAP bipolar	164	AP1G1	0.023967
120 TRAP bipolar	342371	ATXN1L	0.0062399
120 TRAP bipolar	5430	POLR2A	0.010311
120 TRAP bipolar	124925	SEZ6	0.0043555
120 TRAP bipolar	85464	SSH2	0.011055
120 TRAP bipolar	1362	CPD	2.08E-05
120 TRAP bipolar	5469	MED1	0.010373

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	94103	ORMDL3	1.79E-05
120 TRAP bipolar	23131	GPATCH8	0.023838
120 TRAP bipolar	8913	CACNA1G	0.0030403
120 TRAP bipolar	9256	TSPOAP1	0.01387
120 TRAP bipolar	9110	MTMR4	0.024013
120 TRAP bipolar	22843	PPM1E	0.0093131
120 TRAP bipolar	8787	RGS9	0.033736
120 TRAP bipolar	79902	NUP85	5.87E-05
120 TRAP bipolar	23163	GGA3	0.0034411
120 TRAP bipolar	2194	FASN	0.021391
120 TRAP bipolar	1453	CSNK1D	0.033123
120 TRAP bipolar	81929	SEH1L	0.047361
120 TRAP bipolar	6860	SYT4	0.0068314
120 TRAP bipolar	90701	SEC11C	0.001025
120 TRAP bipolar	57614	RELCH	0.031621
120 TRAP bipolar	8192	CLPP	0.044414
120 TRAP bipolar	9817	KEAP1	0.043354
120 TRAP bipolar	9592	IER2	0.041173
120 TRAP bipolar	3337	DNAJB1	0.015198
120 TRAP bipolar	10523	CHERP	0.02622
120 TRAP bipolar	10331	B3GNT3	0.0090065
120 TRAP bipolar	23373	CRTC1	0.0011273
120 TRAP bipolar	5976	UPF1	0.0092178
120 TRAP bipolar	1463	NCAN	5.96E-05
120 TRAP bipolar	57130	ATP13A1	2.22E-05
120 TRAP bipolar	388536	ZNF790	0.02154
120 TRAP bipolar	81	ACTN4	0.0083568
120 TRAP bipolar	478	ATP1A3	0.0099741
120 TRAP bipolar	4858	NOVA2	0.016403
120 TRAP bipolar	57469	PNMA8B	0.042886
120 TRAP bipolar	5178	PEG3	0.012095
120 TRAP bipolar	65982	ZSCAN18	0.031994
120 TRAP bipolar	79673	ZNF329	0.024196
120 TRAP bipolar	10155	TRIM28	4.33E-04
120 TRAP bipolar	83737	ITCH	0.0011548
120 TRAP bipolar	10137	RBM12	0.0042591
120 TRAP bipolar	6185	RPN2	0.0264
120 TRAP bipolar	7150	TOP1	5.46E-04
120 TRAP bipolar	23051	ZHX3	3.42E-04
120 TRAP bipolar	1434	CSE1L	0.0053883
120 TRAP bipolar	84612	PARD6B	0.039299
120 TRAP bipolar	128611	ZNF831	0.024219
120 TRAP bipolar	6874	TAF4	2.48E-04
120 TRAP bipolar	24148	PRPF6	6.88E-04
120 TRAP bipolar	6612	SUMO3	0.046143
120 TRAP bipolar	754	PTTG1IP	0.0056126
120 TRAP bipolar	8888	MCM3AP	0.011362
120 TRAP bipolar	57553	MICAL3	0.018297
120 TRAP bipolar	57591	MRTFA	0.0051638

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar	5905	RANGAP1	0.018365
60 TRAP bipolar	728642	CDK11A	0.0058284
60 TRAP bipolar	9696	CROCC	0.0022841
60 TRAP bipolar	5909	RAP1GAP	0.017956
60 TRAP bipolar	149420	PDIK1L	0.0026844
60 TRAP bipolar	6421	SFPQ	4.98E-04
60 TRAP bipolar	149175	MANEAL	0.011631
60 TRAP bipolar	643314	NA	0.026222
60 TRAP bipolar	54802	TRIT1	0.0012997
60 TRAP bipolar	4802	NFYC	0.032901
60 TRAP bipolar	284716	RIMKLA	9.34E-04
60 TRAP bipolar	2166	FAAH	0.016948
60 TRAP bipolar	79699	ZYG11B	0.031335
60 TRAP bipolar	3725	JUN	0.011754
60 TRAP bipolar	84251	SGIP1	0.003107
60 TRAP bipolar	26135	SERBP1	0.03169
60 TRAP bipolar	26289	AK5	0.011454
60 TRAP bipolar	84146	ZNF644	0.0056845
60 TRAP bipolar	55119	PRPF38B	0.043202
60 TRAP bipolar	128338	DRAM2	0.025561
60 TRAP bipolar	204851	HIPK1	0.0056171
60 TRAP bipolar	333932	H3C15	0.014018
60 TRAP bipolar	9900	SV2A	0.048865
60 TRAP bipolar	51177	PLEKHO1	6.12E-07
60 TRAP bipolar	9129	PRPF3	5.86E-04
60 TRAP bipolar	4170	MCL1	0.0016341
60 TRAP bipolar	405	ARNT	0.01798
60 TRAP bipolar	10500	SEMA6C	0.03909
60 TRAP bipolar	57198	ATP8B2	0.0042103
60 TRAP bipolar	4580	MTX1	0.016212
60 TRAP bipolar	23623	RUSC1	0.037686
60 TRAP bipolar	477	ATP1A2	6.77E-04
60 TRAP bipolar	93185	IGSF8	0.0014372
60 TRAP bipolar	8682	PEA15	0.026221
60 TRAP bipolar	4720	NDUFS2	1.91E-04
60 TRAP bipolar	5999	RGS4	0.010114
60 TRAP bipolar	7143	TNR	1.19E-04
60 TRAP bipolar	64326	COP1	0.011923
60 TRAP bipolar	777	CACNA1E	0.032627
60 TRAP bipolar	1660	DHX9	0.0080168
60 TRAP bipolar	8707	B3GALT2	0.043641
60 TRAP bipolar	163486	DENND1B	0.0027524
60 TRAP bipolar	2848	GPR25	0.0025989
60 TRAP bipolar	55705	IPO9	0.024888
60 TRAP bipolar	5929	RBBP5	0.014037
60 TRAP bipolar	25778	DSTYK	0.006077
60 TRAP bipolar	9911	TMCC2	0.0048273
60 TRAP bipolar	55699	IARS2	0.0015922
60 TRAP bipolar	142	PARP1	0.0045376

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	9804	TOMM20	0.0040895
60 TRAP bipolar	56776	FMN2	0.020705
60 TRAP bipolar	9859	CEP170	0.015801
60 TRAP bipolar	6664	SOX11	0.049368
60 TRAP bipolar	3777	KCNK3	0.0073201
60 TRAP bipolar	64838	FNDC4	0.015331
60 TRAP bipolar	8491	MAP4K3	0.033511
60 TRAP bipolar	6546	SLC8A1	0.020454
60 TRAP bipolar	9655	SOCS5	0.001746
60 TRAP bipolar	9378	NRXN1	1.14E-04
60 TRAP bipolar	57142	RTN4	3.81E-04
60 TRAP bipolar	53335	BCL11A	0.001006
60 TRAP bipolar	10097	ACTR2	0.010195
60 TRAP bipolar	5534	PPP3R1	0.028876
60 TRAP bipolar	23233	EXOC6B	0.043881
60 TRAP bipolar	10322	SMYD5	2.32E-04
60 TRAP bipolar	1961	EGR4	0.0068944
60 TRAP bipolar	1844	DUSP2	2.66E-07
60 TRAP bipolar	56910	STARD7	5.54E-07
60 TRAP bipolar	10865	ARID5A	3.22E-04
60 TRAP bipolar	23505	TMEM131	3.12E-04
60 TRAP bipolar	3631	INPP4A	0.0015235
60 TRAP bipolar	11320	MGAT4A	0.017154
60 TRAP bipolar	9669	EIF5B	0.0011727
60 TRAP bipolar	9392	TGFBRAP1	0.039335
60 TRAP bipolar	79074	C2orf49	0.035828
60 TRAP bipolar	54520	CCDC93	0.048944
60 TRAP bipolar	23332	CLASP1	0.017851
60 TRAP bipolar	4929	NR4A2	0.0012562
60 TRAP bipolar	10716	TBR1	2.76E-05
60 TRAP bipolar	57282	SLC4A10	3.14E-04
60 TRAP bipolar	9360	PPIG	0.00398
60 TRAP bipolar	151230	KLHL23	3.72E-04
60 TRAP bipolar	100131390	SP9	0.001465
60 TRAP bipolar	151556	GPR155	0.0096594
60 TRAP bipolar	3676	ITGA4	0.017123
60 TRAP bipolar	2487	FRZB	0.0015166
60 TRAP bipolar	10787	NCKAP1	0.001689
60 TRAP bipolar	23451	SF3B1	2.82E-05
60 TRAP bipolar	23314	SATB2	0.0099068
60 TRAP bipolar	205327	C2orf69	7.52E-04
60 TRAP bipolar	471	ATIC	0.0072063
60 TRAP bipolar	26058	GIGYF2	0.011441
60 TRAP bipolar	8864	PER2	0.019105
60 TRAP bipolar	547	KIF1A	3.06E-05
60 TRAP bipolar	9881	TRANK1	5.31E-11
60 TRAP bipolar	9852	EPM2AIP1	2.12E-04
60 TRAP bipolar	2803	GOLGA4	0.0061435
60 TRAP bipolar	10289	EIF1B	0.040593

