The Role of Schizophrenia Susceptibility Genes in Hippocampal-Dependent Long-Term Memory in the Adult Rat

A dissertation submitted for the degree of

Doctor of Philosophy

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Abstract

This thesis investigates whether genes that have been associated with schizophrenia have a role in hippocampal-dependent long-term memory. Schizophrenia is psychiatric disorder in which individuals experience, amongst other symptoms, cognitive difficulties including long-term memory (LTM) impairments. The functional roles of many schizophrenia susceptibility genes remain unknown. In this study, a selection of schizophrenia susceptibility genes and their splice variants, in particular *Neuregulin 1* (*Nrg1*), *Dysbindin 1* (*Dtnbp1*), *Disrupted-in-schizophrenia 1* (*Disc1*) and *Early growth response factor 3* (*Egr3*), were hypothesized to be regulated in association with the processes of LTM. The contextual fear conditioning behavioural paradigm in which a rat associates an electric footshock with a distinct context was used to investigate hippocampal-dependent LTM.

Firstly the exonic structure of all known splice variants of *Nrg1*, *Dtnbp1* and *Disc1* were determined using NCBI SPIDEY mRNA to genomic alignment software and oligonucleotide probes were designed to detect *Nrg1* type I, II, III and IV splice variants, *Disc1* I and *Disc1* Lv splice variants, and *Dtnbp1* exons 1, 5, 8 and 9 in an attempt to identify *Dtnbp1* splice variants. Pan probes were also designed to detect all splice variants of *Nrg1*, *Dtnbp1* and *Disc1*, and the only identified transcripts of *Egr3*, and its repressors *Nab1* and *Nab2*. Whole brain expression patterns were characterized by *in situ* hybridization for all probes except for the Nrg1 type IV probe and all of the Disc1 probes as no specific labeling could be observed despite trying two or three different probes and probing in amphetamine-activated brain tissue.

The level of expression of the genes and splice variants to which specific labeling had been achieved, were assayed in regions of the adult rat brain known to be involved LTM processes, including the hippocampus, amygdala and medial prefrontal cortex, during the consolidation of contextual fear memory (CFM). *Egr3* expression in the CA1 region of the hippocampus and in the dorsolateral nucleus of the amygdala, and *Nrg1* type I expression in the CA3 region of the hippocampus was upregulated in association with the consolidation of CFM. Infusion of antisense into the dorsal hippocampus to knockdown EGR3 expression during the consolidation of CFM was performed and it was determined that EGR3 expression in the dorsal hippocampus was not necessary for the consolidation of CFM. Further investigation of the expression levels of *Egr3* and *Nab2* as well as *Egr1* during reconsolidation and extinction of CFM in the same brain regions, identified that *Egr3*, *Egr1* and *Nab2* expression was

upregulated in the hippocampus, *Egr3* and *Egr1* expression were upregulated in the amygdala, and *Egr1* was upregulated in the medial prefrontal cortex in association with reconsolidation and extinction of CFM. Finally, infusion of antisense into the dorsal hippocampus to knockdown EGR3 expression during the reconsolidation of CFM was performed and it was determined that EGR3 expression in this region was not necessary for the reconsolidation of CFM.

It was concluded that some of the schizophrenia susceptibility genes were regulated in association with hippocampal-dependent LTM processes but no schizophrenia susceptibility genes were identified to be causally involved in hippocampal-dependent LTM processes. It was discussed that considering the large number of schizophrenia susceptibility genes and range of cognitive impairments in schizophrenia, this approach of investigating the functional roles of schizophrenia susceptibility genes would benefit from many more schizophrenia susceptibility genes in a range of behavioural paradigms, analogous to the range of schizophrenia endophenotypes, would expedite the rate at which functional roles for these genes would be discovered.

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ABBREVIATIONS

Anatomical

BLA	Basolateral nucleus
CA1	Cornu Ammons 1
CA3	Cornu Ammons 3
CeN	Central nucleus
Cg	Cingulate 1 cortex
DG	Dentate Gyrus
DLA	Dorsolateral nucleus
IL	Infralimbic cortex
LA	Lateral nucleus
mPFC	medial prefrontal cortex
PFC	prefrontal cortex
PrL	Prelimbic cortex

Behavioural and Methods

CFC	Contextual Fear Conditioning
CR	Conditioned Response
CS	Conditioned Stimulus
MECS	Maximal Electroconvulsive Shock
LI	Latent Inhibition
LTD	Long-Term Depression
LTM	Long-Term Memory
LTP	Long-Term Potentiation
STM	Short Term Memory
US	Unconditioned Stimulus

Chemical

ddH2O	Double distilled water
DTT	DL-Dithiothreitol
PB	Phosphate Buffer
PBS	Phosphate Buffer Saline
PFA	Paraformaldehyde
SDS	Sodium Dodecyl Sulphate
SSC	Saline Sodium Citrate

Biochemical

ASO	Antisense Oligodeoxynucleotide
DNA	Deoxyribonucleic Acid
IEG	Immediate Early Gene
MSO	Missense Oligodeoxynucleotide
mRNA	messenger Ribonucleic Acid
ODN	Oligodeoxynucleotide

Molecular

BDNF	Brain Derived Neurotrophic Factor
CaMK	Calcium-Calmodulin Dependent Kinase
cAMP	Cyclic Adenosine Monophosphate
CRE	cAMP response-element
CREB	CRE binding protein
DISC1	Disrupted-in-schizophrenia1
DTNBP1	Dysbindin1

EGR	Early Growth Response factor
EGR3	Early Growth Response Factor 3
ERBBR	Tyrosine kinase-type cell surface receptor
GABA	γ-aminobutyric acid
IEG	Immediate Early Gene
MAPK	Mitogen-associated protein kinase
NMDA	N-methyl-D-aspartate
NMDAR	NMDA receptor
NRG1	Neuregulin1
PKA	cAMP-dependent protein kinase

Statistical

ANOVA	Analysis of variance
F	Variance ration of the ANOVA
p	Probability level of the sample statistic
SE	Standard error
CV	Coefficient of Variation

Measurements

g	Gram
h	Hour
kg	Kilogram
M	Molar
mg	Milligram
min	Minutes
ml	Millilitre
mm	Millimetre

mM	Millimolar
nCi	Nano-curies
nmol	Nanomole
s	Second
µg	Microgram
µl	Microlitre
µm	Micrometre

NOTE: The above represent the most commonly used abbreviations. Infrequently used abbreviations are defined in the text.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Schizophrenia

Schizophrenia affects approximately one in every 150 individuals worldwide (Flint et al., 2010). Schizophrenia is a heterogeneous syndrome that has been characterised by three main groups of symptoms known as positive, negative and cognitive symptoms (Barch, 2005). Positive symptoms include delusions (defined as fixed, unshakeable false beliefs not shared by people from the same cultural background) and hallucinations (defined as involuntary false perceptions occurring concurrently with real perceptions and having qualities of real perceptions) (Chiu, 1989). Most hallucinations are auditory perceptions in the absence of stimuli, but in approximately one sixth of cases visual perceptions in the absence of stimuli are experienced. Negative symptoms include emotional and social withdrawal and lack of motivation. Cognitive symptoms are wide ranging and include impairments in learning and memory, central executive function and attention (Barch, 2005). Recent studies suggest that the cognitive deficits are present early in the course of schizophrenia and are comparable to those detected in the chronic phase of the disorder (Sponheim et al., 2009; Reichenberg et al., 2010). Schizophrenic patients differ in the combination of these symptoms from which they suffer. The severity of some of the cognitive symptoms have been associated with the severity of disorganised thought (a positive symptom) and negative symptoms (Aleman et al., 1999; Barch, 2005). Cognitive impairment in young individuals is thought to be a marker of biological vulnerability to schizophrenia (Jones et al., 1994; Cornblatt et al., 1999; Niendam et al., 2003; Delawalla et al., 2006).

Schizophrenia lacks any consistent physiological identifiable marker, therefore it is diagnosed in the process of consultation through identification of the clinical symptoms described in the Diagnostic and Statistical Manual of Mental Disorders (DSM) of which the most recent version

is DSM-IV. An updated DSM-V is being developed at present. Currently there is discussion over the usefulness of the term schizophrenia covering such a varied combination of symptoms. In order to ease treatment of different symptoms and to help research into the different phenotypes, the possibility of new nosology has been suggested (Owen et al., 2007). The onset of schizophrenia seems to occur in two stages. Behavioural changes, including some cognitive and negative symptoms, can be present in childhood, while the positive symptoms are rarely observed before puberty. This is in agreement with schizophrenia being a neurodevelopmental disorder (Weinberger, 1987; Murray & Lewis 1987).

While no biomarkers for schizophrenia have been identified, there have been neuropathological observations that are associated with schizophrenia. These findings have been frequently inconsistent and incomplete. Nevertheless, some of the most consistent findings include ventricular enlargement, volume reduction in some brain regions, and the presence of smaller pyramidal cell bodies with reduced dendritic spines and arborizations in both the hippocampus and neocortex (Harrison & Weinberger, 2005). Evidence of hippocampal abnormalities in schizophrenia include findings of altered hippocampal shape in schizophrenic patients, including bilateral deformity of the head of the hippocampus and the loss of normal hippocampal asymmetry (Csernansky et al., 2002). Meta-analysis of structural magnetic resonance imaging (MRI) studies investigating regional brain volumes in schizophrenia have commonly found reductions in hippocampal volume in individuals with schizophrenia compared to control individuals (Nelson et al., 1998; Wright et al., 2000; Honea et al., 2005). Functional neuroimaging studies have also detected abnormal activity in the hippocampus in schizophrenic individuals when they are at rest, experiencing auditory hallucinations, and performing memory retrieval tasks (Heckers, 2001). Meta-analysis of structural MRI studies investigating regional brain volumes in individuals with schizophrenia have also found reductions in volume of the prefrontal cortex (PFC) in general, and more particularly in the dorsolateral PFC (DLPFC) and medial PFC compared to control individuals (Shenton et al., 2001; Wright et al., 2000; Honea et al., 2005; Glahn et al., 2008). Other cortical differences found in individuals with schizophrenia include cortical thinning in the orbitofrontal cortices bilaterally, the inferior frontal cortex on the left, and the medial frontal cortices on the right (Kuperberg et al., 2003) and reduced cortical gyrification (Kulynych et al., 1997; Harris et al., 2004; Harris et al., 2007). Furthermore thalamic volume was shown to be reduced in schizophrenic individuals compared to control subjects (Konick & Friedman, 2001). Finally, white matter abnormalities have been detected in many diverse regions of the brain in

schizophrenia patient, but frontal and temporal white matter regions have been most consistently implicated (Kyriakopoulos et al., 2008). White matter abnormalities detected between the hippocampus and cortex in schizophrenic patients has been suggested to contribute to evidence of aberrant structural hippocampal-cortical connectivity in schizophrenia (Qiu et al., 2010).

As schizophrenia lacks any consistent physiologically identifiable biomarker an alternative approach used to understand the pathophysiology of schizophrenia has included the identification of intermediate phenotypes or endophenotypes. Endophenotypes are defined as measurable components unseen by the unaided eye along the pathway between disease and distal genotype (Gottesman & Gould, 2003). An endophenotype can be distinguished from biomarkers in general by the following criteria (Gould & Gottesman, 2006):

1. The endophenotype must be associated with the illness.
2. The endophenotype must be heritable.
3. The endophenotype must be state-independent (but may require challenge/provocation).
4. Within families, endophenotype and illness cosegregate.
5. The endophenotype identified in patients can also be found in their unaffected relatives at a higher rate than in the general population.

As both the behavioural phenotypes and genetic basis of schizophrenia are complex, the identification of endophenotypes of schizophrenia is thought to constrain the heterogeneity of the behavioral phenotype and therefore increase the chances of efficiently identifying the genetic and neurobiological underpinnings of that endophenotype. This reductionist approach enables the identification of some of the pathways to which therapeutic drugs could be developed for treatment of that component of the disorder. The more endophenotypes of schizophrenia that are studied the more the neurobiological underpinnings of schizophrenia will be understood. The complex behavioural phenotypes observed in psychiatry have been reduced into components at the neurophysiological, biochemical, endocrine, neuroanatomical, cognitive or neuropsychological levels (Gould & Gottesman, 2006). In schizophrenia poor attention and other cognitive deficits (Niendam et al., 2003) and decreased social drive (Laurent et al., 2000) are examples of endophenotypes that have been identified.

The cause of schizophrenia is not understood but both genetic and environmental components contribute to its development. Environmental factors associated with the disorder include maternal infection and maternal starvation during pregnancy, hypoxia during birth, acute stress during childhood and drug abuse during puberty (Susser et al., 1996; St Clair et al., 2005; Hoek et al., 1998; Gearon et al., 2003). Substantial evidence for schizophrenia having a genetic component that predisposes an individual to the disorder has been accrued through the study of twins since as long ago as 1966 (Gottesman & Shields, 1966; McGuffin et al., 1994). Different studies have estimated the percentage of the genetic contribution to developing schizophrenia to different degrees, this is likely due to variation in the definition of the disorder (Portin & Alanen, 1997). However the highest heritability has been estimated at approximately 80% between monozygotic twins (McGuffin et al., 2004). The unusual pattern of heritability of schizophrenia reflects the heterogeneous and complex genetic architecture of schizophrenia combined with environmental factors (Owen et al., 2010).

1.1.1 Molecular Mechanisms of Schizophrenia

At the systems level, glutamatergic, dopaminergic, γ-aminobutyric acid related (GABAergic) and cholinergic neurotransmission have all been identified as being disrupted in schizophrenia (Lisman et al., 2008).

1.1.1.1 Glutamate Hypothesis of Schizophrenia

The glutamate hypothesis of schizophrenia was first put forward in 1980 and has received much greater support more recently (Kim et al., 1980; Moghaddam, 2003). Glutamate is a major excitatory neurotransmitter used by 40% of all synapses in the brain. It is released from pyramidal cells in the cerebral cortex and limbic system regions of the brain that have been implicated in schizophrenia (Tsai and Coyle, 2002). Glutamate neurotransmission was initially identified as being disrupted in schizophrenic patients through a study that detected low glutamate levels in the cerebrospinal fluid (Kim et al., 1980; but see Moghaddam, 2003). Glutamate binds to four groups of receptors; metabotrophic glutamate receptors, and the ionotropic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate and N-methyl-D-aspartic acid (NMDA) receptors (Coyle et al., 2001). Particular evidence for a role of NMDA receptor (NMDAR) hypofunction in schizophrenia has accumulated over the last three

decades. Antagonism of the NMDAR by phencyclidine (PCP) or ketamine in healthy individuals produces "schizophrenia-like" symptoms (Javitt et al., 1991; Krystal et al., 1994), and worsens symptoms in schizophrenia patients (Lahti et al., 1995). In addition, reduced NMDAR binding in the hippocampus of schizophrenic patients has been identified using single photon emission computed tomography (SPECT) imaging (Pilowsky et al., 2005). Furthermore, many of the genes that have been associated with susceptibility to developing schizophrenia have been shown to interact with NMDAR signalling or modulation of NMDAR activity, such as *Neuregulin 1* (*NRG1*), *Dysbindin* (*DTNBP1*), *D-Amino Acid Oxidase Activator* (*DAOA*), *Metabotropic Glutamate Receptor 3* (*GRM3*) and Disrupted-In-Schizophrenia 1 (*DISC1*). This has been suggested to be evidence supporting the glutamatergic hypothesis of schizophrenia as these genes show a degree of convergence on the glutamate system (Hall et al., 2009). Disruption in the expression of these genes has been proposed as a mechanism by which glutamatergic activity in schizophrenic patients could be altered without causing complete disruption to glutamate neurotransmitter activity across the whole brain (Moghaddam, 2003). Other studies supporting disruption to glutamate neurotransmission in schizophrenia include studies that show the levels of substrates, enzymes and products involved in glutamate synthesis were altered in schizophrenic patients (Tsai et al., 1995), and postmortem studies have identified alterations in glutamate receptor binding and decreases in expression of glutamate receptor subunits in the hippocampus and frontal cortical areas in schizophrenia patients (Harrison et al., 2003; Clinton & Meador-Woodruff, 2004). As a result of the glutamate hypothesis therapeutic drugs have been designed to address NMDAR dysfunction and reports from initial clinical trials are promising (Lindsley et al., 2006).

1.1.1.2 Dopamine Hypothesis of Schizophrenia

The dopamine hypothesis was initially proposed in 1966 (Rossum, 1966). Evidence supporting the dopamine hypothesis of schizophrenia has been drawn from pharmacological, postmortem and imaging studies (Toda & Abi-Dargham, 2007). Pharmacological studies have repeatedly shown that drugs that modulate dopamine release, such as amphetamines, produce paranoid psychotic symptoms in healthy individuals (Griffith et al., 1968; Angrist & Gershon, 1970; Bell, 1973). In addition, the vast majority of antipsychotic treatments for schizophrenia, with the exception of the most recently developed, have been shown to be dopamine D2 receptor antagonists (Talbot & Laruelle, 2002). Postmortem studies have assayed the presence of dopamine and its metabolites, tyrosine hydroxylase, D1, D2, D3 and D4 receptors and

dopamine transporters in schizophrenia with mixed results (Toda & Abi-Dargham, 2007). However an increase in D2 receptors in the striatum in schizophrenia has been consistently observed in 17 separate studies (Owen et al., 1978; Dean et al., 1997; see Toda & Abi-Dargham, 2007). Eighteen imaging studies revealed a small elevation of striatal D2 receptors in schizophrenic patients indicating that a hyperdopaminergic state is present in the striatum in schizophrenia (Weinberger & Laruelle, 2001; Yang et al., 2004). Also in the striatum, three studies have suggested that there is an increase in synaptic dopamine or an increase in affinity of D2 receptors for dopamine, following amphetamine challenge in schizophrenic patients compared to healthy individuals (Laruelle et al., 1996; Breier et al., 1997; Abi-Dargham et al., 1998). Additionally, abnormal activity in the mesolimbic and mesostriatal systems, thought to represent a hyperdopaminergic state, has been shown to correlate with reward learning and psychosis (Murray et al., 2008). In contrast indirect evidence from multiple studies suggest that a hypodopaminergic state may be present in the DLPFC in schizophrenia and could contribute to the manifestation of the cognitive symptoms (Toda & Abi-Dargham, 2007). Finally a selection of known schizophrenia susceptibility genes are involved in dopamine signalling, such as *Catechol-O-Methyltransferase* (*COMT*), *Dopamine Receptor D2* (*DRD2*) and *Protein Phosphatase 1 Regulatory Subunit 1B* (*PPP1R1B*), thereby implicating altered dopamineric neurotransmission in schizophrenia pathophysiology (Hall et al., 2009).

1.1.1.3 GABA Hypothesis of Schizophrenia

The GABA hypothesis of schizophrenia posits that GABA release is reduced in regions of the brain in schizophrenia. Postmortem investigations have consistently found that GABA concentrations are lower in the amygdala in schizophrenia (Spokes et al., 1980; Korpi et al., 1987; Kutay et al., 1989). A decrease in induced GABA release from the temporal and frontal cortex regions has also been observed in schizophrenia (Sherman et al., 1991). Additionally, two isoenzymes of glutamate decarboxylase (GAD), a biosynthetic enzyme that converts glutamic acid to GABA, have been shown to be differentially expressed in schizophrenia (Blum & Mann, 2002). In the PFC, lower *GAD67* mRNA expression (Akbarian et al., 1995; Volk et al., 2000; Guidotti et al., 2000) and lower GAD67 protein expression (Impagnatiello et al., 1998; Todtenkopf & Benes, 1998) have been associated with schizophrenia, while GAD65 protein levels do not appear to be altered in schizophrenia (Todtenkopf & Benes 1998; Benes et al., 2000). Thus less GABA may be synthesized in a subpopulation of cells in the PFC. Further postmortem investigations have shown that GABA-A receptor binding was increased in

schizophrenia in cortical and hippocampal regions in schizophreni patients (Hanada et al., 1987; Benes et al., 1992; Benes et al., 1996a; Benes et al., 1996b; Dean et al., 1999), with the exception of one study where no change in frontal cortex was found (Bennett et al., 1979). Increased binding at GABA-A receptors could be a compensatory response to reduced GABA release (Blum & Mann, 2002). There are different subpopulations of GABAergic interneurons in the prefrontal cortex. These include chandelier and wide-basket GABA neurons, identifiable by positive labelling for parvalbumin, that synapse onto the initial axon segments of pyramidal cells and on the cell bodies and dendrites of pyramidal cells respectively. The GABA transporter GAT-1 can be detected in distinctive vertical arrays of chandelier axons known as cartridges (Blum & Mann, 2002). Fewer GAT-1 axon cartridges are present in schizophrenia (Woo et al., 1998; Pierri et al., 1999; Volk et al., 2001; Ohnuma et al., 1999). The reduction in the number of GABA transporters in chandelier neurons supports the hypothesis of less GABAergic inhibition of pyramidal cells. Disruption to the GABAergic activity in the prefrontal cortex could contribute to the cognitive impairments in schizophrenia (Goldman-Rakic, 1996), the positive symptoms in schizophrenia (Grace, 1991) and the inability of schizophrenics to appropriately filter excessive or irrelevant stimuli (Carlsson et al., 2001).

1.1.1.4 Acetylcholine Hypothesis of Schizophrenia

The cholinergic hypothesis of schizophrenia suggests that alteration of the cholinergic system, and particularly signalling via the G protein coupled muscarinic receptors, is associated with schizophrenia pathogenesis (Raedler et al., 2007). Pharmacological evidence suggests that cholinergic neurotransmission is disrupted in schizophrenia because anticholinergic treatment in healthy individuals causes cognitive dysfunction (Ellis et al., 2006), and at higher doses can induce delirium and vivid hallucinations (Perry & Perry, 1995). In schizophrenia patients, anticholinergics have been used in conjunction with first-generation antipsychotics to alleviate motor side effects. This adjunct treatment worsened psychosis and cognitive impairments and had only a modest improvement of negative symptoms (Johnstone et al., 1983; Singh et al., 1987; Chouinard et al., 1987, Tandon et al., 1991; Tandon et al., 1992; Minzenberg et al., 2004). Additionally adjunct treatment with cholinesterase inhibitors have had mixed results (Raedler et al., 2007). However initial studies on the use of muscarinic agonists for treating schizophrenia are promising (Sullivan et al., 2000; Shekhar et al., 2008). Postmortem studies have shown region-specific decreases in specific types of muscarinic receptors in schizophrenic brains; in regions of the cortex (Dean et al., 2000; Crook et al., 2001; Dean et al.,

2002; Zavitsanou et al., 2004; Deng & Huang, 2005; Mancama et al., 2003), in the hippocampus (Crook et al., 2000; Scarr et al., 2007) and in the caudate and putamen (Dean et al., 1996; Crook et al., 1999). Complimenting the decrease in muscarinic receptor activity suggested by the postmortem studies, a single photon emission computed tomography (SPECT) imaging study in schizophrenic patients has shown that muscarinic receptor availability was reduced in the cortex and basal ganglia (Raedler et al., 2003). The pharmacological, postmortem and imaging studies combined support the hypothesis that cholinergic neurotransmission is involved in schizophrenia pathogenesis.

1.1.1.5 Synthesis of Current Hypotheses of Schizophrenia

As these systems all interact in different ways in different regions of the brain it is possible that disruption to one system could lead to disruption of the other three neurotransmitter systems. Lisman and colleagues (2008) have proposed a circuit-based framework for understanding the role and interaction of four neurotransmitter systems in Schizophrenia (Fig. 1.1) (Lisman et al., 2008). Central to this framework is NMDAR hypofunction that reduces glutamatergic neurotransmission through disinhibition of GABAergic activity leading to a hyperdopaminergic state. Cholinergic activity in interneurons can enhance GABA release and thereby modulate glutamatergic and consequently dopaminergic activity (Lisman et al., 2008).

1.1.2 Schizophrenia Susceptibility Genes

Schizophrenia is a highly heritable disorder (Flint et al., 2010). Therefore researchers have tried to identify the allelic variants in the DNA of individuals with schizophrenia that makes them more susceptible to developing the disorder. The allelic variants have become known as the schizophrenia susceptibility genes. In the past schizophrenia susceptibility genes have been identified using: (1) microscopic chromosomal abnormalities, (2) linkage studies, and (3) gene association studies (Harrison & Weinberger, 2005; MacIntyre et al., 2003).

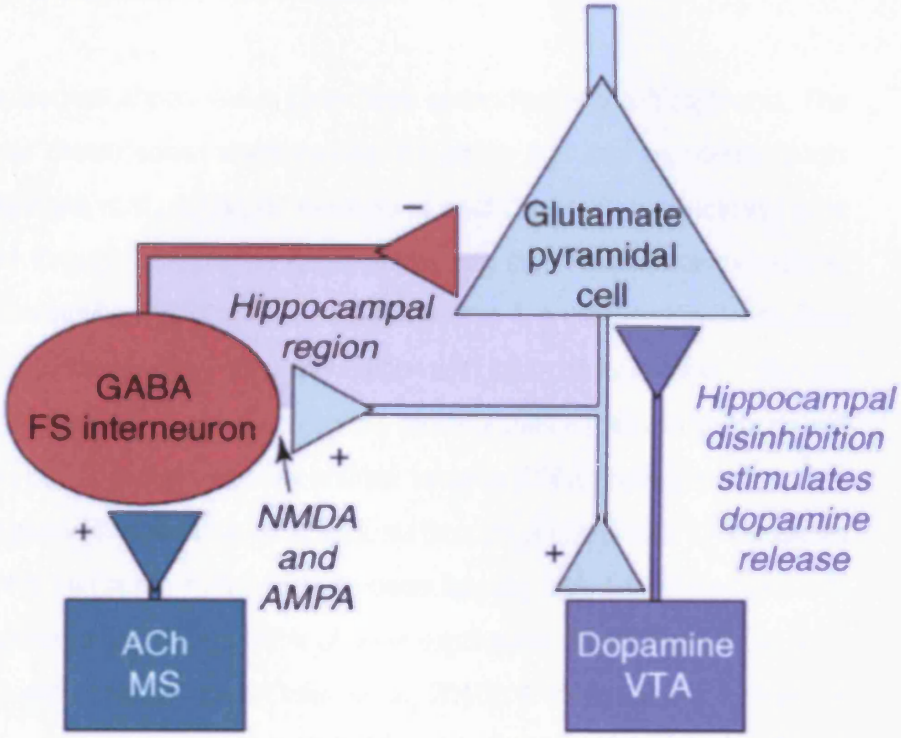


Figure 1.1. Schematic of the circuit based framework proposed by Lisman and colleagues (2008) for understanding the interaction between the glutamate, dopamine, GABA and acetylcholine neurotransmitter systems in schizophrenia (adapted from Lisman et al., 2008). NMDA receptor hypofunction in GABA FS interneurons could lead to less inhibition of glutamate pyramidal cells in the hippocampus that could lead to overstimulation of dopamine VTA neurons and a hyperdopaminergic state within the brain. Reduced excitatory output from cholinergic MS cells to GABA FS interneurons could lead to further reductions in the level of inhibition of glutamate activity in the hippocampal pyramidal cells. +, excitatory neurotransmitter release; -, inhibitory neurotransmitter release; Ach, acetylcholine; FS, fast-spiking; MS, medial septal region; VTA, ventral tegmental area.

1.1.2.1 Microscopic Chromosomal Abnormalities

Many microscopic chromosomal abnormalities have been associated with schizophrenia. The genes disrupted by these chromosomal abnormalities are genes that may be schizophrenia susceptibility genes (MacIntyre et al., 2003). An example of a schizophrenia susceptibility gene that has been identified through observation of a microscopic chromosomal abnormality is *DISC1*. In a large Scottish family, *DISC1* located to chromosome 1 is disrupted by a balanced reciprocal translocation with chromosome 11 in association with schizophrenia, bipolar disorder and major depressive disorder (Millar et al., 2000). More recently submicroscopic deletions and duplications of segments of DNA known as copy number variants (CNVs) have been identified as important sources of genomic variation in all individuals through genome-wide CNVs studies (Sebat et al., 2009). CNVs can result in disruption to gene function including altered levels of gene dosage and disruption of normal regulation of gene expression. Some CNVs have been found to be associated with schizophrenia (Owen et al., 2010). A chromosomal deletion at 22q11.2 has been associated with an approximately 20-fold increase in risk of schizophrenia (Murphy et al., 1999). Two other loci, deletions of chromosomes 1q21.1 and 15q13.3, have been associated with schizophrenia in two different studies (International Schizophrenia Consortium, 2008; Stefansson et al., 2008). At present there is evidence for an increase of large (>100 kb), rare (frequency <1%) CNVs in schizophrenia. However as to which of the multiple genes present within these CNVs are relevant to schizophenia pathophysiology remains unknown with one exception (Owen et al., 2010). The gene *Neurexin1* (*NRXN1*) at chromosome 2p16.3 has been disrupted by deletion CNVs in multiple studies (Kirov et al., 2009).

1.1.2.2 Linkage Studies

Genetic linkage analysis is a tool that can detect chromosomal locations contributing to the physiology of a disease through genotyping marker DNA sequences. Within these chromosomal regions is located the gene or genes that are irregularly expressed or abnormally functioning in the disease (Pulst, 1999). Linkage studies have identified at least 18 chromosomal regions that differ between schizophrenic and control populations (Kohn & Lerer, 2002). Two examples of schizophrenia linkage regions include 6p23 (Wang et al., 1995) and 8p21 (Blouin et al., 1998). Investigation of the genes that are located in these regions and gene

association studies on allelic variants that are present within these regions lead to the identification of two schizophrenia susceptibility genes, *DTNBP1* at 6p22.3 (Straub et al., 2002) and *NRG1* at 8p22-p11 (Stefansson et al., 2002). However the findings from linkage analysis studies have not been consistent and a recent meta-analysis on 32 independent genome-wide linkage scans suggested that fewer linkage regions were significantly different between individuals with schizophrenia and the control population (Ng et al., 2009).

1.1.2.3 Gene Association Studies

Gene association studies initially genotyped a number of single nucleotide polymorphisms (SNPs) within a length of DNA, usually across a gene of interest, in order to try and identify allelic variants that associated with schizophrenia. Due to low sample sizes, many gene association studies have only had the power to identify relatively common and highly penetrant allelic variants (Risch & Merikangas, 1996). At odds with the power of these initial gene association studies recent allelic variant architecture hypotheses of schizophrenia suggest that there are common variants of small effect and rare variants of large effect that contribute to schizophrenia (Craddock et al., 2007). This helps to explain the many inconsistent findings from the schizophrenia gene association studies. There have been thousands of gene association studies published, many of which are replication studies. More than 900 genes have been investigated through association studies across a range of populations with commonly negative, or both positive and negative, findings for each gene (Allen et al., 2008). However the veracity of the positive findings are not necessarily tempered by the negative findings as the studies vary in the genetic heterogeneity and size of the sample population and in the genetic variation coverage investigated (Alaerts et al., 2009; Owen et al., 2010). Harrison and Weinberger suggested that candidate schizophrenia susceptibility genes worthy of further focused investigations should have greater than three separate positive gene association studies, should be present in a schizophrenia linkage region, should have evidence for altered expression in the schizophrenic postmortem brain, and finally they should have biological plausibility in relation to current hypotheses of schizophrenia pathogenesis. In 2005 *NRG1*, *DTNBP1* and *DISC1* were three of the most well supported schizophrenia susceptibility genes using this criteria (Harrison & Weinberger, 2005). Two other genes that have a moderate level of evidence suggesting that they contribute to schizophrenia pathophysiology and that have also been shown to be involved in long-term memory are *Early Growth Response factor 3* (*EGR3*) and *Brain Derived Neurotrophic Factor* (*BDNF*). The aim of this thesis is to identify if

these genes have a role in hippocampal-dependent long-term memory. The evidence for these genes as schizophrenia susceptibility genes is discussed below.

1.1.2.3.1 *NRG1*

NRG1 was first identified as a schizophrenia susceptibility gene in 2002 through a gene association study (Stefansson et al., 2002). Since then there have been 21 positive case-control studies and 17 negative case-control studies, 10 positive family-based studies and 10 negative family-based studies. These studies were performed in a range of ethnic groups including Caucasian, Asian, Hispanic, and African descent, using different selections of single nucleotide polymorphisms (SNPs) within the gene, and varying numbers of schizophrenic patients and controls (SchizophreniaGene Database on Schizophrenia Research Forum website as of July 2010, see Allen et al., 2008). Therefore the inconsistent findings may be due to genetic heterogeneity, low genetic variation coverage with a lack of linkage disequilibrium between the SNPs used in the studies and the actual susceptibility allele, and/or insufficient power in the studies to detect risk variants with small effects. It is also possible that *NRG1* is a susceptibility gene in some populations but not in others (Alaerts et al., 2009). Three meta-analyses of the association of *NRG1* with schizophrenia found that *NRG1* was positively associated with schizophrenia (Li et al., 2006; Munafo et al., 2006; Munafo et al., 2008). In addition *NRG1* is positioned on chromosomal region 8p22-p11 which is a replicated linkage loci for schizophrenia (Kendler et al., 1996; Pulver et al., 2000; Ng et al., 2009). *NRG1* has also been implicated in schizophrenia through two postmortem studies on schizophrenic individuals in which an increase in *NRG1* expression was detected in the PFC and hippocampus in individuals with schizophrenia (Hashimoto et al., 2004; Law et al., 2006). Finally, in addition to the evidence from genetic association, linkage analysis and postmortem studies, *NRG1* also has a functional role in processes hypothesized to be involved in schizophrenia pathophysiology including neurodevelopment, myelination, regulation of neurotransmitter receptor expression and synaptic plasticity (Corfas et al., 2004).

1.1.2.3.2 *DTNBP1*

DTNBP1 was first identified as a schizophrenia susceptibility gene in 2002 by a gene association study (Straub et al., 2002). Since then there have been 14 positive and 21 negative case-control studies, and 8 positive and 8 negative family-based studies (SchizophreniaGene

Database on Schizophrenia Research Forum website as of July 2010, see Allen et al., 2008). The inconsistent findings are likely due to genetic heterogeneity, low genetic variation coverage, and/or insufficient power in the studies as discussed for *NRG1* (see 1.1.2.3.1). A meta-analysis of *DTNBP1* association studies published before May 2006 concluded that one SNP in *DTNBP1* was weakly associated with schizophrenia but it was no longer significant after multiple testing (Li & He, 2007). In contrast, another meta-analysis using a novel approach to take into account the allelic heterogeneity in the *DTNBP1* association studies found significant evidence for a mixture of association distributions in multiple loci (Maher et al., 2010). *DTNBP1* is located in the region 6p24-22, a region that has been linked to schizophrenia in multiple studies (Straub et al., 1995; Moises et al., 1995; Schwab et al., 2000; Maziade et al., 2001). Postmortem studies in schizophrenic individuals have found *DTNBP1* mRNA expression to be reduced in the dorsolateral prefrontal cortex (DLPFC) (Weickert et al., 2004) and in the hippocampus (Weickert et al., 2007). In addition, DTNBP1 protein expression was reduced in the DLPFC (Straub et al., 2004) and in the hippocampus (Talbot et al., 2004) in postmortem studies of schizophrenic cases. A recent study that investigated different transcripts and isoforms of DTNBP1 in the DLPFC found that of the three isoforms investigated only dysbindin-1C protein expression was reduced in schizophrenic cases, while in contrast dysbindin-1A and dysbindin-1B mRNA expression was upregulated in schizophrenic cases (Tang et al., 2009a). Finally, DTNBP1 has a role in neurotransmitter release and synaptic plasticity (Numakawa et al., 2004; Davis & Dickman, 2009; Tang et al., 2009b); both processes involved in current hypotheses of schizophrenia.

1.1.2.3.3 *DISC1*

DISC1 was first identified in 2000 as a schizophrenia susceptibility gene as it was a gene disrupted by a genomic translocation that co-segregated with schizophrenia (Millar et al., 2001). The first genetic association study correlating *DISC1* with schizophrenia was in 2001 (Devon et al., 2001), and since then there have been 20 positive and 8 negative case-control studies, and 8 positive and 1 negative family-based studies (SchizophreniaGene Database on Schizophrenia Research Forum website as of July 2010, see Allen et al., 2008). Meta-analysis of 9 different schizophrenia samples of European ancestry has identified a common risk interval within *DISC1* positively associating *DISC1* with schizophrenia (Schumacher et al., 2009). Supporting the gene association findings, multiple linkage studies have found that 1q42, the region within which *DISC1* is located, has been associated with schizophrenia (Hovatta et

al., 1999; Ekelund et al., 2001; Ekelund et al., 2004; Hwu et al., 2003; Hamshere et al., 2005). One postmortem study assaying *DISC1* mRNA expression has been performed, but no differences in the levels of *DISC1* expression was found between schizophrenic cases and controls (Rastogi et al., 2009). Further postmortem studies are required before any conclusions can be drawn as to whether *DISC1* expression levels are abnormal in schizophrenia. Using inducible transgenic mice it was shown that *Disc1* expression on postnatal day 7 was necessary for normal basal neurotransmission in the same mice when they were adults (Li et al., 2007). This shows that *Disc1* is necessary for neurodevelopmental processes. *Disc1* is a multifunctional scaffold protein that has also been shown to be involved in neuronal migration, cortical layering and hippocampal formation (Hennah et al., 2006). The *Disc1* cellular function findings support a functional role for *Disc1* in neurodevelopmental processes hypothesized to underlie schizophrenia pathophysiology.

1.1.2.3.4 *EGR3*

EGR3 was first identified as a schizophrenia susceptibility gene in 2007 through a family-based and a case-control gene association study (Yamada et al., 2007). Subsequently, there have been 1 positive and 3 negative case-control studies, and 1 positive family-based study (SchizophreniaGene Database on Schizophrenia Research Forum website as of July 2010, see Allen et al., 2008). In support of the positive genetic association findings, *EGR3* is found in genomic region 8p23-21 which is a region that has been linked to schizophrenia in multiple studies (Takahashi et al., 2005; Suarez et al., 2006; Walss-Bass et al., 2006; Holliday et al., 2008; Wiener et al., 2009). In addition, *EGR3* was expressed at reduced levels in the DLPFC in a postmortem study of schizophrenic individuals (Yamada et al., 2007). Finally, *EGR3* is a transcription factor that has a functional role in neuronal development and synaptic plasticity, both processes hypothesized to be abnormal in schizophrenia (O'Donovan et al., 1999; Li et al 2007).

1.1.2.3.5 *BDNF*

BDNF was first identified as a schizophrenia susceptibility gene in 2000 through a gene association study (Krebs et al., 2000). There have been 8 positive and 30 negative case-control studies, and 2 positive and 2 negative family-based studies (SchizophreniaGene Database on Schizophrenia Research Forum website as of July 2010, see Allen et al., 2008).

BDNF located at chromosomal position 11p13 is in a region that has been associated with schizophrenia in one linkage study (Suarez et al., 2006). There have been 4 postmortem studies of BDNF expression in schizophrenia. One postmortem study in schizophrenic patients showed that BDNF protein expression was upregulated in the anterior cingulate cortex and hippocampus of schizophrenic patients (Takahashi et al., 2000). In contrast, two other postmortem studies showed that BDNF mRNA and protein expression was downregulated in the prefrontal cortex of schizophrenic patients (Weickert et al., 2003; Issa et al., 2010). While another postmortem study found upregulation of BDNF protein expression in cortical areas and a decrease in BDNF protein expression in the hippocampus (Durany et al., 2001). Therefore it could be concluded that abnormal BDNF expression is present in individuals with schizophrenia. Finally, BDNF has an important functional role in neurodevelopment and synaptic plasticity, both processes hypothesized to be involved in schizophrenia pathophysiology (Shoval & Weizman, 2005).

1.1.2.4 Genome Wide Association Studies (GWAS)

Due to the development of array platforms, hundreds of thousands of SNPs from the human genome can now be assayed in parallel for association with schizophrenia enabling the identification of schizophrenia susceptibility genes in larger samples. These studies are known as genome-wide association studies (GWAS) (O'Donovan et al., 2008; Psychiatric GWAS Consortium Coordinating Committee, 2009). GWAS are advantageous in comparison to the original gene association studies as the larger case and control sample numbers allows for the many genes of small effect and rarer genes of larger effect to be detected. The recent GWAS studies have identified four schizophrenia susceptibility genes that are robustly supported by rigorous statistical analysis and these are *ZNF804A*, *MHC*, *NRGN* and *TCF4* (O'Donovan et al., 2008; Stefansson et al., 2009). While these approaches have identified susceptibility genes, comprehensive whole exome sequencing and whole genome sequencing is expected to start being applied to this field of research in the near future. This holds great promise for identifying further susceptibility genes that are not included in the array of genes that are probed for in the GWAS microarray studies (Psychiatric GWAS Consortium Coordinating Committee, 2009).

1.1.2.5 Contributions of Schizophrenia Susceptibility Genes to Schizophrenia Pathophysiology

How these susceptibility genes contribute to the disorder is under intense investigation, but it is thought that no particular constellation of genes will be characteristic of most ill individuals. Even when the same causative allele is present, the resulting phenotype may vary depending on the genetic background. It is thought that there are particular key pathways that these susceptibility genes contribute to, that when disrupted lead to pathogenesis of the disorder. The schizophrenia susceptibility gene *NRG1* that indirectly affects localisation of NMDA receptors at the postsynaptic membrane is an example of a schizophrenia susceptibility gene that regulates glutamate neurotransmission, a pathway that is hypothesised to be disrupted in individuals with schizophrenia. Disruption of glutamate neurotransmission activity via aberrant NRG1 expression supports the glutamate hypothesis of schizophrenia pathogenesis. However the molecular roles of many of the identified candidate susceptibility gene products were unknown upon first identification and are still being investigated now. This combined with the prevailing hypothesis that there are common alleles of small effect and rare alleles of a larger effect that contribute to the genetic component of schizophrenia (Craddock et al., 2007), has lead to an emphasis on research into the functional roles of the genes in order to identify converging functional pathways. Determining a functional role for these schizophrenia susceptibility genes to schizophrenia pathogenesis will lead to improved prospects in treatment of the disorder (Harrison & Weinberger, 2005). Along the same line of reasoning some genes such as *NRG1* (Hall et al., 2006), *DISC1* (Burdick et al., 2005) and *DTNBP1* (Burdick et al., 2006; DeRosse et al., 2006) are now being associated with particular intermediate phenotypes of schizophrenia in an attempt to understand part of what is a very complex pathophysiology.

Hall and colleagues (2009) have proposed a hypothesis that risk for psychosis in schizophrenia could be explained by combining the schizophrenia susceptibility gene findings with the current knowledge on associative learning theory. It was suggested that the schizophrenia susceptibility genes discovered so far could be divided into two broad classes. Those that have direct effects on synaptic plasticity mediated through glutamatergic synapses and those that impact on meso-limbic dopamine signalling. It is suggested that genes affecting NMDA-receptor-mediated plasticity and dopamine signalling might interact to produce alterations in meso-limbic dopamine signalling resulting in inappropriate stimuli gaining motivational importance (Hall et al., 2009).

1.1.3 Cognitive Function in Schizophrenia

A wide range of impairments in cognitive function have been identified in individuals with schizophrenia. This has been achieved through performing batteries of neuropsychological tests on individuals with schizophrenia and matched control individuals (Heinrichs & Zakzanis, 1998). The cognitive impairments in schizophrenia include deficits in learning and memory, executive functions, attention, and processing speed (Saykin et al., 1991; Palmer et al., 1997). Some findings suggest that impairment in different cognitive functions might be due to generalized cognitive dysfunction as opposed to deficits in specific cognitive functions (Blanchard & Neale, 1994; Mohamed et al., 1999). Other studies concur with the findings of generalized cognitive impairments but also suggest that there are more subtle differential deficits that are present amongst a background of generalized cognitive deficit (Saykin et al., 1991; Bilder et al., 2000; Dickinson et al., 2007; Titone et al., 2004). The cognitive deficits are thought to be the product of either widespread disturbances in intrinsic cortical circuitry or disturbances in frontolimbic systems and possibly brainstem systems (Bilder et al., 2000). It has been hypothesised that the cognitive deficits observed in schizophrenic individuals may be a consequence of more fundamental "core" impairments, such as working memory and verbal learning and memory (Reichenberg & Harvey, 2007; Zanelli et al., 2010). However, it has been argued that memory impairments are not secondary to attentional dysfunction in schizophrenic individuals as their ability to perform the backward digit span test is the same as in the forward digit span test (Aleman et al., 1999). Nevertheless, meta-analysis of neuropsychological studies performed on individuals with schizophrenia between 1980 and 1997 found that out of the nine categories of cognitive impairments in schizophrenia, verbal learning and memory was most impaired (Heinrichs & Zakzanis, 1998).

1.1.3.1 Memory Function in Schizophrenia

Substantial memory impairment has been identified in individuals with schizophrenia. A meta-analysis of studies published between 1975 and 1998 investigated memory function in schizophrenia (Aleman et al., 1999). It concluded that individuals with schizophrenia performed worse than healthy controls in both delayed and immediate verbal and nonverbal recall memory, verbal and nonverbal recognition memory and short-term memory. The most impaired form of memory was long-term recall memory. As deficits in verbal and nonverbal memory

were of similar magnitudes, it has been suggested that the memory impairments in schizophrenia are not modality specific (Aleman et al., 1999). Additionally, a more recent study has found long-term associative/relational memory impairments in schizophrenic patients (Titone et al., 2004). There are memory impairments in both medicated and medication-naïve schizophrenia patients in comparison to healthy controls (Goldberg & Weinberger, 1996; Saykin et al., 1994) showing that medication is not causally related to memory dysfunction. While there is evidence that anticholinergic medication given to treat side effects of antipsychotic medication may cause memory impairments (Frith, 1984), there is other evidence that the antipsychotic treatments may ameliorate memory impairments (Green et al., 1997; Mortimer, 1997). Other potential confounds such as age, duration of illness, severity of psychopathology and positive symptoms do not appear to be associated with the memory impairments in individuals with schizophrenia (Aleman et al., 1999). In addition, symptom severity has been shown to correlate with level of neuropsychological impairment in schizophrenia (Bornstein et al., 1990). In general the extent and stability of the association between schizophrenia and memory impairment suggests that memory dysfunction is a trait rather than a state-dependent characteristic (Aleman et al., 1999).

1.1.3.2 Hippocampal-Dependent Memory in Schizophrenia

Of all of the memory tests used, a large number of studies have focused on investigating whether episodic memory is disturbed in schizophrenia, in particular in relation to dysfunction in the hippocampus (Barch, 2005). Episodic memory is a type of associative long-term memory. It is the memory of an event, combining information on where and when it took place. A meta-analysis comparing performance on associative and item memory tests concluded that individuals with schizophrenia had a 20% greater impairment in associative memory than in item memory (Achim & Lapage, 2003). A meta-analysis from imaging studies that investigated regions of the brain that consistently showed abnormal activity during memory tasks in people with schizophrenia, found that differential activation was consistently found in the medial temporal cortex bilaterally, left inferior prefrontal cortex, left cerebellum and in other prefrontal and temporal lobe regions (Achim & Lepage, 2005). These regions have also been shown to be involved in episodic memory. Episodic memory is a hippocampal-dependent process so some studies have investigated whether there is an association between hippocampal processes and schizophrenia (Achim & Lepage, 2005; Boyer et al., 2007). Deficits in contextual binding and hippocampal abnormalities are both associated with schizophrenia suggesting that

long-term contextual association memory could be disrupted in schizophrenia (Boyer et al., 2007). Relational memory, as tested using the transitive inference test, showed schizophrenic individuals to be impaired in high level memory processes. Relational memory networks are supported by activity in the hippocampus (Titone et al., 2004). Schizophrenic patients typically perform worse on recall tasks, which rely on the hippocampus, compared to recognition tasks, which are hippocampal independent (Aleman et al., 1999). Individuals with schizophrenia have also been found to have deficits in recall memory with a significant loss of the emotional enhancement of recognition memory. The hippocampus and amygdala are both engaged in the emotional processes supporting the enhancement of recognition memory. This evidence contributes to the view that medial temporal lobe function is abnormal in schizophrenia (Hall et al., 2007; Hall et al., 2010).

1.1.3.3 Pavlovian Conditioning and Associative Long-Term Memory in Schizophrenia

Another test that has been used to investigate long-term associative memory in individuals with schizophrenia is Pavlovian conditioning. The galvanic skin responses (GSR) measured during Pavlovian conditioning to electric shock indicated that there was less conditioning in individuals with schizophrenia than in healthy individuals (Peters & Murphee, 1954). Another two studies also found impaired conditioning in schizophrenic individuals compared to healthy individuals through measuring finger-sweating responses and vascular responses, in addition to the GSR (Ax et al., 1970; Gorham et al., 1978). Non-aversive avoidance learning (avoiding a particular shape in a succession of shapes on a computer screen by clicking a button), and aversive avoidance learning (a loud buzzer sound, the aversive stimulus, was avoided by clicking a button upon observing a shape, amidst a succession of shapes, on a computer screen) were compared in both schizophrenic and healthy control individuals. It was found that schizophrenic individuals demonstrated relatively intact non-aversive avoidance learning. However in the aversive avoidance learning test, half of the schizophrenic individuals failed to learn how to avoid the aversive stimulus, while the other half learned to avoid the aversive stimulus more quickly than in the non-aversive avoidance learning tests. Poor performance on the aversive avoidance learning test was more likely in individuals with an earlier age of illness onset. It was suggested that increased arousal associated with the buzzer may have interfered with the ability to use feedback constructively, and therefore disrupted appropriate modification of behaviour from taking place (Kosmidis et al., 1999). Finally, an aversive avoidance learning

test was combined with functional magnetic resonance imaging (fMRI) and it was found that individuals with schizophrenia could not distinguish between a conditioned stimulus and similar neutral stimuli, but showed a high GSR to both conditioned and neutral stimuli indicative of abnormal associative learning. This abnormal associative learning in schizophrenic individuals was correlated with activation in the ventral striatum, upon presentation of the neutral stimuli, that was not present in healthy controls (Jensen et al., 2007). These findings of abnormal associative learning in schizophrenic individuals suggests that investigation of the schizophrenia susceptibility genes of interest in hippocampal-dependent aversive Pavlovian conditioning in rats will be an appropriate model for investigating the role of schizophrenia susceptibility genes in hippocampal-dependent long-term memory.

1.1.3.4 Cognitive Endophenotypes in Schizophrenia

Many cognitive impairments in schizophrenia have been identified (see 1.1.3) and specific neuropsychological tests have been tested on schizophrenic patients, unaffected relatives of schizophrenic patients and on the general population in order to identify cognitive endophenotypes (Gur et al., 2007). Long-term memory impairments are among the range of cognitive endophenotypes identified in schizophrenic patients. More specifically verbal, face and spatial memory impairments have been observed in schizophrenic patients and to a lesser degree in their unaffected relatives (Cannon et al., 1994; Faraone et al., 1995; Egan et al., 2001; Gur et al., 2007). Other identified cognitive endophenotypes include working memory, attention, emotion identification, flexibility and prepulse inhibition (Gur et al., 2007; Amann et al., 2010).

Investigation of all these cognitive endophenotypes will contribute to the elucidation of the neurobiological underpinnings of the cognitive impairments in schizophrenia. This study focuses on the investigation of the neurobiological underpinnings of LTM. Support for investigating LTM includes that in addition to being identified as one of the schizophrenia cognitive endophenotypes, LTM has also been shown to be impaired in individuals with schizophrenia compared to controls in many more studies (see 1.1.3.1, 1.1.3.2 and 1.1.3.3). Furthermore the hippocampus and prefrontal cortex regions of the brain that are involved in LTM function have been shown by histological and structural neuroimaging methods to have reduced volume and altered shape in schizophrenic patients (see 1.1). Functional neuroimaging studies have also identified abnormal activity in the hippocampus in patients performing a memory retrieval task (Heckers, 2001). Finally many of the genes associated with

schizophrenia appear to be involved in synaptic plasticity and neurotransmission at glutamatergic synapses (Hall et al., 2009). Glutamate is released from the pyramidal cells in the hippocampus and prefrontal cortex and glutamate neurotransmission in these regions is important for LTM function (Hall et al., 2009). Thus there is converging evidence from behavioural, histological, neuroimaging and biochemical studies supporting investigation of schizophrenia susceptibility genes in LTM.

1.2 Long-term Memory (LTM)

Memory is represented by a collection of neural changes known as an engram. An engram is distributed across many neural systems that when combined represent the event being committed to memory. Specific aspects of the memory are localized in different brain systems. The study of humans with memory pathology has lead to the identification of different brain systems in different types of memory. Short-term memory (STM) is a capacity-limited immediate memory that is intact in amnesic patients and is thought to be distributed in regions within the cerebral cortex. Long-term memory is a more long-lasting type of memory that is impaired in amnesic patients and is thought to be distributed across a greater number of brain regions including the medial temporal lobe and the cerebral cortex (Squire, 1986). A frequently cited example of an individual suffering from a long-term memory deficit is the patient H.M. who sustained a bilateral resection of the medial temporal lobes which resulted in profound difficulty in establishing new memories (Scoville & Milner, 1953; Corkin, 2002). STM is typically described as having a duration that ranges from the immediate to a number of hours, and does not depend on RNA or protein synthesis that can be expressed immediately. While LTM may persist for the duration of an individual's lifetime, is thought to be dependent on new RNA and protein synthesis and is assumed to be mediated by changes in synaptic efficacy (McGaugh, 2000). There are distinct types of LTM that involve the processing of different kinds of information called declarative and nondeclarative memory (Fig. 1.2). Declarative memory, also known as explicit memory, is accessible to conscious awareness. There are two types of declarative memory; episodic memory (specific time-and-place events) and semantic memory

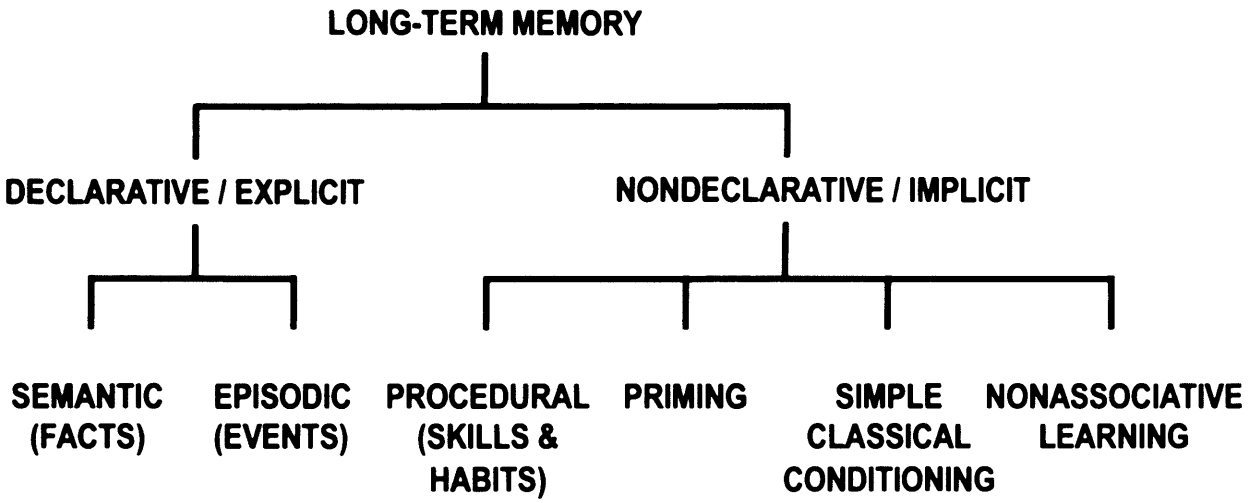


Figure 1.2. A taxonomy of LTM systems (adapted from Squire & Zola, 1996).

(facts and general information gathered in the course of specific experiences). Nondeclarative memory, also known as implicit memory, is only accessible through performance that engages the skills or operations in which the memory is embedded. There are four types of nondeclarative memory:- procedural memory, priming, simple classical conditioning and nonassociative learning. Nondeclarative memory is considered phylogenetically older than declarative memory (Squire, 1986). LTM is suggested by some to be an independent process from STM such that they run in parallel (McGaugh, 1966; Agranoff et al., 1965), while other evidence suggests that they are not independent but that they are serially linked (Alloway & Routtenberg, 1967).

In 1900 Muller and Pilzecker proposed the memory consolidation hypothesis that new memories consolidate slowly over time. This hypothesis was based on the findings that in humans the memory of newly learned information was disrupted by the learning of other information shortly after the original learning (Muller & Pilzecker, 1900; McGaugh, 2000). The consolidation theory was further developed during the 1960s when it was hypothesized that electroconvulsive shocks (ECS) given prior to or following learning training lead to conditioned inhibition of the training through competition between learning training and associative learning with the ECS. While this hypothesis did not directly explain engram disruption or consolidation, it did change future approaches to investigating engram consolidation and suggested that consolidation was vulnerable to disruption by interference with the acquisition of another association (Lewis & Adams, 1963; Lewis & Mahers, 1965). The first evidence suggesting that consolidation of LTM was protein synthesis dependent came from studies of mice and fish (Flexner et al., 1963; Agranoff et al., 1965). Many studies since have produced data consistent with this idea and consequently *de novo* protein synthesis dependent consolidation is now the most common model used for studies investigating the consolidation of LTM memories (McGaugh, 2000). However, very recently the necessity of protein synthesis to LTM has been challenged and post-translational modification of proteins has been proposed to be the critical substrate for long-lasting memory (Routtenberg & Rekart, 2005). Exactly how long is required for a memory to consolidate is not clear but a positron emission tomography (PET) imaging study indicated that brain activity following motor skill learning persisted for six hours; initially in the prefrontal regions of the cortex and then in the premotor, posterior parietal and cerebellar cortex structures (Shadmehr & Holcomb, 1997). In addition most studies investigating memory consolidation have investigated the effects of treatments administered within several hours after training (McGaugh, 2000). However recently the window of change theories have been

challenged as infusion of a PKMz inhibitor into the cortex 3 days following conditioned taste aversion training resulted in associative LTM vanishing for at least several weeks. Therefore it is proposed that persistence of memory is dependent on ongoing activity of a protein kinase long after the memory has been considered consolidated into a stable form (Shema et al., 2007) (Fig. 1.3). This concurs with the hypothesis put forward by Routtenberg and Rekart in 2005 (Routtenberg & Rekart, 2005).

The reconsolidation or the extinction of associative memories can occur following retrieval of the memory depending on the conditions under which the memory is recalled. The concept of reconsolidation of LTM was first introduced in 1968 following experiments that involved disrupting a memory by first reactivating it and then immediately administering an electric shock. This resulted in retrograde amnesia for the reactivated memory suggesting that the memory became vulnerable to change after reactivation (Misanin et al., 1968). Reconsolidation of LTM was first shown to be dependent on *de novo* protein synthesis in 2000 (Nader et al., 2000). A previously consolidated fear memory is thought to return to a labile state after reactivation and then become reconsolidated over the next 6 hours via *de novo* protein synthesis (Nader et al., 2000). Most studies agree that protein synthesis is necessary for memory reconsolidation (Debiec et al., 2002; Pedreira et al., 2002; Lee et al., 2004; Inda et al., 2005), but at least one study did not find any loss of memory when protein synthesis inhibitors were used during reactivation (Biedenkapp & Rudy, 2004; Lattal & Abel, 2004). In some cases the proteins implicated in reconsolidation differ to those implicated in consolidation (Taubenfeld et al., 2001), while in other cases the activity of the same proteins have been shown to be involved in both processes (Kida et al., 2002; Kelly et al., 2003; Lee et al., 2006). Also there are some different cellular processes and signalling mechanisms that appear to be used in reconsolidation in comparison to consolidation (Lee et al., 2004; Barnes et al., in press). Therefore the molecular mechanisms underlying consolidation and reconsolidation are dissociable but not mutually exclusive.

Extinction is the apparent loss of a consolidated memory that occurs following reactivation of a memory but in the absence of the reinforcer. For example, the predictive value of a context with respect to the occurrence of an aversive event that had occurred in that context will be compromised following exposure to the context in the absence of the event (Myers & Davis, 2007). In 1927 extinction was first studied experimentally in Pavlov's study of appetitive conditioned responses in dogs (Pavlov, 1927; Quirk & Mueller, 2008). The process of extinction

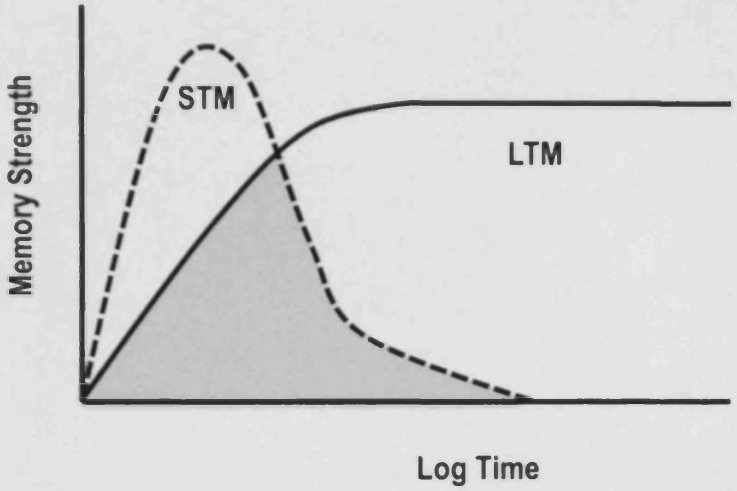


Figure 1.3. Phases of memory consolidation. STM can last seconds to hours while LTM can last hours to a lifetime. STM, short-term memory (represented by the dotted line); LTM, long-term memory (represented by the solid line); pale gray region represents the time period in which STM and LTM may be running serially or in parallel.

does not result in permanent loss of the memory as an extinguished memory can be retrieved under certain conditions including reinstatement, renewal and spontaneous recovery. Furthermore, extinction is now generally considered a form of new learning (Myers & Davis, 2007). The molecular basis of extinction has only been studied in the last 10 years and mainly in the well established fear conditioning paradigm (Quirk & Mueller, 2008). Most studies have shown that extinction is protein synthesis dependent (Vianna et al., 2001; Lin et al., 2003; Bahar et al., 2003; Pedreira & Maldonado, 2003; Inda et al., 2005; Barnes & Thomas, 2008), however other studies have found that extinction can occur in the absence of protein synthesis (Lattal & Abel, 2001), or indeed that extinction is actually enhanced by the inhibition of protein synthesis (Fischer et al., 2004).

Research in different animals using multiple behavioural paradigms, in addition to research in human subjects, have been used to investigate long-term memory (Goldman-Rakic, 1996). Behavioural paradigms commonly used include cued-fear conditioning, contextual fear conditioning (CFC), conditioned taste aversion (CTA), inhibitory avoidance (IA) and appetitive learning (for examples see Nader & Hardt, 2009). The range of animals used in the study of LTM covers animals from throughout the phyla including rodents, monkeys, flies, bees, fish, worms, snails, rabbits, cats, dogs, pigeons and crabs (LeDoux, 2000; Bitterman et al., 1983; Pedreira et al., 1996).

1.2.1 Contextual-Fear Conditioning (CFC)

Explicit LTM, as described previously, requires conscious recollection and therefore cannot be modeled in animals as we are unable to know if they have a conscious experience. Implicit memory however can be modeled in animals for the purposes of research (Squire et al., 1993). People with schizophrenia have impairments in their hippocampal-dependent emotional associative LTM (see 1.1.6). Therefore an appropriate behavioural paradigm in which to study these memory processes in healthy rats is the hippocampal-dependent contextual-fear conditioning (CFC) paradigm. Out of the behavioural tasks testing LTM that can be performed by a rat, such as fear-conditioning, Morris water maze, novel object recognition and conditioned taste aversion, the contextual fear conditioning paradigm was chosen because very similar versions of this task have been investigated in schizophrenic patients revealing impaired associative LTM (see 1.1.3.3). Aside from the translatable aspect of this paradigm, the brain regions, neurocircuitry and molecules involved in contextual fear conditioning in rats

is highly studied and well characterized which is helpful when deciding which brain regions and time points to investigate in the rat following behavioural conditioning. Also this characterization shows that the brain regions important for CFM in the rat are similar to those that are relevant to schizophrenia pathophysiology. Finally a major advantage of using the CFC paradigm is that the rat only requires one-trial of conditioning to form the associative memory which enables gene expression to be investigated at accurate timepoints post-acquisition of the associative memory.

The CFC paradigm consists of an electric footshock (an unconditioned stimulus (US)) being given in a novel, distinct environment which acts as a conditioned stimulus (CS). An associative LTM is formed between the CS and the US. Whether the rats have formed a CS-US memory, known as a contextual fear memory (CFM) memory, following conditioning, can be tested by measuring the level of freezing behaviour displayed by the rats upon re-exposure to the CS in the absence of the US. Freezing behaviour is a conditioned response (CR) that involves complete cessation of movement in the rat with exception of breathing (Blanchard & Blanchard, 1969). The presence of freezing behaviour is an index that the CFM memory has been recalled (Fanselow, 1980). Consolidation of a CFM memory takes place in the hours immediately following the conditioning procedure (McGaugh, 2000). Reconsolidation of a CFM takes place in the hours immediately following a short retrieval session (eg. 2 min), while extinction of a CFM takes place in the hours immediately following a long retrieval session (eg. 10 min) (Barnes & Thomas, 2008).

1.2.2 Consolidation of CFC

1.2.2.1 Brain Regions Involved in Consolidation of CFM

The hippocampus has been shown to be necessary for contextual fear conditioned responses but not for cued fear conditioned responses, therefore the hippocampus is thought to have a sensory relay role in CFC (Phillips & LeDoux, 1992). It also is involved in forming a configural representation of the stimuli in the environment (Rudy & O'Reilly, 2001). Electrolytic lesions in the dorsal hippocampus of rats both pre-acquisition and shortly after acquisition of the CFM lead to deficits in freezing behaviour during CS re-exposure 24 hours post-acquisition of the CFM, despite the presence of freezing behaviour immediately after acquisition of the memory (Kim et al., 1993; Kim & Fanselow, 1992). Pharmacological lesion of the hippocampus before

CFM acquisition indicated that the hippocampus was not required for CFM acquisition but pharmacological lesion post-acquisition indicated that the hippocampus did have a role in CFM consolidation. Therefore it was concluded that CFC could occur in the absence of the dorsal hippocampus. The authors proposed a conceptual model suggesting that in the absence of the dorsal hippocampus the rat may associate the US with one cue from the context, and fear learning could be completed using a hippocampal-independent pathway (Maren et al., 1997). In this case the associative memory formed would not be a contextual fear memory but would be a cued fear memory. Additional studies have also concluded that systems other than the hippocampus can acquire context fear, but do so less efficiently than the hippocampus, as the hippocampus has been determined to be important in integrating multiple stimuli in a memory trace (Matus-Amat et al., 2004; Wiltgen et al., 2006; Moses et al., 2007). More recently gene expression studies are being used to try to identify roles of the different hippocampal subregions in consolidation of CFM (Kubik et al., 2008). The three main regions of interest within the hippocampus are the Cornu Ammonis area 1 (CA1), Cornu Ammonis area 3 (CA3) and dentate gyrus (DG). The CA1 and CA3 regions are mainly populated by large densely packed glutamatergic pyramidal neurons in addition to some smaller GABAergic interneurons (Klausberger & Somogyi, 2008). The DG region is mainly populated by small and densely packed glutamatergic granule cells in addition to some GABAergic basket cell interneurons, and at most 6% of the cells in the DG are neural progenitor cells (Treves et al., 2008; Cameron & McKay, 2001).

There have been many lesion studies providing evidence for the importance of the amygdala to the consolidation of CFC (LaLumiere et al., 2003; Sacchetti et al., 1999; Vazdarjanova & McGaugh, 1999). Studies have shown that the role of the lateral (LA) and basolateral (BLA) nuclei is distinct from the role of the central nucleus (CeN). Lesion studies in cued-fear conditioning studies have shown that the CeN is necessary for the expression of fear (Kim et al., 1993). In contrast, lesion of the BLA nucleus did not prevent CFM consolidation (Berlau & McGaugh, 2003) and rats could acquire a CFM with an inactivated BLA nucleus if intensive overtraining was used (Ponnusamy et al., 2007). These findings were used to suggest that there is a primary more efficient BLA-dependent pathway and alternate compensatory pathways capable of mediating fear (Ponnusamy et al., 2007). There is evidence that both the BLA and LA nucleus of the amygdala are sites where synaptic plasticity underlying the association of the CS and US take place (Fanselow & LeDoux, 1999; Kwon & Choi, 2009).

Furthermore, the amygdala has been shown to have a memory-modulating role in consolidation (McGaugh, 2004).

The regions of interest within the prefrontal cortex are the cingulate cortex, prelimbic cortex and infralimbic cortex; these are collectively known as the medial prefrontal cortex (mPFC). The differential contribution of the different regions was elucidated through the comparison of two separate cued-fear conditioning studies. Lesions of the ventral mPFC (ventral prelimbic and infralimbic regions) resulted in a prolonged fear response upon reintroduction to the CS (Morgan et al., 1993), whereas lesions of the dorsal mPFC (cingulate and dorsal prelimbic regions) resulted in increased freezing behaviour, thus indicating an increased fear response to the CS (Morgan & LeDoux, 1995). It has been suggested that the ventral mPFC is responsible for the updating of behavioural responses to changing stimuli, and that the dorsal mPFC could be responsible for the regulation or suppression of fear reactivity, for blocking out irrelevant stimuli or for discriminating whether a CS is present, thus making the whole episode more fear provoking (Morgan & LeDoux, 1995).

1.2.2.2 Neurocircuitry Supporting Consolidation of CFM

Memories are thought to be stored in the brain in neural networks. The neurocircuitry supporting consolidation of CFM has been moderately well characterized. Generally, information about the CS and US is projected to the BLA nucleus of the amygdala. Information associating the CS and US is then passed onto the central nucleus of the amygdala from which fear expression responses are controlled (LeDoux, 2000). In CFC the context information of the novel environment passes from the sensory regions of the cortex via the entorhinal cortex to the hippocampus. Here the different sensory inputs describing the context are processed and a representation of the spatial component of the CFC experience is encoded. Axons project from neurons of the entorhinal cortex to neurons in the DG, CA3 and CA1 regions of the hippocampus separately (perforant pathways). Within the hippocampus DG axons project to the CA3 (Mossy fibres) and then, in turn, CA3 axons project to the CA1 (Schaffer collaterals). Recurrent networks are present in the DG and the CA3 regions. The DG recurrent network is more complicated than that in the CA3 as the DG granule cells excite mossy cells, also in the DG, that then make modifiable excitatory connections back onto the granule cells. The recurrent network in the CA3 results from axons of CA3 pyramidal neurons exciting other CA3 pyramidal cells. The recurrent networks in the DG and CA3 are reciprocally connected as the

CA3 pyramidal cells have axon branches that produce excitatory feedback to the DG (see Lisman, 1999). Assignment of specific functions to the synaptic modifications occurring at all of the hippocampal synapses is not yet fully understood. However the monosynaptic pathway (entorhinal cortex to CA1) has been shown to be sufficient for incremental spatial learning but the full trisynaptic pathway (entorhinal cortex to DG to CA3 to CA1) is required for rapid one-trial contextual fear learning (Nakashiba et al., 2008). Lisman (1999) has proposed a model of hippocampal neural activity in LTM function. The CA1 has been proposed to have two functions. The CA1 compares the sensory reality arriving through the perforant pathway to the processed sensory input arriving through the Schaffer collateral from the CA3 region to identify match/mismatch of information. The CA1 also converts the hippocampal representation to a cortical one to enable cortical interpretation. A proposed function of the CA3 is to link different memories that occur at different times via a heteroassociative network. The autoassociative network in the DG has been proposed to enable accurate sequence recall (Lisman, 1999).

The CA1 and subiculum regions of the hippocampus project to the BLA nucleus of the amygdala (Canteras & Swanson, 1992). The BLA is thought to be a location of sensory convergence, between spatial information (CS) processed in the hippocampus and footshock (US) related information (LeDoux, 2000). Footshock information reaches the BLA through inputs from the posterior thalamus which is the terminal region of the spinothalamic tract (LeDoux, 1990a; LeDoux 1987). BLA neurons project via the central nucleus to brainstem regions that control the expression of fear (LeDoux, 2000) such as the ventral periaqueductal gray that triggers the expression of fear as indexed by freezing behaviour (LeDoux, 1988). Information representing the association between the spatial representation and the fear representation is also passed back to the hippocampus from the BLA for further processing that, in turn, leads to the storage of the CFM (Ponnusamy et al., 2007; McGaugh, 2004; Simons & Spiers, 2003).

The CA1 and subiculum are the only regions of the hippocampus to project to the mPFC, specifically the prelimbic, infralimbic and medial orbital cortex regions. The entire rostrocaudal extent of the prelimbic and infralimbic cortex receives projections from the CA1 and subiculum, while the medial orbital cortex only receives projections to its caudal region (Jay et al., 1989; Jay & Witter, 1991). Hippocampal projections to regions of the mPFC innervate all cortical cell layers, but are more densely distributed in layers V and VI of the dorsal region of the prelimbic cortex (Jay & Witter, 1991), where approximately 40% of the neurons can be activated by

hippocampal stimulation. In comparison, only 6% of the neurons in the cingulate cortex respond to hippocampal stimulation. The single-cell recordings in the cingulate cortex have longer latencies than observed in the prelimbic cortex, suggesting indirect polysynaptic activation is required for hippocampal stimulation of the neurons in the cingulate cortex (Laroche et al., 1990). The hippocampal excitatory inputs to the PFC have been shown to be glutamatergic (Jay et al., 1992; Carr & Sesack, 1996). During associative learning training a delayed increase in synaptic transmission has been detected in the hippocampo-prefrontal cortex pathway (Doyere et al., 1993). It has been suggested that the delayed changes at the hippocampal to prefrontal cortex synapses may correlate to a late consolidation process in which the hippocampus stabilizes a cortical representation of the learned event (Laroche et al., 1995). Indeed, imaging of activity-dependent genes have shown that the anterior cingulate cortex is activated by remote memory (Frankland et al., 2004). The hippocampo-prefrontal cortex circuits are active during a critical phase of spatial learning (Davis et al., 1998) and are therefore likely to be important for the executive processing and possibly modulation of the spatial representation propagated from the hippocampus during consolidation. Bidirectional regulation of synaptic strength in the CA1 and subiculum projections to the mPFC increases the range of processing of the hippocampal spatial representation (Laroche et al., 2000).

All of the neurocircuitry outlined within and between the hippocampus, amygdala and mPFC is thought to make up part of the neural network activated during the acquisition and/or consolidation of the CFM (Figure 1.4).

1.2.2.3 Molecular Mechanisms of the Consolidation of CFM

Molecular mechanisms involved in associative LTM have been determined by studying animals undergoing behavioural paradigms including CFC, cued-fear conditioning (Cued-FC), inhibitory avoidance (IA), conditioned taste aversion (CTA), appetitive (drug) conditioning, spatial memory, olfactory memory, object recognition memory and startle response (see 1.2). However while some of the brain regions involved in the process of LTM formation in the different behavioural paradigms are the same, the complete set of brain regions over which the LTM is distributed differs between the paradigms. The molecular mechanisms of LTM discussed here will focus on those determined from CFC studies, but will also include molecular findings from studies using other behavioural paradigms when the molecular activity identified was in the hippocampus, amygdala or prefrontal cortex. There are three main groups

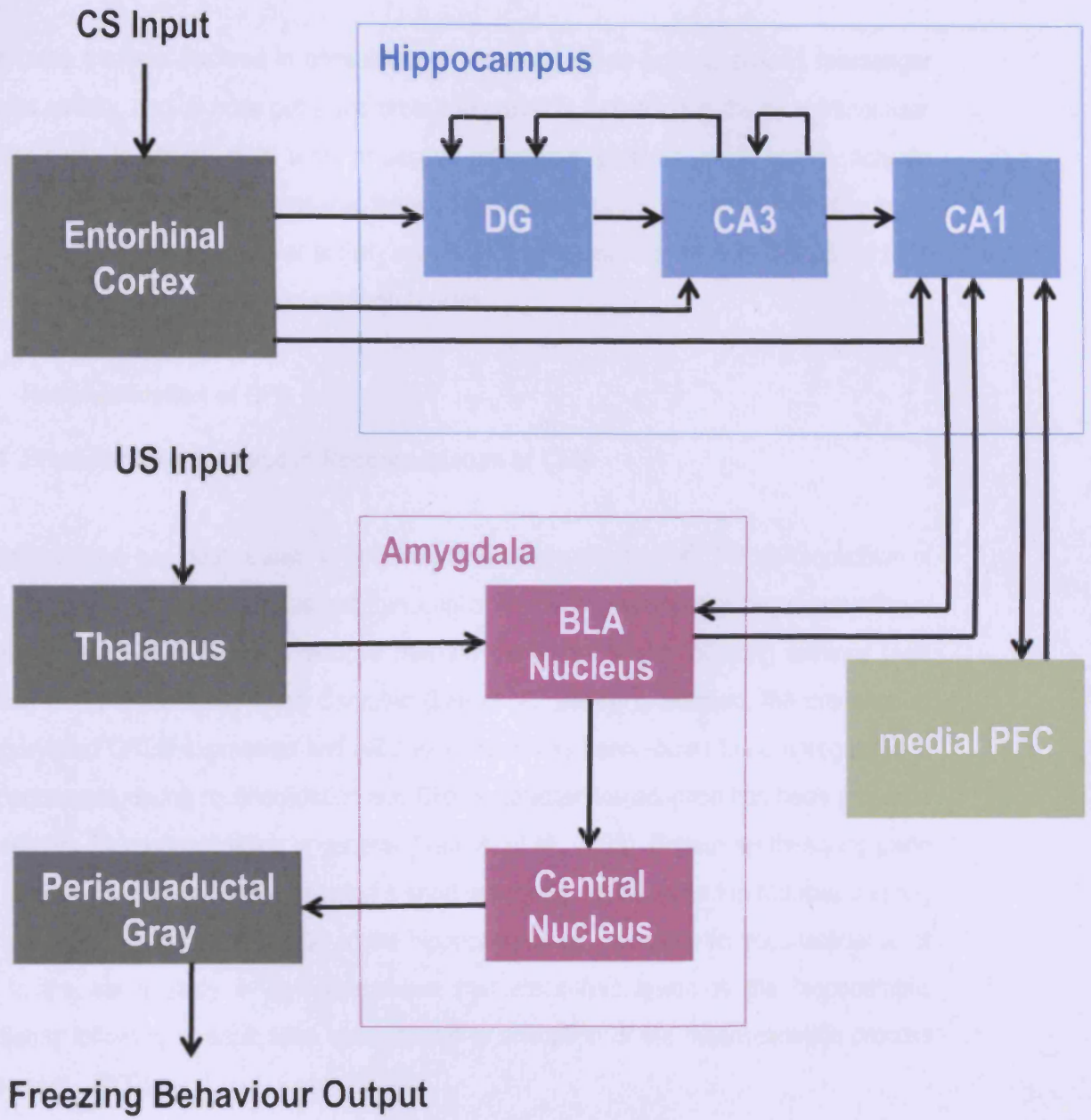


Figure 1.4. Neurocircuitry supporting consolidation of CFM. The entorhinal cortex receives environmental CS information from sensory cortex projections. The entorhinal cortex then projects to the DG, CA3 and CA1, known as the perforant pathways. The DG projects projects to the CA3 via the mossy fibres and the CA3 projects to the CA1 via the Schaffer collaterals. Recurrent networks are present in both the DG and CA3, and branches of CA3 axons also project back to the DG. The CA1 projects the processed CS information to the BLA nucleus of the amygdala, which also receives thalamic projections relaying aversive sensory US information. The integrated CS-US information is then passed along projections to the central nucleus and to the periaquaductal gray that initiates freezing behaviour, and other regions of the hypothalamus and brainstem to orchestrate other behavioural and autonomic changes. Integrated CS-US information is also projected back to the CA1 and then medial PFC for comparison to previously stored memories and storage of the CS-US memory.

of molecules that are involved in consolidation; receptor related activity, second messenger molecular activity, and *de novo* gene and protein expression. Activation of the neurotransmitter receptors leads to activity in an array of second messenger proteins, which in turn, activate downstream nuclear substrates that lead to *de novo* gene and protein expression (Rodrigues et al., 2004). Table 1.1 lists molecular activity required for or correlating with consolidation of LTM in the hippocampus, amygdala and prefrontal cortex.

1.2.3 Reconsolidation of CFC

1.2.3.1 Brain Regions Involved in Reconsolidation of CFM

The hippocampus has been shown to be involved in reconsolidation of CFM as knockdown of *Zif268* expression, protein synthesis and transcription in the hippocampus during reactivation of a consolidated CFM resulted in reduced freezing behaviour in the following retrieval tests indicating that the CFM had been disrupted (Lee et al., 2004). In addition, the presence of phosphorylated CREB expression and ARC expression has been shown to be upregulated in the hippocampus during reconsolidation and CREB-mediated transcription has been shown to be necessary for reconsolidation in general (Mamiya et al., 2009). Protein synthesis inhibition in the hippocampus immediately following a short reactivation trial resulted in reduced freezing behaviour thereby implicating activity in the hippocampus as necessary for reconsolidation of CFM. In the same study it was also shown that electrolytic lesion to the hippocampus immediately following a reactivation session lead to disruption of the reconsolidation process (Debiec et al., 2002).

The amygdala was first found to be necessary for reconsolidation using a cued-fear conditioning paradigm (Nader et al., 2000). Since then the amygdala has been implicated as having a role in reconsolidation of CFM as phosphorylated CREB expression has been shown to be upregulated in the amygdala during reconsolidation and CREB-mediated transcription has been shown to be necessary for reconsolidation in general (Mamiya et al., 2009). A cued-fear conditioning study also supports the involvement of the amygdala in the reconsolidation, as infusions of a partial agonist of NMDA receptors into the amygdala resulted in potentiation of reconsolidation (Lee et al., 2006).

Table 1.1. Molecules involved in the consolidation of LTM in the hippocampus, amygdala and prefrontal cortex.

MOLECULAR ACTIVITY	TASK	CAUSAL ROLE?	REFERENCE
HIPPOCAMPUS			
Receptor Related Activity			
NMDAR activity	Spatial M	✓	Bannerman et al., 1995
Increased proteolysis of proBDNF	CFM	✓	Barnes & Thomas, 2008
Second Messenger Molecular Activity			
Anchoring of PKA to AKAPs	CFM	✓	Nijholt et al., 2008
Glucocorticoid activation of MAPK cascade	Cued-FC	✓	Revest et al., 2005
Increased cAMP levels	IA	✓	Bernabeu et al., 1997
Increased PKA levels	IA	✓	Bernabeu et al., 1997
P38 MAPK activity	IA	✓	Rossato et al., 2006
Increase in C/EBP beta & delta activity	IA	✓	Taubenfeld et al., 2001
PKC activity	Spatial M	✓	Bonini et al., 2007
alpha CaMKII	Spatial M	✓	Mayford et al., 1996
De Novo Gene & Protein Expression			
CREB	Spatial M	✓	Guzowski & McGaugh, 1997
Increased CRE-mediated gene expression	CFM	✓	Impey et al., 1998
BDNF expression	CFM	✓	Lee et al., 2004
Increased *NGFI-B* expression	CFM	✓	Von Hertzen & Giese, 2005
Increased Arc expression	Spatial M	✓	Guzowski et al., 2001
Increased c-Fos expression	Spatial M	-	Guzowski et al., 2001
Increased Egr1 expression	Spatial M	-	Guzowski et al., 2001
EGR1 expression	CFM	✗	Lee et al., 2004
AMYGDALA			
Receptor Related Activity			
NMDAR activity	Cued-FC	✓	Rodrigues et al., 2001
Movement of AMPARs to post-synaptic spines	Cued-FC	?	Rumpel et al., 2005
Second Messenger Molecular Activity			
Persistant PKM zeta actitivity	Cued-FC	✓	Serrano et al., 2008
PI-3K activity	Startle R	✓	Lin et al., 2003
MAPK activity	Startle R	✓	Lin et al., 2003
De Novo Gene & Protein Expression			
Increased CRE-mediated gene expression	CFM	✓	Impey et al., 1998
Increased Egr1 expression	CFM	-	Malkani & Rosen, 2000
Increased Bdnf transcripts with exons I & III	Cued-FC	-	Rattiner et al., 2004

Table 1.1. Continued.

MOLECULAR ACTIVITY	TASK	CAUSAL ROLE?	REFERENCE
PREFRONTAL CORTEX			
Receptor Related Activity			
NR2B subunit of the NMDAR	CFM	✓	Zhao et al., 2005
NMDAR activity	Olfactory M	✓	Tronel & Sara, 2003
AMPAR activity	IA	✓	Izquierdo et al., 2007
Dopamine D1 receptor activity	IA	✓	Izquierdo et al., 2007
Noradrenaline activity	Olfactory M	✓	Tronel et al., 2004
Reduced serotonin R mRNA expression	Pav/Instrl M	-	Huerta-Rivas et al., 2010
Second Messenger Molecular Activity			
alpha CaMKII	Spatial M	✓	Mayford et al., 1996
De Novo Gene & Protein Expression			
Increased *NCAM* expression in ventral PFC	Spatial M	-	Ter Horst et al., 2008
Reduced *NCAM* expression in ventral PFC	Spatial M	-	Ter Horst et al., 2008
Increased c-FOS expression	Cued-FC	✓	Morrow et al., 1999
Increased Syntaxin 1B expression	Spatial M	-	Davis et al., 1998

At present the only evidence that may suggest a role for the medial PFC region in reconsolidation of CFM is that inducible repression of CREB activity in the cortex (in addition to the hippocampus, amygdala and stratum) during reconsolidation impaired subsequent freezing behaviour upon re-exposure to the CS (Kida et al., 2002), and upregulation of *Egr1* has been observed in the prefrontal cortex following a short retrieval session (Thomas et al., 2002). However it has since been shown that the presence of phosphorylated CREB expression in the prelimbic and infralimbic cortex does not change in association with reconsolidation (Mamiya et al., 2009). Thus far, evidence for the involvement of the medial prefrontal cortex in the reconsolidation of CFM is inconclusive. However NMDA receptor activity in the prefrontal cortex has been shown to be involved in the reconsolidation of object recognition memory (Akirav & Maroun, 2006).

1.2.3.2 Neurocircuitry Supporting Reconsolidation of CFM

The neurocircuitry supporting consolidation of CFM has not yet been characterized. However speculation on the neurocircuitry underlying reconsolidation could be made by comparison to the neurocircuitry underlying consolidation as both the hippocampus and amygdala have been implicated in both consolidation and reconsolidation of CFM. Information related to the CS may pass from the sensory regions of the cortex via the entorhinal cortex to the hippocampus where a representation of the spatial component related to the CS exposure may be encoded. Connectivity between neurons of the amygdala and the hippocampus may be active during reconsolidation. Information relating to the CS-US association may be present in the BLA nucleus of the amygdala and output from the central nucleus of the amygdala may control fear expression responses. By comparison to consolidation the prefrontal cortex may be involved in a comparator/modulatory role and might be a site of storage for the reconsolidated memory but currently there is inconclusive findings as to whether the prefrontal cortex is even activated during reconsolidation.

1.2.3.3 Molecular Mechanisms of the Reconsolidation of CFM

Table 1.2 lists molecular activity required for or correlating with reconsolidation of LTM in the hippocampus, amygdala and prefrontal cortex.

Table 1.2. Molecules involved in the reconsolidation of LTM in the hippocampus, amygdala and prefrontal cortex.