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	9045	RPL14	0.041297
60 TRAP bipolar	29072	SETD2	0.028273
60 TRAP bipolar	8927	BSN	0.0053692
60 TRAP bipolar	79012	CAMKV	0.017158
60 TRAP bipolar	10181	RBM5	0.0037869
60 TRAP bipolar	80335	WDR82	6.14E-05
60 TRAP bipolar	55193	PBRM1	7.39E-11
60 TRAP bipolar	5580	PRKCD	0.0041303
60 TRAP bipolar	166336	PRICKLE2	0.011629
60 TRAP bipolar	5067	CNTN3	0.029006
60 TRAP bipolar	214	ALCAM	0.0010726
60 TRAP bipolar	11343	MGLL	0.035092
60 TRAP bipolar	55764	IFT122	0.032522
60 TRAP bipolar	5912	RAP2B	0.022245
60 TRAP bipolar	23007	PLCH1	8.87E-06
60 TRAP bipolar	1981	EIF4G1	2.17E-05
60 TRAP bipolar	1487	CTBP1	0.0091057
60 TRAP bipolar	339983	NAT8L	0.02205
60 TRAP bipolar	6002	RGS12	6.42E-04
60 TRAP bipolar	116984	ARAP2	0.019077
60 TRAP bipolar	10463	SLC30A9	0.031789
60 TRAP bipolar	2557	GABRA4	0.0013838
60 TRAP bipolar	285527	FRYL	6.39E-04
60 TRAP bipolar	132299	OCIAD2	0.0077153
60 TRAP bipolar	3815	KIT	0.0017485
60 TRAP bipolar	5530	PPP3CA	0.01733
60 TRAP bipolar	56884	FSTL5	0.04575
60 TRAP bipolar	11275	KLHL2	0.034489
60 TRAP bipolar	54888	NSUN2	0.0027856
60 TRAP bipolar	11044	TENT4A	0.042234
60 TRAP bipolar	108	ADCY2	2.55E-07
60 TRAP bipolar	10409	BASP1	1.76E-05
60 TRAP bipolar	2255	FGF10	0.034112
60 TRAP bipolar	10884	MRPS30	3.06E-04
60 TRAP bipolar	3156	HMGCR	0.020521
60 TRAP bipolar	9456	HOMER1	9.78E-05
60 TRAP bipolar	10085	EDIL3	0.0025779
60 TRAP bipolar	64839	FBXL17	0.004598
60 TRAP bipolar	27089	UQCRQ	1.36E-04
60 TRAP bipolar	9879	DDX46	0.010354
60 TRAP bipolar	1958	EGR1	3.39E-06
60 TRAP bipolar	3313	HSPA9	2.69E-05
60 TRAP bipolar	54882	ANKHD1	0.0026414
60 TRAP bipolar	3550	IK	1.73E-04
60 TRAP bipolar	8641	PCDHGB4	0.021901
60 TRAP bipolar	56097	PCDHGC5	0.032394
60 TRAP bipolar	10915	TCERG1	0.0015679
60 TRAP bipolar	6534	SLC6A7	0.02759
60 TRAP bipolar	221692	PHACTR1	1.46E-04

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	29116	MYLIP	0.038309
60 TRAP bipolar	51439	FAM8A1	0.019862
60 TRAP bipolar	9856	KIAA0319	0.0021154
60 TRAP bipolar	81688	C6orf62	0.011827
60 TRAP bipolar	8359	H4C1	4.73E-05
60 TRAP bipolar	3006	H1-2	0.0031833
60 TRAP bipolar	8365	H4C8	3.82E-06
60 TRAP bipolar	79692	ZNF322	1.79E-04
60 TRAP bipolar	8294	H4C9	2.05E-06
60 TRAP bipolar	79897	RPP21	6.84E-07
60 TRAP bipolar	5514	PPP1R10	0.04796
60 TRAP bipolar	221545	C6orf136	0.011458
60 TRAP bipolar	57176	VARS2	1.49E-05
60 TRAP bipolar	534	ATP6V1G2	0.0025683
60 TRAP bipolar	80736	SLC44A4	4.85E-06
60 TRAP bipolar	6048	RNF5	5.78E-06
60 TRAP bipolar	5089	PBX2	2.45E-06
60 TRAP bipolar	51596	CUTA	7.46E-04
60 TRAP bipolar	23787	MTCH1	0.031498
60 TRAP bipolar	114781	BTBD9	0.011044
60 TRAP bipolar	25844	YIPF3	0.010547
60 TRAP bipolar	2729	GCLC	0.0039522
60 TRAP bipolar	23469	PHF3	0.00292
60 TRAP bipolar	22999	RIMS1	9.83E-07
60 TRAP bipolar	9892	SNAP91	3.47E-05
60 TRAP bipolar	114792	KLHL32	0.0013097
60 TRAP bipolar	26235	FBXL4	0.0037014
60 TRAP bipolar	57673	BEND3	0.013135
60 TRAP bipolar	135112	NCOA7	0.047329
60 TRAP bipolar	10370	CITED2	0.0373
60 TRAP bipolar	134957	STXBP5	0.025368
60 TRAP bipolar	23345	SYNE1	2.59E-07
60 TRAP bipolar	57492	ARID1B	0.010644
60 TRAP bipolar	6950	TCP1	0.041928
60 TRAP bipolar	84629	TNRC18	0.028474
60 TRAP bipolar	54664	TMEM106B	0.0033718
60 TRAP bipolar	6671	SP4	3.80E-08
60 TRAP bipolar	23242	COBL	0.006077
60 TRAP bipolar	7532	YWHAG	0.020006
60 TRAP bipolar	27445	PCLO	3.98E-06
60 TRAP bipolar	154661	RUNDC3B	8.92E-04
60 TRAP bipolar	889	KRIT1	0.036165
60 TRAP bipolar	2845	GPR22	0.019975
60 TRAP bipolar	4897	NRCAM	8.41E-04
60 TRAP bipolar	7982	ST7	0.0052975
60 TRAP bipolar	83992	CTTNBP2	8.55E-04
60 TRAP bipolar	51530	ZC3HC1	0.0064391
60 TRAP bipolar	23008	KLHDC10	4.47E-04
60 TRAP bipolar	26047	CNTNAP2	0.037968

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60 TRAP bipolar	9601	PDIA4	0.016348
60 TRAP bipolar	9690	UBE3C	0.014634
60 TRAP bipolar	26260	FBXO25	0.04685
60 TRAP bipolar	9258	MFHAS1	9.46E-05
60 TRAP bipolar	66036	MTMR9	0.02453
60 TRAP bipolar	55140	ELP3	0.0038258
60 TRAP bipolar	9530	BAG4	4.20E-04
60 TRAP bipolar	115294	PCMTD1	0.042233
60 TRAP bipolar	54332	GDAP1	0.012416
60 TRAP bipolar	734	OSGIN2	0.011741
60 TRAP bipolar	862	RUNX1T1	7.68E-04
60 TRAP bipolar	3788	KCNS2	0.042985
60 TRAP bipolar	7071	KLF10	0.033243
60 TRAP bipolar	9699	RIMS2	0.019273
60 TRAP bipolar	11236	RNF139	0.036662
60 TRAP bipolar	9373	PLAA	0.032945
60 TRAP bipolar	54926	UBE2R2	0.011328
60 TRAP bipolar	26267	FBXO10	0.040925
60 TRAP bipolar	23287	AGTPBP1	0.016931
60 TRAP bipolar	65268	WNK2	0.0026575
60 TRAP bipolar	23196	FAM120A	0.0016501
60 TRAP bipolar	5253	PHF2	0.012854
60 TRAP bipolar	9568	GABBR2	0.0046665
60 TRAP bipolar	10592	SMC2	0.048813
60 TRAP bipolar	8818	DPM2	0.047946
60 TRAP bipolar	54461	FBXW5	0.016726
60 TRAP bipolar	221061	FAM171A1	2.06E-04
60 TRAP bipolar	23283	CSTF2T	0.0043465
60 TRAP bipolar	220965	FAM13C	0.038595
60 TRAP bipolar	288	ANK3	3.73E-06
60 TRAP bipolar	84890	ADO	7.63E-05
60 TRAP bipolar	1959	EGR2	1.65E-04
60 TRAP bipolar	55749	CCAR1	0.0019405
60 TRAP bipolar	657	BMPR1A	0.030809
60 TRAP bipolar	56889	TM9SF3	0.043076
60 TRAP bipolar	8945	BTRC	0.024582
60 TRAP bipolar	30819	KCNIP2	0.041797
60 TRAP bipolar	8861	LDB1	0.025601
60 TRAP bipolar	81603	TRIM8	6.34E-04
60 TRAP bipolar	22986	SORCS3	2.74E-04
60 TRAP bipolar	57678	GPAM	0.026841
60 TRAP bipolar	64429	ZDHHC6	0.0096944
60 TRAP bipolar	57700	FHIP2A	0.010887
60 TRAP bipolar	118987	PDZD8	0.016102
60 TRAP bipolar	2018	EMX2	0.020352
60 TRAP bipolar	84435	ADGRA1	0.036763
60 TRAP bipolar	406	ARNTL	9.96E-07
60 TRAP bipolar	8534	CHST1	1.38E-04
60 TRAP bipolar	8525	DGKZ	0.0013755

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60 TRAP bipolar	9793	CKAP5	0.012209
60 TRAP bipolar	55709	KBTBD4	0.0080685
60 TRAP bipolar	23788	MTCH2	0.022329
60 TRAP bipolar	747	DAGLA	7.90E-06
60 TRAP bipolar	10963	STIP1	0.0037899
60 TRAP bipolar	56834	GPR137	0.0021984
60 TRAP bipolar	9379	NRXN2	9.53E-05
60 TRAP bipolar	10992	SF3B2	7.60E-07
60 TRAP bipolar	266743	NPAS4	1.56E-05
60 TRAP bipolar	78999	LRFN4	6.66E-07
60 TRAP bipolar	22941	SHANK2	2.52E-06
60 TRAP bipolar	283219	KCTD21	0.0073622
60 TRAP bipolar	4684	NCAM1	2.56E-06
60 TRAP bipolar	51092	SIDT2	0.015829
60 TRAP bipolar	53826	FXYD6	0.02124
60 TRAP bipolar	6230	RPS25	0.030931
60 TRAP bipolar	2597	GAPDH	0.0036248
60 TRAP bipolar	1822	ATN1	0.006463
60 TRAP bipolar	55729	ATF7IP	0.017735
60 TRAP bipolar	6660	SOX5	0.012836
60 TRAP bipolar	83857	TMTC1	0.015863
60 TRAP bipolar	10526	IPO8	0.019993
60 TRAP bipolar	636	BICD1	0.032337
60 TRAP bipolar	23109	DDN	9.03E-05
60 TRAP bipolar	5571	PRKAG1	9.38E-05
60 TRAP bipolar	10376	TUBA1B	0.025483
60 TRAP bipolar	23416	KCNH3	0.047388
60 TRAP bipolar	3164	NR4A1	0.01002
60 TRAP bipolar	5502	PPP1R1A	0.023916
60 TRAP bipolar	3798	KIF5A	0.01644
60 TRAP bipolar	4922	NTS	3.68E-04
60 TRAP bipolar	8411	EEA1	0.0052873
60 TRAP bipolar	7184	HSP90B1	0.045703
60 TRAP bipolar	5781	PTPN11	0.04476
60 TRAP bipolar	23389	MED13L	0.041247
60 TRAP bipolar	55884	WSB2	0.0101
60 TRAP bipolar	1337	COX6A1	6.00E-04
60 TRAP bipolar	51367	POP5	0.022674
60 TRAP bipolar	121665	SPPL3	7.28E-04
60 TRAP bipolar	65082	VPS33A	0.02249
60 TRAP bipolar	6249	CLIP1	0.0063249
60 TRAP bipolar	10959	TMED2	0.010142
60 TRAP bipolar	80212	CCDC92	0.0014172
60 TRAP bipolar	144348	ZNF664	0.001491
60 TRAP bipolar	9612	NCOR2	5.39E-04
60 TRAP bipolar	55504	TNFRSF19	0.0056644
60 TRAP bipolar	10208	USPL1	0.0057116
60 TRAP bipolar	10808	HSPH1	0.014497
60 TRAP bipolar	27253	PCDH17	0.0050952

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	64062	RBM26	1.51E-04
60 TRAP bipolar	22873	DZIP1	0.041349
60 TRAP bipolar	9358	ITGBL1	0.024537
60 TRAP bipolar	7174	TPP2	3.07E-05
60 TRAP bipolar	1948	EFNB2	0.01809
60 TRAP bipolar	84945	ABHD13	0.0097728
60 TRAP bipolar	8451	CUL4A	2.00E-08
60 TRAP bipolar	57680	CHD8	0.037688
60 TRAP bipolar	2290	FOXG1	1.14E-04
60 TRAP bipolar	9472	AKAP6	0.048021
60 TRAP bipolar	10484	SEC23A	0.016923
60 TRAP bipolar	23116	TOGARAM1	0.010737
60 TRAP bipolar	161357	MDGA2	3.07E-04
60 TRAP bipolar	8814	CDKL1	0.031648
60 TRAP bipolar	145407	ARMH4	0.011653
60 TRAP bipolar	55860	ACTR10	8.58E-04
60 TRAP bipolar	83851	SYT16	4.35E-05
60 TRAP bipolar	10243	GPHN	0.033228
60 TRAP bipolar	2353	FOS	0.019489
60 TRAP bipolar	145567	TTC7B	0.021762
60 TRAP bipolar	1735	DIO3	0.0068821
60 TRAP bipolar	55778	ZNF839	0.0055287
60 TRAP bipolar	7187	TRAF3	0.0018926
60 TRAP bipolar	388021	TMEM179	0.0065711
60 TRAP bipolar	114791	TUBGCP5	0.0012929
60 TRAP bipolar	2562	GABRB3	0.023422
60 TRAP bipolar	23359	FAM189A1	5.64E-04
60 TRAP bipolar	27079	RPUSD2	0.0087301
60 TRAP bipolar	3706	ITPKA	0.049203
60 TRAP bipolar	23339	VPS39	2.19E-04
60 TRAP bipolar	9836	LCMT2	0.027048
60 TRAP bipolar	7158	TP53BP1	0.019908
60 TRAP bipolar	9101	USP8	0.044917
60 TRAP bipolar	79811	SLTM	5.01E-05
60 TRAP bipolar	54778	RNF111	0.031662
60 TRAP bipolar	388125	C2CD4B	0.01476
60 TRAP bipolar	23060	ZNF609	0.037803
60 TRAP bipolar	4088	SMAD3	5.19E-04
60 TRAP bipolar	57611	ISLR2	0.012341
60 TRAP bipolar	1445	CSK	0.023459
60 TRAP bipolar	8120	AP3B2	1.96E-04
60 TRAP bipolar	9455	HOMER2	1.39E-04
60 TRAP bipolar	53339	BTBD1	5.15E-06
60 TRAP bipolar	4122	MAN2A2	3.74E-05
60 TRAP bipolar	1106	CHD2	0.010436
60 TRAP bipolar	23162	MAPK8IP3	0.035118
60 TRAP bipolar	7249	TSC2	0.0077213
60 TRAP bipolar	5310	PKD1	0.021134
60 TRAP bipolar	79641	ROGDI	0.0070442

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	91949	COG7	0.023041
60 TRAP bipolar	124454	EARS2	0.0073493
60 TRAP bipolar	5579	PRKCB	3.96E-07
60 TRAP bipolar	26470	SEZ6L2	1.55E-05
60 TRAP bipolar	8479	HIRIP3	1.00E-04
60 TRAP bipolar	83723	TLCD3B	0.0048484
60 TRAP bipolar	226	ALDOA	0.0068236
60 TRAP bipolar	5432	POLR2C	0.023328
60 TRAP bipolar	3801	KIFC3	0.034373
60 TRAP bipolar	23019	CNOT1	7.19E-04
60 TRAP bipolar	8883	NAE1	0.010583
60 TRAP bipolar	9114	ATP6V0D1	1.58E-04
60 TRAP bipolar	146206	CARMIL2	2.42E-04
60 TRAP bipolar	65057	ACD	2.84E-05
60 TRAP bipolar	55512	SMPD3	0.001127
60 TRAP bipolar	84916	UTP4	1.01E-04
60 TRAP bipolar	10725	NFAT5	0.033617
60 TRAP bipolar	7571	ZNF23	0.010782
60 TRAP bipolar	161882	ZFPM1	0.029253
60 TRAP bipolar	29123	ANKRD11	7.08E-05
60 TRAP bipolar	8558	CDK10	0.0027105
60 TRAP bipolar	22980	TCF25	0.0012999
60 TRAP bipolar	7531	YWHAE	2.55E-07
60 TRAP bipolar	1398	CRK	4.07E-04
60 TRAP bipolar	9905	SGSM2	0.017505
60 TRAP bipolar	84254	CAMKK1	0.02113
60 TRAP bipolar	56919	DHX33	0.0096187
60 TRAP bipolar	83394	PITPNM3	0.014069
60 TRAP bipolar	2256	FGF11	0.021937
60 TRAP bipolar	51701	NLK	0.015046
60 TRAP bipolar	124925	SEZ6	0.0043555
60 TRAP bipolar	399687	MYO18A	5.05E-04
60 TRAP bipolar	28964	GIT1	0.0055577
60 TRAP bipolar	4763	NF1	0.043796
60 TRAP bipolar	84152	PPP1R1B	0.030659
60 TRAP bipolar	94103	ORMDL3	1.79E-05
60 TRAP bipolar	5709	PSMD3	1.17E-05
60 TRAP bipolar	4836	NMT1	0.0023073
60 TRAP bipolar	113026	PLCD3	0.011502
60 TRAP bipolar	8913	CACNA1G	0.0030403
60 TRAP bipolar	51747	LUC7L3	1.63E-04
60 TRAP bipolar	3131	HLF	1.84E-06
60 TRAP bipolar	4591	TRIM37	0.011874
60 TRAP bipolar	9772	TMEM94	0.010597
60 TRAP bipolar	55666	NPLOC4	4.29E-04
60 TRAP bipolar	9146	HGS	0.012006
60 TRAP bipolar	6182	MRPL12	0.029757
60 TRAP bipolar	1453	CSNK1D	0.033123
60 TRAP bipolar	9229	DLGAP1	0.009864