MOLECULAR ACTIVITY	TASK	CAUSAL ROLE?	REFERENCE
HIPPOCAMPUS			
Receptor Related Activity			
NR2B subunit labilisation	Cued-FC	✓	Ben Mamou et al., 2006
IL-1 receptor activity	CFM	✓	Barnes et al., *
Second Messenger Molecular Activity			
PKC activity	Spatial M	✓	Bonini et al., 2007
De Novo Gene & Protein Expression			
CREB activity	CFM	✓	Mamiya et al., 2009
EGR1 activity	CFM	✓	Lee et al., 2004
Increased *IL6* expression	CFM	-	Barnes et al., *
Increased IL1a expression	CFM	-	Barnes et al., *
Increased *Rara* expression	CFM	-	Barnes et al., *
Increased *Rgs1* expression	CFM	-	Barnes et al., *
Increased *Trrp5* expression	CFM	-	Barnes et al., *
Increased *Grpr* expression	CFM	-	Barnes et al., *
Decreased *Actg2* expression	CFM	-	Barnes et al., *
Increased *SGK3* expression	CFM	-	Von Hertzen & Giese, 2005
AMYGDALA			
Receptor Related Activity			
NMDAR activity	Cued-FC	✓	Lee et al., 2006
beta-Adrenergic activity	Cued-FC	✓	Debiec & Ledoux, 2004
CB1 receptor activity	Cued-FC	✓	Bucherelli et al., 2006
Second Messenger Molecular Activity			
ERK activity	Cued-FC	✓	Duvarci et al., 2005
PKA activity	Cued-FC	✓	Tronson et al., 2006
De Novo Gene & Protein Expression			
CREB activity	CFM	✓	Mamiya et al., 2009
EGR1 activity	Drug M	✓	Lee et al., 2005
C/EBP beta activity	IA	✓	Tronel et al., 2005

Table 1.2. Continued.

MOLECULAR ACTIVITY	TASK	CAUSAL ROLE?	REFERENCE
PREFRONTAL CORTEX			
Receptor Related Activity			
NMDAR activity	Obj R M	-	Akirav & Maroun, 2006
Second Messenger Molecular Activity			
De Novo Gene & Protein Expression			
CREB activity	CFM	✓	Kida et al., 2002
Increased Egr1 expression	CFM	-	Thomas et al., 2002

1.2.4 Extinction of CFC

1.2.4.1 Brain Regions Involved in Extinction of CFM

Evidence that the hippocampus is involved in extinction of CFM includes the finding that pharmacological inactivation of the dorsal hippocampus using muscimol prior to extinction training attenuated extinction learning (Corcoran et al., 2005). Molecular activity in the hippocampus has also implicated the hippocampus in extinction of CFM. Inhibition of actin rearrangement and extracellular regulated kinases 1 and 2 (ERK1/2) activity in the hippocampus leads to impaired extinction of CFM, and inhibition of protein synthesis in the hippocampus lead to enhanced extinction of CFM (Fischer et al., 2004; Fischer et al., 2007). Also implicating the hippocampus in extinction of CFM is the finding that decreased proteolysis of proBDNF in the hippocampus has been shown to be necessary for the extinction of CFM (Barnes and Thomas, 2008). In addition, inhibition of histone deacetylase (HDAC) in the hippocampus resulted in enhanced extinction of CFM (Lattal et al., 2007), and hippocampal endocannabinoid activity is necessary for the extinction of CFM (De Oliveira Alvares et al., 2008). Finally the hippocampus has also been shown to be involved in extinction as inhibition of Src-family tyrosine kinases (SFKs) and Src homology 2-containing protein-tyrosine phosphatases 1 and 2 (SHP1/2) in the dorsal hippocampus lead to facilitated and suppressed extinction of CFM respectively (Isosaka et al., 2009; Isosaka & Yuasa, 2010).

The amygdala has been implicated in extinction of CFM through multiple studies. Inactivation of populations of neurons in the BLA nucleus of the amygdala has been shown to prevent CFM extinction expression (Herry et al., 2008). An important recent finding from a study using the cued-fear conditioning paradigm has shown that the lesioning or activation of the intercalated cell masses (ICM) in the amygdala impair or facilitate extinction respectively (Likhtik et al., 2008; Jungling et al., 2008). Molecular activity that implicates the amygdala in extinction of CFM includes the finding that phosphorylated CREB expression has been shown to be upregulated in the amygdala during extinction and CREB-mediated transcription has been shown to be necessary for extinction in general. In addition blocking protein synthesis in the amygdala prevented the formation of extinction memory (Mamiya et al., 2009). In addition, pharmacological enhancement of AMPA receptor activity in the amygdala has also been shown to facilitate extinction of CFM (Yamada et al., 2009), and inactivation of the BLA nucleus of the

amygdala by a GABA A agonist immediately after an extinction session resulted in disruption to the extinction of CFM process (Laurent & Westbrook, 2008).

The prefrontal cortex has been implicated in extinction of CFM through both pharmacological studies and studies identifying molecular activity. Inactivation of the infralimbic cortex (IL) region of the mPFC using a cannabinoid antagonist has been shown to impair extinction of CFM (Laurent & Westbrook, 2009) and extinction of cued-fear conditioning was impaired when the IL region was inactivated by a dopamine D1 antagonist (Hikind & Maroun, 2008). Inactivation of the prelimbic cortex (PrL) region of the mPFC has been shown to reduce expression of conditioned fear to contextual and auditory stimuli but have no effect on plasticity related to extinction (Laurent & Westbrook, 2008). Molecular activity that implicates the mPFC in extinction includes the finding that phosphorylated CREB expression was upregulated in the mPFC during extinction and CREB-mediated transcription was required for extinction of CFM. Also inhibition of protein synthesis in the prefrontal cortex prevented formation of extinction (Mamiya et al., 2009). In addition, activation of the NMDAR containing the NR2B subunit in the mPFC is necessary for CFM extinction, and inactivation of the mPFC by a GABA-A agonist immediately after an extinction session disrupted CFM extinction (Laurent & Westbrook, 2008). In support of these findings implementing the PFC in extinction of CFM, lesioning of the ventral mPFC impaired extinction of cued-fear conditioning (Morgan et al., 1993).

1.2.4.2 Neurocircuitry Supporting Extinction of LTM

The neurocircuitry supporting extinction of CFM is in the process of being characterized. As discussed above, there is evidence for activity in the amygdala, prefrontal cortex and hippocampus during extinction of LTM. In extinction of CFM, as in consolidation of CFM, the central nucleus receives information from the BLA nucleus of the amygdala and then sends it to regions of the brainstem controlling the expression of fear. As memory becomes extinguished, reduced freezing behaviour can be observed in the animal upon re-exposure to the context (Sah & Westbrook, 2008). Recently a subpopulation of neurons in the BLA, called extinction neurons, have been discovered to selectively respond to a conditioned stimulus undergoing extinction. The activity of these neurons has been shown to be crucial for extinction learning. These extinction neurons are connected to the mPFC in both directions (Herry et al., 2008). Neurons from the mPFC have been shown to densely innervate clusters of inhibitory neurons known as the intercalated cell masses (ICMs) (McDonald et al., 1996). It has been

shown that a decrease in activation of these ICM neurons can result in reactivation of an extinguished fear response and so the ICM neurons have been proposed to be necessary for the expression of learned extinction (Likhtik et al., 2008). These findings are consistent with the finding that stimulation of the IL region of the mPFC prevents Ce nucleus neurons from being activated by BLA nucleus inputs (Quirk et al., 2003). Therefore the mPFC is thought to modulate the level of activity in the amygdala leading to reduced output from the central nucleus of the amygdala during extinction (Sah & Westbrook, 2008). In addition, protein synthesis and gene expression in the mPFC has been observed following an extinction session suggests that the mPFC is a site of consolidation and storage of fear extinction (Santini et al., 2004; Herry & Mons, 2004). The hippocampus, as in consolidation of CFM, processes the sensory input to encode the spatial information present during the extinction session. The hippocampus projects this information to the mPFC and amygdala to process the specificity of the extinction to a particular context (Sah & Westbrook, 2008).

All of the neurocircuitry outlined within and between the hippocampus, amygdala and mPFC is thought to make up part of the neural network activated during the extinction of the CFM (Figure 1.5).

1.2.4.3 Molecular Mechanisms of the Extinction of CFM

Table 1.3 lists molecular activity required for or correlating with extinction of LTM in the hippocampus, amygdala and prefrontal cortex.

1.2.5 Comparison of Consolidation, Reconsolidation and Extinction of LTM

There are both similarities and differences between consolidation, reconsolidation and extinction of LTM in terms of the brains regions, neurocircuitry and underlying molecular mechanisms involved. The hippocampus, amygdala and prefrontal cortex are involved in consolidation, reconsolidation and extinction of LTM (see 1.2.2.1, 1.2.3.1 and 1.2.4.1). The neurocircuitry differs between consolidation, reconsolidation and extinction as each process is triggered by different circumstances (Nader & Hardt, 2009). An example of differing neurocircuitry between consolidation and extinction of CFM is the activation of fear neurons in the BLA during consolidation only, and the activation of extinction neurons in the BLA during extinction only (Herry et al., 2008). While consolidation and reconsolidation both involve

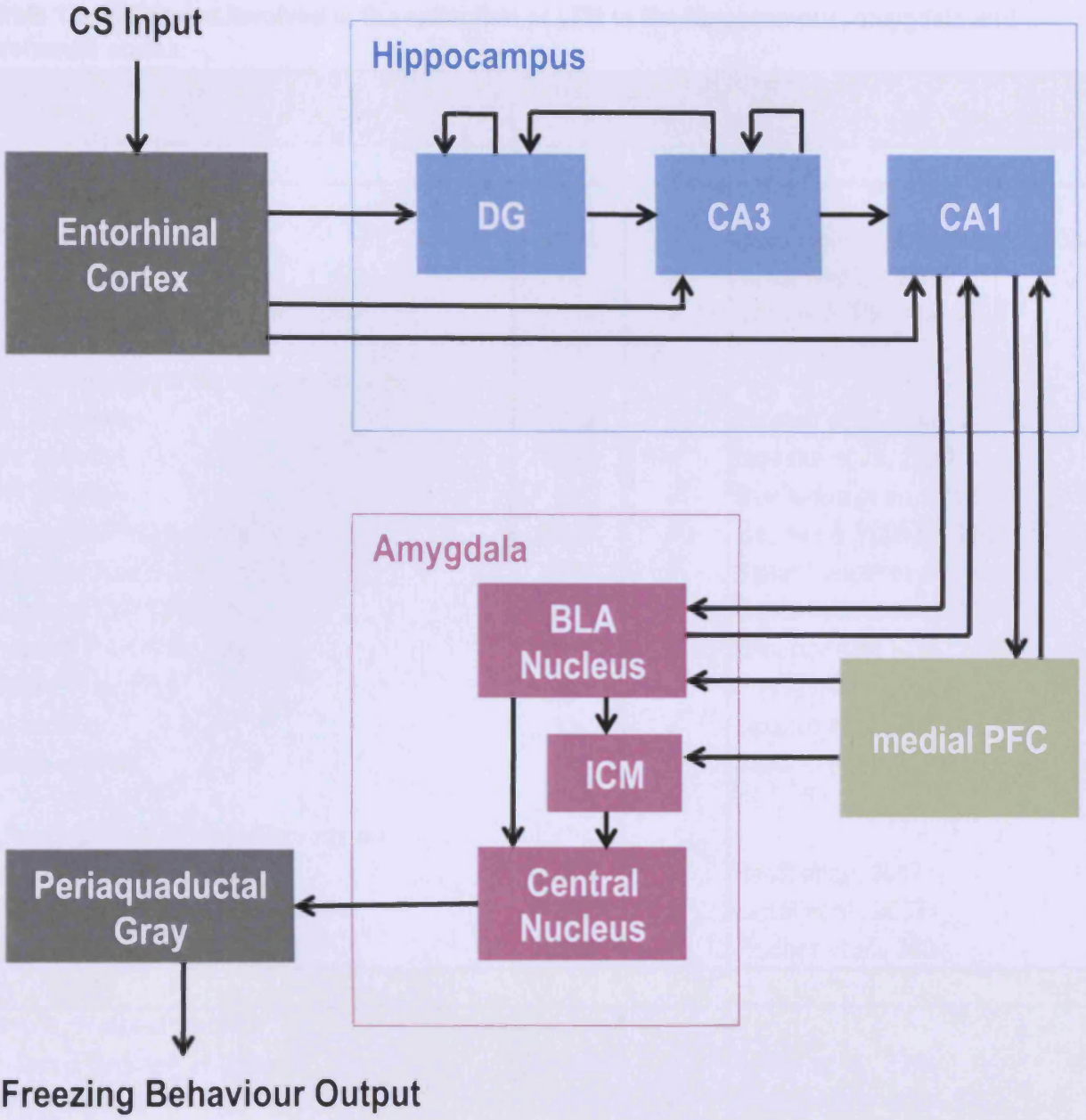


Figure 1.5. Neurocircuitry supporting extinction of CFM. The entorhinal cortex receives environmental CS information from sensory cortex projections. The entorhinal cortex then projects to the DG, CA3 and CA1, known as the perforant pathways. The DG projects projects to the CA3 via the mossy fibres and the CA3 projects to the CA1 via the Schaffer collaterals. Recurrent networks are present in both the DG and CA3, and branches of CA3 axons also project back to the DG. The CA1 projects the processed CS information to the BLA nucleus of the amygdala. Stored integrated CS-US information is then passed along projections to the central nucleus and to the periaquaductal gray that initiates freezing behaviour, and other regions of the hypothalamus and brainstem to orchestrate other behavioural and autonomic changes. In the absence of an aversive sensory US, extinction neurons within the BLA project to the ICM, from which inhibitory neurons project to the central nucleus, inhibiting the expression of fear. Neurons in the medial PFC project to the extinction neurons in the BLA nucleus of the amygdala and to the ICM to modulate their activity. Integrated CS-US information is also projected back to the CA1 and then medial PFC for comparison to previously stored memories and storage of the CS-US memory.

Table 1.3. Molecules involved in the extinction of LTM in the hippocampus, amygdala and prefrontal cortex.

MOLECULAR ACTIVITY	TASK	CAUSAL ROLE?	REFERENCE
HIPPOCAMPUS			
Receptor Related Activity			
Endocannabinoid activity	CFM	✓	De Oliveira Alvares et al., 2008
NMDAR activity	IA	✓	Szapiro et al., 2003
Decreased proteolysis of proBDNF	CFM	✓	Barnes & Thomas, 2008
Second Messenger Molecular Activity			
ERK1/2 activity	CFM	✓	Fischer et al., 2007
SFKs activity	CFM	✓	Isosaka et al., 2009
SFKs activity	IA	✓	Bevilaqua et al., 2003
Reduced SHP1/2 activity	CFM	✓	Isosaka & Yuasa, 2010
Decreased Rac-1 activity	CFM	✓	Sananbenesi et al., 2007
Decreased Cdk5 activity	CFM	✓	Sananbenesi et al., 2007
Increased PAK-1 activity	CFM	✓	Sananbenesi et al., 2007
P38 MAPK activity	IA	✓	Rossato et al., 2006
PKA activity	IA	✓	Szapiro et al., 2003
CaMKII activity	IA	✓	Szapiro et al., 2003
De Novo Gene & Protein Expression			
BDNF activity	CFM	✓	Heldt et al., 2007
Inhibition of HDAC	CFM	✓	Lattal et al., 2007
Actin rearrangement	CFM	✓	Fischer et al., 2004
AMYGDALA			
Receptor Related Activity			
Increased GABA-A activity	Cued-FC	✓	Akirav et al., 2006
GRPR activity	Cued-FC	✓	Shumyatsky et al., 2002
NMDAR activity	Startle R	✓	Lin et al., 2003
Second Messenger Molecular Activity			
PSA-NCAM activity	Cued-FC	-	Markram et al., 2007
PI-3K activity	Startle R	✓	Lin et al., 2003
MAPK activity	Startle R	✓	Lin et al., 2003
De Novo Gene & Protein Expression			
CREB activity	CFM	✓	Mamiya et al., 2009
CREB activity	Startle R	-	Lin et al., 2003
Increased insertion of GABA-A R into synapse	Cued-FC	✓	Lin et al., 2009
Increased *Bdnf* mRNA expression	Cued-FC	-	Chhatwal et al., 2006
Increased c-FOS protein expression	Cued-FC	-	Herry & Mons, 2004
Increased EGR1 protein expression	Cued-FC	-	Herry & Mons, 2004
Increased Calcineurin expression	Startle R	-	Lin et al., 2003

Table 1.3. Continued.

MOLECULAR ACTIVITY	TASK	CAUSAL ROLE?	REFERENCE
PREFRONTAL CORTEX			
Receptor Related Activity			
AMPAR activity	CFM	✓	Zushida et al., 2007
NMDAR activity	CFM	✓	Suzuki et al., 2004
NMDAR activity	Cued-FC	✓	Burgos-Robles et al., 2007
CB1R activity	CFM	✓	Suzuki et al., 2004
L-VGCC activity	CFM	✓	Suzuki et al., 2004
GABA-A activity	Cued-FC	✓	Akirav et al., 2006
beta-Adrenergic activity	Cued-FC	✓	Mueller et al., 2008
Second Messenger Molecular Activity			
MAPK activity	Cued-FC	✓	Hugues et al., 2004
PKA activity	Cued-FC	✓	Mueller et al., 2008
De Novo Gene & Protein Expression			
CREB activity	CFM	✓	Mamiya et al., 2009
HDAC inhibition increased BDNF activity	Cued-FC	✓	Bredy et al., 2007

stabilization of a labile memory, the molecular mechanisms underlying these processes have been shown to be both similar (Kida et al., 2002) and doubly dissociable (Lee et al., 2004). Reconsolidation and extinction are both activated by retrieval of a memory, but have been shown to have distinct temporal and biochemical signatures (Suzuki et al., 2004) as well as similarities (Mamiya et al., 2009). Consolidation and extinction are both considered to be new learning, but again there are both similarities and difference in the molecular mechanisms underlying the two processes (Lin et al., 2003). Finally, within a memory process the molecular activity can vary between brain regions that are part of the engram (Tronel et al., 2005; Mamiya et al., 2009).

1.3 Synaptic Plasticity

1.3.1 Molecular Mechanisms of LTM and Synaptic Plasticity

Changes in neuronal molecular activity in regions of the brain including the hippocampus, amygdala and mPFC are necessary for the consolidation, reconsolidation and extinction of associative LTM (see 1.2.2.3, 1.2.3.3 and 1.2.4.3). In particular, *de novo* gene expression and protein synthesis takes place following new learning and retrieval of memories that is thought to modulate the strength of some of the activated synaptic connections within the engram. The ability for the efficacy of communication between neurons to change at the site of the synapse when neurons are active together is one form of what is generally known as synaptic plasticity. In 1949, Hebb postulated that 'when an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased' (Hebb, 1949). In 1973 Bliss and Lomo discovered an artificial form of plasticity in which brief, high-frequency electrical stimulation of the excitatory perforant pathway (neurons originating from the entorhinal cortex and terminating on neurons in the dentate gyrus) produced a long-lasting enhancement in the strength of the stimulated synapses (Bliss & Lomo, 1973). This type of synaptic plasticity became known as long-term potentiation (LTP). In 1982 the BCM theory was posited suggesting that synaptic plasticity could be bidirectional. In contrast to the strong postsynaptic depolarization that was necessary for LTP to take place, long-lasting weakening in the strength of stimulated synapses was predicted to take place if the postsynaptic cell was only weakly depolarized (Bienenstock et al., 1982). Long-lasting weakening of the strength of

synapses was experimentally induced by prolonged, low frequency electrical stimulation of the excitatory Schaffer collaterals (neurons originating from the CA3 and terminating on neurons in the CA1) (Stanton & Sejnowski, 1989). This type of synaptic plasticity became known as long-term depression (LTD). While low level depolarisation results in LTD at the active synapses, depolarisation above a certain level, known as the modification threshold, results in LTP at the active synapses.The physiological substrate of this modification threshold is proposed to be the NMDA receptor, and the threshold is proposed to change dynamically to homeostatically regulate the excitability of the cell (Stanton, 1996). LTP and LTD have been detected to take place in neurons in other regions of the brain including in neurons of the prefrontal cortex (Laroche et al., 1990; Takita et al., 1999) and neurons found in some nuclei of the amygdala (Chapman et al., 1990; Wang & Gean, 1999). However while some molecular mechanisms found to be underlying LTP in different brain regions appear to be the same, differences have also been found (Bear et al., 2007). LTP and LTD, most commonly investigated in the hippocampus, have become models for studying the molecular mechanisms underlying the consolidation, reconsolidation and extinction of LTM. The synaptic plasticity and memory (SPM) hypothesis posits that 'activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which the plasticity is observed' (Martin et al., 2000). However the research effort to prove or disprove the SPM hypothesis has been pursued for the last 22 years and so far there is no conclusive evidence either way (Neves et al., 2008).

LTP studies in glutamatergic synapses in the hippocampus have shown that glutamate released from pre-synaptic cells binds to AMPA receptors in the membrane of post-synaptic cell dendritic spines. The AMPA receptor ion channels open and Na+ ions pass into the postsynaptic cell. As positively charged ions enter into the post-synaptic cell it becomes depolarized. If glutamate binds to the NMDA receptor ion channel, at the same time as the post-synaptic cell becomes sufficiently depolarized such that the Mg^{2+} ion no longer blocks the ion pore, then Ca^{2+} ions flood into the dendritic spine along the membrane potential. In this way the NMDA receptor acts as a coincidence detector. The increase in the concentration of intracellular Ca^{2+} then leads to second messenger activity, such as the phosphorylation of kinases in the dendritic spine, that may result in more AMPA receptors being inserted into the dendritic spine membrane. The presence of more AMPA receptors in the dendritic spine will increase the sensitivity of the spine to glutamate release in the future and therefore the

strength of the synapse becomes increased. Insertion of AMPA receptors into the spine is positively correlated with the spine size. *De novo* gene expression is activated to support the structural changes within the dendritic spine and the modifications to signal transduction (LeDoux, 2000; Bear et al., 2007; Yuste & Bonhoeffer, 2001). The products of *de novo* gene expression in the nucleus diffuse throughout the neuron and are thought to be "captured" only by the activated synapses that have a molecular tag. This hypothesis is known as the synaptic tagging and capture hypothesis (Frey & Morris, 1997; Barco et al., 2008). In addition to postsynaptic modifications, presynaptic modifications that alter the quantity of neurotransmitter released and the rate of reuptake of neurotransmitter from the synaptic cleft have also been proposed to alter the strength of a synaptic connection during synaptic plasticity (Bliss & Collingridge, 1993). The molecular mechanisms underlying the strength of the synapses in LTP may also underlie the strength of the synapses that are part of the memory engram.

A number of molecules that have been shown to be necessary for both LTP and LTM in the post-synaptic density include the kinases CamKII (Malinow et al., 1989; Izquierdo et al., 2000), PKA (Frey et al., 1993; Izquierdo et al., 2000), PKC (Malinow et al., 1989; Izquierdo et al., 2000), PKMζ (Osten et al., 1996; Shema et al., 2007), adenylyl cyclase (Wong et al., 1999), PI-3K (Man et al., 2003; Sui et al., 2008), MAPK (English & Sweatt, 1997; Izquierdo et al., 2000), ERK1/2 (Jones et al., 1999; Berman et al., 1998), and the scaffolding protein PSD-95 (Migaud et al., 1998). Genes that are expressed rapidly and transiently as part of the *de novo* gene response are known as the immediate early genes (IEG). It has been predicted that there are 30 – 40 IEG, and that 10 - 15 of these IEG would be transcription factors. Other IEG have been shown to include growth factors and to be involved in signal transduction and cytoskeletal rearrangement (Lanahan & Worley, 1998). Genes that are expressed less rapidly but still transiently are also expressed as part of the *de novo* gene response. *De novo* gene expression is generally thought to be completed within 8 hours post stimulation of the neuron. *De novo* gene expression that has been shown to be necessary for both LTP and LTM includes CREB (Bourtchuladze et al., 1994), EGR1 (Cole et al., 1989; Jones et al., 2001), BDNF (Patterson et al., 1996; Alonso et al., 2002) and ARC (Guzowski et al., 2000). However while genes necessary for LTP may have also been shown to be necessary in LTM studies, there are occasions where genes have been shown to be required for LTP but not required for LTM of a particular task. For example *Egr1* and *syntaxin 1B* expression has been shown to be necessary for LTP but not for spatial learning in a water maze task. It is suggested that the genes activated following LTP are likely to be more general and more spatially extensive than the

genes activated following a single learning episode. Therefore it was suggested that future comparisons between LTP and LTM should be considered in respect to specific LTM paradigms separately (Richter-Levin et al., 1998). Comparison of LTP and LTM findings indicate that molecules that have been implicated in synaptic plasticity are reasonable molecules to investigate for having a role in LTM, but no molecule found to be necessary for LTP could be interpreted as also being necessary for LTM.

1.3.2 Schizophrenia Susceptibility Genes and Synaptic Plasticity

Abnormal neuronal development leading to abnormal neuronal circuitry and function has been implicated in schizophrenia (Weinberger, 1987; Waddington, 1993). Schizophrenia susceptibility genes that are known to have a functional role in synaptic plasticity, in addition to being correlated with schizophrenia through gene association, linkage and postmortem studies, are considered to be the genes most likely to contribute to schizophrenia pathophysiology (Harrison & Weinberger, 2005). The involvement of the schizophrenia susceptibility genes of interest for this thesis, *Nrg1*, *Dtnbp1*, *Disc1*, *Egr3* and *Bdnf*, in synaptic plasticity is discussed below.

1.3.2.1 *Nrg1* and Synaptic Plasticity

Several studies suggest that *Nrg1* is involved in synaptic plasticity. In glutamatergic synapses presynaptic neuregulin1 protein (NRG1) is proteolytically cleaved and the N-terminal fragment of the protein binds to postsynaptic receptor tyrosine kinases ErbB3 or ErbB4 modulating the localisation of NMDA receptors to the postsynaptic membrane (Huang et al., 2000; Falls, 2003). Activation of the ErbB receptors by NRG1 can also lead to postsynaptic gene transcription via the PI3K pathway, while presynaptic gene transcription can be activated by proteolytic cleavage of the NRG1 C-terminal domain (Falls, 2003; Mei & Xiong, 2008; see Fig. 1.6). Bath application of *Nrg1* has been shown to suppress induction of LTP in the hippocampal CA1 region without affecting basal synaptic transmission, paired pulse facilitation or disrupting NMDAR mediated currents, suggesting a postsynaptic role downstream of NMDAR activation (Huang et al., 2000). Also in the CA1 *Nrg1* has been shown to depotentiate LTP by reducing surface AMPA receptor expression in an activity and time dependent manner (Kwon et al., 2005). Disruption of NRG1/ERBB signalling in the hippocampal CA3-CA1 pathway has been shown to destabilize synaptic AMPA receptors and lead to a loss of synaptic NMDA currents

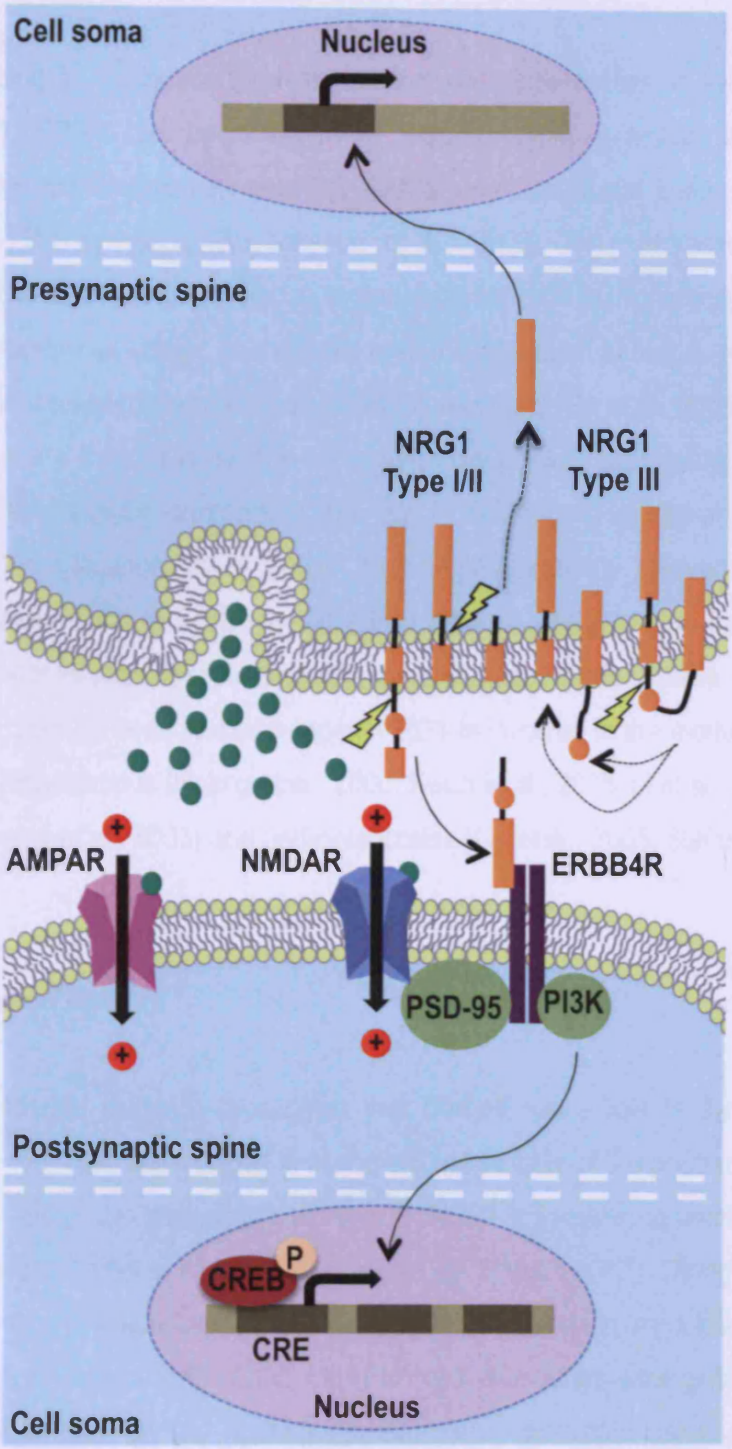


Fig. 1.6. NRG1 cellular interactions. As glutamate is released from the presynaptic terminal it binds to AMPARs and NMDARs that may lead to synaptic plasticity. NRG1 acts as a bidirectional neuromodulator. Proteolytic cleavage of the extracellular domain of NRG1 type I and II isoforms leads to this N-terminal domain fragment binding to ERBB2, ERBB3 or ERBB4 receptors in the postsynaptic membrane. Secondary messengers downstream of PI3K that is activated upon NRG1 binding to the ERBB receptors leads to gene transcription in the nucleus of the postsynaptic cell that will modulate the synapse. The intracellular C-terminal domain in the presynaptic neuron can also be proteolytically cleaved and lead to gene transcription in the presynaptic cell that will modulate the synapse. PSD-95 is a scaffolding protein that binds to the intracellular domain of the ERBB receptors upon NRG1 binding to the extracellular domain of the ERBB receptors. PSD-95 also binds to NMDARs and therefore NRG1 indirectly modulates the cellular localisation of NMDARs in the postsynaptic density.

and dendritic spines, leading to glutamatergic hypofunction and impairments in synaptic plasticity (Li et al., 2007). NRG1 has been shown to regulate synaptic activity in the hippocampus by potentiating transmission at entorhinal-dentate synapses and suppressing transmission at entorhinal-CA1 synapses (Roysommuti et al., 2003). *Nrg1* expression is upregulated in the DG and CA3 following stimulation of the perforant path that produced LTP (Eilam et al., 1998). In the prefrontal cortex, an increase in NRG1 has been shown to reduce NMDA receptor currents and increased internalisation of NMDA receptors (Gu et al., 2005). In contrast, Nrg1 +/- mice, that are thought to have a decreased level of *Nrg1* expression, have fewer functional NMDA receptors in the prefrontal cortex than in WT mice (Stefansson et al., 2002). NRG1 is a synaptic transmembrane protein that is proteolytically cleaved. The extracellular N-terminal domain binds to ErbB receptors in a paracrine or autocrine fashion. *Nrg1* acts as a neuromodulator through synaptic bidirectional release and binding (Falls, 2003; Ozaki et al., 2004; Li et al., 2007). These studies suggest NRG1 is involved in the modulation of synaptic plasticity in the hippocampus (Huang et al., 2000; Kwon et al., 2005; Li et al., 2007; Eilam et al., 1998; Roysommuti et al., 2003) and prefrontal cortex (Gu et al., 2005; Stefansson et al., 2002).

1.3.2.2 *Dtnbp1* and Synaptic Plasticity

Multiple studies have contributed evidence to suggest that *Dtnbp1* has a role in synaptic plasticity. Dysbindin protein (DTNBP1) is engaged in assembly and stability of the postsynaptic density in dendritic spines, and in the presynaptic terminal DTNBP1 is involved in priming of synaptic vesicles for exocytosis (Talbot et al., 2006; Tian et al., 2005; see Fig 1.7). *Dtnbp1* has been shown to be required presynaptically for the retrograde, homeostatic modulation of neurotransmssion (Dickman & Davis, 2009). Changes in *Dtnbp1* expression alter glutamate neurotransmission suggested that *Dtnbp1* influences exocytotic glutamate release via upregulation of the molecules in the pre-synaptic machinery (Numakawa et al., 2004). Larger vesicle size, slower quantal release, lower release probability and a smaller total population of the readily releasable vesicle pool have been found in mice with a *Dtnbp1* deletion (Chen et al., 2008). Also in mice with a *Dtnbp1* deletion there is an increase in expression of the NR2A subunit of NMDA receptors at the neuronal surface and an increase in NMDA-mediated synaptic currents and LTP at CA1 synapses in hippocampal slices (Tang et al., 2009b).

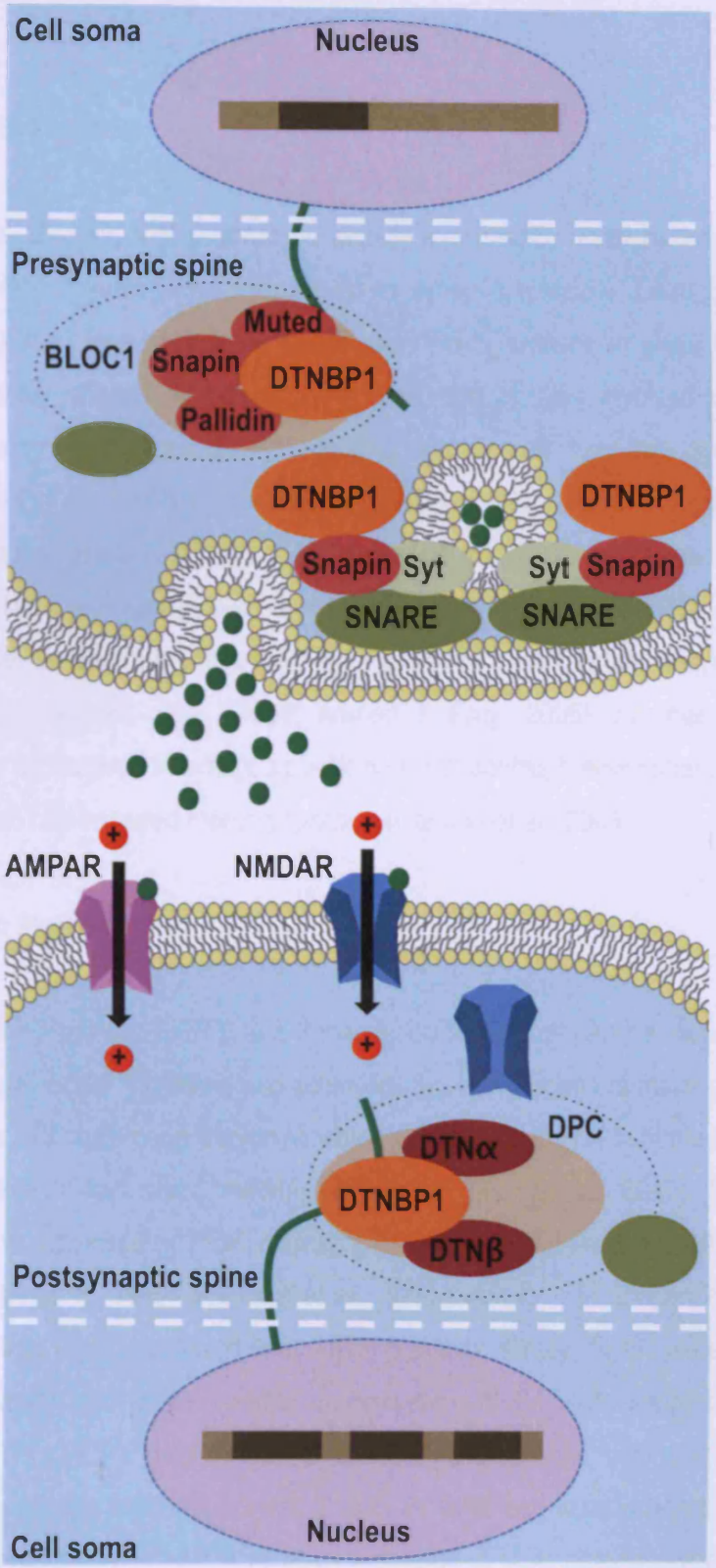


Fig. 1.7. DTNBP1 cellular interactions. As glutamate is released from the presynaptic terminal it binds to AMPARs and NMDARs that may lead to synaptic plasticity. DTNBP1 acts both presynaptically and postsynaptically to traffick proteins for maintenance or modification of the synapse. DTNBP1 in the postsynaptic density is part of the DPC which is thought to be involved in the trafficking and tethering of receptors, such as the NMDAR, and of signal transduction proteins (Harrison & Weinberger, 2005). DTNBP1 has two roles in the presynaptic terminal. It is part of the BLOC1 complex that is involved in protein trafficking and it is also involved in priming synaptic vesicles for release (Chen et al., 2008).

1.3.2.3 *Disc1* and Synaptic Plasticity

There is no direct evidence showing that *Disc1* is necessary for synaptic plasticity, however there is some evidence that suggests *Disc1* is involved in synaptic plasticity. Disrupted-in-schizophrenia 1 protein (DISC1) is a scaffolding protein that holds proteins in place in the postsynaptic density to allow efficient molecular responses and is also involved in the microtubule-associated transport of proteins and mitochondria to and from the synapse (Porteous et al., 2006; see Fig.1.8). DISC1 interacts with PDE4B such that in a resting cell DISC1 sequesters phosphodiesterase (PDE4B), but in response to cellular adenosine 3', 5'-monophosphate (cAMP) DISC1 releases PDE4B and PDE4B inactivates cAMP (Millar et al., 2005). Changes in cAMP levels and in the levels of PDE4B activity are important in synaptic plasticity (Frey et al., 1993; Ahmed et al., 2004; Ahmed & Frey, 2005). Another study suggesting that *DISC1* may be involved in synaptic plasticity is that schizophrenia patients with a particular *DISC1* allele also had impaired memory function (Cannon et al., 2005).

1.3.2.4 *Egr3* and Synaptic Plasticity

Early growth response factor 3 protein (EGR3) is a transcription factor that can be expressed as a result of both NRG1 and BDNF signalling and promotes the transcription of many genes including those that encode ARC, p75 neurotrophin receptor, GABA receptor α4 subunit, Nerve Growth Factor receptor, EGR3 and some miRNA sequences (Guo et al., 2010). EGR3 transcriptional activity can be repressed by NGFI-A binding proteins 1 and 2 (NAB1 and NAB2) in neuronal cells (O'Donovan et al., 1999; Sevetson et al., 2000; see Fig. 1.9). Evidence from two different studies show that *Egr3* is involved in synaptic plasticity. Firstly, *Egr3*$^{-/-}$ mice, that had normal brain development and basal synaptic transmission in CA3-CA1 hippocampal neurons, had disrupted LTP in CA1 neurons (Li et al., 2007). Secondly, high frequency stimulation of the perforant pathway in the hippocampal granule cells lead to induction of *Egr3* mRNA expression that was mediated by NMDA receptor activation in glutamatergic synapses. In the same study *Egr3* was also upregulated in the hippocampus in response to maximal electroconvulsive seizure (MECS) with the first signs of mRNA detected at 30 min, peak levels of expression detected after 1-2 hours and remaining elevated up to 8 hours after stimulation (Yamagata et al., 1994).

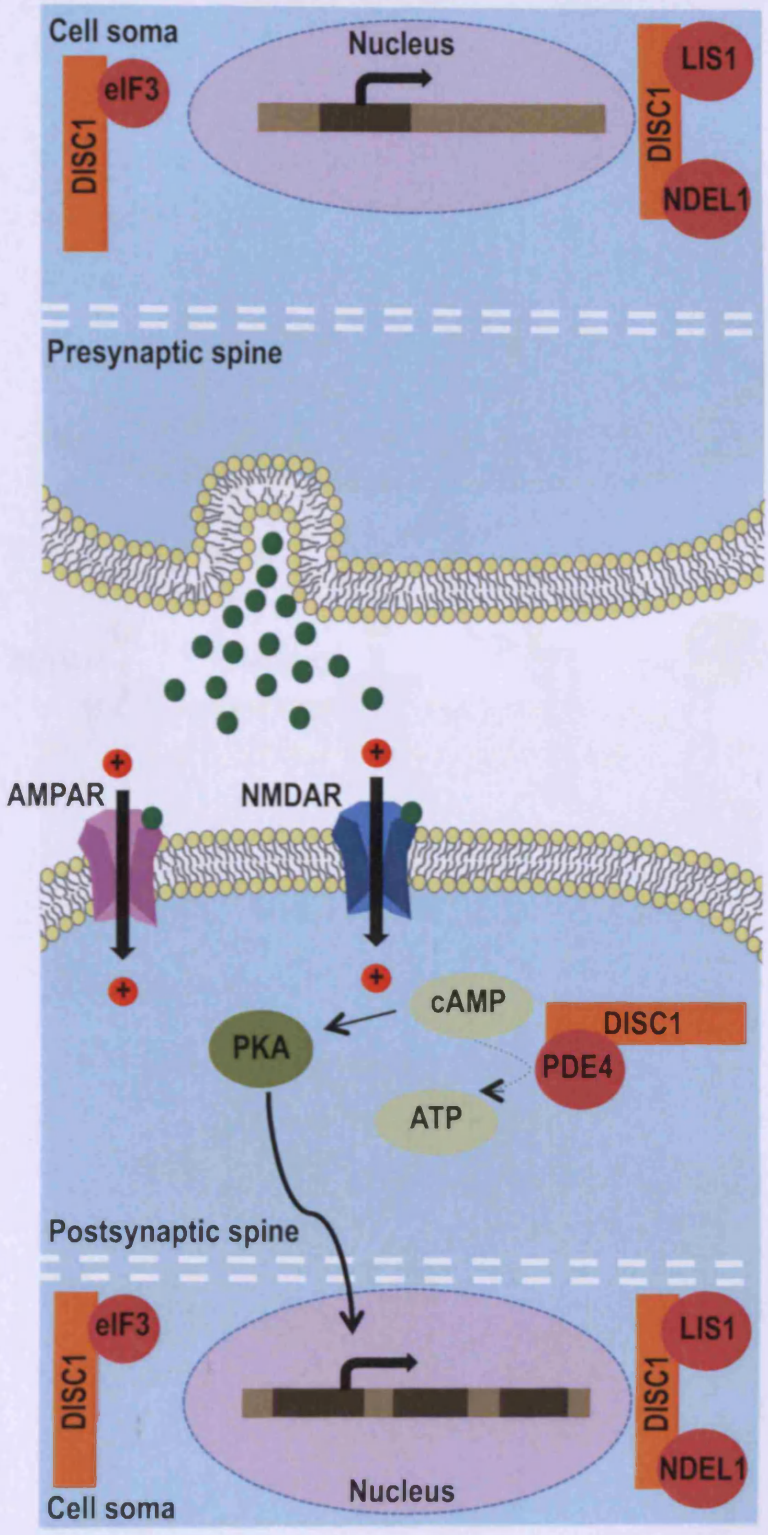


Fig. 1.8. DISC1 cellular interactions. As glutamate is released from the presynaptic terminal it binds to AMPARs and NMDARs that may lead to synaptic plasticity. DISC1 is a scaffolding protein that when complexed with PDE4 regulates cAMP levels and thereby PKA activity which is part of the second messenger cascade modulating gene expression. The DISC1/NDEL1/LIS1 complex is involved in neuronal migration and the DISC/eIF3 complex is thought to regulate translation (Porteous et al., 2006).

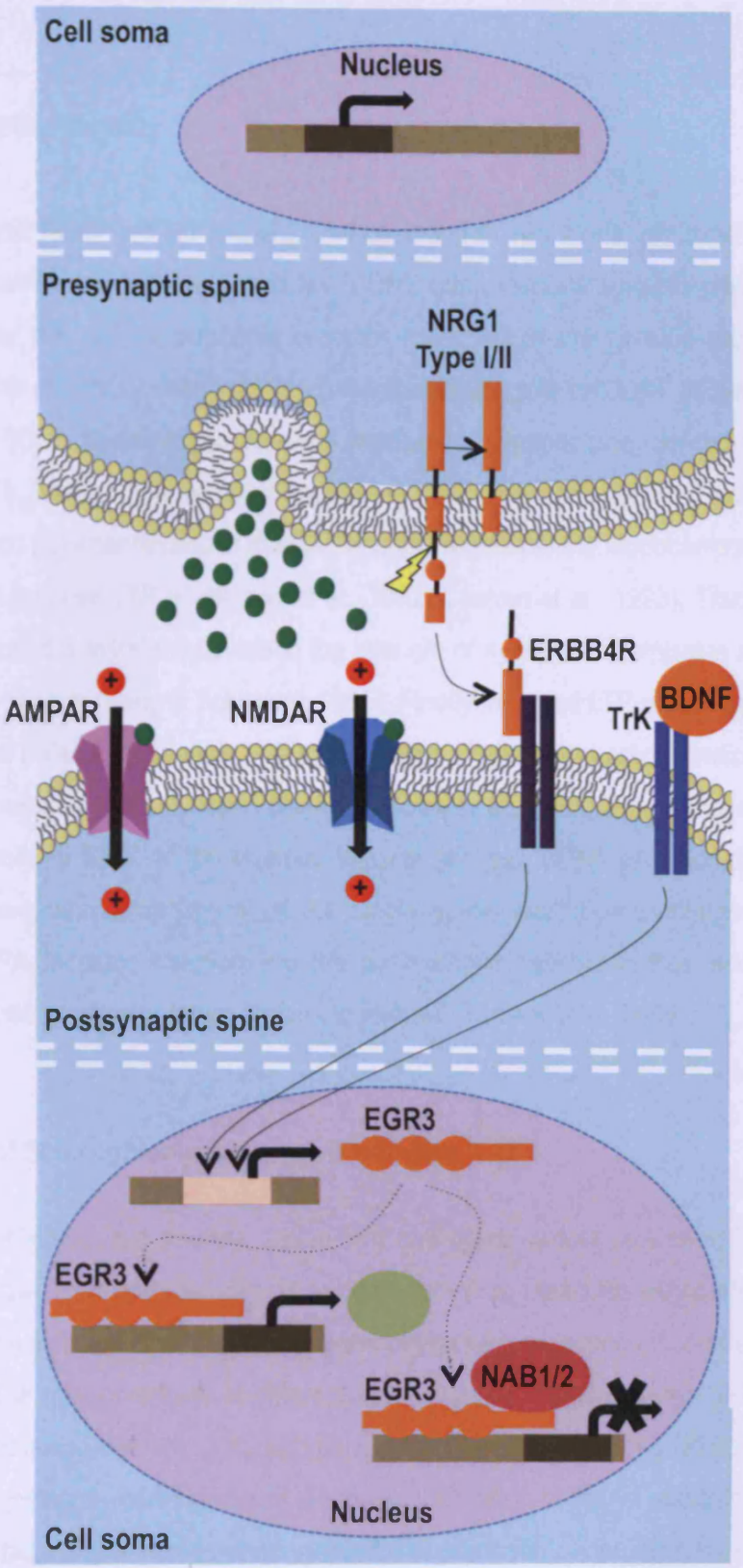


Fig. 1.9. EGR3 cellular interactions. As glutamate is released from the presynaptic terminal it binds to AMPARs and NMDARs that may lead to synaptic plasticity.NRG1 binding to ERBB receptors and BDNF binding to TrK receptors activate second messenger cascades that lead to transcriptionof EGR3. The transcription factor EGR3 promotes transcription of genes that encode ARC, p75 neurotrophin receptor, GABA receptor α4 subunit, Nerve Growth Factor receptor, EGR3 and some miRNA sequences. NAB1 or NAB2 can bind to EGR3 to modulate its transcriptional activity. In neurons NAB1 and NAB2 repress EGR3 transcriptional activity.

1.3.2.5 *Bdnf* and Synaptic Plasticity

Brain derived neurotrophic factor protein (BDNF), can be found in two forms, proBDNF and mature BDNF, both of which are signalling proteins. BDNF can modulate synaptic plasticity through binding to either the p75 neurotrophin receptor (p75 ^{NTR}) or the tyrosine receptor kinase B (TrkB) (Cunha et al., 2010). Many studies have identified a role for BDNF in synaptic plasticity and LTM, and BDNF has emerged as a key regulator of synaptic plasticity (Lu et al., 2008; Cunha et al., 2010). Some of the evidence implicating *Bdnf* in synaptic plasticity includes the findings that increased *Bdnf* expression in the CA1 and DG regions of the hippocampus followed stimulation that induced LTP (Patterson et al., 1992; Castren et al., 1993). Transient application of BDNF induced a marked increase in the strength of synaptic transmission in the CA1 region of the hippocampus (Kang & Schuman, 1995). Finally, reduced LTP was observed in *Bdnf* homozygous and heterozygous mice in the CA1 region of the hippocampus indicating that *Bdnf* expression is necessary for synaptic plasticity (Korte et al., 1995). Recent research has identified a cellular role for BDNF in the synaptic plasticity process. BDNF secretion during LTP is necessary for long-term enlargement at the single spine level. Spine enlargement supports additional AMPA receptor insertion into the postsynaptic membrane that leads to increased sensitivity of that synapse to future glutamate release (Tanaka et al., 2008).

1.4 Splice Variants of Schizophrenia Susceptibility Genes

Recent studies have highlighted that in some cases only one splice variant or a selection of splice variants out of the total number of splice variants of a particular schizophrenia susceptibility gene may be involved in schizophrenia pathophysiology, or indeed different splice variants of the same gene may contribute in different ways. A neuroimaging study identified that a SNP that had been associated with schizophrenia in the promoter region for *NRG1* type IV splice variants was significantly correlated with decreased activation of frontal and temporal lobe regions and premorbid IQ, and increased development of psychotic symptoms (Hall et al., 2006). This same SNP has also been associated with a 49% increase in the mRNA expression levels of *NRG1* type IV splice variants in the hippocampus of individuals with schizophrenia compared to individuals with schizophrenia that did not have the SNP (Law et al., 2006). Law and colleagues (2006) also assayed the mRNA expression levels of *NRG1* type I, II and III

splice variants in the hippocampus of individuals with schizophrenia. There was no difference in the level of *NRG1* type II and III expression between control and schizophrenic individuals but the level of expression of *NRG1* type I splice variants was increased by 34% in the schizophrenic individuals compared to the control individuals (Law et al., 2006). Another study that investigated the levels of expression of three different splice variants of the schizophrenia susceptibility gene *DTNBP1* found that two splice variants were at significantly increased levels in schizophrenic individuals compared to controls while there was no difference in the levels of expression of the other splice variant between schizophrenic and control individuals (Tang et al., 2009). Thus when investigating or discussing the involvement of different schizophrenia susceptibility genes in schizophrenia pathophysiology, the role of the different splice variants of those genes should be considered.

1.5 Outline of Experiments

The experiments presented in this thesis were designed to investigate whether the schizophrenia susceptibility genes *Nrg1*, *Dtnbp1*, *Disc1* and *Egr3* are involved in hippocampal-dependent contextual fear long-term memory processes in the adult rat. In order to address these issues the following experiments were performed:

1. **The exonic structures of the schizophrenia susceptibility genes of interest were determined, probes were designed to detect the genes and gene splice variants of interest, and the whole brain basal expression profile for each gene and gene splice variant of interest was characterized (Chapter 3).**

   The exonic structure of each gene was determined by aligning the experimentally determined mRNA sequences with the gene's genomic sequence using the NCBI SPIDEY mRNA to genomic alignment software. The probes were designed to complement parts of the mRNA sequences to detect the genes or gene splice variants of interest. The adult rat whole brain basal expression profile for each probe was assayed using *in situ* hybridization (ISH) with radioactively labelled probes, and visual inspection of the autoradiographic images. Further analysis of the *Nrg1* splice variants was performed at the regional level, using image densitometry, and at the cellular level, using silver grain counting from emulsion-dipped sections. Probes not detectable

under basal conditions were assayed by ISH in rat brains that had had widespread activity induced by amphetamine.

2. **It was hypothesised that the schizophrenia susceptibility genes of interest would be regulated in association with consolidation of CFM. Therefore the expression levels of the schizophrenia susceptibility genes of interest were investigated after CFC (Chapter 4).**

   The well established CFC paradigm was used to investigate the consolidation of CFM. CFC of adult rats consisted of a footshock administered after the rat had been exposed to the context for 2 min followed by removal of the rat from the context 1 min later. Full behavioural controls were used. The freezing behaviour of the three groups was measured to determine whether a CFM had formed. The expression levels of the schizophrenia susceptibility genes of interest, and *Bdnf* as a positive control, were assayed by ISH 2 hours post-training in some of the brain regions known to be involved in the consolidation of CFM, including the hippocampus, amygdala and prefrontal cortex. Analysis of gene expression was initially performed at both the regional and cellular level. *Egr3* expression in the CA1 region of the hippocampus 2 hours following CFC was additionally investigated using QPCR. The time profile of the regulation of *Egr3* expression in the CA1 region of the hippocampus following CFC was also assayed using ISH and analysed at the regional level.

3. **It was hypothesised that EGR3 was required for consolidation of CFM. Therefore the effect of intrahippocampal infusions of *Egr3* antisense on CFC was investigated (Chapter 4).**

   Bilateral indwelling cannulae were placed in the hippocampus by stereotaxic surgery. *Egr3* antisense oligodeoxynucleotides and control scrambled missense oligodeoxynucleotides were infused into the hippocampus through the cannulae 90 min prior to CFC. The freezing behaviour of the rats was measured 24 hours, 15 days and 22 days post-CFC to determine whether the consolidation of the CFM had been disrupted in either of the *Egr3* antisense or missense groups.

4. **It was hypothesised that *Egr3*, *Egr1* and *Nab2* would be regulated in reconsolidation and extinction of CFM. Therefore the expression of *Egr3*, *Egr1***

and *Nab2* was investigated after short or long recall of a CFM. It was also hypothesised that EGR3 was required for reconsolidation of CFM. Therefore the effect of intrahippocampal infusions of *Egr3* antisense on short recall of a CFM was investigated (Chapter 5).

The CFC paradigm was also used to investigate reconsolidation and extinction of CFM. Rats that had been fear conditioned to a particular context were re-exposed to that context for either a short recall test or long recall test 4 days post-CFC. Freezing behaviour was measured during conditioning and during the retrieval sessions to determine whether conditioning had occurred. Freezing behaviour was also measured during two LTM tests to determine whether the short and long retrieval tests resulted in reconsolidation and extinction of the CFM respectively. The expression levels of the schizophrenia susceptibility gene *Egr3*, a member of the same gene family, *Egr1*, and a repressor of *Egr3* and *Egr1* transcriptional activity, *Nab2*, were assayed by ISH 30 min post-retrieval of both short and long retrieval sessions and in a behavioural control group. Gene expression was analysed at the cellular level in some of the brain regions known to be involved in the reconsolidation and extinction of CFM, including the hippocampus, amygdala and prefrontal cortex. To investigate whether EGR3 had a causal role in reconsolidation of CFM, bilateral indwelling cannulae were placed in the hippocampus by stereotaxic surgery, and *Egr3* antisense oligodeoxynucleotides and control scrambled missense oligodeoxynucleotides were infused into the hippocampus through the cannulae 90 min prior to short retrieval of a CFM. The freezing behaviour of the rats was measured in a STM and LTM tests to determine whether the reconsolidation of the CFM had been disrupted in either the *Egr3* antisense or missense groups.

CHAPTER 2

GENERAL METHODS

2.1 Subjects and Housing

One hundred and fifty one adult male Listar hooded rats (250-300g; Charles River, UK) were used in total for all experiments. The rats were housed in pairs and kept in a holding room at 21°C under reverse light-dark conditions (12 hours light: 12 hours dark; lights off at 10 am). Therefore the rats were in their active state for handling, behavioural experiments and at the time they were killed. The rats were allowed *ad libitum* access to food (rodent laboratory chow, Purina, UK) and water. The rats were killed by CO_2 asphyxiation. Animal testing, surgery, infusions and care were conducted in accordance with the Animals (Scientific Procedures) Act of 1986 and local ethical guidelines.

2.2 Behavioural Protocols

2.2.1 Investigating Consolidation of Contextual-Fear Memory (CFM) using Contextual-Fear Conditioning (CFC)

Fear-conditioning is a behavioural paradigm that has been commonly used by researchers since 1959 (Baron, 1959), and is a well-established method used to investigate associative long-term memory in rodents (Fanselow, 1980). Conditioning in these studies involves the rat receiving an electric footshock (2 s, 0.5 mA shock), the unconditioned stimulus (US), 2 min after being placed in a novel conditioning chamber context, conditioned stimulus (CS), and returning the rat to its home cage 1 min later. This conditioning protocol has been used previously and is well-established (Hall et al., 2000; Lee et al., 2004; Barnes & Thomas, 2008). The interior size of the conditioning chamber was 30.5 cm L x 24.1 cm W x 29.2 cm H. The

chamber had aluminium panelled side walls and clear polycarbonate rear panel and front door (0.6 cm thick) (Med Associates Ltd, Vermont, USA). The conditioning chamber was placed within a sound-attenuating cubicle that had an interior size of 55.9 cm x 55.9 cm x 35.6 cm (Med Associates Ltd, Vermont, USA). The electric footshock was generated by the standalone aversive stimulater/scrambler (Med Associates Ltd, Vermont, USA) and delivered through 19 grid floor bars evenly distributed raised 1.6 cm above the floor tray (Med Associates Ltd, Vermont, USA). Conditions in the conditioning chamber were controlled using Med-PC version IV research control and data acquisition system (Med Associates Ltd, Vermont, USA). The group that was conditioned was called the CS-US group, as the rat forms a long-term memory (LTM) of the CS being associated with the US. In order to determine whether any changes in gene expression observed in the CS-US group were correlated with the formation of a CS-US association, as opposed to the presence of the CS or US in isolation, two behavioural control groups were also investigated. The CS-only control group controlled for the presence of the CS. The latent inhibition (LI) group controlled for the presence of the US as a US was received but the CS was no longer novel or distinct when the US was received resulting in no CS-US association. This phenomenom of prolonged exposure to a CS resulting in no CS-US association forming upon US exposure in the presence of the CS is known a latent inhibition (Lubow & Moore, 1959; McLaurin et al., 1963). LI control groups have been used previously to control for the US component of CS-US conditioning (Impey et al., 1998; Hall et al., 2000).

All rats were handled for 5 -10 min each, for 3 consecutive days, to enable the rats to become familiar with being handled. On day 4, the rats in the CS-US group were conditioned by being placed in the conditioning chamber for 2 min, then receiving an electric footshock (2 s, 0.5 mA shock) before being returned to their home cage 1 min later. The rats in the CS-only control group were placed in the conditioning chamber for 3 min and then returned to their home cage. Rats in the LI control group were placed in the conditioning chamber for 8 h and then received an electric footshock (2 s, 0.5 mA shock) before being returned to their home cage 1 min later. Retrieval tests, that consisted of re-exposing the rats to the CS for 2 min, were performed both 24 hours (LTM1 test) and 3 weeks (LTM2 test) later.

2.2.2 Investigating Reconsolidation and Extinction of CFM using CFC

Using a protocol previously established by Thomas & Barnes (2008), each rat was conditioned in two different conditioning chamber contexts. Context A had wallpaper (white background

with bold back stars), no house light on and lavender oil (Boots, UK), while context B had clean sawdust (IPS Ltd, UK) in the floor tray and the house light on. Rats were habituated to the contexts A and B for 20 min for 3 days. Exposure to each context was always separated by a minimum of 4 hours to further distinguish the two contexts. On day 4 half the rats were conditioned to context A and the other half to context B. On day 5 rats were conditioned in the context that they had not already been conditioned in. Two days after each conditioning trial the rats were re-exposed to the contexts that they had been conditioned to, half for 2 min (the Short Recall group) and the other half for 10 min (the Long Recall group). Two days post-conditioning was the time point used for extinction sessions because consolidation should be complete by this time point so CFM consolidation will not be disrupted by the extinction sessions. The order of the contexts that the rats were exposed during the recall tests was the same as during conditioning. The LTM1 test was performed 4 days later to test whether the CS-US association had been committed to long-term memory, and a LTM2 test was performed 14 days later to test whether the CS-US association long-term memory had persisted. The LTM tests consisted of placing the rats in one of the conditioned contexts for 2 min followed the next day by 2 min in the other conditioned context. The order to which each rat was exposed to the two contexts in the LTM tests was the same as in the conditioning training.

The CFC paradigm with short and long retrieval sessions described above was adapted to investigate whether gene expression correlates with reconsolidation and extinction of CFM. Rats were habituated to a single context (either A or B) for 20 min for 3 days. On day 4 they were conditioned in that context. Four days after conditioning, the Short Recall group of rats were re-exposed to the context to which they were conditioned for 2 min, and the Long Recall group of rats were re-exposed to the context to which they were conditioned for 10 min. After the Short and Long Recall tests the rats were returned to their home cages and killed 30 min later by CO_2 asphyxiation. A control No Recall group of rats were conditioned but remained in their home cages and were not exposed to any retrieval sessions before being killed by CO_2 asphyxiation 4 days after conditioning. The brain was immediately excised and rapidly frozen on dry ice before storage at - 80°C. These brains were then used to investigate whether the genes of interest were regulated 30 min after short and long retrieval in association with reconsolidation and extinction of a CFM.

2.2.3 Behavioural Analysis

The freezing behaviour of the rats in the boxes was digitally recorded (JSP Electronics Ltd., China) and viewed using Numeroscope software (Viewpoint, France). The freezing behaviour was scored by observation and recording the presence or absence of freezing behaviour every 10 s. Freezing behaviour was scored throughout the 2 min pre-US and 1 min post-US periods for the CS-US and LI group rats and in the equivalent first 2 min and final 1 min period for the CS-only group rats. Freezing behaviour was also recorded and scored for the 2 min LTM1 and LTM2 tests.

2.3 Antisense Inhibition of Gene Expression

Antisense oligodeoxynucleotides (ODNs) are synthetic short single strands of nucleotides mimicking natural single stranded DNA (Szklarczyk & Kaczmarek, 1997). The sequence of nucleotides of the antisense ODNs is designed to complement strands of mRNA transcribed from the gene of interest. The antisense ODNs bind to the mRNA and this interaction prevents the mRNA strand from being translated into a protein, thereby knocking down the level of protein expression of the gene of interest (Milligan et al., 1993). There is evidence to suggest that translation is disrupted through a combination of steric hindrance and RNase H-like cleavage (Eder et al., 1993). Antisense EC-ODNs can be infused directly into the brain where they rapidly diffuse and are suggested to enter neurons by receptor-mediated endocytosis (Ogawa et a., 1995; Loke et al., 1989; Yakubov et al., 1989). Intrahippocampal infusions of ODNs into the dorsal CA1 diffuse throughout the hippocampus by 90 min and were cleared from the hippocampus by 24 hours (Lee et al., 2004).

Antisense ODNs are extremely susceptible to hydrolysis by nucleases and so chemical analogues derived from the phosphodiesters have been developed (Cook, 1993). The most common modification for brain research is introducing sulphur in place of non-bridging oxygen, known as antisense phosphorothioate ODNs (S-ODN) (Szklarczyk & Kaczmarek, 1997). However some findings suggest that the highly active thiol group of S-ODN leads to general cytotoxicity. A stable antisense ODN with minimal toxicity has been developed by only introducing a sulphur in place of the non-bridging oxygen in the phosophodiesters at the 3'- and

5'- terminal ends of the antisense ODN and these are known as antisense end-capped phosphorothioate ODNs (EC-ODN) (Hoke et al., 1991; Gao et al., 1992; Ehrlich et al., 1994).

2.3.1 Design of Antisense and Missense Oligodeoxynucleotides

Antisense (ASO) oligodeoxynucleotide probes (ODNs) were designed to detect the GOI in the rat. Two different ASO ODNs were designed; ASOI and ASOII (see Table 2.1). A missense (MSO) ODNs that has the same 18 nucleotides as ASOI but in a scrambled order was designed and infused into the control group of rats (see Table 2.1). This was to identify whether the response observed in the rats receiving the ASO was due to the knockdown of the GOI or a neurotoxic effect related to the infusion of the ODNs. All sequences underwent a NCBI BLAST search to check that the ASO ODNs only had positive matches to the target GOI mRNA sequences and that the MSO ODN had no positive matches to any rat mRNA sequences. The ODNs used were PAGE-purified phosphorothioate end-capped 18-mer sequences (Sigma-Genosys, UK). The ODNs were resuspended in sterile PBS to 1 or 2 nmol/μl and stored at -20°C until required. These two concentrations of ODNs were used because previous studies have shown that 2 nmol/μl *Egr1* antisense was sufficient to knock down EGR1 protein expression and prevent reconsolidation of a CFM from taking place (Lee et al., 2004) and 1 nmol/μl *Bdnf* antisense was sufficient to knock down BDNF protein expression and prevent consolidation of a CFM from taking place (Lee et al., 2004).

2.3.2 Surgical Placement of Indwelling Hippocampal Cannulae

In accordance with local regulations rats were administered analgesia (500 mg paracetamol; Bristol Laboratories Ltd, UK) in their water bottles (~ 200ml) on the day proceeding surgery, on the day of surgery and for the two days following surgery. The rats were initially anaesthetised for 5 minutes in a box using approximately 5% isofluorane (Abbott, UK) with an oxygen flow rate of 0.8 l/min. The rat was assessed throughout surgery to ensure that it was always anaesthetised by monitoring the claw-pinch and eye-blink reflexes. An electronic razor (Wella Contura, UK) was used to prepare the skull for surgery and the rat secured immediately in a stereotaxic frame (Model 900, David Kopf, USA). The front two teeth were placed over the incisor bar, set at 3.3 mm below the interaural line, and the nose bar secured in place. The rat continued receiving gaseous anaesthesia throughout surgery (approximately 2-3% isofluorane

Table 2.1. Sequences of two antisense oligodeoxyribonucleotide (ODN) probes, designed to detect *Egr3*, and a missense ODN control sequence. The ODN probe sequences, the reference sequences from which the ODN probes were designed and the specific nucleotide position in the reference sequence to which the ODN probe was designed is provided for the two antisense (ASO) ODN probes (ASOI and ASOII). The MSO ODN probe was a scrambled version of the ASOI sequence so only the sequence is provided.

Oligodeoxynucleotide	Sequence	PubMed sequence
Egr3 antisense I (ASO I)	5'- ACCGATGTCCATCACATT -3'	NM_017086, nt 157-174
Egr3 antisense I (ASO II)	5'- ACAGATTGTCAGGCAATT -3'	NM_017086, nt 56-73
Egr3 missense (MSO)	5'- ATCACATCTATCTAGCGC -3'	

with an oxygen flow rate of 0.8 l/min and a nitrogen flow rate of 0.6 l/min) and the exhaled gases were removed with a scavenging system. Blunted ear bars were used to fix the head in the appropriate position. A midline incision was made using a scalpel to expose the cranium. The position of the cannulae placement was measured with respect to Bregma. A bilateral indwelling cannulae (22 gauge, 3.8 mm centre-to-centre, 3 mm below pedestal; Plastics One, Semat, UK) was implanted targeting the dorsal hippocampus at the coordinates - 3.5 mm anterior-posterior, ± 1.9 mm medio-lateral and - 2.5 mm dorso-ventral to Bregma. Burr-holes were produced with a dental drill (Harvard Microtorque II Drill System, UK) at the two determined cannulae entry locations. Four skull screws (Plastics One, Semat, UK) were attached to the skull bilaterally, anterior and posterior to the cannulae holder. The cannulae holder was lowered into position flush with the cranium and dental acrylic (Kemdent simplex rapid powder and liquid, UK) was used to fix the cannulae to the skull surface and skull screws. The incision was closed using Mersilk 5 - 0 non-absorbable surgical sutures (Ethicon Inc, UK) and stainless steel wire stylets (12 mm in length; Plastics One, Semat, UK) were inserted into the cannulae in order to prevent the cannulae from becoming blocked. A screw cap (Plastics One, Semat, UK) covered the top of the cannulae holder to protect the stylets from removal. The rat was then removed from the stereotaxic frame. After surgery the rats were placed individually in Plexiglas fan heated cages (Vet Tech Solutions Ltd, UK) at 22°C for 30 min – 1h hour to recover before being returned to their home cages. A minimum of 7 days recovery period was allowed before infusions and behavioural experiments began.

2.3.3 Infusion Procedure

Injectors, 28 gauge and 1 mm longer than guide cannulae, (Plastics One, Semat, UK) were connected via polyethylene tubing to two 5 μl Hamilton syringes (Hamilton, Switzerland) fixed into a syringe pump (Harvard Apparatus 11 Plus, UK). The screw cap and stylets were removed from the cannulae before inserting the injectors into the cannulae in the awake rats. Infusion of 2 μl of PBS solution for the practice infusions, or 2 μl of ASO/MSO in PBS solution for the knockdown experiments, into the dorsal hippocampus took place over 8 min at a constant rate of 0.125 μl/min; 1 μl was infused through each cannulae. The injectors were left in place for two minutes following the completion of infusion before being removed to allow for diffusion of the injected solution away from the injector tips. The stylets and screw cap were put back in place and the rat returned to its home cage.

Histology was performed to identify the site of infusion of ASO and MSO. The tissue sections were Nissl stained with thionin and viewed through a light microscope (Leica DMLB, Germany) to determine the end-point site of the injectors and therefore the site of infusion. Photomicrographs of typical cannulae placements are shown in Figure 2.1.

2.4 Histology

The Nissl method used thionin to stain the RNA and rough endoplasmic reticulum blue in the cell bodies of the brain tissue. Firstly the tissue was prepared. Brain sections 14 μm thick were cut coronally from the brain region(s) of interest using a cryostat (Leica CM1900, Germany) with chamber temperature at - 18°C, and the stage temperature at - 20°C. The sections were thaw-mounted onto poly-L-lysine coated glass slides (slides dipped in 5 mg/ml solution in ddH_2O; Sigma P1524, UK) and allowed to air dry at RT. The slides were placed in glass racks and the sections fixed by immersion in 4% ice - cold paraformaldehyde for 5 min, followed by 1x PBS (1.3 M sodium chloride, 70mM disodium phosphate, 30 mM monosodium phosphate) for 1min and 70% ethanol for 4 min. For the thionin staining the sections were were rinsed in distilled water before being placed in succession of solutions; 1% Thionin (10 min), distilled water (1 min), 70% ethanol (5min), 70% ethanol plus 2.5ml 1M acetic acid (until an appropriate level of staining was obtained), 95% ethanol (2 x 10 min), 100% ethanol (2 x 10 min) and Histoclear (1 x 10 min & 1 x 30 min; National Diagnostics, UK). Immediately after removal from Histoclear, DePeX (BDH Laboratory Supplies, UK) mounting medium was used to seal the glass coverslips (Raymond Lamb, UK) on top of the sections.

2.5 *In situ* Hybridisation

In situ hybridisation (ISH) was performed to characterize the expression of the genes of interest throughout the adult rat brain under basal conditions and to quantify changes in gene expression in different brain regions following CFC, and short and long retrieval sessions. Short oligonucleotide probes were used as they are easily available and can penetrate cells more easily and thereby increase the sensitivity of the ISH experiment. All reagents were molecular grade and supplied by Sigma, UK, unless otherwise stated.

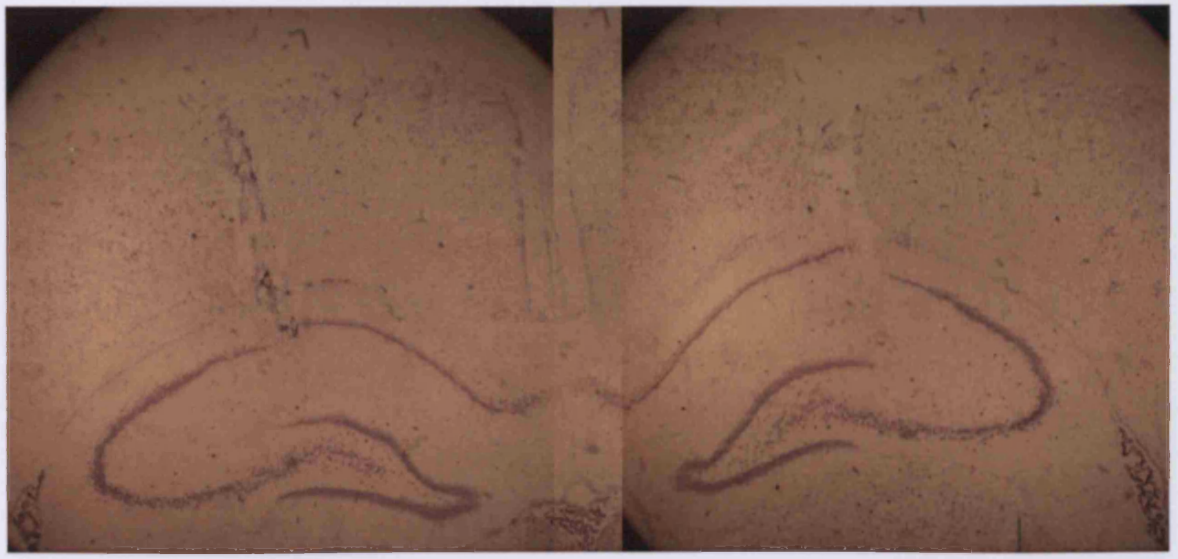


Figure 2.1. Typical cannula placements in the dorsal hippocampus. Three photomicrographs taken at 2.5X magnification covering the hippocampus and the cortical region dorsal to the hippocampus have been merged together to show the bilateral cannula endpoints from which the antisense and missense oligodeoxynucleotides were infused into the dorsal hippocampus.

2.5.1 Design of Oligonucleotide Probes

Probes were designed to complement approximately 45 nucleotides of the mRNA sequence within the gene of interest. Comparison of the gene structure of known transcripts in the human and in the rat enabled the design of probes that would differentiate between different splice variants of a gene, and enable the design of probes that would detect all known splice variants of a gene (pan probe). Accumulation of information on gene structure was done through obtaining a list of all the known experimentally derived transcripts for a gene from NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/gene). The appropriate list of sequences was found on this website by searching the gene name and from the output selecting the organism of interest; in this study both rat and human sequences were investigated for all genes except *Nab1* and *Nab2* for which only rat sequences were investigated.

In NCBI Entrez gene for each gene in each organism they categorise mRNA transcripts into experimentally determined sequences, Reference Sequences (RefSeq) and Expressed Sequence Tags (ESTs). Experimentally determined sequences are sequences that have been determined in a laboratory by researchers who input their findings into this database having had a peer-reviewed journal article relating to that sequence published. Experimentally determined sequences are denoted by accession numbers that start with one or two alphabetical letters eg. AY995222 and U02324. RefSeq sequences are experimentally determined transcripts that have been verified using other publicly available databases by NCBI. RefSeq sequences are denoted by accession numbers that start with 'NM_' eg NM_022856. EST sequences are short single-read transcript sequences produced by one-shot sequencing of a cloned mRNA that represent portions of expressed genes. EST were not used in this study as they only represented small parts of the transcripts of interest, with the exception of CN603655 which was used to provide an example of the 5'-terminal fragment of a *NRG1* type V splice variant as no other transcript sequences have been discovered for this splice variant. In this study the RefSeq sequences were characterised to identify the exonic structure of splice variants of the genes of interest as these sequences have been verified. In some cases, for *Nrg1* in the rat and *DTNBP1* in humans, the exonic structures of the experimentally determined sequences were also characterised in order to search for more splice variants.

For each transcript that had its exonic structure characterised, the RNA sequence was accessed from the NCBI Entrez nucleotide website (http://www.ncbi.nlm.nih.gov/nuccore) by searching the accession number for that transcript. The RNA sequence was then copied into the mRNA sequence window in an mRNA to genomic alignment software program called NCBI SPIDEY (http://www.ncbi.nlm.nih.gov/spidey). The genomic sequence required for this alignment was obtained from NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/gene), or in the case of *Nrg1* in the rat the genomic sequence was obtained from the UCSC Genome Browser (http://genome.ucsc.edu) as the sequence obtained from NCBI gene did not extend far enough 5' in order to detect *Nrg1* type IV. The genomic sequence for the gene, from which the query mRNA sequence was transcribed, was copied into the genomic sequence window in NCBI SPIDEY. Large intron sizes and then 'Align' was selected. Schematics of the exonic structure output was then made in Microsoft powerpoint.

Probes were designed to detect a selection of *Nrg1*, *Dtnbp1* and *Disc1* exons specific to different splice variants and to detect exons in each gene that should detect all splice variants for that gene. Probes were also designed to detect *Egr3*, *Nab1*, *Nab2* and *Bdnf* transcripts. These oligonucleotide probes were approximately 45 nucleotides long, were approximately GC:AT equal and near the beginning of the exon if possible. An RNA alignment tool called Vector NTI Align X (Invitrogen) was used to align human and rat sequences in the exons of interest in order to ascertain regions of high homology. If a sequence of 45 nucleotides, abiding to the criteria mentioned previously, could be selected in the rat sequence in regions of high homology to the human sequence, then this was carried out. Probes were BLASTed in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), UCSC (http://genome.ucsc.edu/cgi-bin/hgBlat) and ENSEMBL (http://www.ensembl.org/Multi/blastview) to confirm that there were no matches of a significant identity level to other genes within the rat genome. The reverse complement of all 45mer sequences was determined and these sequences synthesised commercially (Sigma-Genosys, UK).

2.5.2 Radioactive Labelling of Oligonucleotide Probes

All water was Diethylpyrocarbonate (DEPC)-treated to make it RNase-free before use in labelling probes and ISH. This was to prevent RNA degradation in the brain sections that would alter the levels of mRNA randomly between tissue samples and render semi-quantification of the level of expression of the GOI inaccurate. To tail the oligonucleotides with radioactive

sulphur-35 (^{35}S; half-life of 87 days) the following reagents were added in order into a 1.5 ml Eppendorf tube: 2.5 µl of 5x Tailing buffer (Promega, UK), 5.0 µl of DEPC-treated water, 2.0 µl of oligonucleotide (5ng/µl), 1.5 µl of terminal deoxytransferase (Tdt) enzyme (Promega, UK) and 1.0 µl of ^{35}S-dATP (Perkin-Elmer, UK). These reagents were spun down briefly in a centrifuge to mix and incubated at 30°C in a waterbath for 1 hour. A Sephadex G-25 Spin column was made; two autoclaved glass balls (2.5-3.5 mm, BDH Laboratory Supplies, UK) were placed into a sterile 1 ml syringe (Fisher, UK) placed in a 15 ml Falcon tube, the syringe was then completely filled with Sephadex G-25 (dry bead diameter: 20 – 80 µm, Sigma, UK) in TNES (0.14 mM Sodium chloride, 20 mM Tris, 5 mM EDTA, 0.1% SDS) slurry and centifuged at RT for 2 min at 2000 rpm (rotations per minute). Buffer caught in the falcon tube was discarded, a 1.5 ml Eppendorf tube with no lid was placed into the Falcon tube and the spin column replaced. After 1 hour of incubation the reaction was stopped by adding 38 µl of DEPC-treated water. The 50 µl reaction mixture was transferred to the top of the spin column to be centrifuged at RT for 2 min at 2000 rpm. The labelled oligonucleotide was collected in the Eppendorf tube and 2 µl of 1 M DTT added. The specific activity of the labelled probe was determined; 2 µl were pipetted into a scintillation vial containing 2 ml of scintillation fluid (Perkin-Elmer, UK) and the decays per minute (dpm) assessed for 1 min in a liquid scintillation analyzer (Perkin-Elmer Tri-Carb 2800 TR, UK) using the QuantaSmart software (Perkin-Elmer, UK). Optimal specific activity was between 150 000 dpm/µl and 400 000 dpm/µl indicative of the ^{35}S labelling having correctly labelled the ODNs. The labelled oligonucleotides were stored at - 20°C until use.

2.5.3 Preparation of Tissue Sections

Each brain was cut coronally in 14 µm sections using a cryostat (Leica CM1900, Germany) with chamber temperature at - 18°C, and the stage temperature at - 20°C. The sections were thaw-mounted onto poly-L-lysine coated glass slides (slides dipped in 5 mg/ml solution in ddH_2O; Sigma P1524, UK) and allowed to air dry at RT. The slides were placed in glass racks and the sections fixed by immersion in 4% ice - cold paraformaldehyde for 5 min, followed by 1x PBS (1.3 M sodium chloride, 70mM disodium phosphate, 30 mM monosodium phosphate) for 1min and 70% ethanol for 4 min. Fixation of the tissue makes the mRNA present in the tissue more accessible to the probes. The sections were submerged in 95% ethanol in an airtight storage box and stored at 4°C.

2.5.4 Hybridisation and Washing of Slides

Hybridisation of the oligonucleotide probe to the mRNA results from hydrogen bonding between complementary bases and base-pair stacking using the same principles that underlie the thermodynamic stability of DNA (Cimino et al., 1989). Therefore the level of hybridisation of the probe to the mRNA can be optimised based on the temperature at which the reaction is carried out and on the concentration of charged ions in the hybridisation buffer. Hybridisation buffer was prepared by pipetting the following reagents into a 100 ml Falcon tube: 25 ml of 100% deionised formamide, 10 ml of 20x SSC (3 m sodium chloride, 0.3 M tri-sodium citrate), 2.5 ml of 0.5 M sodium phosphate pH 7.0 (0.5 M disodium phosphate, 0.5 M monosodium phosphate), 0.5 ml of 0.1 M sodium pyrophosphate, 5 ml Denhardt's solution (prepared as a 50 x stock comprising 5 g polyvinylpyrrolidine, 5 g bovine serum albumin and 5 g Ficoll 400 in 500 ml DEPC-treated water), 1 ml of 10 mg/ml acid-alkali hydrolysed salmon sperm DNA (Sigma, UK), 1 ml of 5 mg/ml polyadenylic acid, 50 μl of 120 mg/ml heparin and 5 g of dextran sulphate. The reagents were then shaken vigorously in the Falcon tube, placed at 4°C until the dextran sulphate was dissolved and the solution made up to 50 ml with DEPC-treated water, and the tube wrapped in foil and stored at 4°C. For the ISH the hybridisation buffer was mixed by vortexing and warmed in a hot water bath to 42°C. Slides of sections were removed from storage and left to air dry for a minimum of 30 min before being placed in a bioassay dish humidified by the addition of folded tissue soaked in DEPC-treated water in the corner of the dish. 100 μl per slide of hybridisation buffer was aliquoted into an Eppendorf tube. 2 μl of 1 M dithiothreitol (DTT; Sigma, UK) per slide was also added to the Eppendorf tube. The volume of labelled probe required to be added to the hybridisation buffer per slide was calculated by dividing 250 000 by the specific activity of the labelled probe such that a final specific activity of 250 000 dpm of labelled probe/100 μl hybridisation buffer was attained. The hybridisation mixture was then vortexed and 100 μl pippetted onto each slide. Pre-cut Parafilm (Fisher, UK) strips were used to cover sections. For the non-specific controls 100x excess unlabelled probe was added to each 100 μl of hybridisation mixture containing labelled probe, hybridisation buffer and DTT. The volume of unlabelled probe required to be added to the hybridisation mixture containing labelled probe was calculated by the multiplication of 250 000 by the concentration of the labelled probe (0.2 ng/μl) and 100, and then dividing the product by the product of the specific activity of the labelled probe and the concentration of the probe (5

ng/μl). Finally, the lid was placed on the bioassay dish and the edges sealed with Parafilm and the whole dish wrapped in cling film, to maintain a humid environment to prevent the slides from drying out, before incubating the sections overnight at 42°C.

After incubation, the Parafilm covers were removed from the slides in 1x SSC at RT, and then they were washed in 1x SSC at 55°C for 1 hour; changing the 1x SSC at 30 min. The sections were then dehydrated by placing them in 0.1x SSC (1min), 70% ethanol (1min) and 95% ethanol (brief wash) at RT. The slides were then left to air dry before being placed in a film hypercassette (Amersham Biosciences, UK) and secured in position using double sided cellotape. A ^{14}C micro-scale (RPA 504; Amersham Biosciences, UK) attached to a slide was also secured in position in the film cassette for later image analysis. Under dark room conditions, Kodak BioMax MR film (Sigma, UK) was then apposed to the slides in the film cassette. The films were then developed 3-14 days later, under dark room conditions, in a developer machine (Agfa CURIX 60, Belgium) that passes the film through developer (Agfa G153, Belgium), rapid fixer (Agfa G354, Belgium) and water, and then dried.

2.5.5 Exposure of Slides to Photographic Emulsion

The emulsion dipping procedure was conducted in a dark room lit only by red light (Agar Scientific Ltd Ilford filter safelight 904, UK). Fifty ml of 0.05% glycerol solution in deionised water was warmed to 42°C in a water bath. Ilford K5 nuclear emulsion in gel form (Agar Scientific, UK) was added to the warmed glycerol solution in a 50 ml Falcon tube in a 1:1 ratio and the tube gently inverted to mix. The gel was left to melt at 42°C for 20 - 30 mins. The melted emulsion was gently inverted to mix thoroughly before being decanted into a dipping chamber. The slides, with hybridised sections mounted on them, were individually dipped into the chamber twice to coat them in a thin layer of emulsion. The slides were stood in a rack to dry overnight. Then the coated dry slides were loaded into light tight boxes containing silica gel, sealed with tape and stored at 4°C for 7 times the number of days that the slides were exposed to film in order to obtain clear detectable labelling. At the end of the exposure period the boxes were removed from the fridge and left to equilibrate to RT for about 3 hours. Then in the dark room the coated slides were loaded into glass racks and submerged in Kodak D-19 Developer (18°C, 2 min; Agar Scientific, UK), water (5 s), Ilford HYPAM (diluted 1+4 at RT, 4 min; Agar Scientific, UK) and then placed under running water for 15+ min. This was in order to precipitate silver grains where the radioactive sulphur beta emissions had reacted with the

photographic nuclear emulsion. The hybridised sections were then Nissl stained to enable identification of cells in which to count the number of grains. Histology was performed to identify cells in which the number of grains were counted (see 2.4).

2.5.6 Visual Observation of Gene Expression

The film with the images of gene expression in sections throughout the whole-brain was placed on a light box (Agar Scientific Ltd, UK). The characterization of the whole-brain gene expression pattern was then determined by observing the individual images of the sections by eye, using The Rat Brain atlas (Paxinos & Watson, 2005) to aid regional identification. For each region studied it was recorded in a table whether gene expression was absent (-), expressed at a low level (+), expressed at a medium level (++) or expressed at a high level (+++) relative to the range of expression in all brain regions for each probe.