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	81929	SEH1L	0.047361
60 TRAP bipolar	7572	ZNF24	0.0029333
60 TRAP bipolar	6860	SYT4	0.0068314
60 TRAP bipolar	23335	WDR7	0.031066
60 TRAP bipolar	57614	RELCH	0.031621
60 TRAP bipolar	80148	SLC66A2	0.0043893
60 TRAP bipolar	148252	DIRAS1	9.38E-07
60 TRAP bipolar	29985	SLC39A3	1.26E-05
60 TRAP bipolar	84717	HDGFL2	0.037982
60 TRAP bipolar	4298	MLLT1	0.02787
60 TRAP bipolar	9817	KEAP1	0.043354
60 TRAP bipolar	6597	SMARCA4	0.0019905
60 TRAP bipolar	4784	NFIX	0.0029281
60 TRAP bipolar	9592	IER2	0.041173
60 TRAP bipolar	3337	DNAJB1	0.015198
60 TRAP bipolar	10523	CHERP	0.02622
60 TRAP bipolar	23031	MAST3	0.049066
60 TRAP bipolar	55295	KLHL26	1.32E-04
60 TRAP bipolar	29946	SERTAD3	0.03012
60 TRAP bipolar	478	ATP1A3	0.0099741
60 TRAP bipolar	2931	GSK3A	0.019216
60 TRAP bipolar	4858	NOVA2	0.016403
60 TRAP bipolar	57469	PNMA8B	0.042886
60 TRAP bipolar	57479	PRR12	0.015761
60 TRAP bipolar	126129	CPT1C	0.019876
60 TRAP bipolar	7376	NR1H2	0.035304
60 TRAP bipolar	126119	JOSD2	7.99E-05
60 TRAP bipolar	402665	IGLON5	0.0032877
60 TRAP bipolar	65982	ZSCAN18	0.031994
60 TRAP bipolar	25799	ZNF324	0.0077688
60 TRAP bipolar	58476	TP53INP2	0.0040918
60 TRAP bipolar	26133	TRPC4AP	0.017153
60 TRAP bipolar	140679	SLC32A1	6.29E-05
60 TRAP bipolar	26051	PPP1R16B	2.74E-07
60 TRAP bipolar	7150	TOP1	5.46E-04
60 TRAP bipolar	5335	PLCG1	4.62E-05
60 TRAP bipolar	57580	PREX1	0.0043993
60 TRAP bipolar	1434	CSE1L	0.0053883
60 TRAP bipolar	57169	ZNFX1	0.011758
60 TRAP bipolar	10079	ATP9A	2.04E-04
60 TRAP bipolar	9885	OSBPL2	7.69E-09
60 TRAP bipolar	3785	KCNQ2	4.84E-04
60 TRAP bipolar	80331	DNAJC5	3.35E-04
60 TRAP bipolar	24148	PRPF6	6.88E-04
60 TRAP bipolar	30811	HUNK	0.020577
60 TRAP bipolar	9619	ABCG1	0.0069438
60 TRAP bipolar	1291	COL6A1	0.0067462
60 TRAP bipolar	5594	MAPK1	1.44E-05
60 TRAP bipolar	83999	KREMEN1	0.0013571

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar	4733	DRG1	0.0012827
60 TRAP bipolar	23761	PISD	0.0074878
60 TRAP bipolar	9681	DEPDC5	0.0042411
60 TRAP bipolar	3761	KCNJ4	0.03437
60 TRAP bipolar	25776	CBY1	0.0068222
60 TRAP bipolar	57591	MRTFA	0.0051638
60 TRAP bipolar	150356	CHADL	0.045542
60 TRAP bipolar	5905	RANGAP1	0.018365
120 TRAP bipolar I	149420	PDIK1L	0.011009
120 TRAP bipolar I	8565	YARS1	0.021827
120 TRAP bipolar I	6421	SFPQ	0.0025468
120 TRAP bipolar I	284716	RIMKLA	8.37E-04
120 TRAP bipolar I	3725	JUN	0.043529
120 TRAP bipolar I	55225	RAVER2	0.04203
120 TRAP bipolar I	84251	SGIP1	0.0039109
120 TRAP bipolar I	284613	CYB561D1	0.0015274
120 TRAP bipolar I	57463	AMIGO1	0.04783
120 TRAP bipolar I	51177	PLEKHO1	9.16E-06
120 TRAP bipolar I	23248	RPRD2	2.74E-06
120 TRAP bipolar I	80222	TARS2	1.15E-06
120 TRAP bipolar I	4170	MCL1	0.019931
120 TRAP bipolar I	57459	GATAD2B	0.028719
120 TRAP bipolar I	103	ADAR	0.0081195
120 TRAP bipolar I	23623	RUSC1	0.0085138
120 TRAP bipolar I	55870	ASH1L	0.019758
120 TRAP bipolar I	54856	GON4L	0.039472
120 TRAP bipolar I	23208	SYT11	0.031335
120 TRAP bipolar I	4720	NDUFS2	1.54E-04
120 TRAP bipolar I	25903	OLFML2B	0.018246
120 TRAP bipolar I	5999	RGS4	0.018258
120 TRAP bipolar I	7143	TNR	9.78E-04
120 TRAP bipolar I	23179	RGL1	0.031962
120 TRAP bipolar I	23114	NFASC	0.0041015
120 TRAP bipolar I	6900	CNTN2	0.034103
120 TRAP bipolar I	142	PARP1	0.015146
120 TRAP bipolar I	84886	C1orf198	0.025809
120 TRAP bipolar I	8443	GNPAT	0.048977
120 TRAP bipolar I	6262	RYR2	0.025655
120 TRAP bipolar I	56776	FMN2	0.015727
120 TRAP bipolar I	9859	CEP170	0.0060089
120 TRAP bipolar I	6664	SOX11	0.0062095
120 TRAP bipolar I	57498	KIDINS220	0.0045849
120 TRAP bipolar I	50618	ITSN2	0.021537
120 TRAP bipolar I	6654	SOS1	0.03602
120 TRAP bipolar I	6546	SLC8A1	0.020894
120 TRAP bipolar I	9581	PREPL	0.022909
120 TRAP bipolar I	57142	RTN4	2.50E-05
120 TRAP bipolar I	53335	BCL11A	0.012295
120 TRAP bipolar I	1961	EGR4	0.015861

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar I	55818	KDM3A	0.04598
120 TRAP bipolar I	23020	SNRNP200	0.0052282
120 TRAP bipolar I	26504	CNNM4	0.0015074
120 TRAP bipolar I	23505	TMEM131	3.48E-04
120 TRAP bipolar I	11320	MGAT4A	0.016364
120 TRAP bipolar I	9669	EIF5B	6.68E-04
120 TRAP bipolar I	5455	POU3F3	0.025918
120 TRAP bipolar I	9392	TGFBRAP1	0.0051074
120 TRAP bipolar I	3625	INHBB	0.0019398
120 TRAP bipolar I	53353	LRP1B	0.01918
120 TRAP bipolar I	4929	NR4A2	0.0017941
120 TRAP bipolar I	10716	TBR1	2.93E-04
120 TRAP bipolar I	57282	SLC4A10	6.14E-06
120 TRAP bipolar I	6328	SCN3A	0.015705
120 TRAP bipolar I	6326	SCN2A	0.0038625
120 TRAP bipolar I	6323	SCN1A	0.0076774
120 TRAP bipolar I	4036	LRP2	0.017148
120 TRAP bipolar I	9360	PPIG	0.023283
120 TRAP bipolar I	151230	KLHL23	0.0052398
120 TRAP bipolar I	3676	ITGA4	0.021127
120 TRAP bipolar I	23451	SF3B1	0.0022736
120 TRAP bipolar I	23314	SATB2	0.0054411
120 TRAP bipolar I	205327	C2orf69	1.52E-04
120 TRAP bipolar I	57683	ZDBF2	0.0022563
120 TRAP bipolar I	57574	MARCHF4	0.034091
120 TRAP bipolar I	80309	SPHKAP	0.0043318
120 TRAP bipolar I	26058	GIGYF2	0.0032775
120 TRAP bipolar I	4705	NDUFA10	0.0025524
120 TRAP bipolar I	547	KIF1A	7.55E-04
120 TRAP bipolar I	152330	CNTN4	0.009696
120 TRAP bipolar I	2803	GOLGA4	0.0023025
120 TRAP bipolar I	353274	ZNF445	3.17E-05
120 TRAP bipolar I	29072	SETD2	0.0486
120 TRAP bipolar I	8927	BSN	0.026698
120 TRAP bipolar I	10181	RBM5	0.0015866
120 TRAP bipolar I	2771	GNAI2	0.0081901
120 TRAP bipolar I	11186	RASSF1	0.038241
120 TRAP bipolar I	80335	WDR82	5.09E-04
120 TRAP bipolar I	55193	PBRM1	7.49E-09
120 TRAP bipolar I	200845	KCTD6	0.0050624
120 TRAP bipolar I	166336	PRICKLE2	0.0012086
120 TRAP bipolar I	253559	CADM2	1.91E-07
120 TRAP bipolar I	214	ALCAM	4.57E-04
120 TRAP bipolar I	26137	ZBTB20	0.024205
120 TRAP bipolar I	253461	ZBTB38	0.0024257
120 TRAP bipolar I	9819	TSC22D2	0.025779
120 TRAP bipolar I	9197	SLC33A1	0.0031132
120 TRAP bipolar I	8833	GMPS	0.0080379
120 TRAP bipolar I	1981	EIF4G1	0.022729