2.5.7 Densitometric Image Analysis

Densitometric analysis is a quick way of obtaining the regional expression of the gene of interest. The film of labelled coronal sections images were scanned and saved as tiff files using a scanner (Dell AI0 Printer A940, UK) and software (Dell All-In-One Center, UK). ImageJ (NCBI tool, http://rsbweb.nih.gov/ij/) was used to measure the density of selected regions of the brain.

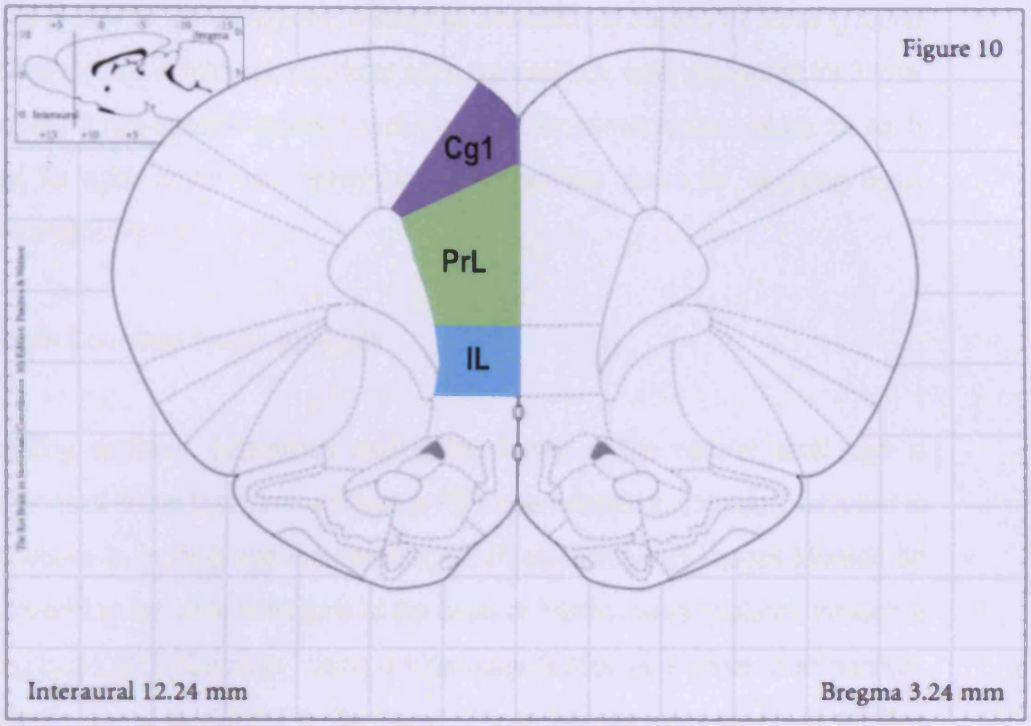
A film was opened in ImageJ on the computer desktop. The zoom in tool was used to view the image made by the microscale at 300%. Regions of interest were sampled and gray values measured of the background and of each of the patches from the microscale image that incrementally increased in level of darkness. Values were assigned to the measured gray values such that the level of gray from the background was made equal to 0 nCi/g, the level of gray from the palest patch from the micro-scale image was made equal to 40 nCi/g, and the level of gray from the darkest patch from the micro-scale image was made equal to 1069 nCi/g, with the patches of increasing darkness between being made equal to 76 nCi/g, 162 nCi/g, 310 nCi/g, 465 nCi/g, 676 nCi/g and 869 nCi/g. In order to create a calibration curve these values were plotted using either a 2^{nd}, 3^{rd} or 4^{th} degree polynomial function, exponential function or $y = a + b * \ln (x - c)$ function. The function to which the plotted values fitted best was used. Densitometric analysis was performed in the hippocampus (CA1, CA3 and DG regions), amydala (lateral, basal and central nuclei) and cerebral cortex (layers I-IV, V and VI) under

basal conditions and in the hippocampus (CA1, CA3 and DG regions), amygdala (dorsolateral, lateral, basolateral nuclei) and prefrontal cortex (cingulate, prelimbic and infralimbic regions) following behavioural training (Fig. 2.2). The regions of interest were viewed at 1600% for densitometric analysis and the densitometric values were read off the calibration curve.

Samples from each region of interest were obtained either by drawing around the region (as performed for the cortex and amygdala) or through taking circular samples within the region of interest (as performed for the hippocampal regions). For densitometric analysis of basal expression, the number of sections from which samples were taken depended on how many of the sections that underwent ISH to analyse the basal whole brain expression pattern had the region of interest present within them. For gene expression under basal conditions measured by densitometry in the hippocampal regions, 5 – 10 circular samples per section, randomly selected bilaterally across the hippocampus, were measured for 15 – 18 total labelled sections and 4 non-specific labelled sections. For gene expression under basal conditions measured by densitometry in the amygdala nuclei, 2 samples selected per section by drawing round the nuclei of interest, one from each hemisphere, were measured for 5 – 8 total labelled sections and 1 - 2 non-specific labelled sections. For gene expression under basal conditions measured by densitometry in the cerebral cortex layers, 2 samples selected per section by drawing round the cortical layers of interest, one from each hemisphere, were measured for 36 – 40 total labelled sections and 7 - 8 non-specific labelled sections. For each region of interest the mean average was calculated from all the measurements assaying the total labelling and the non-specific labelling to obtain a densitometric value for both the level of total labelling and non-specific labelling in that region by that probe. A specific densitometric value was determined by subtracting the non-specific densitometric value from the total densitometric value. The specific densitometric values for regions of interest for a particular probe are all relative to each other if all measurements were determined from the same autoradiographic film.

For gene expression following behavioural training measured by densitometry in the hippocampal regions, 8 – 16 circular samples per section, randomly selected bilaterally across the hippocampus, were measured for 3 – 4 total labelled sections and 2 – 3 non-specific labelled sections. For gene expression following behavioural training measured by densitometry in the amygdala nuclei, 2 samples selected per section by drawing round the nuclei of interest, one from each hemisphere, were measured for 4 total labelled sections and 3 non-specific labelled sections. For gene expression following behavioural training measured by

A



B

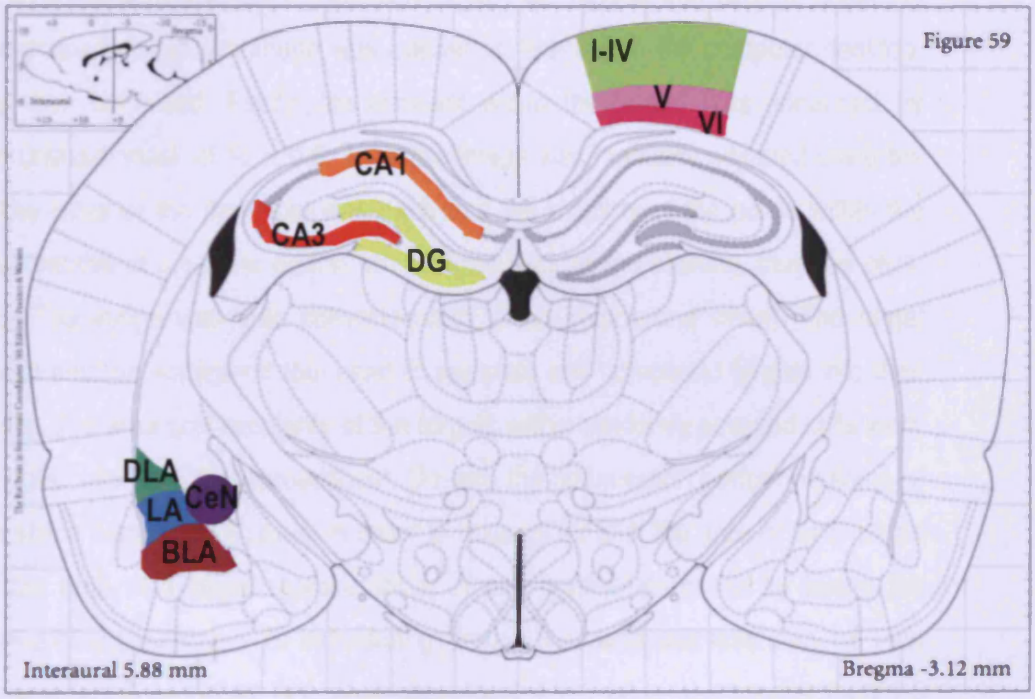


Figure 2.2. Brain regions in which gene expression was assayed. (A) Schematic indicating the cingulate (Cg1), prelimbic (PrL) and infralimbic (IL) regions in the medial prefrontal cortex. (B) Schematic indicating the location of the cornu ammonis 1 (CA1), cornu ammonis 3 (CA3) and dentate gyrus (DG) regions of the hippocampus, examples of the area of the cerebral cortex covered by layers I-IV, V and VI, and the dorsolateral (DLA), lateral (LA), basolateral (BLA) and (CeN) nuclei of the amygdala in the adult rat brain (adapted from Paxinos & Watson, 2005).

densitometry in the prefrontal cortex regions, 2 samples selected per section by drawing round the prefrontal cortex regions of interest, one from each hemisphere, were measured for 3 total labelled sections and 2 non-specific labelled sections. Specific densitometric values for each region of interest for each probe was determined as described above for assaying basal expression densitometrically.

2.5.8 Silver Grain Counting Image Analysis

Silver grain counting analysis determines expression levels at the cellular level and is performed on sections of tissue that have undergone ISH, been dipped in emulsion, exposed to the emulsion for weeks to months and then developed. Photomicrograph images focused on the silver grains overlying the cells in regions of the brain of interest were captured through a light microscope (Leica DMLB, Germany) using a 100x magnification lens under oil immersion, with a digital camera (Leica DFC300 FX, Germany). These images were saved as tiff files using the Leica QWin v3 software (Leica, Germany), and then ImageJ was used to count the number of silver grains per cell. An image was opened in ImageJ on the computer desktop. The image was then optimised. Firstly, the contrast within the image was enhanced by application of an unsharp mask of 10 ± 0.6. Then the image was manually adjusted using the threshold tool. The limits of the threshold was such that the majority of the pixels within the black dots, representative of the silver grains, but minimal background staining, from the cells, were highlighted. The image was then converted into binary (black and white). The target grains were dilated and the watershed tool used to separate any compound targets into their component targets. The area and circularity of the targets within randomly selected cells were measured and copied into an Excel spreadsheet. Despite the optimisation performed some of the targets were still in compound shapes. In order to account for this, the area of each target was divided by the area of a target representative of one grain (determined by taking the average of the area of approximately 20 individual grains), if the value was less than 1.5 then that target was recorded to represent one silver grain, and if the value was greater than 1.5 then the number of silver grains in a target was recorded as the value resulting from the division of the area of each target dot by the area of a target dot representative of one silver grain. The Excel formulae used was:

Number of Silver Grains that a target dot represents = IF((A/a)<1.5,1,(A/a))

A = Area of target

a = Area of a target representative of one silver grain

The number of grains per cell was calculated for each cell, and then the average number of grains per cell was calculated for that particular brain region for that rat. Any grains per cell values that were greater or less than the mean average number of grains per cell ± the standard deviation multiplied by 2 were excluded as outliers. The total number of cells in which the number of grains per cell were measured was such that the standard error of the grain per cell count was less than 10% of the population mean.

For silver grain counting analysis of basal gene expression 12 photomicrographs were taken from 6 total labelled sections and 4 photomicrographs were taken from 2 non-specific labelled sections for all regions of interest. For silver grain counting analysis of basal gene expression in the hippocampal regions and amygdala nuclei, the number of silver grains per cell (SG/cell) were measured in approximately 3 cells that were selected randomly from each total labelled photomicrograph and approximately 6 cells that were selected randomly from each non-specific labelled photomicrograph. For silver grain counting analysis of basal expression in the cerebral cortex, the number of SG/cell were measured in approximately 2 cells that were selected randomly from each photomicrograph for both total labelled sections and non-specific labelled sections. For each region of interest the mean average was calculated from all the measurements assaying the total labelling and the non-specific labelling to obtain a SG/cell value for both the level of total labelling and non-specific labelling in that region by that probe. A specific SG/cell value was determined by subtracting the non-specific SG/cell value from the total SG/cell value. SG/cell values, representing the cellular level of gene expression, are relative only to SG/cell values obtained from tissue that underwent hybridisation with the same probe at the same time and were exposed to nuclear emulsion for the same length of time and developed together.

For silver grain counting analysis of gene expression following behavioural training 6 – 8 photomicrographs were taken for each region of interest from 3 – 4 total labelled sections and 4 – 6 photomicrographs for each region of interest were taken from 2 – 3 non-specific labelled sections. For silver grain counting analysis of gene expression following behavioural training in the hippocampal regions and amygdala nuclei, the number of SG/cell were measured in approximately 3 cells that were selected randomly from each total labelled photomicrograph and approximately 6 cells that were selected randomly from each non-specific labelled photomicrograph. For silver grain counting analysis of gene expression following behavioural training in the prefrontal cortex regions, the number of SG/cell were measured in approximately

2 cells that were selected randomly from each photomicrograph for both total labelled sections and non-specific labelled sections. Therefore in each region of interest, the number of SG/cell was measured in approximately 24 – 36 total labelled cells and ain approximately 18 – 24 non-specific labelled cells. A specific SG/cell value was determined for each probe in each region of interest for each rat as described previously for the basal silver grain counting analysis.

2.5.9 Analysis of Heavily Labelled Cells

Heavily labelled cells in the hippocampal regions of the brain investigated at the cellular level were identified in two ways. Firstly, the heavily labelled cells were identified in digitally captured images taken at 100x magnification under bright-field conditions as cells that were very densely covered with silver grains in comparison to the majority of the surrounding cells. Secondly, heavily labelled cells were identified under dark-field conditions as sparsely distributed clusters of bright points. These clusters of bright points were confirmed to represent high levels of gene expression in cells by switching to light-field conditions without moving the slide. The number of heavily labelled cells per region per section were counted under dark-field conditions using the light microscope at 10x magnification manually. This analysis was performed on 4 total labelled sections and 2 – 3 non-specific labelled sections for the hippocampal regions of interest. In some cases the number of SG/cell in the subjectively identified heavily and non-heavily labelled cells were counted in approximately 3 cells per photomicrograph image (there being 2 photomicrograph images per section) using ImageJ as described in 2.5.8.

2.5.10 Profiling Expression of Genes of Interest in Adult Rat Brains with Amphetamine Induced Activity

For splice variants that could not be visualised in naïve brain, ISH was performed in rats that had increased neuronal activity induced by amphetamine. A sub-cutaneous injection of amphetamine solution was administered to elevate activity in the brain that would lead to increased expression of plasticity-dependent genes (Graybiel et al., 1990). The amphetamine solution was injected subcutaneously at a concentration of 5 mg of amphetamine/kg of rat. The amphetamine was dissolved in 0.1 M PBS. To allow time for the expression of the genes of interest to be regulated the rats were left in their home cages for 2 hours post injection before being killed by CO_2 asphyxiation. The brains were excised immediately, frozen on dry ice and stored at –80°C. ISH was performed, as described in 2.4, at least twice for each probe. For

each attempt, ISH was completed for two total labelled slides and two non-specific labelled slides.

2.6 Real-Time Quantitative-PCR

Real-time quantitative-PCR (RT-QPCR) was performed to quantify changes in gene expression in the CA1 region of the hippocampus following CFC. RNA from the rat hippocampal tissue was firstly reverse transcribed into cDNA in a manner that maintained the relative levels of all different sequences. Forward and reverse primers were designed to complement short sense and antisense sequences of the cDNA of the gene of interest. The samples then underwent Q-PCR amplification. Each cycle of amplification involves denaturation of the double stranded cDNA, annealing of the primers to the complementary sense and antisense sequences and binding of polymerase to the primer-template hybrid to initiate DNA synthesis, and an elongation stage in which the DNA polymerase synthesizes a new DNA strand complementary to the template. Fluorescent SYBR green was intercalated with each new DNA strand synthesized. The increase in amplified product is directly proportional to the increase in fluorescence. Therefore using a standard curve the fluorescence level was used to quantify the relative level of product in each sample. The relative levels of cDNA between the samples could then be compared for Q-PCR reactions that were performed at the same time with the same primers (Tevfik Dorak, 2006).

2.6.1 Dissection of Hippocampus

The brain was placed on glass covered in ddH_20 soaked filter paper on ice. The cerebellum and frontal cortex were cut off using a blade. The brain was turned onto its cortical surface with the rostral brain facing towards the dissector. The hemispheres were teased apart using two pairs of curved forceps. The thalamic and striatal regions were pinched out and discarded, and blood vessels removed from the hippocampal region. The hippocampal region was then rolled out using two pairs of curved forceps, flipped over so that the left hippocampus that was on the left hand side is now on the right had side, and the forceps used to unroll the dorsal DG away from the dorsal CA1. The dorsal CA1 was then dissected from the dorsal CA3/DG using the edge of the forceps. The dorsal CA1 and dorsal CA3/DG dissected tissues were stored in RNAlater (Qiagen, UK) in separately labelled Eppendorf tubes at - 80°C.

2.6.2 RNA Extraction

The total RNA was extracted and purified from the dissected CA1 and CA3/DG tissue using a Qiagen RNeasy protect mini kit (Qiagen, UK) according to the manufacturers instruction. Briefly, 600 µl of buffer RLT (Qiagen, UK) was pipetted into an Eppendorf tube to which 6 µl of 14.3 M beta-mercaptoethanol (Sigma, UK) was added. Into this Eppendorf tube was placed 20 – 30 mg of the dissected tissue that was immediately homogenized using a rotor-stator homogenizer (Ika T18 basic ultra-turrax, Germany) for approximately 5 bursts of 8 sec until the contents of the Eppendorf tube were uniformly homogenous. The lysate was then centrifuged (Hettich Zentrifugen Mikro 200R, Germany) for 3 min at 14 000 rpm. The supernatant was pipetted into another Eppendorf tube, an equal volume of 70% ethanol added, and the pipette used to mix the two solutions. 700 µl of the sample was transferred to a RNeasy spin column (Qiagen, UK) placed in a 2 ml collection tube, the lid closed and centrifuged for 15 s at > 10 000 rpm. The effluent was pipetted back into the column and centrifuged again for 15 s at > 10 000 rpm before discarding the flow-through. If more than 700 µl of sample was available the remainder was pipetted into the same spin column and centrifuged in the same manner, discarding the flow-through after the second centrifugation step. Then 700 µl of buffer RW1 (containing ethanol) (Qiagen, UK) was pipetted into the spin column and centrifuged for 15 s at > 10 000 rpm before discarding the flow-through. 500 µl of buffer RPE (Qiagen, UK) was pipetted into the spin column, centrifuged for 15 s at > 10 000 rpm, the flow-through discarded and the spin column place in a new collection tube. A further 500 µl of buffer RPE was pipetted into the spin column and centrifuged for 2 min at > 10 000 rpm. Then the spin column was placed in a new 1.5 ml Eppendorf tube, 30 µl of RNase-free water (Qiagen, UK) pipetted directly onto the spin column membrane and left for 10 min. The RNA was eluted by centrifuging the Eppendorf tube for 1 min at >10 000 rpm. The RNA containing effluent was stored in 10 µl aliquots. One aliquot was used immediately to measure RNA concentration and the remainder of the aliquots were stored at - 80°C. The concentration of RNA was measured using a Nanodrop machine (Thermo Fisher Scientific ND-2000, UK) and Nanodrop 3.0.1software (Thermo Fisher Scientific, UK). The RNA concentration and absorbance (A) values describing the samples' level of purity (A_{260}/A_{280}) and contaminants (A_{260}/A_{230}) were obtained (Tevfik Dorak, 2006). The purity value should be between 1.9 - 2.1 and the

contaminants value should be greater than 1.8 to ensure that high-quality accurate reverse transcription can take place.

2.6.3 Reverse Transcription of RNA to cDNA

Reverse transcription was performed on the RNA samples to synthesise the first strand of cDNA ready for Q-PCR. All reagents used for the reverse transcription of RNA to cDNA were supplied by Stratagene, UK. Firstly, the volume of reagents required to provide enough sample cDNA to assay the levels of expression of 4 genes of interest was calculated. For one reverse transcription (RT) reaction, 13.2 μl of water, 1 μl of random hexamers, 2 μl of RNA, 0.8 μl of 100 mM deoxyribonucleotide trisphosphate (dNTP), 2 μl of 10x buffer, 2 μl of DTT, and 1 μl of AffinityScript reverse transcriptase are pipetted into a tube. One RT reaction is required to provide enough RNA for creating 1.75 standard curves. One standard curve is necessary for investigating 1 gene of interest. As we were investigating 4 genes of interest, 3x the volumes of reagents for 1 RT reaction were used. As the extracted CA1 hippocampal RNA samples contain different concentrations of RNA, the volume of each RNA sample added to the reagents for reverse transcription varies such that the concentration of each sample is standardised to 2 μl of a sample that has a high concentration of RNA, thereby by minimising the volumes of RNA sample required, but not so high such that the samples with the lowest concentrations are an impractical volume to pipette. The volume of water added to the reverse transcription mixture is varied for each sample to maintain a constant volume. For each sample the appropriate volumes of RNA, water and random hexamers was pipetted into a well of a 96 well plate; duplicates of each RNA sample were investigated. In the initial QPCR experiment the standard curve was created using RNA from one of the naïve group CA1 samples. In the technical replicate QPCR experiment, the standard curve was created using RNA from the CA1 of an additional rat under naïve conditions. Enough of the RNA sample for generating the standard curve for creating 4 standard curves was added to water and random hexamers. Blank RT mix containing double distilled water instead of an RNA sample in addition to the other RT reagents is used as a no template control solution for the Q-PCR experiment and to dilute the RNA for the standard curve. One RT reaction is required to provide enough Blank RT mix for creating 0.5 standard curves. As 4 standard curves were required, 8x the volumes of reagents for 1 RT reaction were pipetted into another well. The contents of each well were mixed by pipetting before incubating the 96 well plate at 65°C for 5 min. The plate was then

removed from the heat block and left on the bench at room temperature for 10 min to allow primer annealing. While incubation and cooling took place the appropriate volumes of the reagents for the second part of the RT reaction (10xbuffer, dNTP, DTT & AffinityScript reverse transcriptase) were pipetted into an Eppendorf tube. After the plate had been at room temperature for 10 min, the appropriate volume of RT Part 2 mix was added to the wells containing the unknown RNA samples. Then appropriate volumes of RT Part 2 reagents were pipetted directly into the well containing RNA being prepared to create a standard curve, and into the well containing the blank RT mix. The plate was covered with an adhesive plate seal (ABgene, UK) and incubated at 54°C for 1 h, followed by 70°C for 15 min. The plate was then spun down in a centrifuge (Eppendorf Centrifuge 5810 R, UK) and placed on ice.

2.6.4 Generating a Standard Curve

To generate a standard curve, appropriate volumes of blank for the number of standard curves required were pipetted into four wells, and serial dilutions of the RNA sample being used for the standard curve were prepared. The resulting standard curve had 1x, 1/3x, 1/9x, 1/27x and 1/81x the RNA sample selected for creating the standard curve.

2.6.5 Real-Time Quantitative-PCR

All reagents used for Q-PCR were supplied by Stratagene, UK. Q-PCR was performed on duplicates of each unknown sample and triplicates of each standard curve dilution for the gene of interest and the reference genes *Hmbs* (*Hydroxymethylbilane synthase*), *Sdha* (*Succinate dehydrogenase complex subunit A*) and *Ubc* (*Ubiquitin C*). The reference genes are the three standard practice genes used in our laboratory (Unpublished observations). The forward and reverse primer sequences for *Egr3*, *Hmbs*, *Sdha* and *Ubc*, along with the respective annealing temperatures are given in Table 2.2. The primers were designed using Beacon Designer 7 to produce amplicons of approximately 200 basepairs in length within the gene of interest that had approximately similar melting temperatures.

For one Q-PCR reaction, 13.375 μl water, 2.5 μl core PCR buffer, 1.25 μl $MgCl_2$, 1.0 μl 20 mM dNTP, 4.0 μl 50% glycerol (Sigma, UK), 0.75 μl DMSO, 1.25μl SYBR green and 0.25 μl Taq DNAse polymerase is required. For each gene of interest 52 reactions were performed,

Table 2.2. *Egr3*, *Hmbs*, *Sdha* and *Ubc* primer sequences. The forward and reverse primer sequences with their melting temperatures (Tm), at which they anneal with the template DNA, and the reference sequence to which they were designed are given.

Gene	Strand	Sequence 5' - 3'	Tm (°C)	Reference Sequence
Egr3	Forward	TCAGATGGCTACAGAGAATG	51.2	NM_017086
	Reverse	CAGTTGGAAGGAGAGTCG	50.8	
Hmbs	Forward	CCTGTTCAGCAAGAAGATG	50.5	NM_013168
	Reverse	TTGACAGCCAGACATAGG	49.9	
Sdha	Forward	GCTCTTTCCTACCCGCTCAC	57.5	NM_130428
	Reverse	GTGTCATAGAAATGCCATCTCCAG	57.4	
Ubc	Forward	CTTTGTGAAGACCCTGAC	49.0	NM_017314
	Reverse	CCTTCTGGATGTTGTAGTC	49.1	

therefore a Q-PCR mastermix was prepared by pipetting the appropriate volumes of the reagents into an Eppendorf tube. Fifty-two microlitres of the primer solutions were then added, and the solution mixed by pipetting. Strip tubes of eight were placed in a 96 well plate cooling block holder. Using a multichannel pipette and tray 22.5 µl of the mastermix containing primers was pipetted into the strip tubes. Then 2.5 µl of cDNA from all the unknown samples, 2.5 µl in triplicate of all the dilutions of the standard curve and 2.5 µl of blank were added. Strips of 8 lids were placed on the strip tubes, the tubes were spun down in a microcentrifuge (Jencons-PLS, UK) to remove any bubbles and the tubes placed in the Q-PCR machine (Stratagene Mx3000P QPCR System, Agilent Technologies UK Ltd, UK). The setup was put into the Q-PCR software programme MxPro – Mx3000P, the annealing temperatures, based on the melting temperatures of the primer sequences, and the elongation temperatures, based on the optimal temperature for DNA polymerase activity (72°C), were put into the cycle and the programme set to run for 40 cycles. On completion of all the cycles the Q-PCR programme automatically produced an amplification plot with a cycle threshold value, a standard curve and a dissociation curve (Appendix Fig. 1 – 7). The amplification plot displays the increasing level of fluorescence as the cDNA is amplified over the 40 cycles. The first few cycles do not provide detectable changes in fluorescence levels and is known as the baseline stage. The earliest detectable signal proceeds at the maximal exponential rate and is known as the exponential stage. Amplification proceeds at a linear rate, known as the linear stage, before the reagents begin to be used up leading to a plateau stage. The dissociation curve displays the melting temperature of any DNA product. Ideally there should only be one peak representing the amplicon amplified from the gene of interest, however smaller peaks that represent primer dimer can sometimes be observed at lower melting temperatures. Other peaks could represent contaminated samples and would require the experiment to be repeated.

Quantification of the *Egr3*, *Hmbs*, *Sdha* and *Ubc* cDNA was achieved through detecting changes in fluorescence emitted as SYBR green becoming incorporated into the amplified product. The standard 40 cycles used for Q-PCR experiments was extended at 38 cycles if some unknown samples or standard curve samples had not reached the linear stage. Ideally each sample amplification plot would show the four main phases of baseline, exponential, linear and plateau. By increasing the number of cycles, complete four phase amplification plots were obtained. The level of expression of each gene of interest was quantified through identification of the cycle number at which the sample crossed the threshold line (Ct value).

The threshold level was automatically determined by the software such that it crossed all sample amplification plots in the experiment within the exponential phase. The standard curve was used to determine the relative amount of cDNA at threshold level for each sample. Outliers from the standard curve triplicate dilutions were removed before using the curve to determine the relative levels of cDNA. To meet the exclusion criteria the range of triplicate values had to be greater than one Ct value. The value that was furthest from the standard curve was excluded. The text report of the raw data was exported into an Excel spreadsheet (Microsoft, UK) before further analysis was performed. A normalisation factor was determined by taking the geometric mean of the three reference genes for each duplicate of each sample. The relative quantities were then multiplied by the normalisation factor to obtain a more accurate normalised gene of interest value. The arithmetic mean of the duplicate normalised gene of interest values was then calculated. Any outliers within the six values within a group were removed, reducing the n value of that group. The arithmetic mean of the remaining samples in each group was calculated. Fold changes were then calculated and the coefficient of variation quotient calculated to give an indication of the variation in the groups. The coefficient of variation was calculated as follows:

$$\text{CV of quotient} = [\,(\text{CV of GOI})^2 + (\text{CV of NF})^2\,]^{1/2}$$

Where CV = Coefficient of variation

GOI = Gene of interest

NF = Normalisation factor

2.7 Statistical Analysis

Statistical tests were conducted using SPSS (version 16.0, SPSS Inc., USA). Repeated measures Analysis of Variance (ANOVA) was used to investigate differences in freezing behaviour between the different behavioural groups in the different tests (Test X Group interaction) and between the two groups overall (Within-Group). Application of the Greenhouse-Geisser correction was used to test for sphericity (the equality of variances of the differences between levels of the repeated measures factor) that is necessary to apply a repeated measures ANOVA test to a dataset. Planned post hoc tests on planned comparisons were performed using Fishers Least Significant Difference (FLSD) statistical test. One-way ANOVA was used to investigate differences between the levels of gene expression determined

using ISH and Q-PCR, and the number of heavily labelled cells, in the different behavioural groups studied. Planned post hoc tests were performed using the FLSD statistical test.

3.1 INTRODUCTION

Due to the evidence implicating the schizophrenia susceptibility genes *Neuregulin 1* (*NRG*
Dysbindin 1 (*DTNBP1*), *Disrupted-in-schizophrenia 1* (*DISC1*) and *Early growth respo*
factor 3 (*EGR3*) in synaptic plasticity (see 1.3.2), these genes were investigated to identif
they were regulated in processes of associative contextual fear memory (CFM). *Nrg1*, *Dtn*
and *Disc1* have multiple splice variants. Therefore before these genes of interest can
investigated in contextual fear conditioning (CFC) in the adult rat, the basal expression prof
for splice variants of these genes must be established. EGR3 transcriptional activity can
repressed by NGFI-A-binding proteins 1 and 2 (NAB1 and NAB2) (Svaren et al., 19
therefore *Nab1* and *Nab2* basal expression profiles were also determined. The exonic struct
and an updated list of splice variants were characterised for each gene of interest (GOI
identify which exons to design probes to in order to detect specific splice variants. Probes w
designed to detect chosen *Nrg1*, *Dtnbp1* and *Disc1* splice variants, and *Egr3*, *Nab1* and *Na*
and basal expression patterns are characterised for all probes in the adult rat brain.

3.1.1 *Nrg1* Structure and Expression

The large and complex *NRG1* gene spans approximately 1.4Mb in the human and 1.1Mb in
rat (Falls, 2003). Many splice variants of *NRG1* have been identified in human and rat,
annotations can be found in NCBI Entrez gene (http://www.ncbi.nlm.nih.gov/nuccore), but
possible that more remain undetected. *NRG1* splice variants have been categorised into ty
I – VI in humans; each type defined by a distinct 5'-exon (Falls, 2003; Steinthorsdottir et
2004; Law et al., 2006). An additional three types of *NRG1* denoted as type VII – IX have b

identified in two different rapid amplification of cDNA ends (RACE) assays but not yet validated by RT-PCR (Steinthorsdottir et al., 2004). In the rat *Nrg1* type I, II and III splice variants have been detected and their adult basal expression pattern have been qualitatively described (Kerber et al., 2003). Whether rats express the type IV, V and VI amino termini is not yet known. Previous to the discovery of the different amino termini in *Nrg1*, the combined expression of most, if not all, *Nrg1* splice variants was characterised in the adult rat brain with some conflicting findings in the cortex, hippocampus and some other rat brain regions (Chen et al., 1994; Pinkas-Kramarski et al., 1994; Corfas et al., 1995; Eilam et al., 1998; Kerber et al., 2003). *Nrg1* splice variants vary as to whether they contain exons encoding immunoglobulin-like domains, spacers, EGF-L α or EGF-L β domains, stalk/juxtamembrane 1, 2, 3, 4 or 5, or different cytoplasmic tails a, b or c (Falls et al., 2003; Kerber et al., 2003). To optimise investigation of a possible role for *Nrg1* in CFM, the different splice variants should be taken into account. However, due to the large number of splice variants of *Nrg1*, it is not feasible to probe for every known *Nrg1* splice variant, therefore a more manageable approach is to investigate the different types of *Nrg1* splice variants. In this study probes are designed and expression patterns determined for *Nrg1* types I, II, III and IV and pan *Nrg1* as they have all been associated with schizophrenia either through association studies or neurobiological functions correlating with schizophrenia pathogenesis hypotheses (Law et al., 2006; Hall et al., 2006; Hashimoto et al., 2004; Stefansson et al., 2002; Chen et al., 2008: Nicodemus et al., 2009).

3.1.2 *Dtnbp1* Structure and Expression

Only one transcript of *Dtnbp1* has been identified in the rat (Benson et al., 2001). In humans two splice variants of *DTNBP1* have been detected by Northern blot analysis (Weickert et al, 2004). Further splice variants of *DTNBP1* have been predicted in a previous study using a program called Aceview that is based on an integrated view of human genes reconstructed by alignment of all publicly available mRNA and expressed sequence tags of the genome sequence. In addition to the known 11 exons, Aceview predicted two further exons and four promoter sites that may lead to four predicted groups of *DTNBP1* splice variants (Williams et al, 2004). Recently, an additional splice variant has been identified such that now three known *DTNBP1* splice variants (A, B and C) are considered in investigations (Talbot et al., 2009; Oyama et al., 2009; Tang et al., 2009). DTNBP1 protein expression has been characterized in the adult mouse brain (Benson et al., 2001) and *DTNBP1* mRNA expression has been

characterized in the adult human brain (Weickert et al., 2004). Additional studies have characterized in more detail the expression of *DTNBP1* mRNA and DTNBP1 protein in the hippocampal formation of the human brain (Talbot et al., 2006; Weickert et al., 2007). All studies identified *DTNBP1* mRNA and DTNBP1 protein to have a very widespread expression pattern. It is thought not to be present in glia, but exclusively expressed in neurons, with particularly high protein expression levels, with respect to itself, in axons, dendrites and synapses (Talbot et al., 2006; Benson et al., 2001). The expression of *DTNBP1* in humans and *Dtnbp1* in mice has not been splice variant specific, with the exception of a recent study investigating the levels of the three different splice variants in schizophrenic patients compared to controls in the dorsolateral prefrontal cortex (Tang et al., 2009). The expression of *Dtnbp1* in the adult rat brain has not yet been characterized.

3.1.3 *Disc1* Structure and Expression

In humans four splice variants of *DISC1* have been identified. They are known as L (long), Lv (long variant), S (short) and Es (extremely short) (Taylor et al, 2003). Only one transcript of *Disc1* has been identified in the rat (Ozeki et al., 2003), while two *Disc1* splice variants have been identified in the mouse (Ma et al., 2002). *Disc1* mRNA expression throughout the adult brain has been characterized in the mouse (Ma et al., 2002; Austin et al., 2004), rat (Miyoshi et al., 2003) and monkey (Austin et al., 2003). *Disc1* mRNA is expressed predominantly in the dentate gyrus of the hippocampus. It is also detected at lower levels in the CA1 - CA3 regions of the hippocampus in the rodent but not monkey brain (Austin et al., 2003; Ma et al., 2002; Miyoshi et al., 2003). *Disc1* mRNA is expressed in the cerebral cortex, olfactory bulbs, Purkinje cell layer of the cerebellum, paraventricular and arcuate nuclei of the hypothalamus and the amygdala (Ma et al., 2002; Austin et al., 2004; Miyoshi et al., 2003). While *Disc1* mRNA is present in the cerebral cortex, it is predominantly expressed in layers II and III of the cortex in mice and rats, but evenly distributed across all layers in the monkey (Ma et al., 2002; Austin et al., 2003; Austin et al., 2004). Additional regions expressing *Disc1* mRNA have been found in the monkey including the septum, interpeduncular nucleus and subthalamic nucleus (Austin et al., 2003). The probes used to detect *Disc1* mRNA in these published studies did not differentiate between different splice variants of *Disc1*. DISC1 protein expression has been characterized in regions of the adult mouse, rat and human brain (Schurov et al., 2004; Meyer & Morris, 2008; Ozeki et al., 2003; Kirkpatrick et al., 2006). Like the *Disc1* mRNA expression found in rodents, DISC1 protein expression in the mouse is detected in the olfactory bulb,

cerebral cortex, hippocampus, Purkinje cell layer of the cerebellum, hypothalamus (Schurov et al., 2004). While layers II and III of the cerebral cortex have predominant labelling of both *Disc1* mRNA and DISC1 protein, predominant labelling of DISC1 protein has been observed in layers V and VI that has not been observed in *Disc1* mRNA expression studies of (Schurov et al., 2004; Ma et al., 2002; Austin et al., 2004). Although *Disc1* mRNA and DISC1 protein are both expressed in the hippocampus *Disc1* mRNA is most heavily expressed in the DG region while DISC1 protein is most heavily expressed in the CA3 region (Ma et al., 2002; Austin et al., 2004; Schurov et al., 2004; Meyer & Morris, 2008).

3.1.4 *Egr3* Structure and Expression

Both human *EGR3* and rat *Egr3* have two exons (Patwardhan et al., 1991; Yamagata et al., 1994). *Egr3* does not have any splice variants but does have at least two isoforms and possibly as many as five isoforms as a result of alternative translational start sites that lead to the production of proteins with distinct transcriptional activation properties (O'Donovan & Baraban, 1999). EGR3 contains an R1 repression domain to which NAB1 and NAB2 can bind, leading to negative regulation of EGR3 transcriptional activity. Only one transcript has been detected for NAB1 and NAB2 (Russo et al, 1995; Svaren et al, 1996; O'Donovan et al, 1999). *Egr3* is expressed in the cerebral cortex, hippocampus, amygdala and basal ganglia (Yamagata et al., 1994). *Nab1* and *Nab2* expression has not been characterized under basal conditions in the brain. However, *Nab1* and *Nab2* expression has been observed in coronal sections taken from 1.5mm anterior to bregma, taken from rats that had received an injection of saline. *Nab2* expression was present in layers I-III and layers V-VI of the cerebral cortex, nucleus accumbens, caudate putamen and olfactory tubercle. *Nab1* expression was present in the same regions as *Nab2* but is expressed at lower levels (Jouvert et al., 2002).

3.1.5 Outline of Experiments

Experiment 1 determined the exonic structure of the genes and splice variants of interest using the NCBI SPIDEY software so that probes could be designed to detect the different splice variants of the GOI. Experiment 2 involved designing probes, testing the specificity of the probes and characterising the basal expression pattern throughout the whole adult rat brain for the schizophrenia susceptibility genes and splice variants of interest. This was done using radioactively labelled probes and *in situ* hybridisation (ISH) on a naïve adult rat brain. The brain

expression patterns were determined by visual inspection. In addition, regional and cellular levels of analysis of *Nrg1* splice variants expression was performed in the hippocampal, amygdala and cerebral cortex brain regions using image densitometry and silver grain counting. Experiment 3 investigated if genes or splice variants, not detectable by ISH in rat brain tissue under basal conditions, were detectable in rat brain that had had widespread neuronal activity induced from a sub-cutaneous injection of amphetamine that increased dopamine neurotransmission (Graybiel et al., 1990).

3.2 METHODS

3.2.1 Subjects

Three male Lister hooded rats (280-350g; Charles River, UK) were used in the experiments for chapter 3. Animals were housed in pairs and kept in a holding room at 21°C under reverse light-dark conditions (lights off at 10am and on at 8pm). Animals were allowed *ad libitum* access to food and water.

3.2.2 Experiment 1: Determining Exonic Structure of Transcripts of Schizophrenia Susceptibility Genes of Interest

3.2.2.1 Identifying Transcripts of Schizophrenia Susceptibility Genes of Interest

The transcripts that were investigated were obtained from NCBI Entrez Gene (http://www.ncbi.nlm.nih.gov/gene) (see 2.5.1) and were mostly Reference Sequences (RefSeq), which are experimentally determined transcripts that have been verified using other publicly available databases by NCBI (denoted by accession numbers that start with 'NM_'). For human *DTNBP1*, rat *Nrg1* and human *NRG1* type V, additional non-RefSeq transcripts also had their exonic structures determined. These transcripts had been experimentally determined but had not been further verified. Additional sequences were characterised for human *DTNBP1* in an attempt to identify the exons predicted by Aceview, while additional sequences were characterised for rat *Nrg1* in an attempt to identify type IV splice variants, and additional sequences were characterised for a human *NRG1* type V sequence as at present there is no RefSeq *NRG1* type V sequence.

3.2.2.2 Determining the Exonic Structure of Transcripts

The NCBI SPIDEY software tool (http://www.ncbi.nlm.nih.gov/spidey/) was used to determine the exonic structure of the transcripts of interest (see 2.5.1). The SPIDEY software aligns the transcript of interest with the genomic sequence for the region of the chromosome in which the gene is found. For *Nrg1* and *Dtnbp1* a composite of the splice variants exonic structures was created to predict the gene exonic structure. This was achieved by comparing all the exons identified in all the splice variants for each gene; the composite was based on the sequence homology of the exons, nucleotide length of the exons and the sequential position of the exons.

3.2.3 Experiment 2: Profiling the Basal Expression of Schizophrenia Susceptibility Genes of Interest in Adult Rat Brain

3.2.3.1 Probe Design

Probes were designed as described in 2.5.1. All designed probe sequences, the NCBI transcript accession numbers and nucleotide numbers that they were designed to, and the presence or absence of specific labelling for the probe are provided in Table 3.1. All probe sequences were put into BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to confirm alignment to the specific gene of interest only.

45mer oligonucleotide probes were designed to detect the 5'-defining exons of *Nrg1* type I, II and III splice variants. The predicted 5'-defining exon of *Nrg1* type IV splice variants is only 187 bp in length and is very GC rich. It was not possible to find a 45 bp stretch of this exon that was approximately GC:AT equal. The GC:AT content of the probe is required to be equal for the hybridization conditions used. This is due to the guanine and cytosine base pairs having three hydrogen bonds as opposed to adenine and thymine base pairs that have only two hydrogen bonds meaning that probes with more GC content than AT content bind more strongly to the RNA and require different hybridisation and washing conditions. Therefore two probes that were GC:AT equal but that were 44 bp and 38 bp in length were designed. A probe was also designed, complementary to the EGF - like domain present in all *Nrg1* transcripts, to detect all rat *Nrg1* splice variants and was denoted as a pan-*Nrg1* probe.

Table 3.1. Sequences of oligonucleotide probes designed to detect *Nrg1*, *Dtnbp1* and *Disc1* splice variants and *Egr3*, *Nab1* and *Nab2* mRNA. The accession number of the reference sequences and nucleotide positions that the probes were designed to are given. The exon(s) within the genes that the probes detect are given. The specific activity of the probes used to carry out whole brain ISH and the number of days that the slides were exposed to film or emulsion is shown. Three different probes were designed to detect *Nrg1* type IV splice variants and two different probes were designed to detect pan *Disc1*. Probes that could not be detected in naïve tissue were tested in rats that had been amphetamine-treated (*).