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar I	23527	ACAP2	0.031489
120 TRAP bipolar I	23284	ADGRL3	0.0043826
120 TRAP bipolar I	2920	CXCL2	0.041801
120 TRAP bipolar I	9348	NDST3	8.34E-04
120 TRAP bipolar I	84162	KIAA1109	8.35E-05
120 TRAP bipolar I	11275	KLHL2	0.01293
120 TRAP bipolar I	8470	SORBS2	0.0035267
120 TRAP bipolar I	108	ADCY2	1.94E-08
120 TRAP bipolar I	55814	BDP1	0.017537
120 TRAP bipolar I	3156	HMGCR	0.0088183
120 TRAP bipolar I	9456	HOMER1	3.55E-04
120 TRAP bipolar I	116068	LYSMD3	8.02E-07
120 TRAP bipolar I	1958	EGR1	2.94E-06
120 TRAP bipolar I	3313	HSPA9	1.53E-05
120 TRAP bipolar I	54882	ANKHD1	2.06E-06
120 TRAP bipolar I	10307	APBB3	6.08E-04
120 TRAP bipolar I	929	CD14	3.63E-06
120 TRAP bipolar I	56124	PCDHB12	7.03E-04
120 TRAP bipolar I	81848	SPRY4	0.041685
120 TRAP bipolar I	51520	LARS1	0.0015408
120 TRAP bipolar I	10915	TCERG1	0.019962
120 TRAP bipolar I	133522	PPARGC1B	3.83E-04
120 TRAP bipolar I	221692	PHACTR1	0.0035713
120 TRAP bipolar I	9856	KIAA0319	0.011936
120 TRAP bipolar I	8968	H3C7	0.0014237
120 TRAP bipolar I	79692	ZNF322	0.0038151
120 TRAP bipolar I	80317	ZKSCAN3	3.74E-04
120 TRAP bipolar I	23787	MTCH1	0.027474
120 TRAP bipolar I	6722	SRF	0.0016531
120 TRAP bipolar I	24149	ZNF318	1.43E-04
120 TRAP bipolar I	4594	MMUT	0.0049861
120 TRAP bipolar I	23469	PHF3	0.040261
120 TRAP bipolar I	577	ADGRB3	0.0084203
120 TRAP bipolar I	3351	HTR1B	0.047649
120 TRAP bipolar I	55603	TENT5A	0.00546
120 TRAP bipolar I	23036	ZNF292	0.048372
120 TRAP bipolar I	4121	MAN1A1	0.024052
120 TRAP bipolar I	23345	SYNE1	3.73E-06
120 TRAP bipolar I	115330	GPR146	0.042872
120 TRAP bipolar I	8887	TAX1BP1	4.70E-05
120 TRAP bipolar I	154807	VKORC1L1	0.0062497
120 TRAP bipolar I	27445	PCLO	9.79E-05
120 TRAP bipolar I	79027	ZNF655	0.0074057
120 TRAP bipolar I	64599	GIGYF1	0.0055112
120 TRAP bipolar I	4897	NRCAM	0.0030019
120 TRAP bipolar I	4189	DNAJB9	9.46E-04
120 TRAP bipolar I	83992	CTTNBP2	0.0049175
120 TRAP bipolar I	27044	SND1	0.040362
120 TRAP bipolar I	9601	PDIA4	0.010541

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar I	64478	CSMD1	0.019327
120 TRAP bipolar I	8658	TNKS	0.010645
120 TRAP bipolar I	5569	PKIA	0.039553
120 TRAP bipolar I	862	RUNX1T1	9.83E-05
120 TRAP bipolar I	7071	KLF10	0.038975
120 TRAP bipolar I	51059	FAM135B	0.0058566
120 TRAP bipolar I	23237	ARC	0.026527
120 TRAP bipolar I	80173	IFT74	0.031228
120 TRAP bipolar I	65268	WNK2	0.0081901
120 TRAP bipolar I	9568	GABBR2	0.0068302
120 TRAP bipolar I	23245	ASTN2	4.27E-04
120 TRAP bipolar I	774	CACNA1B	7.20E-04
120 TRAP bipolar I	221079	ARL5B	0.036263
120 TRAP bipolar I	57512	GPR158	0.049086
120 TRAP bipolar I	23283	CSTF2T	0.02829
120 TRAP bipolar I	288	ANK3	3.95E-04
120 TRAP bipolar I	84159	ARID5B	2.35E-04
120 TRAP bipolar I	1959	EGR2	0.0016249
120 TRAP bipolar I	6585	SLIT1	0.010139
120 TRAP bipolar I	23223	RRP12	0.0028196
120 TRAP bipolar I	8945	BTRC	0.016864
120 TRAP bipolar I	23082	PPRC1	0.013032
120 TRAP bipolar I	1847	DUSP5	0.016852
120 TRAP bipolar I	57698	SHTN1	0.028221
120 TRAP bipolar I	282974	STK32C	0.036813
120 TRAP bipolar I	9646	CTR9	0.030585
120 TRAP bipolar I	627	BDNF	0.0044951
120 TRAP bipolar I	1132	CHRM4	0.037522
120 TRAP bipolar I	1500	CTNND1	0.048257
120 TRAP bipolar I	1937	EEF1G	0.026729
120 TRAP bipolar I	56834	GPR137	0.029894
120 TRAP bipolar I	10992	SF3B2	9.63E-08
120 TRAP bipolar I	55690	PACS1	1.63E-10
120 TRAP bipolar I	266743	NPAS4	3.75E-05
120 TRAP bipolar I	10432	RBM14	0.0056662
120 TRAP bipolar I	5091	PC	2.71E-07
120 TRAP bipolar I	22941	SHANK2	5.19E-05
120 TRAP bipolar I	2915	GRM5	1.69E-04
120 TRAP bipolar I	120114	FAT3	0.015285
120 TRAP bipolar I	85459	CEP295	2.72E-04
120 TRAP bipolar I	8065	CUL5	0.013028
120 TRAP bipolar I	91893	FDXACB1	0.031395
120 TRAP bipolar I	4684	NCAM1	4.23E-06
120 TRAP bipolar I	399979	SNX19	8.52E-04
120 TRAP bipolar I	25900	IFFO1	0.0028325
120 TRAP bipolar I	8078	USP5	8.70E-05
120 TRAP bipolar I	6515	SLC2A3	0.046405
120 TRAP bipolar I	9052	GPRC5A	0.013929
120 TRAP bipolar I	2904	GRIN2B	0.023365

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar I	3164	NR4A1	0.0068034
120 TRAP bipolar I	4141	MARS1	3.10E-05
120 TRAP bipolar I	8089	YEATS4	0.012374
120 TRAP bipolar I	5053	PAH	0.025994
120 TRAP bipolar I	10985	GCN1	0.011623
120 TRAP bipolar I	121665	SPPL3	0.031473
120 TRAP bipolar I	6249	CLIP1	0.0040997
120 TRAP bipolar I	9612	NCOR2	0.0046976
120 TRAP bipolar I	23141	ANKLE2	0.044127
120 TRAP bipolar I	10808	HSPH1	0.002644
120 TRAP bipolar I	11215	AKAP11	0.0019257
120 TRAP bipolar I	22862	FNDC3A	0.0060437
120 TRAP bipolar I	27253	PCDH17	0.020073
120 TRAP bipolar I	4008	LMO7	0.029519
120 TRAP bipolar I	79596	OBI1	0.0020194
120 TRAP bipolar I	64062	RBM26	6.36E-05
120 TRAP bipolar I	3843	IPO5	0.0036996
120 TRAP bipolar I	7174	TPP2	3.59E-04
120 TRAP bipolar I	57680	CHD8	0.036774
120 TRAP bipolar I	2290	FOXG1	6.93E-05
120 TRAP bipolar I	25831	HECTD1	0.021255
120 TRAP bipolar I	9472	AKAP6	0.0059235
120 TRAP bipolar I	253959	RALGAPA1	0.0095601
120 TRAP bipolar I	23116	TOGARAM1	0.025743
120 TRAP bipolar I	161357	MDGA2	0.0041236
120 TRAP bipolar I	10243	GPHN	0.010699
120 TRAP bipolar I	2353	FOS	0.048112
120 TRAP bipolar I	64919	BCL11B	2.50E-07
120 TRAP bipolar I	3320	HSP90AA1	0.0047068
120 TRAP bipolar I	81693	AMN	0.048618
120 TRAP bipolar I	23359	FAM189A1	2.65E-04
120 TRAP bipolar I	56160	NSMCE3	0.035278
120 TRAP bipolar I	6263	RYR3	0.014838
120 TRAP bipolar I	7057	THBS1	0.031188
120 TRAP bipolar I	23312	DMXL2	0.0040872
120 TRAP bipolar I	54778	RNF111	0.041082
120 TRAP bipolar I	54832	VPS13C	0.0053518
120 TRAP bipolar I	388125	C2CD4B	0.046788
120 TRAP bipolar I	8925	HERC1	0.047896
120 TRAP bipolar I	3658	IREB2	0.0052759
120 TRAP bipolar I	8120	AP3B2	0.0061404
120 TRAP bipolar I	53339	BTBD1	2.89E-04
120 TRAP bipolar I	1106	CHD2	0.024008
120 TRAP bipolar I	57585	CRAMP1	0.0014971
120 TRAP bipolar I	23162	MAPK8IP3	0.0056055
120 TRAP bipolar I	21	ABCA3	0.026774
120 TRAP bipolar I	115	ADCY9	0.046204
120 TRAP bipolar I	29035	C16orf72	0.0082898
120 TRAP bipolar I	2903	GRIN2A	1.90E-05

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar I	123876	ACSM2A	0.029054
120 TRAP bipolar I	11273	ATXN2L	0.045146
120 TRAP bipolar I	26470	SEZ6L2	5.82E-04
120 TRAP bipolar I	9344	TAOK2	5.16E-05
120 TRAP bipolar I	23019	CNOT1	0.0024349
120 TRAP bipolar I	1006	CDH8	2.99E-05
120 TRAP bipolar I	1014	CDH16	0.010992
120 TRAP bipolar I	79567	RIPOR1	3.16E-04
120 TRAP bipolar I	23644	EDC4	3.67E-06
120 TRAP bipolar I	164	AP1G1	0.046208
120 TRAP bipolar I	342371	ATXN1L	0.006694
120 TRAP bipolar I	23199	GSE1	0.020792
120 TRAP bipolar I	10514	MYBBP1A	0.030661
120 TRAP bipolar I	5430	POLR2A	0.039432
120 TRAP bipolar I	482	ATP1B2	0.0042753
120 TRAP bipolar I	5187	PER1	0.01564
120 TRAP bipolar I	124925	SEZ6	0.030931
120 TRAP bipolar I	1362	CPD	3.79E-04
120 TRAP bipolar I	94103	ORMDL3	9.40E-06
120 TRAP bipolar I	1659	DHX8	0.048836
120 TRAP bipolar I	8913	CACNA1G	2.00E-04
120 TRAP bipolar I	22843	PPM1E	0.031748
120 TRAP bipolar I	51136	RNFT1	0.042904
120 TRAP bipolar I	8787	RGS9	0.0021628
120 TRAP bipolar I	2186	BPTF	0.038686
120 TRAP bipolar I	79902	NUP85	0.0031939
120 TRAP bipolar I	23163	GGA3	0.043783
120 TRAP bipolar I	10458	BAIAP2	0.043652
120 TRAP bipolar I	81929	SEH1L	0.0078048
120 TRAP bipolar I	6860	SYT4	0.001922
120 TRAP bipolar I	90701	SEC11C	9.12E-04
120 TRAP bipolar I	57614	RELCH	0.022371
120 TRAP bipolar I	8570	KHSRP	0.041737
120 TRAP bipolar I	3726	JUNB	0.017339
120 TRAP bipolar I	9592	IER2	0.032754
120 TRAP bipolar I	3337	DNAJB1	0.029189
120 TRAP bipolar I	10331	B3GNT3	0.0023651
120 TRAP bipolar I	23373	CRTC1	0.011195
120 TRAP bipolar I	5976	UPF1	0.037678
120 TRAP bipolar I	1463	NCAN	1.30E-05
120 TRAP bipolar I	57130	ATP13A1	9.47E-06
120 TRAP bipolar I	147991	DPY19L3	0.047237
120 TRAP bipolar I	478	ATP1A3	0.018048
120 TRAP bipolar I	10155	TRIM28	0.008327
120 TRAP bipolar I	23767	FLRT3	0.03678
120 TRAP bipolar I	83737	ITCH	0.024875
120 TRAP bipolar I	10137	RBM12	0.0026155
120 TRAP bipolar I	7150	TOP1	0.0025456
120 TRAP bipolar I	23051	ZHX3	8.32E-04

gene_set	entrez_ID	Symbol	P-value
120 TRAP bipolar I	1434	CSE1L	3.90E-05
120 TRAP bipolar I	6874	TAF4	0.0015829
120 TRAP bipolar I	24148	PRPF6	0.017042
120 TRAP bipolar I	8888	MCM3AP	0.028113
120 TRAP bipolar I	57553	MICAL3	0.012156
120 TRAP bipolar I	162	AP1B1	0.037166
120 TRAP bipolar I	57591	MRTFA	2.06E-04
60 TRAP bipolar I	728642	CDK11A	0.0065927
60 TRAP bipolar I	5909	RAP1GAP	0.0057519
60 TRAP bipolar I	149420	PDIK1L	0.011009
60 TRAP bipolar I	6421	SFPQ	0.0025468
60 TRAP bipolar I	54802	TRIT1	4.90E-05
60 TRAP bipolar I	284716	RIMKLA	8.37E-04
60 TRAP bipolar I	79654	HECTD3	0.011172
60 TRAP bipolar I	10489	LRRC41	0.021784
60 TRAP bipolar I	2166	FAAH	3.75E-04
60 TRAP bipolar I	260293	CYP4X1	0.043398
60 TRAP bipolar I	3725	JUN	0.043529
60 TRAP bipolar I	84251	SGIP1	0.0039109
60 TRAP bipolar I	26135	SERBP1	0.049443
60 TRAP bipolar I	26289	AK5	0.003995
60 TRAP bipolar I	260425	MAGI3	0.046766
60 TRAP bipolar I	333932	H3C15	0.013076
60 TRAP bipolar I	51177	PLEKHO1	9.16E-06
60 TRAP bipolar I	9129	PRPF3	1.82E-04
60 TRAP bipolar I	4170	MCL1	0.019931
60 TRAP bipolar I	10500	SEMA6C	0.02919
60 TRAP bipolar I	10899	JTB	0.042966
60 TRAP bipolar I	4580	MTX1	0.045322
60 TRAP bipolar I	23623	RUSC1	0.0085138
60 TRAP bipolar I	477	ATP1A2	6.93E-04
60 TRAP bipolar I	93185	IGSF8	0.0013775
60 TRAP bipolar I	4720	NDUFS2	1.54E-04
60 TRAP bipolar I	25903	OLFML2B	0.018246
60 TRAP bipolar I	5999	RGS4	0.018258
60 TRAP bipolar I	7143	TNR	9.78E-04
60 TRAP bipolar I	8707	B3GALT2	0.042745
60 TRAP bipolar I	163486	DENND1B	0.0065252
60 TRAP bipolar I	2848	GPR25	0.02143
60 TRAP bipolar I	55705	IPO9	0.0011954
60 TRAP bipolar I	6900	CNTN2	0.034103
60 TRAP bipolar I	25778	DSTYK	0.049631
60 TRAP bipolar I	55699	IARS2	0.0089172
60 TRAP bipolar I	142	PARP1	0.015146
60 TRAP bipolar I	56776	FMN2	0.015727
60 TRAP bipolar I	9859	CEP170	0.0060089
60 TRAP bipolar I	6664	SOX11	0.0062095
60 TRAP bipolar I	3777	KCNK3	0.020001
60 TRAP bipolar I	64838	FNDC4	0.010993

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60 TRAP bipolar I	8491	MAP4K3	0.0090184
60 TRAP bipolar I	6546	SLC8A1	0.020894
60 TRAP bipolar I	57504	MTA3	0.0088023
60 TRAP bipolar I	9655	SOCS5	0.0055208
60 TRAP bipolar I	9378	NRXN1	0.028508
60 TRAP bipolar I	57142	RTN4	2.50E-05
60 TRAP bipolar I	53335	BCL11A	0.012295
60 TRAP bipolar I	5534	PPP3R1	0.0025844
60 TRAP bipolar I	23233	EXOC6B	4.93E-04
60 TRAP bipolar I	1961	EGR4	0.015861
60 TRAP bipolar I	1844	DUSP2	1.99E-05
60 TRAP bipolar I	56910	STARD7	4.92E-06
60 TRAP bipolar I	10865	ARID5A	0.0029961
60 TRAP bipolar I	23505	TMEM131	3.48E-04
60 TRAP bipolar I	3631	INPP4A	0.0030405
60 TRAP bipolar I	11320	MGAT4A	0.016364
60 TRAP bipolar I	9669	EIF5B	6.68E-04
60 TRAP bipolar I	9392	TGFBRAP1	0.0051074
60 TRAP bipolar I	79074	C2orf49	0.012515
60 TRAP bipolar I	10096	ACTR3	0.030431
60 TRAP bipolar I	54520	CCDC93	0.0047058
60 TRAP bipolar I	4929	NR4A2	0.0017941
60 TRAP bipolar I	10716	TBR1	2.93E-04
60 TRAP bipolar I	57282	SLC4A10	6.14E-06
60 TRAP bipolar I	9360	PPIG	0.023283
60 TRAP bipolar I	151230	KLHL23	0.0052398
60 TRAP bipolar I	3676	ITGA4	0.021127
60 TRAP bipolar I	2487	FRZB	0.013635
60 TRAP bipolar I	10787	NCKAP1	0.040995
60 TRAP bipolar I	23451	SF3B1	0.0022736
60 TRAP bipolar I	23314	SATB2	0.0054411
60 TRAP bipolar I	205327	C2orf69	1.52E-04
60 TRAP bipolar I	57683	ZDBF2	0.0022563
60 TRAP bipolar I	471	ATIC	0.0021705
60 TRAP bipolar I	57574	MARCHF4	0.034091
60 TRAP bipolar I	26058	GIGYF2	0.0032775
60 TRAP bipolar I	547	KIF1A	7.55E-04
60 TRAP bipolar I	9881	TRANK1	4.97E-12
60 TRAP bipolar I	9852	EPM2AIP1	2.90E-04
60 TRAP bipolar I	2803	GOLGA4	0.0023025
60 TRAP bipolar I	29072	SETD2	0.0486
60 TRAP bipolar I	8927	BSN	0.026698
60 TRAP bipolar I	79012	CAMKV	0.0085243
60 TRAP bipolar I	10181	RBM5	0.0015866
60 TRAP bipolar I	80335	WDR82	5.09E-04
60 TRAP bipolar I	55193	PBRM1	7.49E-09
60 TRAP bipolar I	5580	PRKCD	1.36E-04
60 TRAP bipolar I	54899	PXK	0.014515
60 TRAP bipolar I	166336	PRICKLE2	0.0012086