Gene		Oligonucleotide Sequence (5' - 3')	Reference Sequence (exon to which probe is designed as in exonic structure figures)	Nucleotide Position of Probe in Reference Sequence	Specific Labelling Detected	Specific Activity (dpm/µl)	Days on Film	Days on Emulsion
Nrg1	I	CTTCTTGCCCTTCCCCTTGCCTCTGCCTTCTTTGCGCTCAGACAT	NCBI: U02324 (E444)	45 - 89	✔	208 500	11	63
	II	TGGCATCCGGCTCCATGAAGAAGATGTACCTGCTGTCCTCCTTGA	NCBI: AF194994 (E346)	747 - 791	✔	281 500	11	63
	III	CCTCCCGCCAGCTACCTCAGACATGTCTGGGGAATAAATCTCCAT	NCBI: DQ176766 (E1291)	1 - 45	✔	267 500	12	63
	IV a	TGCTAAGCTGCAGTCCGAGTGGGCTGCGGAGATGTACT	UCSC: chr16 (predicted E187)	63,052,728 - 65	✘	264 000	13	
	IV b	TGACTGCTGCAATTGGGAGGACCGGCGGGCTGCTAAGCTGCAGT	UCSC: chr16 (predicted E187)	63,052,752 - 95	✘	285 000	10	
	IV a+b				✘	213 000 + 264 500	7	
	IV a+b *				✘	206 000 + 191 000	15	
	IV c	TTCAATCTGGGAGGCAGTGCGCCCCGTGCCTTGCCGGCCTGTTT	UCSC:chr16 (predicted E187 + E178)	63,052,891 - 916	✘	178 000	10	
				+ 63,983,495 - 514				
	Pan	CGCACTTTATGAGATGGCTGGTCCCAGTCGTGGATGTCGATGTGG	UCSC: chr16 (E130)	64,093,864 - 908	✔	210 000	21	
Dtnbp1	Exon 1	TGAAATCCTGCTGCACGCTCAGCAGCCGCTCGCGCAGGGTCTCCA	NCBI: NM_001037664 (E166)	115 - 159	✔	176 116	14	
	Exon 5	TCTTCTTCTCCCAGTGGGCAGACAGCATGACCACCTCGCTGTCCA	NCBI: NM_001037664 (E133)	337 - 381	✔	244 000	14	
	Exon 8	TGTGCTCCATTTCCAGGATCTTCTGTGCGTGTTCTGTATCGAGTT	NCBI: NM_001037664 (E156)	625 - 669	✔	322 000	14	
	Exon 9	CATCTGCTCCAGCACGTCCACATTCACTTCCATGGAGGACATGCT	NCBI: NM_001037664 (E144)	789 - 833	✔	313 000	14	
Disc1	L	GCACAGTGTGGTAAGGAAGCTGAGTAAGTGTTCCACTCACAGAGT	UCSC: chr19 (gDNA following E208)	55,422,919 - 963	✘	351 500	10	
	L *				✘	148 400	10	
	Lv	TCAGCAAAAACGACATGAGATTCCTGCAAGGGGGACTTTTCCTCT	NCBI: NM_175596 (E208 + E118)	2199 - 2243	✘	362 000	10	
	Lv *				✘	166 000	10	
	Pan a	ACAACCACTGTCCCCAGTCAGCCTCTCGGATTGTCTTCTGTCATT	NCBI: NM_175596 (E974)	400 - 444	✘	469 500	10	
	Pan a *				✘	170 300	10	
	Pan b	GGGAGAGTCGGATGAAGCTGAAGTTGGAAGTAAAGGTGTCTTGGA	NCBI: NM_175596 (E974)	596 - 640	✘	164 800	4	
Egr3	Pan	GATTTAGCAAACTGCTCATGGTCACCGGCAGCTTCTCGGCGAGTT	NCBI: NM_017086 (E154)	11 - 55	✔	360 500	13	
Nab1	Pan	ACAGTTGCTGGACATCATCACCACCTTGTTGGATAAAGGCATCAA	NCBI: NM_022856 (E835)	277 - 321	✔	283 000	13	
Nab2	Pan	CCTGCTCCAACATCAGATTCTGGAGTGCTCTGGCCTGGCCAGATC	NCBI: NM_001134874 (E874)	759 - 803	✔	320 000	13	

To investigate whether rats express splice variants of *Dtnbp1*, probes were designed to detect four different exons in the rat. 45mer oligonucleotide probes were designed to detect the rat equivalent exons of the human exons that are directly downstream from the human predicted promoter regions P1 and P2; these were detected by the *Dtnbp1* exon 1 and exon 5 probes. The absence of exon 1 has been observed in a human *DTNBP1* splice variant but exon 5 is present in all human *DTNBP1* splice variants. Therefore the probe designed to exon 5 is considered a pan-*Dtnbp1* probe. Two other pan-*Dtnbp1* probes were designed; one detected rat exon 8, the equivalent to human exon 11, and the other detected rat exon 9, the equivalent to human exon 12.

A pan-*Disc1* probe was designed to exon 1. L and Lv *Disc1* splice variants have not yet been identified in the rat. In human *DISC1* the L splice variant contains an exon 11 of 199 bp while the LV splice variant contains an exon 11 of 265 bp. *Disc1* exon 10 in the rat is the equivalent of human *DISC1* exon 11 and it is 208 bp in length. To investigate whether *Disc1* splice variants L and Lv could be identified in the rat, as they are in the human, two probes were designed. One probe was designed to detect the last part of exon 10 and the first part of exon 11. Another probe was designed to detect a 45 bp sequence within the 66 bp immediately following exon 10. This sequence was derived from the genomic sequence.

Egr3, *Nab1* and *Nab2* do not have different splice variants therefore only one 45mer probe per gene was designed to detect the expression of *Egr3*, *Nab1* and *Nab2*.

3.2.3.2 Tissue Preparation

A rat was taken from its home cage and killed immediately by CO_2 asphyxiation. The brain was immediately excised, frozen on dry ice and stored at -80°C until ready for sectioning. 14 μm sections throughout the whole brain were collected on poly-L-lysine dipped glass slides using a cryostat. Sections were collected such that consecutive sections on a slide were at approximately 168 μm intervals throughout the brain. Tissue was fixed in 4% paraformaldehyde and the slides stored in racks in 95% ethanol at 4°C (see 2.5.3).

3.2.3.3 *In Situ* Hybridisation (ISH)

ISH was carried out as described in 2.5.4. The specific activity of each of the radioactively labelled probes used is in Table 3.1. ISH was carried out on sections throughout the whole brain. Eighteen slides of sections labelled with radioactively labelled probe (total labelled sections) and 4 slides of sections labelled with radioactively labelled probe plus excess unlabelled probe (non-specific labelled probe) were processed for each variant of interest. Autoradiographic film was laid on top of each set of slides in light-tight cassettes for the number of days listed in Table 3.1 for each probe. A radioactive micro-scale was opposed to each film for calibration. The films were developed as previously described (2.5.4). The films were then placed on a light box and the regional localization and relative level of gene expression determined by eye and recorded in a plus chart. Brain regions of high gene expression relative to the level of expression of the same probe in other brain regions from the same images was denoted by '+++', while '++' and '+' denoted increasingly lower relative levels of expression of that gene.

3.2.3.4 Emulsion Dipping

After films had been developed for the *Nrg1* type I, II and III splice variants, the slides with hybridised sections mounted on them were individually dipped in Ilford K5 nuclear emulsion in a dark room as described in 2.5.5 and left to dry overnight. Then the coated dry slides were loaded into light-tight boxes containing silica gel, sealed with tape and stored at 4°C for 9 weeks (Table 3.1). The slides were then removed and developed, thionin stained and coverslipped as described in 2.5.5 and 2.4.

3.2.3.5 Densitometric Analysis

Regional analysis of gene expression was performed by image densitometry (ID) in selected brain regions from the electronically scanned autoradiographic films using ImageJ as described in 2.5.7. Regional analysis was performed for *Nrg1* type I, II and III splice variants and pan *Nrg1* in the CA1, CA3 and DG regions of the hippocampus, the lateral, basal and central nuclei of the amygdala, and layers I-IV, layer V and layer VI of the cerebral cortex (Fig. 2.2). For densitometric analysis of the hippocampal regions, 15-18 total labelled sections and 4 non-specific labelled sections were analysed. For densitometric analysis of the amygdala nuclei 5-8

total labelled sections and 1-2 non-specific labelled sections were analysed. For densitometric analysis of the cerebral cortex layers 36-40 total labelled sections and 7-8 non-specific labelled sections were analysed. The mean non-specific value was subtracted from the mean total labelled value to determine the mean specific value. For each type of *Nrg1* splice variants, expression levels in the investigated brain regions were described as a percentage of the highest densitometric value observed for that type of splice variant.

3.2.3.6 Silver Grain Image Collection and Analysis

Photomicrographs were captured digitally. The focus was on the silver grains with cells detectable in the background. Photomicrographs were taken through a 100x magnification lens under oil immersion. The regions of interest included the CA1, CA3 and DG of the hippocampus, the lateral, basal and central nuclei of the amygdala, and layers I-IV, layer V and layer VI of the cerebral cortex. For all regions of interest, 12 photomicrographs were taken from 6 total labelled sections and 4 photomicrographs were taken from 2 non-specific labelled sections. For the hippocampal regions and amygdala nuclei, approximately 3 cells were selected randomly from each photomicrograph from total labelled sections and approximately 6 cells were selected randomly from each photomicrograph of non-specific labelled sections. For the cerebral cortex, approximately 2 cells were selected randomly from each photomicrograph for total labelled sections, and approximately 6 cells were selected randomly from each photomicrograph for non-specific labelled sections. Cellular analysis of gene expression was performed using ImageJ as described in 2.5.8. Silver grains were counted over these randomly selected counterstained cells. The specific grain count for each GOI was then calculated for each region by subtracting the mean non-specific labelled grain counts from the mean total labelled grain counts. Expression levels in the investigated brain regions were described as a percentage of the highest silver grain count observed for that type of splice variant.

3.2.4 Experiment 3: Profiling Expression of *Nrg1* type IV Splice Variants and *Disc1* Splice Variants in Adult Rat Brains with Amphetamine Induced Activity

For splice variants that could not be visualised in naïve brain, ISH was performed in rats that had received a sub-cutaneous injection of amphetamine solution. This was administered to elevate activity in the brain that would lead to increased expression of plasticity-dependent genes (Graybiel et al., 1990). The amphetamine solution was injected subcutaneously at a

concentration of 5 mg of amphetamine/kg of rat into two rats that were 290 g and 300 g. The amphetamine was dissolved in 0.1 M PBS. To allow time for the expression of the GOI to be regulated the rats were left in their home cages for 2 hours post injection before being killed by CO_2 asphyxiation. The brains were excised immediately, frozen on dry ice and stored at –80°C. ISH was performed, as described in 2.5.4, at least twice for each probe (see Table 3.1). For each attempt, ISH was completed for two total labelled slides and two non-specific labelled slides.

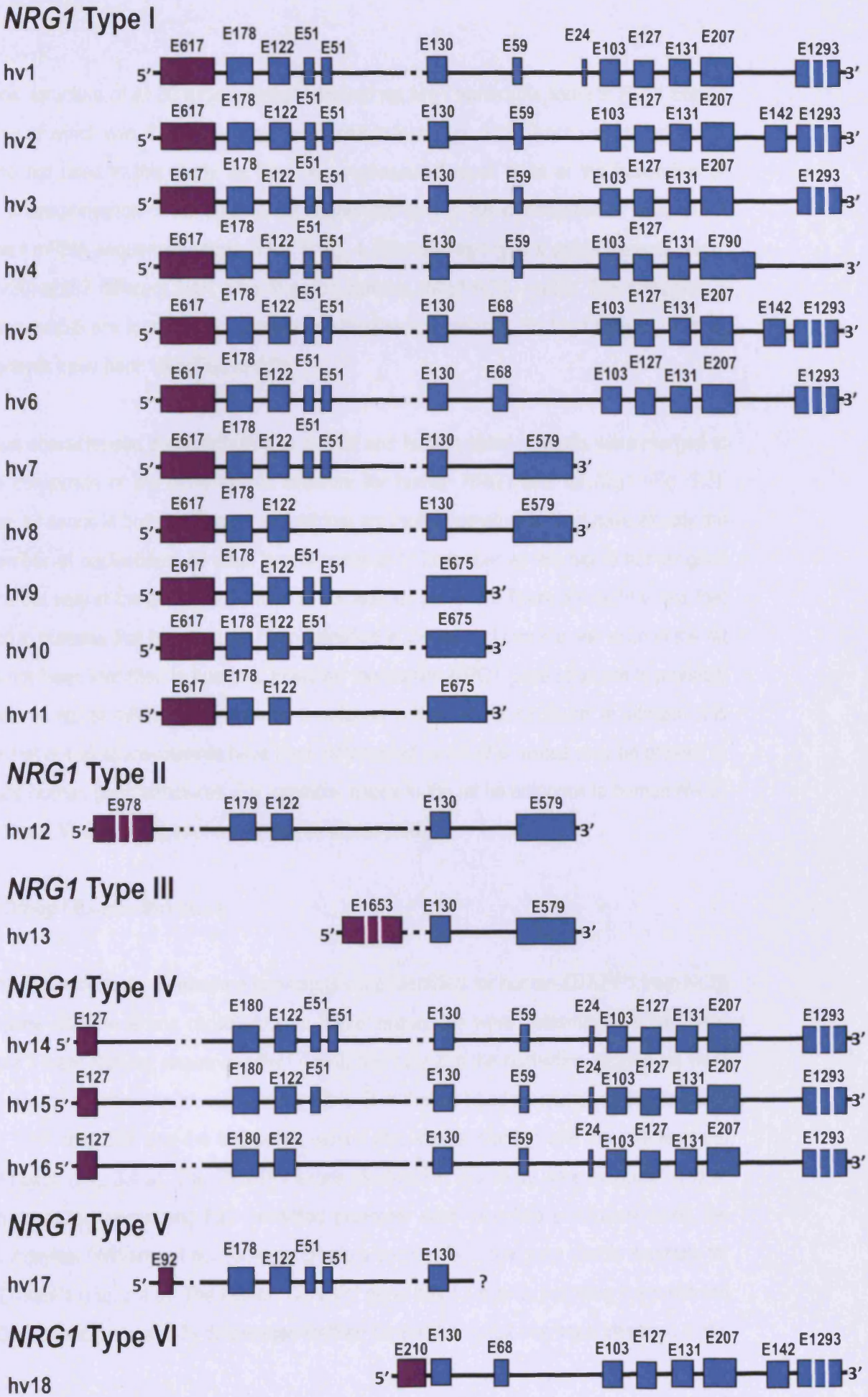
3.3 RESULTS

3.3.1 Experiment 1: Determining Exonic Structure of Transcripts of Schizophrenia Susceptibility Genes of Interest

3.3.1.1 *Nrg1* Exonic Structure

In total 17 RefSeq transcripts were identified for human *NRG1* from NCBI Entrez Gene. The exonic structure of these transcripts was determined using NCBI SPIDEY mRNA to genomic DNA alignment tool. There were 11 different *NRG1* type I splice variants (*NRG1* hv1 - hv11), one *NRG1* type II splice variant (*NRG1* hv12), one *NRG1* type III splice variant (*NRG1* hv13), 3 different *NRG1* type IV splice variants (*NRG1* hv14 - hv16) and one *NRG1* type VI splice variant (*NRG1* hv18). The RefSeq sequences gave at least one example of types I – IV and VI of *NRG1* splice variants but not an example of *NRG1* type V splice variant. There were however another 61 experimentally determined mRNA sequences without verification from other publicly available databases by NCBI and a further 114 EST sequences that were identified as coming from the *NRG1* gene. The exonic structure was only determined for one of these sequences in order to provide an example of an NRG1 type V splice variant (*NRG1* hv17). This sequence was selected from the sequences described in the original paper that discovered the type IV, V and VI 5'-defining exons (Steinthorsdottir et al., 2004). The purpose of determining the exons contributing to human *NRG1* splice variants was purely to demonstrate that *NRG1* has many splice variants that can be divided into six different types of splice variants each defined by a 5'-exon. Figure 3.1 demonstrates the complexity of *NRG1* splice variants and gives examples of all six different "types" of *NRG1* splice variants.

Figure 3.1. Schematic of the exonic structures of a selection of the known human *NRG1* splice variants type I, II, III, IV, V and VI. Boxes represent exons. Purple boxes represent *NRG1* type defining exons. Boxes are approximately relative in width compared to the number of nucleotides in the exon with the exception of one exon in which vertical white lines through the box indicates that the actual exon length is much longer than suggested by the relative width of the box. The number of nucleotides in an exon is given above the exon following the letter E. The length of the line between the boxes is approximately relative to the length of the introns. Vertical white lines through the black line indicate that the actual intron length is much longer than suggested by the relative distance between the exons in that region. mRNA sequences and one Expressed Sequence Tag (EST) (for *NRG1* type V splice variant) were were found in NCBI Entrez gene and sourced from NCBI Entrez nucleotide. NCBI SPIDEY tool was used to determine the exonic structures. The NCBI accession numbers for each transcript are: v1, NM_013956; v2, NM_001160004; v3, NM_013957; v4, NM_001160008; v5, NM_013960; v6, NM_013964; v7, NM_013958; v8, NM_001160005; v9, NM_04495; v10, NM_001160002; v11, NM_001160007; v12, NM_013962; v13, NM_013959; v14, NM_001159999; v15, NM_001159995; v16, NM_001160001; v17, CN603655; v18, NM_001159996.


NRG1 Type I
hv1
E617 E178 E122 E51 E51 E130 E59 E24 E103 E127 E131 E207 E1293
hv2
E617 E178 E122 E51 E51 E130 E59 E103 E127 E131 E207 E142 E1293
hv3
E617 E178 E122 E51 E51 E130 E59 E103 E127 E131 E207 E1293
hv4
E617 E178 E122 E51 E51 E130 E59 E103 E127 E131 E790
hv5
E617 E178 E122 E51 E51 E130 E68 E103 E127 E131 E207 E142 E1293
hv6
E617 E178 E122 E51 E51 E130 E68 E103 E127 E131 E207 E1293
hv7
E617 E178 E122 E51 E51 E130 E579
hv8
E617 E178 E122 E130 E579
hv9
E617 E178 E122 E51 E51 E675
hv10
E617 E178 E122 E51 E675
hv11
E617 E178 E122 E675
NRG1 Type II
hv12
E978 E179 E122 E130 E579
NRG1 Type III
hv13
E1653 E130 E579
NRG1 Type IV
hv14
E127 E180 E122 E51 E51 E130 E59 E24 E103 E127 E131 E207 E1293
hv15
E127 E180 E122 E51 E130 E59 E24 E103 E127 E131 E207 E1293
hv16
E127 E180 E122 E130 E59 E24 E103 E127 E131 E207 E1293
NRG1 Type V
hv17
E92 E178 E122 E51 E51 E130 ?
NRG1 Type VI
hv18
E210 E130 E68 E103 E127 E131 E207 E142 E1293

The exonic structure of all 30 experimentally derived rat *Nrg1* transcripts listed in NCBI Entrez Gene, one of which was RefSeq verified, were determined (Fig. 3.2). There were a further 10 EST were not used in this study as they only represented small parts of the transcripts of interest. Characterisation of the 30 different sequences lead to the identification of 16 different *Nrg1* type I mRNA sequences (*Nrg1* rv1 – rv16), 4 different *Nrg1* type II splice variants (*Nrg1* rv17 – rv20) and 7 different *Nrg1* type III splice variants (*Nrg1* rv21 – rv27). The remaining 3 mRNA sequences are incomplete fragments of *Nrg1* splice variants. No *Nrg1* type IV, V or VI splice variants have been identified to date.

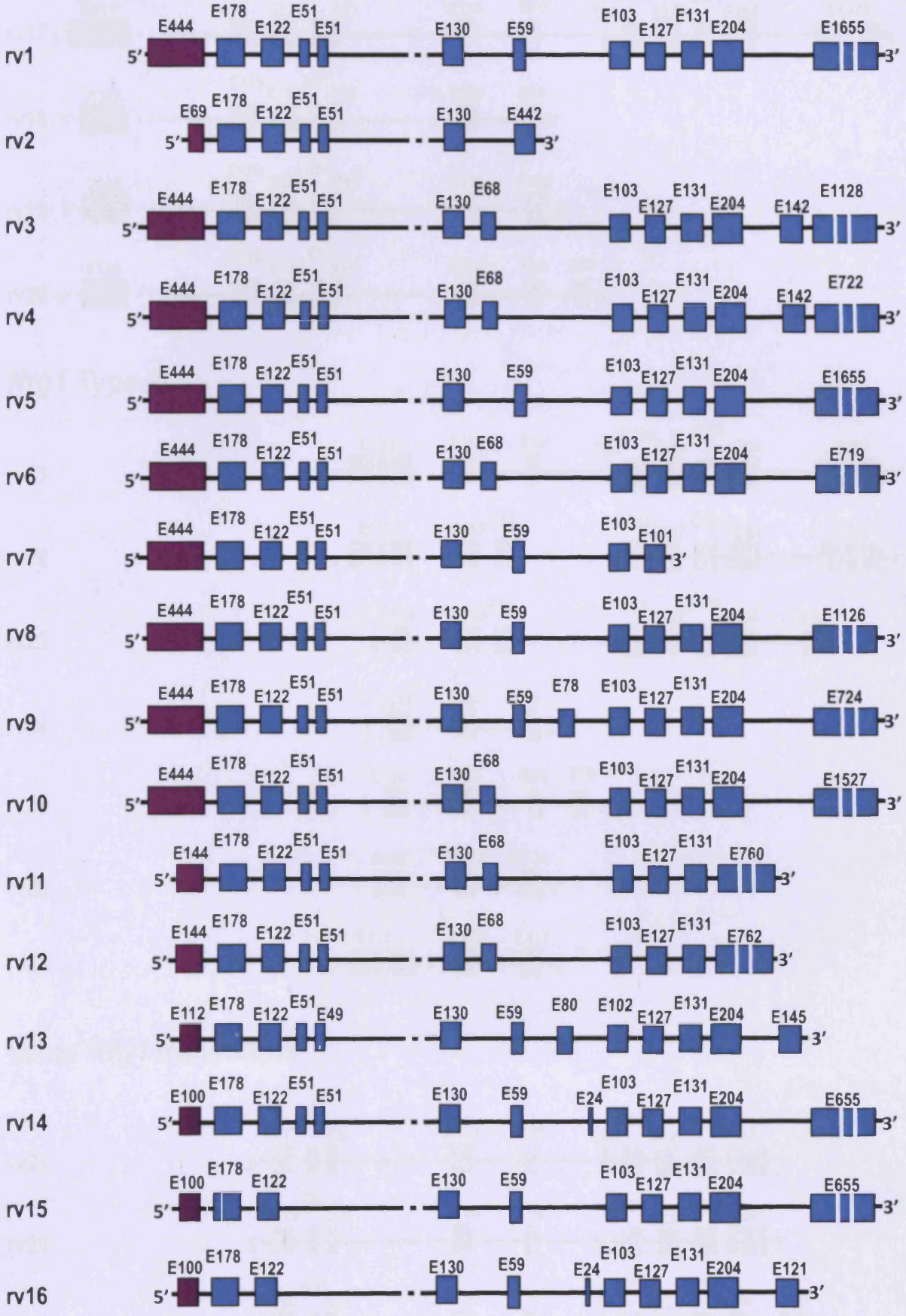
The exons characterised as contributing to the rat and human splice variants were merged to create a composite of the gene exonic structure for human *NRG1* and rat *Nrg1* (Fig. 3.3). There are 12 exons in both the human and rat that are highly homologous and have exactly the same number of nucleotides. All other exons common to both species are highly homologous in regions but vary in the complete number of nucleotides per exon. There are eight exons that are found in humans that have not yet been identified in the rat and there is one exon in the rat that has not been identified in humans. However the human *NRG1* gene structure is probably incomplete as not all mRNA sequences were reduced to their exonic structure. In addition, it is possible that not all splice variants have been determined and further exons may be present in the rat and human gene structures. For example, exons in the rat homologous to human *NRG1* type IV, V and VI 5'-defining exons may still be discovered.

3.3.1.2 *Dtnbp1* Exonic Structure

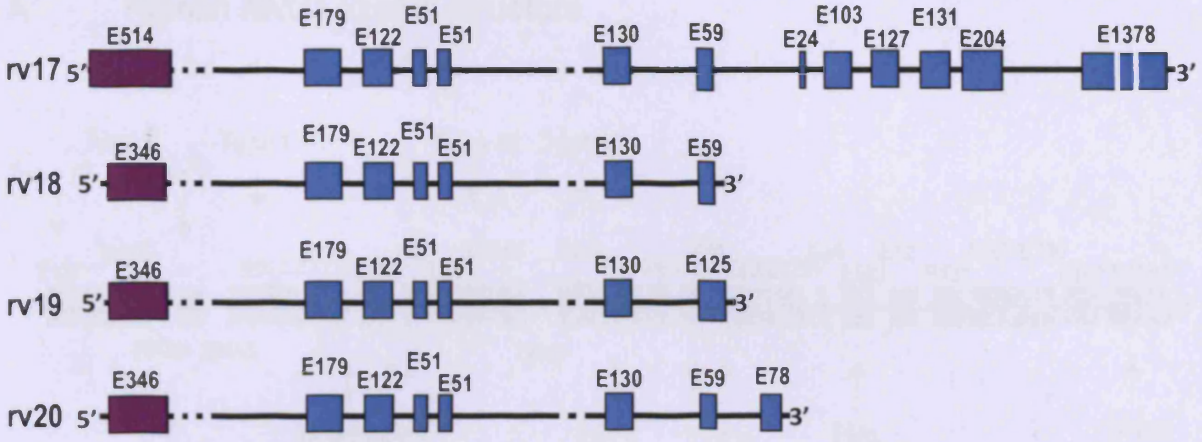
In total 15 experimentally determined transcripts were identified for human *DTNBP1* from NCBI Entrez Gene and the exons contributing to these sequences were determined. From these transcripts 3 were RefSeq sequences (hv1 – hv3) and only 2 of the remaining sequences were different from the RefSeq splice variants (hv4 & hv5) in that hv4 had an additional exon (E139) between E161 and E54, and the hv5 splice variant was shorter starting with the middle of the first E133 exon (Fig. 3.4 a). The *DTNBP1* exons identified in this study were merged together with two predicted exons and four predicted promoter sites identified previously using the program Aceview (Williams et al., 2004), to create a composite of the gene exonic structure for human *DTNBP1* (Fig. 3.4 b). The human *DTNBP1* gene has 13 exons, including the predicted exons. Only one experimentally determined RefSeq *Dtnbp1* transcript has been identified in the

Figure 3.2. Schematic of the exonic structure of all known rat *Nrg1* splice variants. Boxes represent exons. Purple boxes represent *Nrg1* type defining exons. Boxes are approximately relative in width compared to the number of nucleotides in the exon, with the exception of boxes with vertical dotted white lines through the box that indicates that the actual exon length is much longer than suggested by the relative width of the box. The number of nucleotides in an exon is given above the exon following the letter E. The length of the black line between the boxes is approximately relative to the length of the introns. Vertical white lines through the black line indicate that the actual intron length is much longer than suggested by the relative distance between the exons in that region. All mRNA sequences were found in NCBI Entrez gene and sourced from NCBI Entrez nucleotide. NCBI SPIDEY tool was used to determine the exonic structures. The NCBI accession numbers for each transcript are: v1, NM_031588; v2, U02315; v3, U02316; v4, U02317; v5, U02318; v6, U02319; v7, U02320; v8, U02321; v9, U02322; v10, U02323; v11, U02324; v12, M92430; v13, AY973245; v14, AY995221; v15, AY995222; v16, AY995223; v17, AF194993; v18, AF194995; v19, AF194996; v20, AF194997; v21, AF194438; v22, AF194439; v23, AF194440; v24, AF194441; v25, AF194442; v26, AF194443; v27, DQ176766; v28, AY973241; v29, AY973243; v30, AY973244.

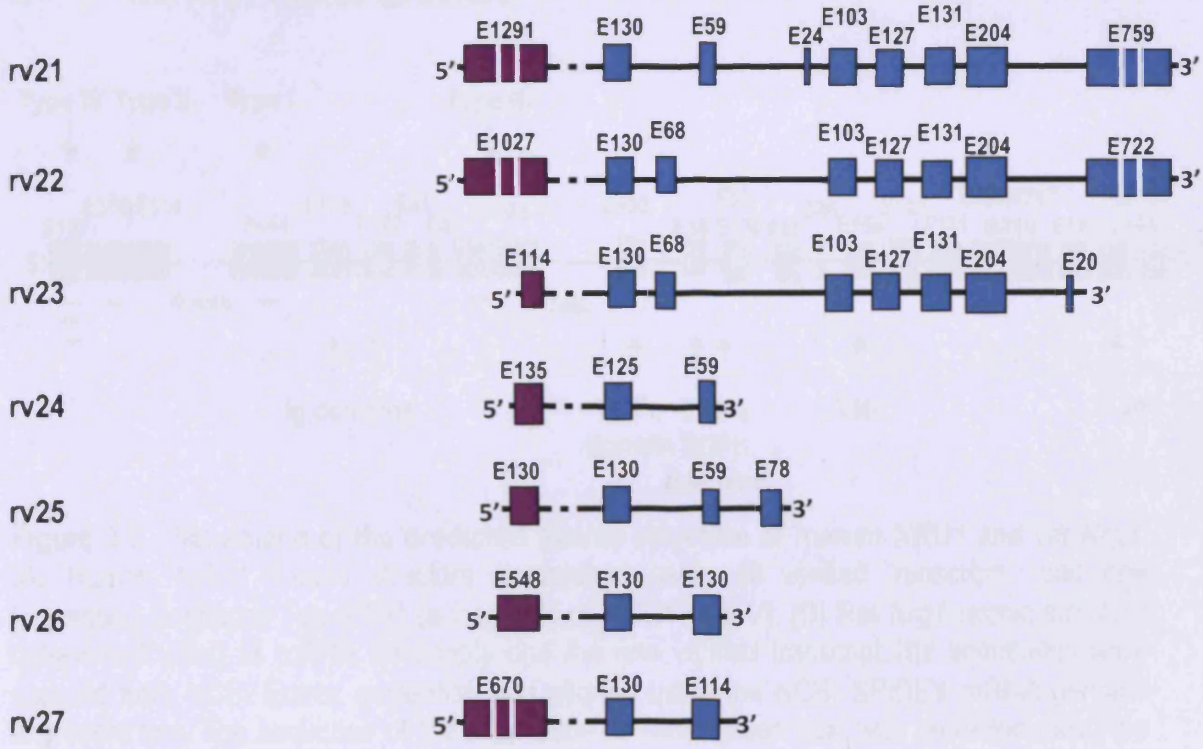
Nrg1 Type I


rv1
5′ E444 E178 E122 E51 E51 E130 E59 E103 E127 E131 E204 E1655 3′
rv2
5′ E69 E178 E122 E51 E51 E130 E442 3′
rv3
5′ E444 E178 E122 E51 E51 E130 E68 E103 E127 E131 E204 E142 E1128 3′
rv4
5′ E444 E178 E122 E51 E51 E130 E68 E103 E127 E131 E204 E142 E722 3′
rv5
5′ E444 E178 E122 E51 E51 E130 E59 E103 E127 E131 E204 E1655 3′
rv6
5′ E444 E178 E122 E51 E51 E130 E68 E103 E127 E131 E204 E719 3′
rv7
5′ E444 E178 E122 E51 E51 E130 E59 E103 E101 3′
rv8
5′ E444 E178 E122 E51 E51 E130 E59 E103 E127 E131 E204 E1126 3′
rv9
5′ E444 E178 E122 E51 E51 E130 E59 E78 E103 E127 E131 E204 E724 3′
rv10
5′ E444 E178 E122 E51 E51 E130 E68 E103 E127 E131 E204 E1527 3′
rv11
5′ E144 E178 E122 E51 E51 E130 E68 E103 E127 E131 E760 3′
rv12
5′ E144 E178 E122 E51 E51 E130 E68 E103 E127 E131 E762 3′
rv13
5′ E112 E178 E122 E51 E49 E130 E59 E80 E102 E127 E131 E204 E145 3′
rv14
5′ E100 E178 E122 E51 E51 E130 E59 E24 E103 E127 E131 E204 E655 3′
rv15
5′ E100 E178 E122 E130 E59 E103 E127 E131 E204 E655 3′
rv16
5′ E100 E178 E122 E130 E59 E24 E103 E127 E131 E204 E121 3′

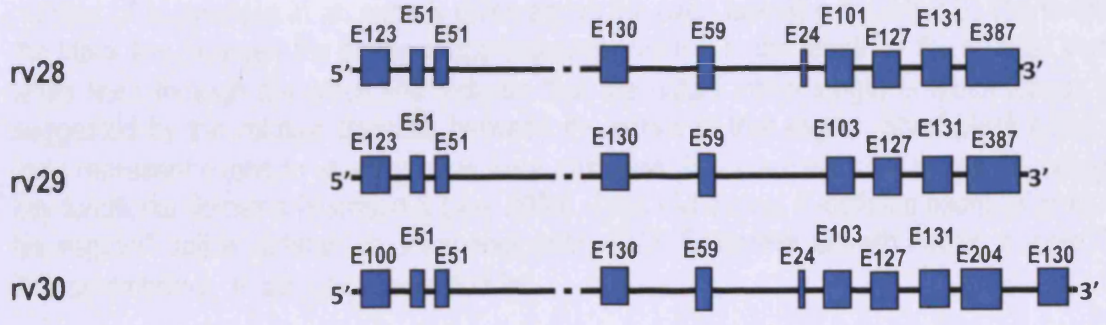
Nrg1 Type II



Nrg1 Type III



Other *Nrg1* transcripts



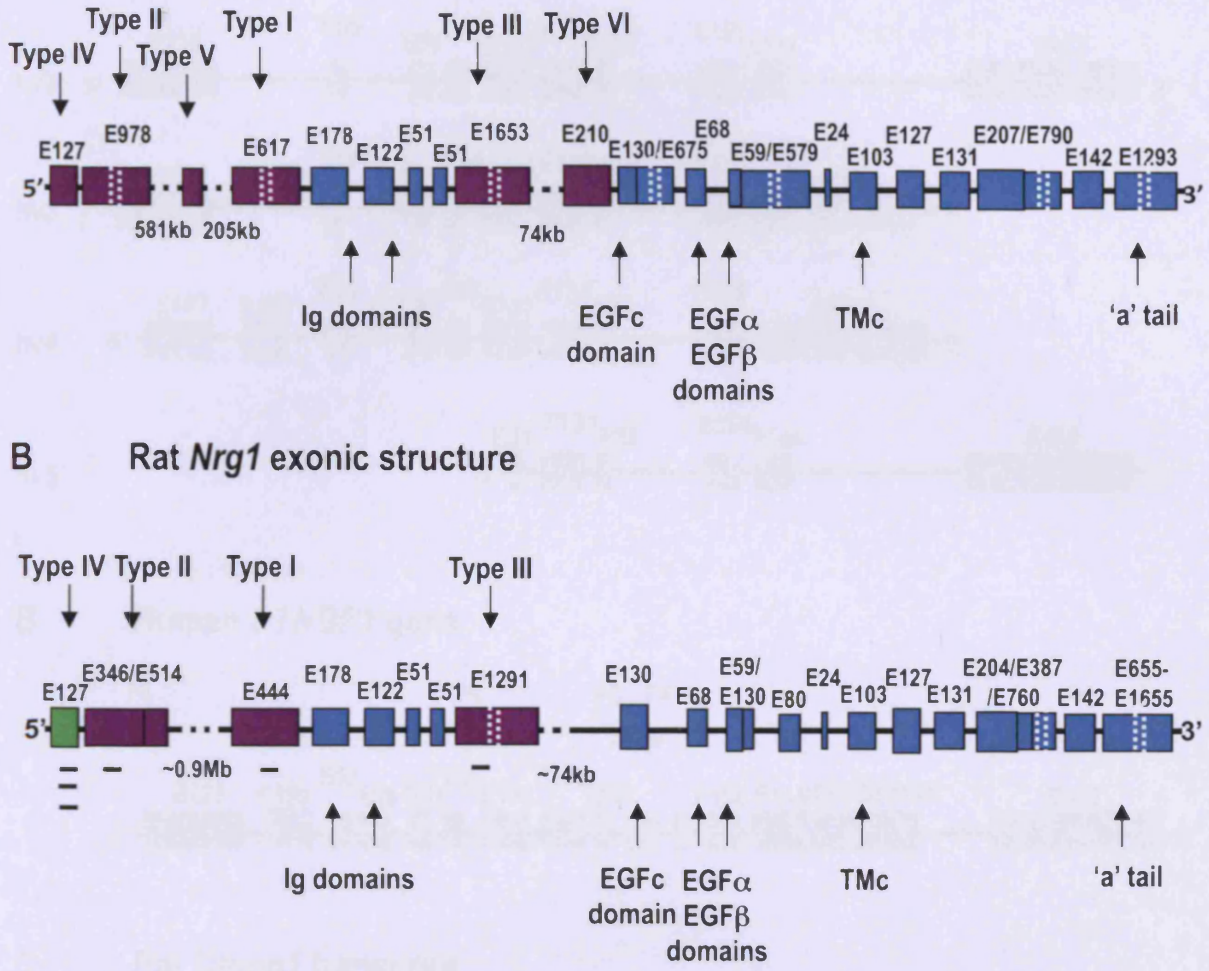


Figure 3.3. Schematic of the predicted exonic structure of human *NRG1* and rat *Nrg1*. (A) Human *NRG1* exonic structure determined using all verified transcripts, and one Expressed Sequence Tag (EST) (a fragment of *NRG1* type V). (B) Rat *Nrg1* exonic structure determined using all mRNA transcripts and the one verified transcript.The sequences were sourced from NCBI Entrez nucleotide and aligned using the NCBI SPIDEY mRNA genome alignment tool. The sequence of the *Nrg1* Type IV exon (green box) was predicted using the USCS exon predict tool. Boxes represent exons. Purple boxes represent exons defining type. Boxes are approximately relative in width compared to the number of nucleotides in the exon, with the exception of boxes with vertical dotted white lines through the box that indicates that the actual exon length is much longer than suggested by the relative width of the box. The number of nucleotides in an exon is given above the exon following the letter E. The length of the black line between the boxes is approximately relative to the length of the introns. Vertical white lines through the black line indicate that the actual intron length is much longer than suggested by the relative distance between the exons in that region. Short black horizontal lines represent exons to which probes were designed. Black arrows label exons of coding for key functional domains (Harrison & Law, 2006). Type I-VI exons, 5'-defining exons of groups of Neuregulin1 splice variants; Ig, Immunoglobulin; EGF, Epidermal Growth Factor; c, core; TM, Transmembrane; 'a' tail, polyadenylated tail.

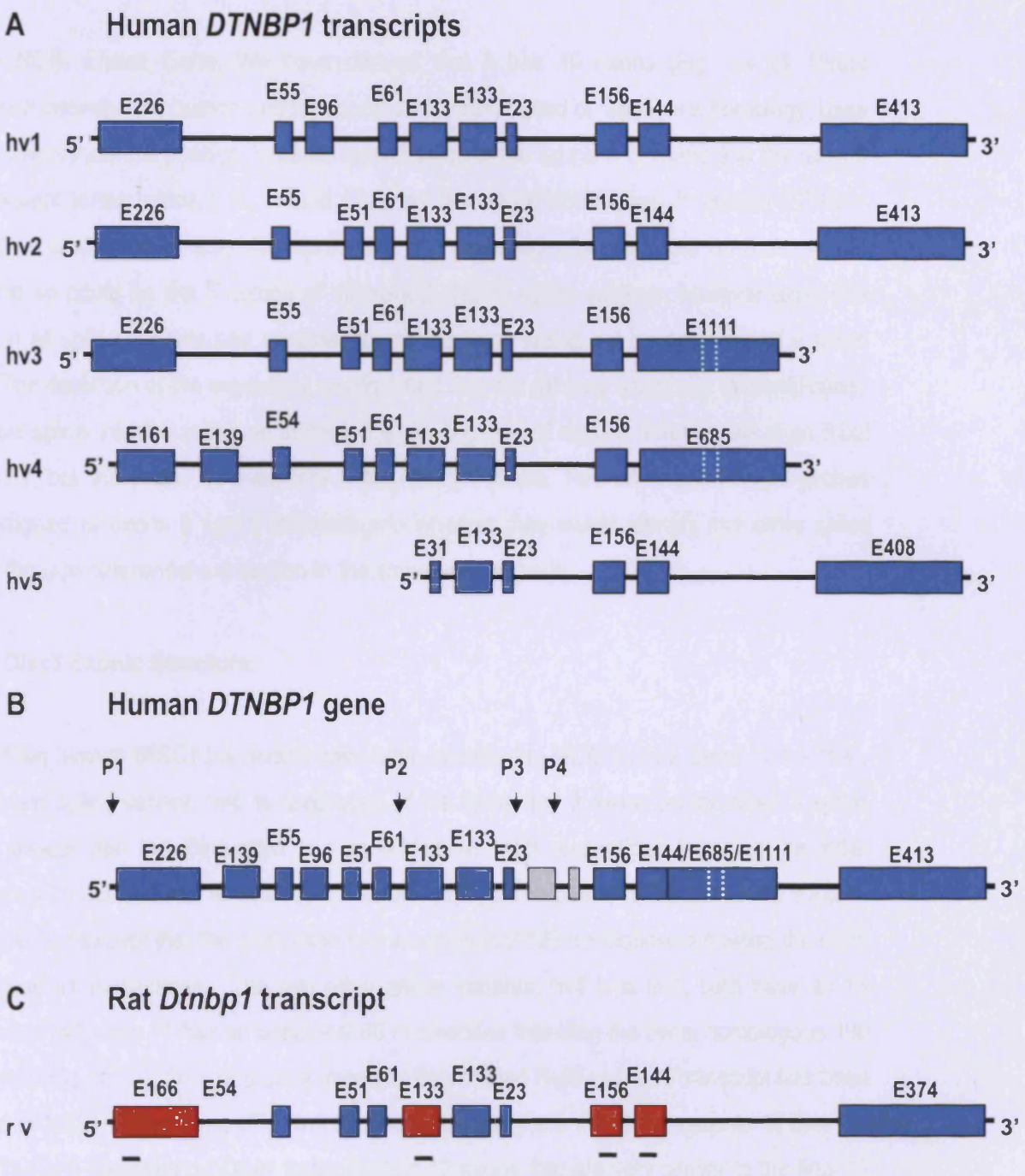


Figure 3.4. Schematic of the exonic structure of all different human *DTNBP1* transcripts, predicted *DTNBP1* gene and the only known rat *Dtnbp1* transcript. (A) Exonic structure of all different human *DTNBP1* splice variants. (B) Exonic structure of human *DTNBP1* gene. Two exons predicted using Aceview (grey boxes) and four predicted promoter sites are included (Williams et al., 2004). (C) Exonic structure of the one known rat *Dtnbp1* transcript with the exons that probes were designed to indicated (red boxes). Boxes represent validated exons. Boxes are approximately relative in width compared to the number of nucleotides in the exon. P1-P4 = Promoter 1-4. Short black horizontal lines represent exons to which probes were designed. NCBI Entrez nucleotide and the NCBI SPIDEY tool were used to determine exonic structure. The NCBI accession numbers for each transcript are: r v, NM_001037664; hv1, NM_183041; hv2, NM_032122; hv3, NM_183040; hv4, AK310590; hv5, AF061734.

rat from NCBI Entrez Gene. We have derived that it has 10 exons (Fig. 3.4 c). Direct comparison between the human and rat exonic structures, based on sequence homology, base pair size and sequential position, enabled identification of the exons 1, 5, 8 and 9 in the rat that are equivalent to the exons 1, 6, 11 and 12 in the human *DTNBP1* gene. In human *DTNBP1* the exons 1 and 6 are directly downstream of two predicted promoter sites (Williams et al., 2004) and so could be the 5' exons of different *DTNBP1* splice variants, however exon 6 is present in all splice variants and so detection of this exon would not be indicative of a splice variant. The detection of the equivalent exons 1 and 5 in the rat may contribute to identification of different splice variants in the rat through the identification of regions that express exon 5 but not exon 1, but the probe for exon 5 is a pan-*Dtnbp1* probe. Two other pan-*Dtnbp1* probes were designed to exons 8 and 9 to investigate whether they would identify any other splice variants through differential expression in the same brain regions.