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60 TRAP bipolar I	214	ALCAM	4.57E-04
60 TRAP bipolar I	11343	MGLL	2.95E-04
60 TRAP bipolar I	23007	PLCH1	3.34E-05
60 TRAP bipolar I	6498	SKIL	0.029116
60 TRAP bipolar I	1981	EIF4G1	0.022729
60 TRAP bipolar I	1487	CTBP1	2.20E-05
60 TRAP bipolar I	6002	RGS12	0.037664
60 TRAP bipolar I	116984	ARAP2	0.0010942
60 TRAP bipolar I	2557	GABRA4	0.0020649
60 TRAP bipolar I	285527	FRYL	0.0021536
60 TRAP bipolar I	132299	OCIAD2	0.0028941
60 TRAP bipolar I	3815	KIT	0.0023691
60 TRAP bipolar I	56884	FSTL5	0.015371
60 TRAP bipolar I	11275	KLHL2	0.01293
60 TRAP bipolar I	8470	SORBS2	0.0035267
60 TRAP bipolar I	54888	NSUN2	1.18E-04
60 TRAP bipolar I	11044	TENT4A	0.039923
60 TRAP bipolar I	108	ADCY2	1.94E-08
60 TRAP bipolar I	10409	BASP1	6.80E-04
60 TRAP bipolar I	10884	MRPS30	3.48E-04
60 TRAP bipolar I	5295	PIK3R1	0.025705
60 TRAP bipolar I	55814	BDP1	0.017537
60 TRAP bipolar I	3156	HMGCR	0.0088183
60 TRAP bipolar I	9456	HOMER1	3.55E-04
60 TRAP bipolar I	10085	EDIL3	0.014645
60 TRAP bipolar I	27089	UQCRQ	3.62E-05
60 TRAP bipolar I	9879	DDX46	0.016679
60 TRAP bipolar I	1958	EGR1	2.94E-06
60 TRAP bipolar I	3313	HSPA9	1.53E-05
60 TRAP bipolar I	54882	ANKHD1	2.06E-06
60 TRAP bipolar I	3550	IK	3.56E-06
60 TRAP bipolar I	10915	TCERG1	0.019962
60 TRAP bipolar I	10667	FARS2	0.033432
60 TRAP bipolar I	221692	PHACTR1	0.0035713
60 TRAP bipolar I	9856	KIAA0319	0.011936
60 TRAP bipolar I	81688	C6orf62	0.0079038
60 TRAP bipolar I	8359	H4C1	0.0024583
60 TRAP bipolar I	3006	H1-2	0.026582
60 TRAP bipolar I	8365	H4C8	2.17E-05
60 TRAP bipolar I	79692	ZNF322	0.0038151
60 TRAP bipolar I	8294	H4C9	9.94E-05
60 TRAP bipolar I	79897	RPP21	1.36E-04
60 TRAP bipolar I	221545	C6orf136	0.025159
60 TRAP bipolar I	57176	VARS2	8.87E-05
60 TRAP bipolar I	80736	SLC44A4	0.0010504
60 TRAP bipolar I	6048	RNF5	5.30E-05
60 TRAP bipolar I	5089	PBX2	1.11E-04
60 TRAP bipolar I	51596	CUTA	0.0018706
60 TRAP bipolar I	23787	MTCH1	0.027474

gene_set	entrez_ID	Symbol	P-value
60 TRAP bipolar I	25844	YIPF3	0.01992
60 TRAP bipolar I	2729	GCLC	0.0032319
60 TRAP bipolar I	23469	PHF3	0.040261
60 TRAP bipolar I	22999	RIMS1	7.71E-06
60 TRAP bipolar I	9892	SNAP91	6.29E-06
60 TRAP bipolar I	23036	ZNF292	0.048372
60 TRAP bipolar I	114792	KLHL32	0.0033419
60 TRAP bipolar I	26235	FBXL4	0.023269
60 TRAP bipolar I	135112	NCOA7	0.033025
60 TRAP bipolar I	134957	STXBP5	0.040804
60 TRAP bipolar I	23345	SYNE1	3.73E-06
60 TRAP bipolar I	6950	TCP1	0.014591
60 TRAP bipolar I	115330	GPR146	0.042872
60 TRAP bipolar I	9265	CYTH3	0.00824
60 TRAP bipolar I	221955	DAGLB	0.049411
60 TRAP bipolar I	54664	TMEM106B	0.010977
60 TRAP bipolar I	6671	SP4	1.66E-07
60 TRAP bipolar I	816	CAMK2B	0.0052948
60 TRAP bipolar I	23242	COBL	0.022369
60 TRAP bipolar I	7532	YWHAG	0.0055774
60 TRAP bipolar I	27445	PCLO	9.79E-05
60 TRAP bipolar I	154661	RUNDC3B	7.18E-05
60 TRAP bipolar I	4897	NRCAM	0.0030019
60 TRAP bipolar I	7982	ST7	0.02274
60 TRAP bipolar I	83992	CTTNBP2	0.0049175
60 TRAP bipolar I	51530	ZC3HC1	5.40E-04
60 TRAP bipolar I	23008	KLHDC10	6.95E-05
60 TRAP bipolar I	9601	PDIA4	0.010541
60 TRAP bipolar I	9690	UBE3C	0.044285
60 TRAP bipolar I	26260	FBXO25	8.23E-04
60 TRAP bipolar I	9258	MFHAS1	0.0028338
60 TRAP bipolar I	66036	MTMR9	0.0052966
60 TRAP bipolar I	55140	ELP3	0.016003
60 TRAP bipolar I	9530	BAG4	0.001243
60 TRAP bipolar I	115294	PCMTD1	0.021116
60 TRAP bipolar I	254778	VXN	0.010105
60 TRAP bipolar I	54332	GDAP1	0.0096385
60 TRAP bipolar I	5569	PKIA	0.039553
60 TRAP bipolar I	862	RUNX1T1	9.83E-05
60 TRAP bipolar I	7071	KLF10	0.038975
60 TRAP bipolar I	23237	ARC	0.026527
60 TRAP bipolar I	4781	NFIB	0.043704
60 TRAP bipolar I	9373	PLAA	0.022495
60 TRAP bipolar I	80173	IFT74	0.031228
60 TRAP bipolar I	54926	UBE2R2	0.0252
60 TRAP bipolar I	65268	WNK2	0.0081901
60 TRAP bipolar I	23196	FAM120A	0.005374
60 TRAP bipolar I	5253	PHF2	0.0069159
60 TRAP bipolar I	9568	GABBR2	0.0068302

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60 TRAP bipolar I	9355	LHX2	0.044639
60 TRAP bipolar I	6136	RPL12	0.017348
60 TRAP bipolar I	8818	DPM2	0.0051964
60 TRAP bipolar I	83549	UCK1	0.037057
60 TRAP bipolar I	221061	FAM171A1	0.0013609
60 TRAP bipolar I	23412	COMMD3	0.038706
60 TRAP bipolar I	23283	CSTF2T	0.02829
60 TRAP bipolar I	220965	FAM13C	0.0058277
60 TRAP bipolar I	288	ANK3	3.95E-04
60 TRAP bipolar I	84890	ADO	0.0055998
60 TRAP bipolar I	1959	EGR2	0.0016249
60 TRAP bipolar I	55749	CCAR1	0.0024333
60 TRAP bipolar I	657	BMPR1A	0.012344
60 TRAP bipolar I	8945	BTRC	0.016864
60 TRAP bipolar I	30819	KCNIP2	0.0078626
60 TRAP bipolar I	8861	LDB1	0.012893
60 TRAP bipolar I	81603	TRIM8	2.14E-04
60 TRAP bipolar I	1847	DUSP5	0.016852
60 TRAP bipolar I	57678	GPAM	0.0060412
60 TRAP bipolar I	64429	ZDHHC6	0.018158
60 TRAP bipolar I	57700	FHIP2A	0.025698
60 TRAP bipolar I	118987	PDZD8	0.011355
60 TRAP bipolar I	84435	ADGRA1	0.0025662
60 TRAP bipolar I	406	ARNTL	2.13E-05
60 TRAP bipolar I	8534	CHST1	2.19E-05
60 TRAP bipolar I	55709	KBTBD4	0.013431
60 TRAP bipolar I	23788	MTCH2	0.034157
60 TRAP bipolar I	747	DAGLA	9.12E-06
60 TRAP bipolar I	10963	STIP1	0.038003
60 TRAP bipolar I	56834	GPR137	0.029894
60 TRAP bipolar I	9379	NRXN2	4.98E-04
60 TRAP bipolar I	10992	SF3B2	9.63E-08
60 TRAP bipolar I	266743	NPAS4	3.75E-05
60 TRAP bipolar I	78999	LRFN4	7.00E-08
60 TRAP bipolar I	22941	SHANK2	5.19E-05
60 TRAP bipolar I	5138	PDE2A	0.043626
60 TRAP bipolar I	408	ARRB1	0.03371
60 TRAP bipolar I	7405	UVRAG	0.025477
60 TRAP bipolar I	283219	KCTD21	0.028535
60 TRAP bipolar I	60496	AASDHPPT	0.032153
60 TRAP bipolar I	8065	CUL5	0.013028
60 TRAP bipolar I	4684	NCAM1	4.23E-06
60 TRAP bipolar I	51092	SIDT2	0.027471
60 TRAP bipolar I	9099	USP2	0.040519
60 TRAP bipolar I	4978	OPCML	0.017381
60 TRAP bipolar I	2597	GAPDH	4.16E-04
60 TRAP bipolar I	1822	ATN1	0.003699
60 TRAP bipolar I	1389	CREBL2	0.025372
60 TRAP bipolar I	6660	SOX5	0.046175

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60 TRAP bipolar I	83857	TMTC1	0.0015096
60 TRAP bipolar I	23109	DDN	3.57E-05
60 TRAP bipolar I	5571	PRKAG1	3.55E-05
60 TRAP bipolar I	91	ACVR1B	0.0012632
60 TRAP bipolar I	3164	NR4A1	0.0068034
60 TRAP bipolar I	3798	KIF5A	4.80E-04
60 TRAP bipolar I	4922	NTS	0.020619
60 TRAP bipolar I	51559	NT5DC3	0.046221
60 TRAP bipolar I	5781	PTPN11	0.0054945
60 TRAP bipolar I	23389	MED13L	0.041891
60 TRAP bipolar I	55884	WSB2	0.0344
60 TRAP bipolar I	1337	COX6A1	8.42E-04
60 TRAP bipolar I	51367	POP5	0.012517
60 TRAP bipolar I	121665	SPPL3	0.031473
60 TRAP bipolar I	65082	VPS33A	0.005243
60 TRAP bipolar I	6249	CLIP1	0.0040997
60 TRAP bipolar I	57605	PITPNM2	6.29E-04
60 TRAP bipolar I	10959	TMED2	0.0033372
60 TRAP bipolar I	80212	CCDC92	0.014045
60 TRAP bipolar I	144348	ZNF664	0.012634
60 TRAP bipolar I	9612	NCOR2	0.0046976
60 TRAP bipolar I	23141	ANKLE2	0.044127
60 TRAP bipolar I	55504	TNFRSF19	0.041537
60 TRAP bipolar I	10208	USPL1	0.0096483
60 TRAP bipolar I	10808	HSPH1	0.002644
60 TRAP bipolar I	22862	FNDC3A	0.0060437
60 TRAP bipolar I	27253	PCDH17	0.020073
60 TRAP bipolar I	64062	RBM26	6.36E-05
60 TRAP bipolar I	2259	FGF14	0.044317
60 TRAP bipolar I	7174	TPP2	3.59E-04
60 TRAP bipolar I	8451	CUL4A	2.16E-06
60 TRAP bipolar I	57680	CHD8	0.036774
60 TRAP bipolar I	2290	FOXG1	6.93E-05
60 TRAP bipolar I	9472	AKAP6	0.0059235
60 TRAP bipolar I	10484	SEC23A	0.013878
60 TRAP bipolar I	23116	TOGARAM1	0.025743
60 TRAP bipolar I	161357	MDGA2	0.0041236
60 TRAP bipolar I	51062	ATL1	0.015489
60 TRAP bipolar I	55860	ACTR10	0.027088
60 TRAP bipolar I	83851	SYT16	8.76E-08
60 TRAP bipolar I	10243	GPHN	0.010699
60 TRAP bipolar I	2353	FOS	0.048112
60 TRAP bipolar I	1735	DIO3	4.08E-04
60 TRAP bipolar I	3320	HSP90AA1	0.0047068
60 TRAP bipolar I	55778	ZNF839	4.49E-04
60 TRAP bipolar I	90135	BTBD6	0.029549
60 TRAP bipolar I	114791	TUBGCP5	0.042969
60 TRAP bipolar I	2562	GABRB3	0.012035
60 TRAP bipolar I	23359	FAM189A1	2.65E-04

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60 TRAP bipolar I	27079	RPUSD2	0.026078
60 TRAP bipolar I	3706	ITPKA	0.020674
60 TRAP bipolar I	23339	VPS39	4.13E-04
60 TRAP bipolar I	54822	TRPM7	0.038179
60 TRAP bipolar I	79811	SLTM	1.64E-05
60 TRAP bipolar I	54778	RNF111	0.041082
60 TRAP bipolar I	388125	C2CD4B	0.046788
60 TRAP bipolar I	23060	ZNF609	0.028977
60 TRAP bipolar I	4088	SMAD3	0.035731
60 TRAP bipolar I	1445	CSK	0.001147
60 TRAP bipolar I	8120	AP3B2	0.0061404
60 TRAP bipolar I	9455	HOMER2	0.0016688
60 TRAP bipolar I	53339	BTBD1	2.89E-04
60 TRAP bipolar I	4122	MAN2A2	0.017333
60 TRAP bipolar I	1106	CHD2	0.024008
60 TRAP bipolar I	23162	MAPK8IP3	0.0056055
60 TRAP bipolar I	7249	TSC2	0.01131
60 TRAP bipolar I	5310	PKD1	0.010082
60 TRAP bipolar I	23274	CLEC16A	0.04306
60 TRAP bipolar I	124454	EARS2	0.037611
60 TRAP bipolar I	5579	PRKCB	1.59E-06
60 TRAP bipolar I	11273	ATXN2L	0.045146
60 TRAP bipolar I	26470	SEZ6L2	5.82E-04
60 TRAP bipolar I	8479	HIRIP3	6.90E-04
60 TRAP bipolar I	83723	TLCD3B	0.013131
60 TRAP bipolar I	226	ALDOA	0.016999
60 TRAP bipolar I	23019	CNOT1	0.0024349
60 TRAP bipolar I	8883	NAE1	0.011612
60 TRAP bipolar I	9114	ATP6V0D1	1.57E-04
60 TRAP bipolar I	146206	CARMIL2	8.93E-05
60 TRAP bipolar I	65057	ACD	5.34E-06
60 TRAP bipolar I	55512	SMPD3	0.0058176
60 TRAP bipolar I	84916	UTP4	7.29E-05
60 TRAP bipolar I	29123	ANKRD11	0.0060827
60 TRAP bipolar I	8558	CDK10	2.72E-04
60 TRAP bipolar I	22980	TCF25	9.56E-04
60 TRAP bipolar I	7531	YWHAE	1.56E-04
60 TRAP bipolar I	1398	CRK	0.0074418
60 TRAP bipolar I	9905	SGSM2	0.016543
60 TRAP bipolar I	84254	CAMKK1	0.0043218
60 TRAP bipolar I	56919	DHX33	0.0011297
60 TRAP bipolar I	83394	PITPNM3	0.022332
60 TRAP bipolar I	440400	RNASEK	0.013739
60 TRAP bipolar I	2256	FGF11	0.0038877
60 TRAP bipolar I	26168	SENP3	0.0014802
60 TRAP bipolar I	482	ATP1B2	0.0042753
60 TRAP bipolar I	51701	NLK	9.33E-04
60 TRAP bipolar I	124925	SEZ6	0.030931
60 TRAP bipolar I	399687	MYO18A	0.0014442

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60 TRAP bipolar I	28964	GIT1	0.0031588
60 TRAP bipolar I	94103	ORMDL3	9.40E-06
60 TRAP bipolar I	5709	PSMD3	3.90E-06
60 TRAP bipolar I	1659	DHX8	0.048836
60 TRAP bipolar I	4836	NMT1	0.0047047
60 TRAP bipolar I	113026	PLCD3	0.0029604
60 TRAP bipolar I	4137	MAPT	0.0067298
60 TRAP bipolar I	4905	NSF	0.0038268
60 TRAP bipolar I	8913	CACNA1G	2.00E-04
60 TRAP bipolar I	51747	LUC7L3	0.0028887
60 TRAP bipolar I	3131	HLF	4.26E-05
60 TRAP bipolar I	4591	TRIM37	0.024754
60 TRAP bipolar I	9772	TMEM94	0.046983
60 TRAP bipolar I	10458	BAIAP2	0.043652
60 TRAP bipolar I	55666	NPLOC4	0.0026556
60 TRAP bipolar I	9146	HGS	0.029179
60 TRAP bipolar I	10939	AFG3L2	0.035222
60 TRAP bipolar I	81929	SEH1L	0.0078048
60 TRAP bipolar I	7572	ZNF24	0.020507
60 TRAP bipolar I	6860	SYT4	0.001922
60 TRAP bipolar I	57614	RELCH	0.022371
60 TRAP bipolar I	80148	SLC66A2	0.021283
60 TRAP bipolar I	148252	DIRAS1	2.05E-05
60 TRAP bipolar I	29985	SLC39A3	1.79E-04
60 TRAP bipolar I	170961	ANKRD24	0.035106
60 TRAP bipolar I	84717	HDGFL2	0.030414
60 TRAP bipolar I	8570	KHSRP	0.041737
60 TRAP bipolar I	79085	SLC25A23	0.029977
60 TRAP bipolar I	6597	SMARCA4	0.0014211
60 TRAP bipolar I	3726	JUNB	0.017339
60 TRAP bipolar I	4784	NFIX	8.74E-04
60 TRAP bipolar I	9592	IER2	0.032754
60 TRAP bipolar I	3337	DNAJB1	0.029189
60 TRAP bipolar I	55295	KLHL26	0.0034958
60 TRAP bipolar I	478	ATP1A3	0.018048
60 TRAP bipolar I	2931	GSK3A	0.034105
60 TRAP bipolar I	57479	PRR12	0.0060224
60 TRAP bipolar I	7376	NR1H2	0.0038423
60 TRAP bipolar I	126119	JOSD2	8.10E-05
60 TRAP bipolar I	402665	IGLON5	0.0010692
60 TRAP bipolar I	25799	ZNF324	0.033076
60 TRAP bipolar I	140885	SIRPA	0.041159
60 TRAP bipolar I	23767	FLRT3	0.03678
60 TRAP bipolar I	58476	TP53INP2	0.039291
60 TRAP bipolar I	26133	TRPC4AP	0.0013233
60 TRAP bipolar I	140679	SLC32A1	4.19E-04
60 TRAP bipolar I	26051	PPP1R16B	1.76E-05
60 TRAP bipolar I	7150	TOP1	0.0025456
60 TRAP bipolar I	5335	PLCG1	2.73E-04

Gene set	EntrezIDs	Symbol	P-value
60 TRAP bipolar I	57580	PREX1	8.77E-04
60 TRAP bipolar I	1434	CSE1L	3.90E-05
60 TRAP bipolar I	57169	ZNFX1	0.0049279
60 TRAP bipolar I	10079	ATP9A	2.36E-04
60 TRAP bipolar I	9885	OSBPL2	4.51E-08
60 TRAP bipolar I	3785	KCNQ2	0.012457
60 TRAP bipolar I	80331	DNAJC5	0.0038973
60 TRAP bipolar I	24148	PRPF6	0.017042
60 TRAP bipolar I	9619	ABCG1	0.0026166
60 TRAP bipolar I	23308	ICOSLG	0.044829
60 TRAP bipolar I	5594	MAPK1	5.10E-04
60 TRAP bipolar I	83999	KREMEN1	0.0071214
60 TRAP bipolar I	4733	DRG1	0.0043114
60 TRAP bipolar I	23761	PISD	0.0021282
60 TRAP bipolar I	9681	DEPDC5	0.0093728
60 TRAP bipolar I	3761	KCNJ4	0.018371
60 TRAP bipolar I	57591	MRTFA	2.06E-04