3.3.1.3 *Disc1* Exonic Structure

Four RefSeq human *DISC1* transcripts have been identified in NCBI Entrez Gene (hv1 – hv4). The shortest splice variant, hv4, is composed of the same first 3 exons as the other 3 splice variants except that the third exon has an additional 1466 nucleotides following the initial homologous 70 nucleotides. Another splice variant (hv3) is composed of the same first 9 exons as hv1 and hv2 except that the ninth exon has an additional 125 nucleotides following the initial homologous 61 nucleotides. The two other splice variants, hv1 and hv2, both have all 13 exons but in hv1 exon 11 has an additional 66 nucleotides following the initial homologous 199 nucleotides (Fig. 3.5 a). Only one experimentally determined RefSeq *Disc1* transcript has been identified in NCBI Entrez gene (Fig. 3.5 b). Therefore there are no splice variants of *Disc1* in the rat. The one identified rat *Disc1* transcript has 12 exons that are very similar to the final 12 exons of the human *DISC1* gene.

3.3.1.4 *Egr3*, *Nab1* and *Nab2* Exonic Structure

Human *EGR3* and rat *Egr3* experimentally derived RefSeq transcripts have two exons, and do not have splice variants (Fig. 3.6 a). While the rat *Egr3* exons are considerably shorter than the human *EGR3* exons, the sequences of the rat *Egr3* exons are homologous to the initial sequences of the human *EGR3* exons. Only one transcript of rat *Nab1* and *Nab2* have been identified and they have 9 and 7 exons respectively (Fig. 3.6 b & c).

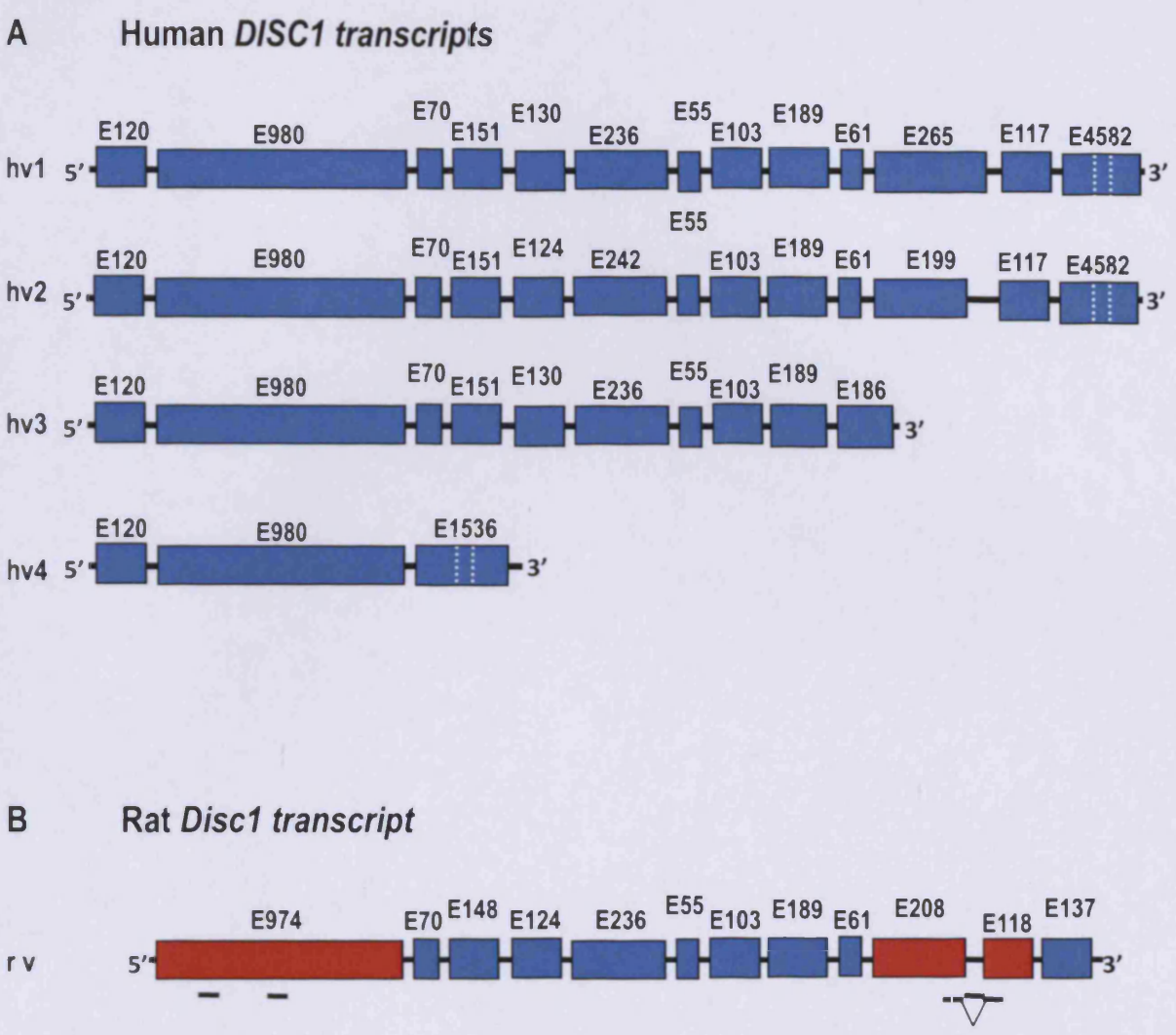


Figure 3.5. Schematic of the exonic structure of human *DISC1* splice variants and a rat *Disc1* transcript. (A) Exonic structure of the four verified mRNA sequences coding for human *DISC1* splice variants L (Long), Lv (Long variant), S (Short) and ES (Extremely Short) for sequences hv1-4 respectively. (B) Exonic structure of the only identified rat *Disc1* transcript. Boxes represent exons. Red boxes represent exons to which probes were designed. Boxes are approximately relative in width compared to the number of nucleotides in the exon with the exception of the boxes with dotted vertical white lines that indicate that the actual exon length is much longer than suggested by the relative width of the box. The number of nucleotides in an exon is given above the exon following the letter E. The black lines between the boxes represent the introns but the lengths are not drawn to scale. Short black horizontal lines represent exons to which probes were designed; the two short lines linked by a V shape represent the probe that crossed the exon boundary between E208 and E118. The sequences were sourced from NCBI Entrez nucleotide. NCBI SPIDEY tool was used to determine exonic structures. The NCBI accession numbers for each transcript are: hv1, NM_018662; hv2, NM_001012957; hv3, NM_001012959; hv4, NM_001012958; rv, NM_175596.

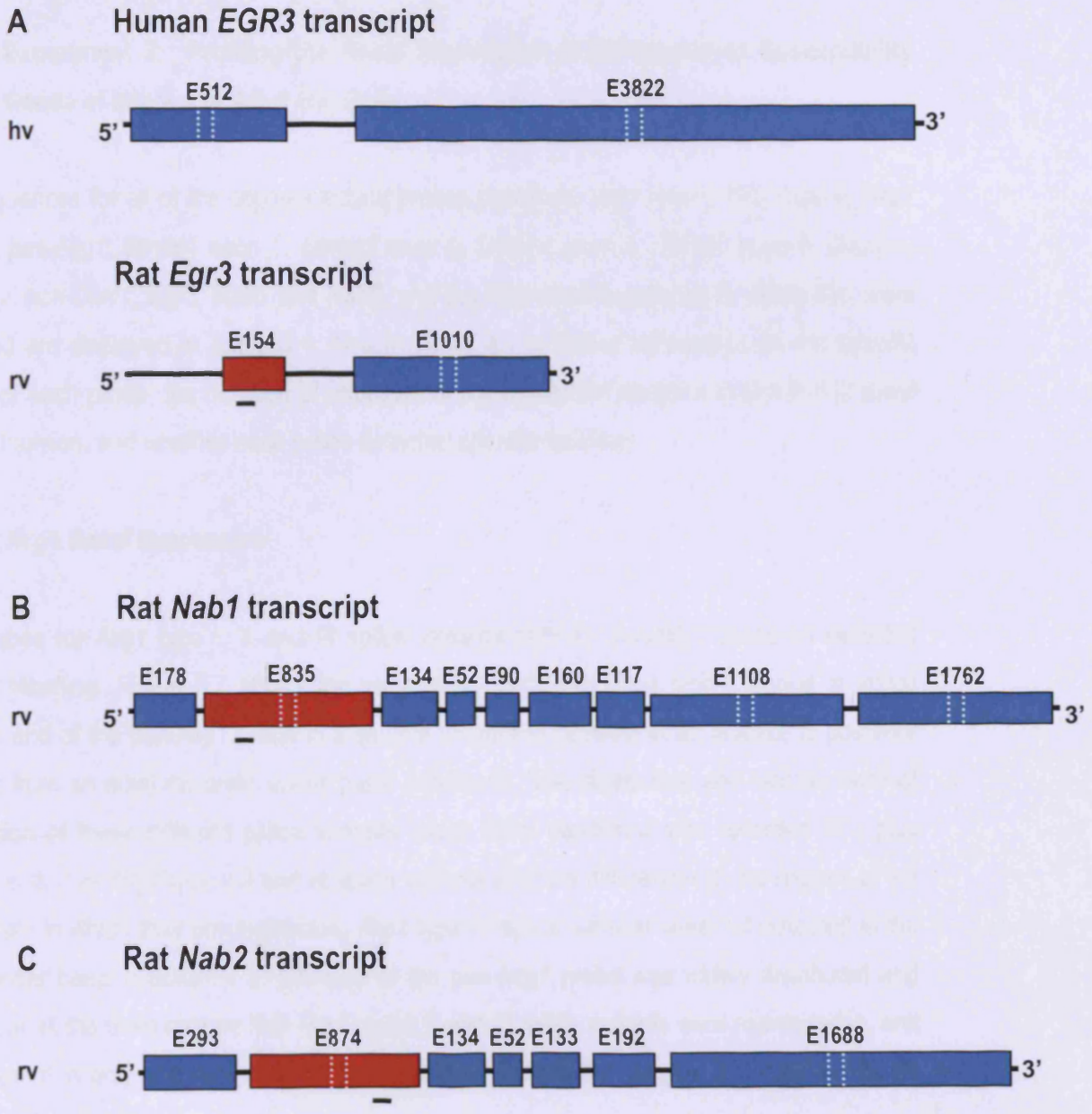


Figure 3.6. Schematic of the exonic structure of the only identified transcripts for human *EGR3* and rat *Egr3*, *Nab1* and *Nab2*. (A) Exonic structure of human *EGR3* and rat *Egr3*. (B) Exonic structure of rat *Nab1*. (C) Exonic structure of rat *Nab2* (C). Boxes represent exons. Red exons represent exons to which probes were designed. Boxes are approximately relative in width compared to the number of nucleotides in the exon with the exception of the boxes with dotted vertical white lines that indicate that the actual exon length is much longer than suggested by the relative width of the box. The number of nucleotides in an exon is given above the exon following the letter E. The black lines between the boxes represent the introns but the lengths are not drawn to scale. Short black horizontal lines represent regions of sequence to which probes were designed. The sequences were sourced from NCBI Entrez nucleotide. NCBI SPIDEY tool was used to determine exonic structures. The NCBI accession numbers for each transcript are: (A) hv, NM_0044304; rv, NM_017086; (B) rv, NM_022856; (C) rv, NM_001134874.

3.3.2 Experiment 2: Profiling the Basal Expression of Schizophrenia Susceptibility Genes of Interest in Adult Rat Brain

The sequences for all of the oligonucleotide probes designed, *Nrg1* type I, *Nrg1* type II, *Nrg1* type III, pan-*Nrg1*, *Dtnbp1* exon 1, *Dtnbp1* exon 5, *Dtnbp1* exon 8, *Dtnbp1* exon 9, *Disc1* L, *Disc1* Lv, pan-*Disc1*, *Egr3*, *Nab1* and *Nab2*, and the reference sequences to which they were designed are displayed in Table 3.1. Also in Table 3.1 is further information on the specific activity of each probe, the duration of exposure of the hybridised sections to film and in some cases emulsion, and whether each probe detected specific labelling.

3.3.2.1 *Nrg1* Basal Expression

The probes for *Nrg1* type I, II and III splice variants and the pan-*Nrg1* probe all detected specific labelling. Figure 3.7 shows the expression pattern of *Nrg1* type I, II and III splice variants and of the pan-*Nrg1* probe in a sample of coronal sections in an anterior to posterior manner from an adult rat brain under basal conditions. The distribution and relative level of expression of these different splice variants under basal conditions was recorded in a plus chart (Table 3.2). *Nrg1* type I, II and III splice variants all show differences in the regions of the adult brain in which they are expressed. *Nrg1* type IV splice variants were not detected in the brain under basal conditions. Expression of the pan-*Nrg1* probe was widely distributed and present in all the brain regions that *Nrg1* type I, II and III splice variants were expressed in, and was present in only one region, the globus pallidus in the basal ganglia, unaccounted for by *Nrg1* type I, II and III.

The highest level of *Nrg1* type I splice variants expression was observed in the hippocampal CA1-CA4 and DG regions, the granule cells of the olfactory bulb, the choroid plexus, bed nucleus of the stria terminus, the medial habenula, some nuclei of the hypothalamus, the pontine nucleus and the Purkinje and granule cell layers of the cerebellum. The mPFC, medial to posterior cerebral cortex and nuclei of the amygdala show medium levels of *Nrg1* type I expression (Fig. 3.7 a and Table 3.2). The expression of *Nrg1* type II splice variants was less widely distributed in the rat brain under basal conditions than *Nrg1* type I splice variants. The highest level of expression is observed in the caudate putamen and nucleus accumbens of the basal ganglia, the reticular nucleus, zona incerta and geniculate nucleus of the thalamus, the

Table 3.2. The expression profiles of *Nrg1* type I, type II, type III and the pan-*Nrg1* probe.
Key: - not detected, + low expression, ++medium expression, +++ high expression.

Brain region	*Nrg1* type I	*Nrg1* type II	*Nrg1* type III	Pan *Nrg1*
Olfactory bulb				
Periglomerular layer	+	+	+	+
External plexiform layer	+	+	+	+
Mitral cells	++	++	+++	+++
Internal plexiform layer	-	-	-	-
Granule cells	+++	+++	++	+++
Ependymal cells	-	-	-	-
Cerebral cortex				
Layer I	++	+	+	++
Layer II	++	+	+	++
Layer III	++	++	+	++
Layer IV	++	-	-	+
Layer V	++	++	++	+++
Layer VI	++	++	++	+++
Amygdalohippocampal area	++	++	++	++
Entorhinal cortex	++	++	+	++
Piriform cortex	++	++	+	++
Olfactory tract nucleus	++	+	+	++
Olfactory tubercle	-	-	-	-
Islands of Calleja	-	-	-	-
Choroid plexus				
Ependymal cells	+++	+++	-	+++
Hippocampus				
CA1 pyramidal cells	+++	-	-	++
CA2 pyramidal cells	+++	-	-	++
CA3 pyramidal cells	+++	-	-	++
CA4 pyramidal cells	+++	-	-	++
DG granule cells	+++	+	-	++
Amygdala				
Cortical amygdaloid nucleus	++	+	+	++
Central amygdaloid nucleus	++	-	+	++
Lateral amygdaloid nucleus	++	-	+	++
Medial amygdaloid nucleus	++	-	+	++
Basolateral amygdaloid nucleus	++	-	+	++
Basal ganglia				
Caudate putamen	+	+++	-	+
Nucleus accumbens	+	+++	-	+
Globus pallidus	-	-	-	+
Claustrum	+	+	+	++
Subthalamic nucleus	+	+	+	+

Table 3.2 Continued.

Substantia nigra				
Pars reticulata	+	+	+	+
Pars compacta	+	+	+	+
Septum				
Lateral septum	-	-	+++	+++
Medial septum	-	-	+++	+++
Diagonal band	++	+	+++	+++
Bed nucleus stria terminus	+++	+	-	+++
Habenula				
Medial	+++	+	+++	+++
Lateral	+	-	-	+
Thalamus				
Paraventricular nucleus	++	+	-	+
Medial dorsal nucleus	++	+	+	+
Anterodorsal nucleus	++	+	-	+
Anteroventral nucleus	++	+	-	+
Lateral nucleus	++	+	-	+
Reticular nucleus	++	+++	+++	+++
Zona incerta	++	+++	+++	+++
Dorsolateral geniculate	++	+++	++	++
Ventrolateral geniculate	++	+++	++	++
Medial geniculate	++	+++	++	++
Rhomboid nucleus	++	+	-	+
Parafascicular nucleus	++	+	-	+
Ventrobasal nucleus	++	-	++	++
Hypothalamus				
Medial preoptic area	++	-	+	+
Anterior nucleus	+++	-	+	+
Paraventricular nucleus	++	+	+++	+++
Arcuate nucleus	+++	+	+	+
Ventromedial nucleus	+++	+	+	++
Dorsomedial nucleus	++	+	+	++
Cerebellum				
Molecular cell layer	+	+	+	++
Purkinje cell layer	+++	+	++	+++
Granule cell layer	+++	+	++	+++
Pons				
Pontine nuclei	+++	+++	+++	+++
Transverse fibres of the pons	-	-	-	-
Longitudinal fasciculus of the	-	-	-	-
Medial Lemniscus	-	-	-	-

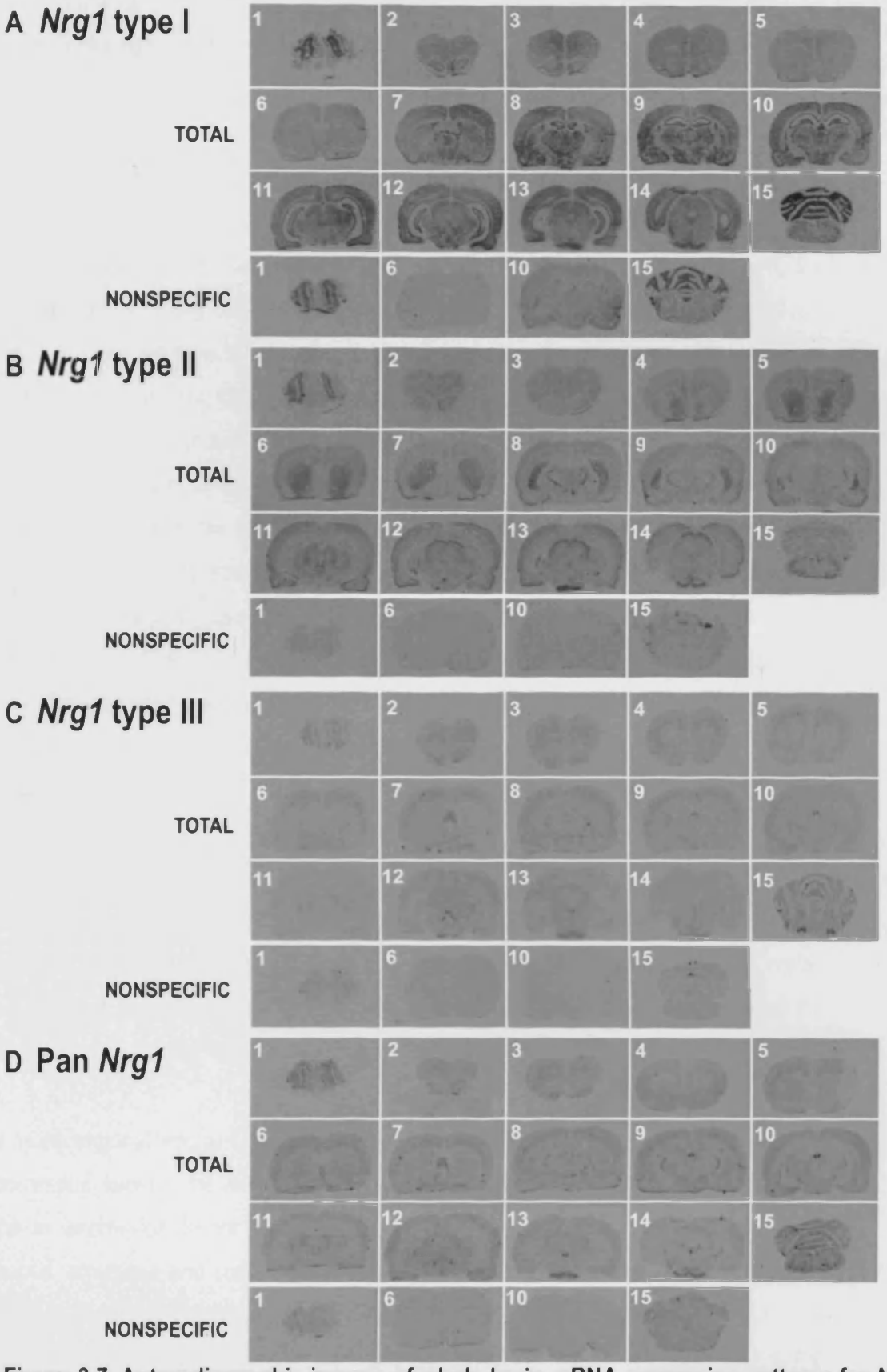


Figure 3.7. Autoradiographic images of whole brain mRNA expression patterns for *Nrg1* type I, type II and type III splice variants and pan *Nrg1* transcripts determined using ISH. (A) *Nrg1* type I expression pattern. (B) *Nrg1* type II expression pattern. (C) *Nrg1* type III expression pattern. (D) Pan *Nrg1* expression pattern. Numbers in ascending order represent sections anterior - posterior through the adult rat brain. Nonspecific labelling is shown in four sections for each probe, numbered to match similar sections with total labelling.

granule cells of the olfactory bulb, the choroid plexus and the pontine nuclei. Layer IV in the mPFC and cerebral cortex shows high expression of Nrg1 type II (Fig. 3.7 b and Table 3.2). The expression of *Nrg1* type III splice variants was also less widely distributed in the rat brain under basal conditions than *Nrg1* type I splice variants. The highest level of expression is detectable in the mitral cells of the olfactory bulb, the lateral and medial septum, the diagonal band of the septum, the medial habenula, the reticular nucleus and zona incerta of the thalamus, the paraventricular nucleus of the hypothalamus and the pontine nuclei (Fig. 3.7 c and Table 3.2). The *Nrg1* type IV splice variants were undetectable in the adult rat brain under basal conditions despite using three different probes. The expression of pan *Nrg1* showed wide distribution in the rat brain under basal conditions. The highest level of expression is detectable in mitral and granule cells of the olfactory bulb, layers V and VI of the cerebral cortex, the choroid plexus, the septum, the medial habenula, the reticular nucleus and zona incerta of the thalamus, the paraventricular nucleus of the hypothalamus, the pontine nuclei and the Purkinje and granule cell layers of the cerebellum. Of all the regions of the brain in which expression of *Nrg1* type I, II or III splice variants was detected, expression of the pan *Nrg1* also detected expression. The pan *Nrg1* probe was also detected in the globus pallidus of the basal ganglia, but *Nrg1* type I, II or III splice variants were not (Table 3.2).

3.3.2.2 *Nrg1* Expression at the Regional Level

More detailed study of *Nrg1* types I, II and III splice variants and pan-*Nrg1* in regions of the hippocampus (CA1, CA3 & DG), amygdala (LA, BLA and CeN nuclei) and cerebral cortex (layers I-IV, V & VI), using image densitometry gave semi-quantitative expression values at the regional level of analysis (Fig. 3.8).

Analysis at the regional level showed that the relative expression of *Nrg1* type I was highest in the hippocampus, lower in the amygdala and lower still in the cerebral cortex. There was little difference in expression levels between the different regions, nuclei and layers of the hippocampus, amygdala and cortex respectively (Fig. 3.8 a). The expression of *Nrg1* type II was highest in the DG of the hippocampus with the CA1 and CA3 both expressing less. The expression level of *Nrg1* type II in layers I-IV of the cerebral cortex was nearly as high as in the DG. Cortical layers V and VI had slightly less *Nrg1* type II expression than layers I-IV. Expression levels in the nuclei of the amygdala had approximately the same level of expression as in the CA1 and CA3 regions of the hippocampus (Fig. 3.8 b). The expression of

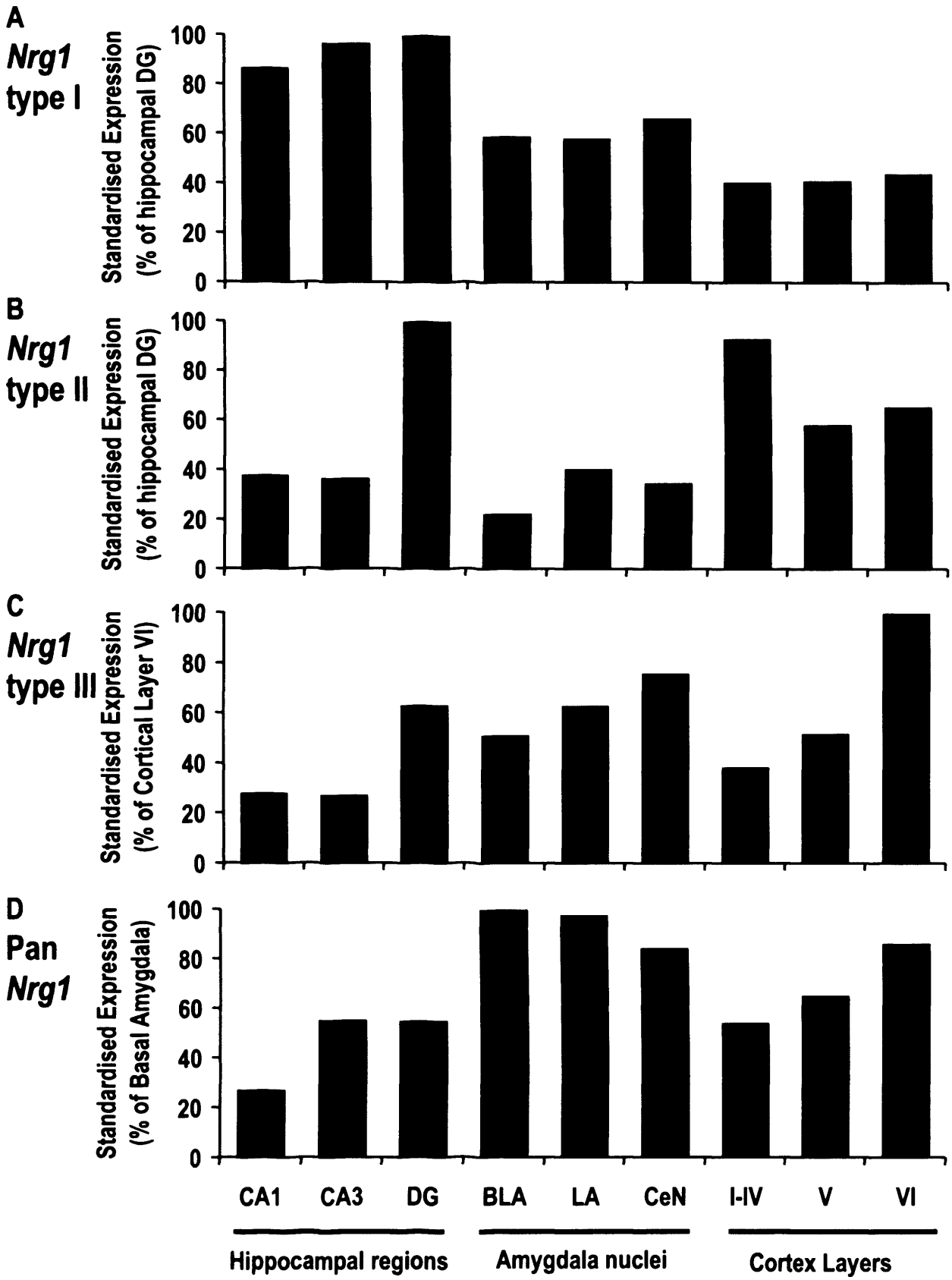


Figure 3.8. *Nrg1* type I, II and III splice variants and pan *Nrg1* expression distribution determined using image densitometry. (A) *Nrg1* type I expression distribution. (B) *Nrg1* type II expression distribution. (C) *Nrg1* type III expression distribution. (D) Pan *Nrg1* expression distribution. Image densitometry was used to determine expression in the hippocampus (CA1, CA3 & DG regions), amygdala (basal, lateral & central nuclei) and cerebral cortex (layers I-IV, V & VI) of the adult rat brain (n=1). Expression (nCi/mg tissue): (A) DG, 76; (B) DG, 24; (C) Layer VI, 4; (D) Basal nucleus, 8.

Nrg1 type III was highest in layer VI of the cerebral cortex with layers I-IV and layer V expressing quite a lot less. Expression of *Nrg1* type III was similar in all nuclei of the amygdala, and expression in the amygdala was less than in layer VI of the cortex but more than in cortical layers I-IV and V. The DG of the hippocampus had a similar level of expression to that measured in the amygdala. Like *Nrg1* type II expression patterns, *Nrg1* type III splice variants showed substantially less expression in the CA1 and CA3 than in the DG regions of the hippocampus (Fig. 3.8 c). Unlike any of the *Nrg1* splice variants probed for the relative expression of the pan-*Nrg1* probe was highest in the amygdala. The expression of the pan-*Nrg1* probe was lowest in the hippocampus with levels in the cortex falling between the two. The different nuclei of the amygdala had similar levels of expression of the pan-*Nrg1* probe. The expression of the pan-*Nrg1* probe was lower in the CA1 compared to CA3 and DG regions of the hippocampus. In the cerebral cortex, the highest level of expression of the pan-*Nrg1* probe was measured in layer VI, with layers I-IV and V expressing less (Fig. 3.8 d).

3.3.2.3 *Nrg1* Expression at the Cellular Level

Cellular level expression of *Nrg1* type I, II and III splice variants in the cerebral cortex layers I-IV, V and VI, and hippocampal regions CA1, CA3 and DG, is shown in a sample of photomicrographs of ISH-labelled emulsion-dipped sections counterstained with thionin (Fig. 3.9). Examples of non-specific labelled photomicrographs are also provided for comparison. The density of silver grains/cell was measured (Fig. 3.10).

Cellular level analysis showed that the expression of *Nrg1* type I was highest in cortex layer V, least in cortex layer VI, and layers I-IV showed approximately half the level of expression of layer V. The amygdala nuclei all had similar expression levels to cortex layers I-IV. The hippocampal CA3 region had the second highest level of expression and the CA1 and DG of the hippocampus expressed approximately half that expressed in the CA3 region (Fig. 3.10 a). Cellular level analysis showed that the expression of *Nrg1* 1 type II was highest in the lateral nucleus of the amygdala. The basal and central nuclei of the amygdala and layers I-IV and layer V of the cerebral cortex all showed similarly high levels of *Nrg1* type II expression. Layer VI of the cortex showed slightly less *Nrg1* type II expression than was measured in layer V. *Nrg1* type II expression in the hippocampal CA1 and CA3 regions was undetectable while the DG showed low level *Nrg1* type II expression (Fig. 3.10 b). Cellular level analysis showed that the relative expression of *Nrg1* type III was highest in the CA3 of the hippocampus with quite a

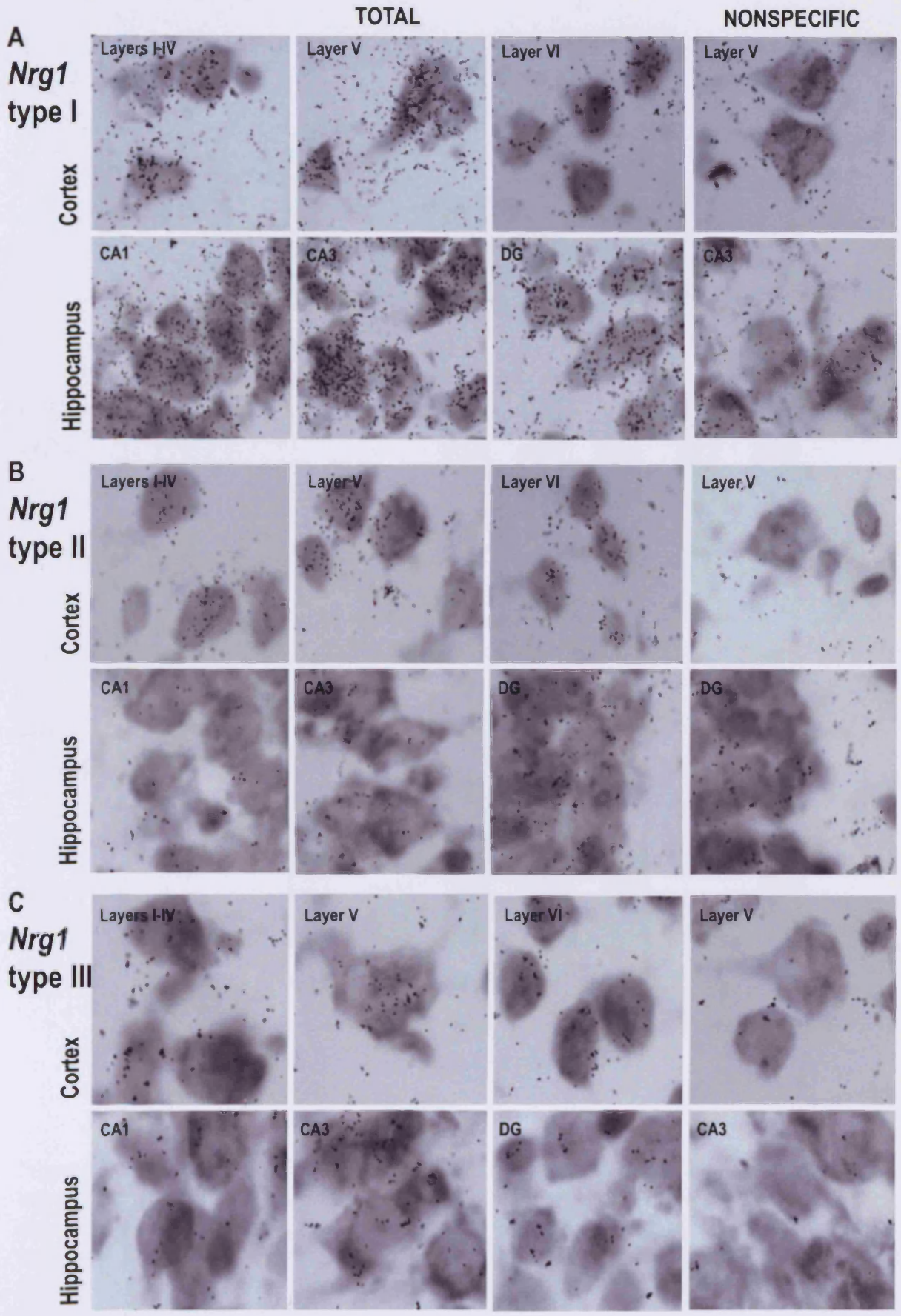


Figure 3.9. Images of silver grains, identifying *Nrg1* type I, type II and type III mRNA, overlying individual cells in different hippocampal and cortical regions of the adult rat brain. (A) *Nrg1* type I cellular expression. (B) *Nrg1* type II cellular expression. (C) *Nrg1* type III cellular expression. Nonspecific labelling for each probe in hippocampal and cortical regions are given.

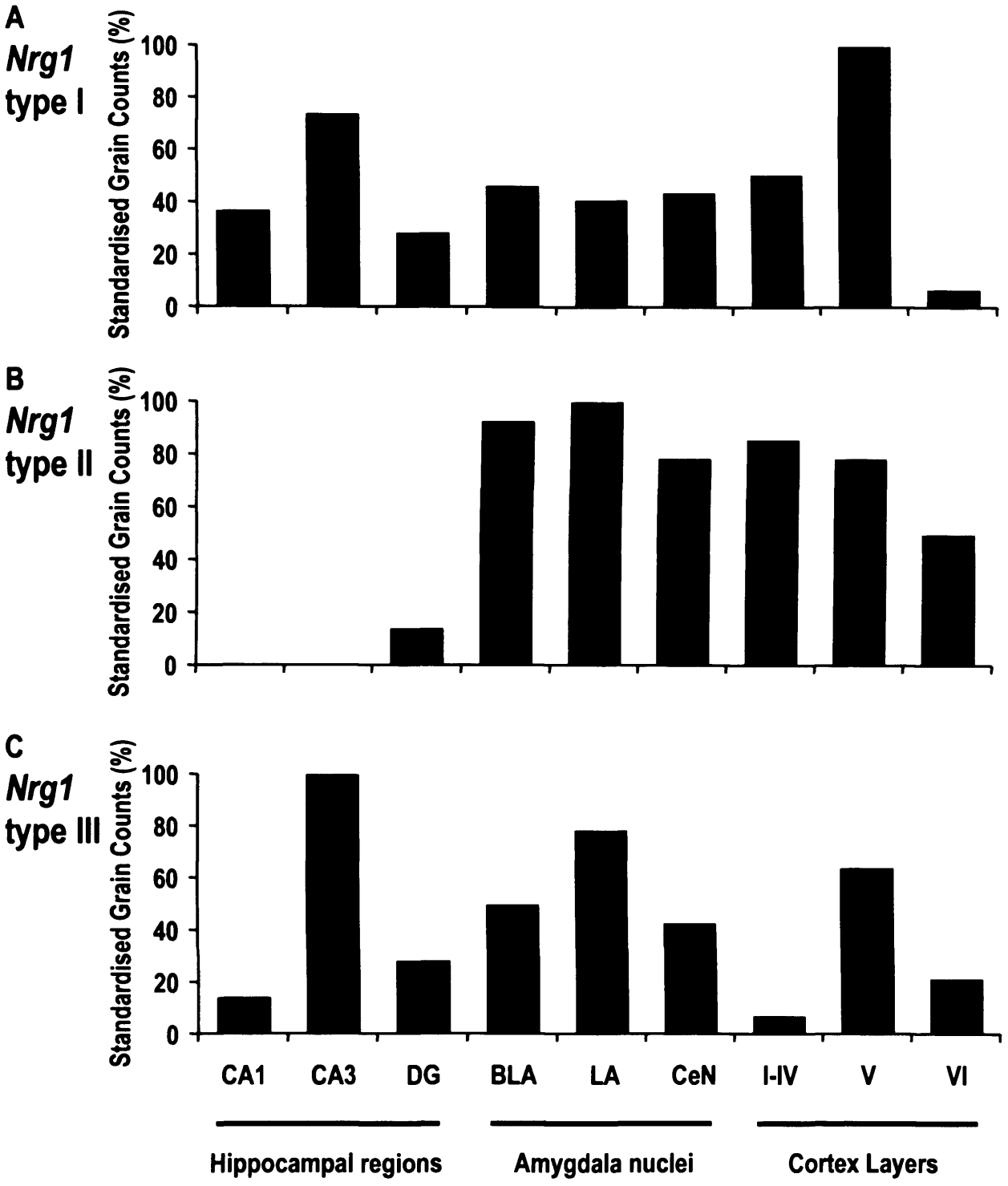


Figure 3.10. The distribution of *Nrg1* type I, II and III splice variants expression measured using silver grain counts. Nrg1 type I (A) *Nrg1* type I expression distribution. (B) *Nrg1* type II expression distribution. (C) *Nrg1* type III expression distribution. Silver grain counting was used to determine expression in the hippocampus (CA1, CA3 & DG regions), amygdala (basal, lateral & central nuclei) and cerebral cortex (layers I-IV, V & VI) of the adult rat brain (n=1). Expression (Grains/cell): (A) Layer V, 73; (B) Lateral nucleus, 14; (C) CA3, 14.

lot less expression measured in the CA1 and DG. The second highest level of expression was found in the lateral nucleus of the amygdala, with the basal and central nuclei expressing nearly half as much. The cerebral cortex layer V had a similar level of expression to that observed in the lateral nucleus of the amygdala, expressed at least three times as much *Nrg1* type III than layers I-IV and layer VI of the cerebral cortex (Fig. 3.10 c).

The expression of *Nrg1* type III splice variants was measured at the cellular level in each of the cortical layers II, III, IV, V and VI in order to confirm that expression in layer V was greater than that measured in layers I-IV and VI as had been observed. There was much higher levels of expression of *Nrg1* type III splice variants in layer V compared to the expression levels detected in layers II, III, IV and VI (Fig. 3.11). This is a similar pattern to that seen when layers were grouped together as layers I-IV, V and VI (Fig. 3.10 c).

3.3.2.4 *Dtnbp1* Basal Expression

Probes designed to detect exons 1, 5, 8 and 9 from the only identified *Dtnbp1* transcript in the rat showed variation in their expression patterns in the adult rat brain, and variation in the relative level of expression in some brain regions. The expression patterns detected for *Dtnbp1* exons 1, 5, 8 and 9 in a selection of coronal sections throughout the adult rat brain are shown in Figure 3.12. The distribution patterns of *Dtnbp1* exons 1, 5, 8 and 9 expression in regions of the adult rat brain are presented in Table 3.3. Due to there not being enough brain sections from the rat brain used for characterization of whole brain gene expression under basal conditions, *Dtnbp1* exon 1 basal expression could only be investigated in a limited selection of rat brain regions determined from the naïve brain sections used as controls in the behavioural experiment performed in Chapter 4.

The highest level of *Dtnbp1* exon 1 expression was observed in layer IV of the cerebral cortex, the piriform cortex, the choroid plexus, the hippocampal CA1 region, and the zona incerta, reticular nucleus and geniculate nuclei of the thalamus (Fig. 3.12 a and Table 3.3). The highest level of *Dtnbp1* exon 5 expression was observed in the granule cells of the olfactory bulb, layer IV of the cerebral cortex, the piriform cortex, the choroid plexus, the hippocampal CA1 region, and the zona incerta, reticular nucleus and geniculate nuclei of the thalamus (Fig. 3.12 b and Table 3.3). The highest level of *Dtnbp1* exon 8 expression was observed in the granule cells of the olfactory bulb, layer IV of the cerebral cortex, the amygdalohippocampal area, the piriform

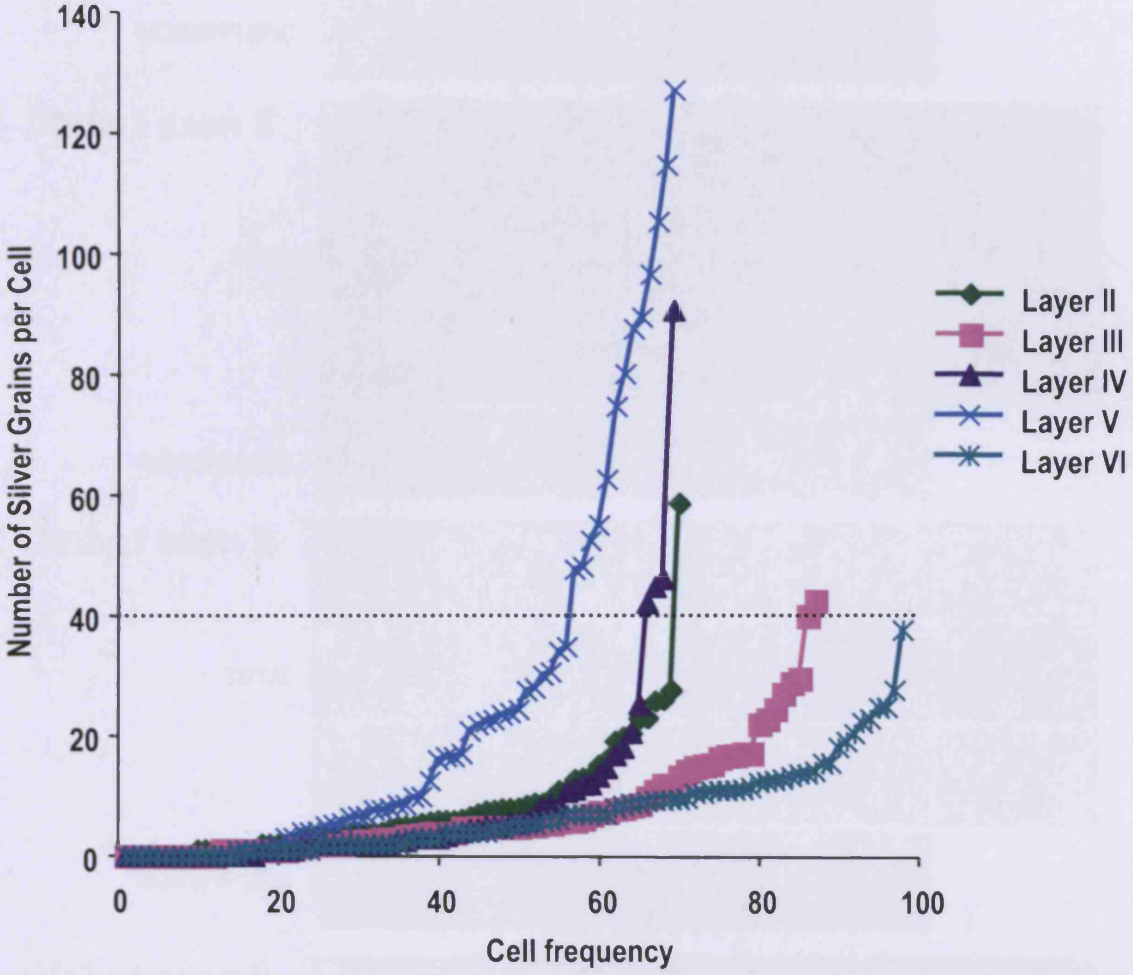


Figure 3.11. Number of silver grains per cell in different cortical layers for *Nrg1* type III in the adult rat brain. Layer V differs from layers II, III, IV and VI as there are many more cells in this layer that have more than 40 silver grains per cell eg. 19% of the cells in layer V compared to 1%, 2%, 6% and 0% of the cells in layers II, III, IV and VI respectively.

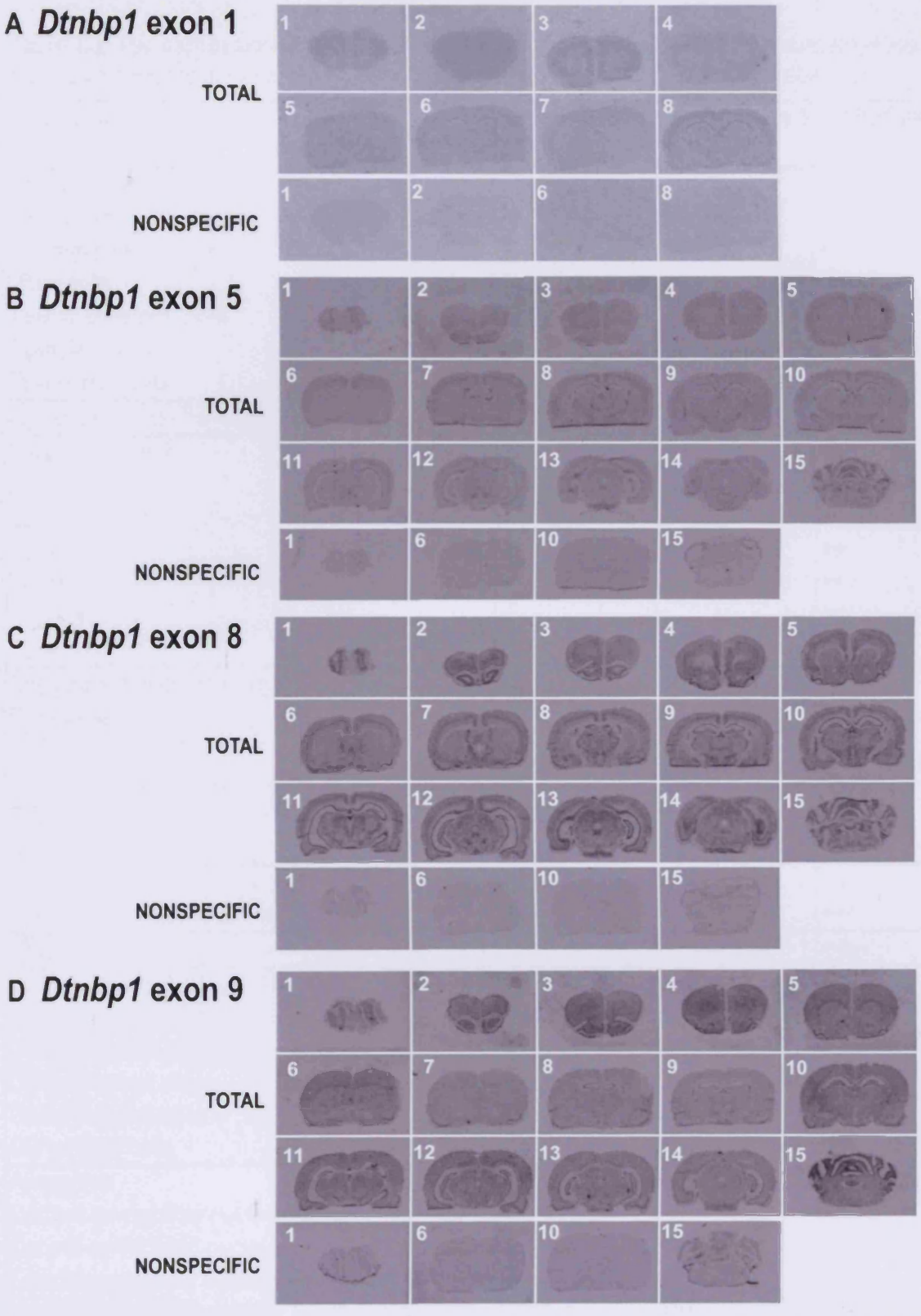


Figure 3.12. Autoradiographic images of whole brain mRNA expression patterns for predicted *Dtnbp1* splice variants determined using ISH. (A) *Dtnbp1* exon 1 expression pattern. (B) *Dtnbp1* exon 5 expression pattern. (C) *Dtnbp1* exon 8 expression pattern. (D) *Dtnbp1* exon 9 expression pattern. Numbers in ascending order represent sections anterior - posterior through the adult rat brain. Nonspecific labelling is shown in four sections for each probe, numbered to match similar sections with total labelling.

Table 3.3. The expression profiles of *Dtnbp1* exon 1, 5, 8 and 9. Key: - not detected, + low expression, ++medium expression, +++ high expression and na tissue not available.

Brain region	*Dtnbp1* exon 1	*Dtnbp1* exon 5	*Dtnbp1* exon 8	*Dtnbp1* exon 9
Olfactory bulb				
Periglomerular layer	na	+	+	+
External plexiform layer	na	+	+	+
Mitral cells	na	++	++	++
Internal plexiform layer	na	-	-	-
Granule cells	na	+++	+++	+++
Ependymal cells	na	-	-	-
Prefrontal cortex- Forceps minor	-	-	-	+++
Cerebral cortex				
Layer I	-	-	+	++
Layer II	-	-	+	++
Layer III	-	-	+	++
Layer IV	+++	+++	+++	+++
Layer V	-	-	+	++
Layer VI	-	-	+	++
Amygdalohippocampal area	+	-	+++	++
Entorhinal cortex	+	-	+	+
Piriform cortex	+++	+++	+++	+++
Olfactory tract nucleus	na	++	++	++
Olfactory tubercle	-	-	-	-
Islands of Calleja	-	-	-	-
Choroid plexus				
Ependymal cells	+++	+++	+++	+++
Corpus callosum	-	-	-	+++
Hippocampus				
CA1 pyramidal cells	+++	+++	+++	+++
CA2 pyramidal cells	++	++	+++	+++
CA3 pyramidal cells	++	++	+++	+++
CA4 pyramidal cells	++	++	+++	+++
DG granule cells	++	++	+++	+++
Amygdala				
Cortical amygdaloid nucleus	++	-	+	+
Central amygdaloid nucleus	+	+	+++	++
Lateral amygdaloid nucleus	+	+	+++	++
Medial amygdaloid nucleus	+	+	+++	++
Basolateral amygdaloid nucleus	+	+	+++	++
Basal ganglia				
Caudate putamen	-	-	+	+
Nucleus accumbens	-	-	+	+
Globus pallidus	-	-	+	+
Claustrum	+	+	+	+
Subthalamic nucleus	+	+	++	++

Table 3.3 Continued.

Substantia nigra				
Pars reticulata	na	+	++	++
Pars compacta	na	++	++	+
Septum				
Lateral septum	na	+	+	+
Medial septum	na	+	++	++
Diagonal band	na	+	++	++
Bed nucleus stria terminus	na	+	+	+
Habenula				
Medial	++	++	+++	+++
Lateral	+	+	+	+
Thalamus				
Paraventricular nucleus	+	+	+	+
Medial dorsal nucleus	+	+	++	++
Anterodorsal nucleus	+	+	+	+
Anteroventral nucleus	+	+	+	+
Lateral nucleus	+	+	++	+
Reticular nucleus	+++	+++	+++	++
Zona incerta	+++	+++	+++	++
Dorsolateral geniculate	+++	++	+++	+
Ventrolateral geniculate	+++	++	+++	+
Medial geniculate	+++	++	+++	+
Rhomboid nucleus	+	++	++	+
Parafascicular nucleus	+	+	++	+
Ventrobasal nucleus	++	+	+	+
Hypothalamus				
Medial preoptic area	na	-	++	+
Anterior nucleus	+	-	++	+
Paraventricular nucleus	+	-	++	+
Arcuate nucleus	-	-	++	+
Ventromedial nucleus	-	+	+++	+
Dorsomedial nucleus	-	+	+++	+
Cerebellum				
Molecular cell layer	na	-	-	-
Purkinje cell layer	na	++	++	+++
Granule cell layer	na	++	++	++
Pons				
Pontine nuclei	na	++	++	++
Transverse fibres of the pons	na	-	-	++
Longitudinal fasciculus of the	na	-	-	++
Medial Lemniscus	na	-	-	++

cortex, the choroid plexus, the hippocampal CA1-CA4 and DG regions, the amygdala, the medial habenula, zona incerta, and reticular nucleus of the thalamus, and the ventromedial and dorsomedial nuclei of the hypothalamus (Fig. 3.8 c and Table 3.3). The highest level of *Dtnbp1* exon 9 expression was observed in the granule cells of the olfactory bulb, forceps minor of the prefrontal cortex, layer IV of the cerebral cortex, the piriform cortex, the choroid plexus, the corpus callosum, the hippocampal CA1-CA4 and DG regions, the medial habenula and the Purkinje cell layer of the cerebellum (Fig. 3.8 d and Table 3.3).

A summary of the different combinations of exons that were expressed together in a region, from all the regions investigated, is presented in Table 3.4. Unexpectedly these findings suggest that there are at least 5 splice variants of *Dtnbp1* expressed in the adult rat brain. These five different splice variants and their patterns of expression are: 1) All four Dtnbp1 exons present (exons 1, 5, 8 and 9) eg. detected in the cerebral cortex in layer IV, piriform cortex, ependymal cells of the choroid plexus, hippocampus, amygdala, claustrum and sub-thalamic nucleus of the basal ganglia, habenula and the thalamus. 2) *Dtnbp1* exons 5, 8 and 9 but not exon 1 eg. detected in the ventromedial and dorsomedial nuclei of the hypothalamus. *Dtnbp1* exons 5, 8 and 9 were also all detected in the same subregions of the olfactory bulb, olfactory tract nucleus, septum, substantia nigra, purkinje and granular cell layers of the cerebellum and the pontine nuclei. *Dtnbp1* exon 1 may have been expressed in these regions but this was not investigated. 3) *Dtnbp1* exons 1, 8 and 9 but not exon 5 eg. detected in the amygdalohippocampal area, cortical amygdaloid nucleus, entorhinal cortex, and the anterior and paraventricular nuclei of the hypothalamus. 4) *Dtnbp1* exons 8 and 9 but not exons 1 and 5 eg. detected in layers I – III and layers V – VI of the cerebral cortex, the caudate putamen, nucleus accumbens and globus pallidus of the basal ganglia, and the arcuate nucleus of the hypothalamus. 5) *Dtnbp1* exon 9 but not exons 1, 5 and 8 was detected in the corpus callosum and forceps minor. *Dtnbp1* exon 9 in the absence of exons 5 and 8 was detected in the transverse fibres of the pons, longitudinal fasciculus of the pons and in the medial lemniscus. *Dtnbp1* exon 1 may have been expressed in these regions but this was not investigated. Another difference between *Dtnbp1* exon 9 expression and the other exons is that there were equal levels of *Dtnbp1* exon 9 expression across the different layers of the prefrontal cortex and posterior cerebral cortex. Prominent labelling of *Dtnbp1* exon 9 was however detected in layer IV of the central cerebral cortex, similar to *Dtnbp1* exons 1, 5 and 8.

Table 3.4. Predicted *Dtnbp1* exon splice vaiants. Deduction from the combinations of *Dtnbp1* exons 1, 5, 8 and 9 in any one region suggests that there are at least 5 splice variants of *Dtnbp1* expressed in the adult rat brain. This study has only investigated *Dtnbp1* exons 1, 5, 8 and 9 so other exons which will most likely be part of these splice variants have not been accounted for in this table. Key: - Exon absent, + Exon present.

Dtnbp1 splice variants	*Dtnbp1* exon 1	*Dtnbp1* exon 5	*Dtnbp1* exon 8	*Dtnbp1* exon 9
1	+	+	+	+
2	-	+	+	+
3	-	-	+	+
4	-	-	-	+
5	+	-	+	+

3.3.2.5 *Disc1* Basal Expression

None of the three probes designed to detect *Disc1* detected any specific labelling.

3.3.2.6 *Egr3*, *Nab1* and *Nab2* Basal Expression

The probes for *Egr3*, *Nab1* and *Nab2* all detected specific labelling (Table 3.1). The expression patterns detected for *Egr3, Nab1* and *Nab2* in a selection of coronal sections throughout the adult rat brain are shown in Figure 3.13. The distribution patterns of *Egr3, Nab1* and *Nab2* expression in regions of the adult rat brain is presented in Table 3.5. *Egr3*, *Nab1* and *Nab2* have similar and relatively restricted expression patterns. All three genes were expressed in the same subregions of the olfactory bulb, layers I – III and layers V – VI of the cerebral cortex, entorhinal cortex, piriform cortex, amygdalohippocampal area, ependymal cells of the choroid plexus, hippocampus, amygdala, claustrum and sub-thalamic nucleus of the basal ganglia, lateral septum and the purkinje and granular cell layers of the cerebellum. *Nab2*, but not *Nab1*, was expressed in all the regions that *Egr3* was detected in including the caudate putamen and nucleus accumbens of the basal ganglia, and the bed nucleus stria terminus. The regions in which both *Nab1* and *Nab2* were detected in the absence *Egr3* included layer IV of the cerebral cortex, the medial habenula, the medial dorsal nucleus of the thalamus and the pons. The regions in which *Nab1* was detected in the absence of *Egr3* and *Nab2* included the medial septum, the diagonal band, and the anterodorsal, anteroventral, lateral, parafascicular and ventrobasal nuclei of the thalamus. The regions in which *Nab2* was detected in the absence of *Egr3* and *Nab1* included the reticular nucleus and the zona incerta.

3.3.3 Experiment 3: Profiling Expression of *Nrg1* type IV Splice Variants and *Disc1* Splice Variants in Adult Rat Brains with Amphetamine Induced Activity

The *Nrg1* type IV splice variants were undetectable in amphetamine treated rat brain despite repeating the ISH test using three different probes and trying an additional ISH test combining two of the probes. Pan *Disc1* and *Disc1* L and Lv splice variants were undetectable in amphetamine treated rat brain despite repeating the ISH test using two different probes for pan *Disc1*.

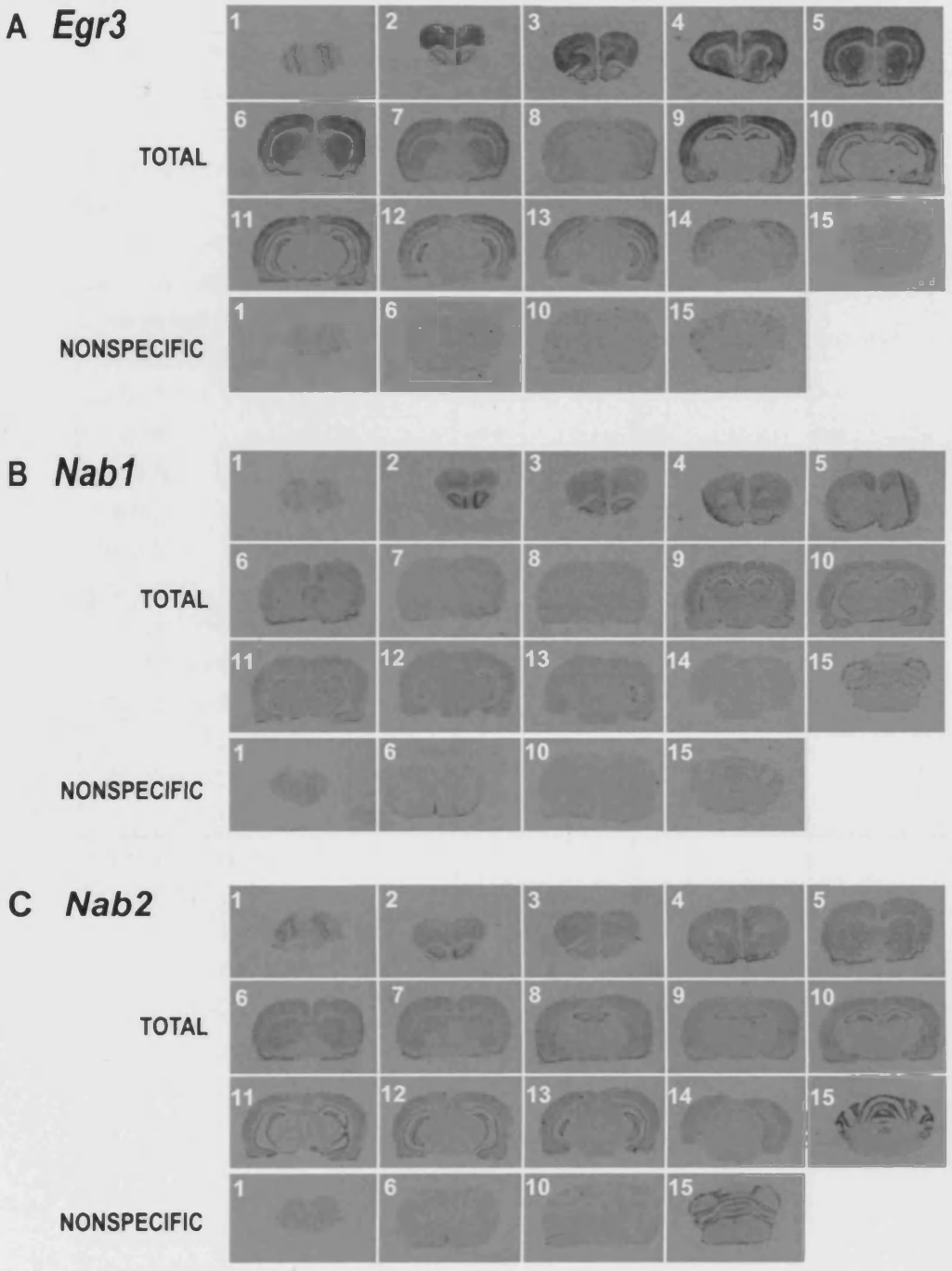


Figure 3.13. Autoradiographic images of whole brain mRNA expression patterns for *Egr3*, *Nab1* and *Nab2* determined using ISH. (A) *Egr3* expresseion pattern. (B) *Nab1* expression pattern. (C) *Nab2* expression pattern. Numbers in ascending order represent sections anterior - posterior through the adult rat brain. Nonspecific labelling is shown in four sections for each probe, numbered to match similar sections with total labelling.

Table 3.5. The expression profiles of *Egr3*, *Nab1* and *Nab2*. Key: - not detected, + low expression, ++medium expression, +++ high expression.

Brain region	*Egr3*	*Nab1*	*Nab2*
Olfactory bulb			
Periglomerular layer	+	+	+
External plexiform layer	+	+	+
Mitral cells	+	+	+
Internal plexiform layer	-	-	-
Granule cells	++	++	++
Ependymal cells	-	-	-
Cerebral cortex			
Layer I	+++	++	++
Layer II	+++	++	++
Layer III	+++	++	++
Layer IV	-	++	++
Layer V	+++	++	++
Layer VI	+++	++	++
Amygdalohippocampal area	++	++	++
Entorhinal cortex	+	+	+
Piriform cortex	+++	+++	+++
Olfactory tract nucleus	-	-	-
Olfactory tubercle	-	-	-
Islands of Calleja	-	-	-
Choroid plexus			
Ependymal cells	+	+	+
Hippocampus			
CA1 pyramidal cells	+++	+++	+++
CA2 pyramidal cells	++	+++	+++
CA3 pyramidal cells	+++	+++	+++
CA4 pyramidal cells	+++	+++	+++
DG granule cells	+++	+++	+++
Amygdala			
Cortical amygdaloid nucleus	+	+	++
Central amygdaloid nucleus	+	+	++
Lateral amygdaloid nucleus	++	+	++
Medial amygdaloid nucleus	++	+	++
Basolateral amygdaloid nucleus	++	+	++
Basal ganglia			
Caudate putamen	+++	-	++
Nucleus accumbens	+++	-	++
Globus pallidus	-	-	-
Claustrum	++	+	+
Subthalamic nucleus	++	+	+

Table 3.5 Continued.

Substantia nigra			
Pars reticulata	-	-	-
Pars compacta	-	-	-
Septum			
Lateral septum	+	+	++
Medial septum	-	++	-
Diagonal band	-	++	-
Bed nucleus stria terminus	++	-	++
Habenula			
Medial	-	+	+
Lateral	-	-	-
Thalamus			
Paraventricular nucleus	-	-	-
Medial dorsal nucleus	-	++	++
Anterodorsal nucleus	-	+	-
Anteroventral nucleus	-	+	-
Lateral nucleus	-	+	-
Reticular nucleus	-	-	+
Zona incerta	-	-	+
Dorsolateral geniculate	-	-	-
Ventrolateral geniculate	-	-	-
Medial geniculate	-	-	-
Rhomboid nucleus	-	-	-
Parafascicular nucleus	-	+	-
Ventrobasal nucleus	-	+	-
Hypothalamus			
Medial preoptic area	-	-	-
Anterior nucleus	-	-	-
Paraventricular nucleus	-	-	-
Arcuate nucleus	-	-	-
Ventromedial nucleus	-	-	-
Dorsomedial nucleus	-	-	-
Cerebellum			
Molecular cell layer	-	-	-
Purkinje cell layer	+	++	++
Granule cell layer	+	++	++
Pons			
Pontine nuclei	-	+	+
Transverse fibres of the pons	-	+	+
Longitudinal fasciculus of the	-	+	+
Medial Lemniscus	-	+	+

3.4 DISCUSSION

3.4.1 Characterisation and Expression of *Nrg1* Splice Variants in the Adult Rat Brain

The characterization of the exonic structure of all rat *Nrg1* transcripts and creation of a gene exonic structure is novel. A similar approach has been carried out previously for human *NRG1* by Steinthorsdottir and colleagues. They performed mRNA to genomic alignment for more transcripts than completed in this study and using a different alignment approach (using NCBI BLAST) explaining why they found exons that were not identified in this study and why the exact number of nucleotides differed in some exons (Steinthorsdottir et al., 2004). They did not present their findings graphically, but Harrison and Law (2006) did produce a graphic of *NRG1* gene exonic structure based on Steinthorsdottir and colleagues findings combined with findings from two other papers (Falls, 2003; Petryshen et al., 2005). There was convergence between the rat and human gene exonic structures derived in this study with the exons in the rat *Nrg1* exonic structure being present in the human *NRG1* exonic structure, with the exception of one exon, such that both the rat and human sequences contained the exons encoding the key EGF, Ig and TMc domains, in addition to 5'-defining exons for the type I, II and III splice variants. However the 5'-defining exons for the type IV, V and VI splice variants were absent in the rat gene exonic structure suggesting that they are not expressed in the rat like they are in humans.

The probes designed to detect *Nrg1* type I, II and III splice variants, and the pan-*Nrg1* probe all identified specific labelling but showed varied expression patterns from visual inspection of the autoradiographic film. This supports the hypothesis that they have functionally distinct roles (Poirier et al., 2008). A comparison of the five other studies that have investigated the expression of *Nrg1* in the adult rat brain is presented in Table 3.6 (Chen et al., 1994; Pinkas-Kramarski et al., 1994; Corfas et al., 1995; Eilam et al., 1998; Kerber et al., 2003). These studies differed in either the probes or techniques used, or the regions analysed. The techniques used included ISH with qualitative analysis at the silver grain level, Northern blots, RT-PCR, immunohistochemistry and immunoblotting. Only one other study has investigated the expression of *Nrg1* type I, II and III splice variants in addition to a pan-*Nrg1* probe (Kerber et al., 2003). Three previous studies used a pan-*Nrg1* probe designed to detect the EGF-L domain (Pinkas-Kramarski et al., 1994; Eilam et al., 1998; Corfas et al., 1995) and one of these

Table 3.6. Meta-analysis of *Nrg1* mRNA expression in the rat brain from the current literature. + expression present, - no expression, blank spaces indicate that this region of the brain was not discussed in the study.

Brain Region	Chen et al., 1994	Pinkas-Kramarski et al., 1994	Corfas et al., 1995		Eilam et al., 1998	Kerber et al., 2003			
						Type			
	TM+EGF-L	EGF-L	EGF-L	Ig+EGF-L	EGF-L	I	II	III	EGF-L
Olfactory bulb		+							
Cerebral cortex			+	+	+			+	+
Layer I	+				-	+	+		+
Layer II					+	+	+		+
Layer III					+	+	+		+
Layer IV					+	+	+		+
Layer V	+				+	+	+		+
Layer VI					+	+	+		+
Prefrontal cortex	+				+				
Piriform cortex		+			+				
Entorhinal cortex		+							
Frontal cortex		+							
Forelimb cortex		+							
Hindlimb cortex		+							
Parietal cortex		+							
Motor cortex		+							
Occipital cortex		+							
Olfactory tubercle		+							
Retrosplenial granular cortex			+	+					
Perirhinal cortex			+	+					
Corpus Callosum		+			-				
Hippocampus									
CA1 pyramidal cells		+	-	-	+	+	+		+
CA2 pyramidal cells		+				+	+		+
CA3 pyramidal cells		+	+	+	+	+	+		+
CA4 pyramidal cells						+	+		+
DG granule cells			+	+	+	+	+		+
Hippocampal fissure			+	+					
Subiculum		+							
Amygdala	+	+			+				
Lateral amygdaloid nucleus			+	+					
Medial amygdaloid nucleus			+	+					
Basolateral amygdaloid nucleus			+	+					
Basal ganglia									
Caudate putamen			+	+					
Nucleus accumbens			+						
Ventral pallidum		+	+	+					

Table 3.6 Continued.

Septum									
Lateral septum	+								
Medial septum	+	+	+	+					
Diagonal band	+	+	+	+					
Basal nucleus of Meynert	+		+	+					
Septofimbrial nucleus			+	+					
Bed nucleus stria terminus			+	+					
Choroid plexus - Ependymal cells			+	+					
Thalamus					+				
Medial habenula	+	+	+	-					
Lateral habenula	-		-	-					
Paraventricular nucleus	+								
Reticular nucleus	+		+	+					
Entopeduncular nucleus			+	-					
Hypothalamus			+		+				
Preoptic nucleus		+							
Paraventricular nucleus		+	+	-					
Ventromedial nucleus									
Dorsomedial nucleus			+	-					
Periventricular region	+		+	-					
Mamillary body		+							
Suprachiasmatic nuclei	+								
Supraoptic nuclei (SO)	+	+	+	-					
Retrochiasmatic SO	+								
Cerebellum		+		-					
Molecular cell layer	-					+	+		+
Purkinje cells	-	+	-			+	+		+
Granule cell layer	+	+	+			+	+		+
Fibrillary astrocyte						+	-	-	+
Basket cell			+						
Bergman glia									+
Golgi type II cell	+		+						
Brainstem						+	+	+	+
Substantia nigra			-	-		-	+	+	+
Pontine nuclei	+	+	+	+					
Lateral lemniscus			+	-					

studies also used a probe that detected all splice variants containing both an immunoglobulin domain and an EGF-L domain (Corfas et al., 1995). Finally, a probe that detected all splice variants containing both a transmembrane domain and EGF-L domain was used in another study (Chen et al., 1994).

At the level of autoradiography, the pan-*Nrg1* probe identified the expression of *Nrg1* in similar regions to previously published studies including the cerebral cortex, regions of the hippocampus, amygdala, septum, brainstem and regions of the cerebellum (Chen et al., 1994; Pinkas-Kramarski et al., 1994; Eilam et al., 1998; Corfas et al., 1995; Kerber et al., 2003). Of note we also saw similar gradients of expression in the cortical layers and habenula, with higher expression in layer V compared to the other cortical layers, and heavier labelling in the medial versus lateral habenula (Chen et al., 1994; Eilam et al., 1998). In contrast to our findings other studies did not describe a differentiation in the expression of *Nrg1* between cortical layers (Pinkas-Kramarski et al., 1994; Corfas et al., 1995; Kerber et al., 2003), the presence of *Nrg1* mRNA in the CA1 (Corfas et al., 1995), or the absence of *Nrg1* in the corpus callosum (Pinkas-Kramarski et al., 1994). These discrepancies may reflect the mRNA regions that different probes were targeting and highlight the complexity of the regulation of the *Nrg1* gene and the large number splice variants that result.

Nrg1 type I, II or III splice variants were expressed in all region of the brain that *Nrg1* mRNA was identified by the pan *Nrg1* probe with the exception of in the globus pallidus. This suggests other types of splice variants possibly including type V and VI variants are expressed in the globus pallidus of the adult rat brain.

In comparison to the widespread expression of *Nrg1* type I, we found a more restricted expression of *Nrg1* type II and *Nrg1* type III splice variants. For example *Nrg1* type I was observed in nearly all the brain regions in which *Nrg1* type II and *Nrg1* type III were observed, but *Nrg1* type I and *Nrg1* type III were not observed in as many of the hippocampal, amygdala or thalamic regions as *Nrg1* type I. Kerber and colleagues (2003) described *Nrg1* type I and II splice variants as having a ubiquitous expression in the adult rat brain, while expression of *Nrg1* type III splice variants showed a more restricted distribution. Again, this difference in findings may reflect the complexity of *Nrg1* regulation and the number of splice variants transcribed. Kerber and colleagues (2003) did not give details of the type-specific ribonucleotide probes so direct comparisons of the sequences used to detect the *Nrg1*

transcripts in between the two studies cannot be carried out. Nevertheless, their study identified further differential expression of splice variants of *Nrg1* containing different combinations of amino termini (I, II or III), EGF-L domains (α or β) and juxtamembrane domains (1, 2, 3, 4 or 5) in a limited selection of brain regions (Kerber et al., 2003). Together these results indicate the complex regulation of *Nrg1* expression in the brain and suggest that *Nrg1* splice variants may have different roles in different brain functions.

Nrg1 type IV splice variants were undetectable in the adult rat brain despite using three different probes under basal conditions and using two of these probes in amphetamine-treated rat brain. Amphetamine treatment causes increased dopaminergic activity in the brain that induces activity-regulated expression of genes (Graybiel et al., 1990). *Nrg1* has been shown to be involved in activity-dependent synaptic plasticity (see 1.3.2.1). Therefore, amphetamine treatment may regulate the expression of *Nrg1* type IV splice variants. The absence of expression after amphetamine may indicate that *Nrg1* type IV expression is (i) not expressed in the rat brain or (ii) below the detectable range of the ISH technique used to assay it's expression. *NRG1* type IV expression has been detected in the human adult hippocampus and in human fetal whole brain using Q-PCR (Law et al., 2006; Tan et al., 2007), and in cDNA libraries from human hippocampus, hypothalamus and total brain using rapid amplification of cDNA ends (RACE) and RT-PCR (Steinthorsdottir et al., 2004). It has not been characterised by ISH in the rat or human brain and has not been detected in the rat by any other techniques. However, other researchers have also not been able to detect *Nrg1* type IV variants in the rat brain (personal communication, Dr. Amanda Law). These preliminary data using complimentary experimental approaches suggest that *Nrg1* type IV splice variants are present in the human but not rat brain. The absence of *Nrg1* type IV in the rat but presence in the human could be indicative that *Nrg1* type IV splice variants have a function that is specific to humans. As schizophrenia is a disorder that is thought to be specific to humans, and the evidence that a variant in *Nrg1* type IV has been associated with particular symptoms of schizophrenia (Hall et al., 2006) and the same variant has been shown to have increased levels in schizophrenic individuals (Law et al., 2006) supports this hypothesis. ISH in human adult hippocampal tissue would determine of the efficiency of the hybridisation of the *Nrg1* type IV probes we used. This experiment may be unsuccessful because the sequence used in the rat probe(s) to identify *Nrg1* Type IV variant in the rats brain may not be complementary to the corresponding human exon sequence. The percentage identity between the human and rat

Nrg1 type IV 5' defining exon is only 63%. The first 40 bp and last 80 bp are homologous, but there is very little homology in the central section of the exon.

3.4.2 Semiquantitative Levels of *Nrg1* Splice Variants Expression in Regions of Hippocampus, Cortex and Amygdala in the Adult Rat Brain

Regional and cellular semi-quantitative analysis of *Nrg1* splice variants expression was performed in subregions of the hippocampus, cortex and amygdala to resolve conflicting findings in these regions in our study here and previous studies (see above).

Two previous studies found that the different regions of the hippocampus either expressed equal levels of *NRG1* expression (Law et al., 2006) or had more *NRG1* expression in the CA3 relative to the CA1 and DG regions (Law et al., 2004). In this study type I, II and III splice variants were assayed in addition to a pan probe. Our findings measured at the regional level for the pan-probe showed regional variation in the level of *Nrg1* expression, with both the CA3 and the DG having higher *Nrg1* expression than the CA1. The findings for the different splice variants all showed variation in the level of expression between the different regions of the hippocampus, with the exception of *Nrg1* type I expression measured at the regional level. From our findings and those of Law and colleagues (2004) it can be concluded that the different regions of the hippocampus should be measured separately when investigating *Nrg1* expression.

For the analysis of the cortex, cortical layers I-IV were grouped together, and cells in layers V and VI analysed separately in an attempt to clarify whether expression in layer V was higher than other cortical layers (this study as measured by autoradiography, Chen et al., 1994; Eilam et al., 1998), or not (Pinkas-Kramarski et al., 1994; Corfas et al., 1995; Kerber et al., 2003). At the cellular level of analysis, we confirmed that *Nrg1* type I and III splice variants, were both expressed at higher levels in layer V relative to layers I-IV and layer VI as previously observed in rats (Chen et al., 1994; Eilam et al., 1998) and humans (Law et al., 2004). In contrast, *Nrg1* type II splice variants, were not more heavily expressed in layer V of the cerebral cortex. Results at the regional level of analysis differed to those at the cellular level of analysis.

The cortex layers were originally grouped according to patterns of expression as determined by visual inspection; layer V and VI appeared to show different expression levels while layers I-IV

showed similar levels of expression for all types of Nrg1 splice variants. Silver grain count analysis for cells in each of layers II-VI for detected Nrg1 type III variants confirmed that the appropriate grouping of layers had been used. Furthermore, analysis using scatter graphs that displayed the number of grains per cell for all cells counted in cortical layers II, III, IV, V and VI, suggested that the number of grains per cell could fall into two groups; Low (0-20 grains/cell) or moderate-to-high (20-120 grains/cell). Layer V has many more cells belonging to the moderate-to-high group than the other layers. This may represent labelling in two different types of cells in the different cortical layers. For example, small GABAergic versus large pyramidal cells especially as pyramidal cells are predominant in layer V (Kandel, 2000). Indeed, photomicrograph images of the cells with overlaying silver grains are predominantly pyramidal-shaped cells in layer V compared to a mixture of cell shapes in layers I-IV and layer VI. The pyramidal-shaped cells also appear to have many more silver grains than the other types of cells.

The expression of *Nrg1* type I and II splice variants and expression detected by the pan-*Nrg1* probe did not vary much between nuclei of the amygdala, but the expression of *Nrg1* type III splice variants appeared to be expressed at a higher level in the lateral nucleus of the amygdala, relative to the basal and central nuclei of the amygdala, as measured by silver grain counting.

3.4.3 Comparison of Expression Levels Determined at the Regional and Cellular Level

Regional analysis by image densitometry, and cellular analysis by silver grain counting, showed two different relative expression patterns of *Nrg1* type I, II and III splice variants between regions of the hippocampus, nuclei of the amygdala and layers of the cerebral cortex. The difference between the results from these two kinds of analysis is possibly due to cell density being a factor that contributes to expression measured by densitometry, but not by silver grain counting. For example, the expression of *Nrg1* type I splice variants in cortical layers V and VI determined by densitometry showed approximately equal expression in these two layers, while expression levels determined by silver grain counting showed that the cells in layer V had approximately 90% more silver grains per cell than the cells in layer VI. The cell density in layer V was 17 cells/photo while the cell density in layer VI was 25 cells/photo. This means that image densitometry is more useful for detecting broad regional changes in expression, while silver grain counting will detect more accurate levels in individual cells. In

addition to the increase in spatial resolution, silver grain counting is likely to be a more sensitive measure of gene expression if mRNA is restricted or regulated in specific cell types, or in a small number of cells in a particular brain region.

3.4.4 Characterisation and Expression of *Dtnbp1* Splice Variants in the Adult Rat Brain

The exonic structure for the only experimentally determined transcript of *Dtnbp1* in the rat was characterised and 10 exons were identified. These 10 exons were found to be homologous to 10 of the 13 exons characterized in human *DTNBP1*. Probes were designed to detect rat homologues of two exons found in human transcripts that could lead to the identification of two different splice variants of *DTNBP1* and another two probes were designed to detect exons that were expected to be present in all *DTNBP1* splice variants. Through comparison of the expression detected by the four probes deigned to exons 1, 5, 8 and 9, different combinations of the exons expressed in any one region lead to the identification of at least five different splice variants of *Dtnbp1* in the adult rat brain (Table. 3.4). These are novel findings. *Dtnbp1* splice variants containing exons 1, 5, 8 and 9 were expressed in most regions of the adult rat brain, while *Dtnbp1* splice variants containing exons 5, 8 and 9, *Dtnbp1* splice variants containing exons 1, 8 and 9, *Dtnbp1* splice variants containing exons 8 and 9, and *Dtnbp1* splice variants containing exon 9, were only exclusively expressed in a few brain regions each.

Dtnbp1 splice variants containing exons 1, 5, 8 and 9 were expressed heavily in layer IV of the cerebral cortex and all regions of the hippocampus. DTNBP1 protein was expressed in these two regions of the mouse brain but there was no mention of variation in the levels of DTNBP1 expression in the different layers of the cortex (Benson et al., 2001). However expression studies in the human cortex using a pan probe showed that, like in the rat, there was variability in the levels of *DTNBP1* expression between the layers. However the variation in labelling between the different layers was different in the human entorhinal cortex from in the human temporal neocortex, and neither of these patterns were the same as the layer IV heavy labelling detected in the rat (Weickert et al., 2004). Recently, three *DTNBP1* splice variants have been confirmed in the human and of these, *DTNBP1*-1A is most heavily expressed in the DLPFC, while much less expression of *DTNBP1*-1B and *DTNBP1*-1C was detected in this region. No differential expression in the DLPFC layers could be detected for the three different splice variants as expression levels were determined from dissected DLPFC using Q-PCR (Tang et al., 2009). The differences in cortical *DTNBP1* and *Dtnbp1* expression in humans, rats

and mice is likely due to the probes used in each study detecting different combinations of splice variants. Future characterisation and confirmation of all splice variants in rats and mice through northern blot analysis, and design of probes that detect each *DTNBP1* splice variant exclusively in humans, rats and mice will lead to clarification of which splice variants are expressed in which layers of the cortex. It will also identify if there are species-specific splice variants that may also account for the difference in cortical layer labelling between the rat, mouse and human.

3.4.5 *Disc1* Splice Variants and Absence of Expression Detection

Expression of *Disc1* splice variants could not be detected in adult rat brain tissue under basal conditions despite two different pan-*Disc1* probes, a *Disc1* L and *Disc1* Lv probe being designed and used. Expression of the two different pan-*Disc1* probes could also not be detected in amphetamine-treated adult rat brain tissue. This may be because *Disc1* is (i) not expressed in the rat brain or (ii) below the detectable range of the ISH technique used to assay it's expression even after amphetamine-treatment. Alternatively, the probes may have unusual kinetics or tertiary structure preventing binding to the target mRNA. *Disc1* mRNA expression has been detected and characterised, and DISC1 protein expression characterized, in the adult rat brain (Ozeki et al., 2003; Miyoshi et al., 2003). Future experiments could involve designing and testing additional probes complementary to *Disc1* mRNA and using them in combination to increase the signal to background level. Alternatively, *Disc1* could be measured using a technique based on signal amplification such as RT-PCR to aid the detection of specific transcripts with very low levels of expression or expression restricted to small numbers of cells within a region.

3.4.6 Characterisation and Expression of *Egr3*, *Nab1* and *Nab2* in the Adult Rat Brain

In agreement with previous studies, the exonic structure for *Egr3* in the rat has two exons and the exonic structure for *EGR3* in the human also has two exons (Patwardhan et al., 1991; Yamagata et al., 1994). The length of the exons were much shorter in the rat than in the human, but the sequence was highly homologous to regions within the human exons. *Nab1* and *Nab2* were determined to have 9 and 7 exons respectively. In support of our analysis, *Nab2* in the mouse has been similarly shown to have 7 exons (Svaren et al., 1996). The determination of these detailed exonic structures in the rat are novel findings. Interestingly, the

first exon of *Nab1* and the final 178 nucleotides of the first exon of *Nab2* are the same and are likely to be analogous to the *Nab* conserved domain 1 (NCD1) identified previously (Svaren et al., 1996). The third and fourth exons of *Nab1* and *Nab2* are also the same and are likely to be analogous to the *Nab* conserved domain 2 (NCD2) also identified in the in the mouse *Nab* sequences. NCD1 has been identified to be sufficient for NAB1 and NAB2 interactions with EGR proteins, and NCD2 has been suggested to be a likely candidate for mediating EGR transcriptional repression (Svaren et al., 1996).

In agreement with a previous study, *Egr3* expression was detected in the superficial and deep layers of the cerebral cortex, hippocampus, amygdala and most regions of the basal ganglia (Yamagata et al., 1994). Expression of *Egr3* in additional regions of the adult rat brain were also described in this study. These include regions of the olfactory bulb and cerebellum. *Nab1* and *Nab2* expression has not been characterized under basal conditions in the rat brain before. In all regions in which *Egr3* was expressed, *Nab2* was also expressed and *Nab1* was expressed in most of the regions in which *Egr3* was expressed. This supports the findings that EGR3 activity in all regions of the adult rat brain can be regulated by NAB1 and NAB2. Nevertheless, some regions express either *Nab1* or *Nab2* such as in the caudate putamen and nucleus accumbens of the basal ganglia, some regions of the septum and some regions of the thalamus. This supports the idea that NAB1 and NAB2 can have different functional roles. For example, *Nab2* expression is activity-dependent while *Nab1* expression is not (Jouvert et al., 2002). *Nab1* and *Nab2* were also expressed in regions of the brain that *Egr3* was not expressed in. It is likely that NAB1 and NAB2 may function in these regions to regulate the activity of other EGR family member that have a Nab conserved domain 1 and 2 (NCD1 and NCD2 domains), such as EGR1 (O'Donovan et al., 1999).

3.4.7 Conclusions

The expression profiles and semi-quantitative results are all drawn from one rat brain. Future experiments could include repeating ISH for each gene or splice variant on more rat brains to improve the reliability of our findings. *Nrg1 and Nrg1* type I, II and III splice variants, *Dtnbp1* splice variants containing exons 1, 5, 8 and 9, *Egr3*, *Nab1* and *Nab2* show differential patterns of expression, but all are expressed in the hippocampus, amygdala and prefrontal cortex. These are regions that form part of the neural circuitry for fear-associated memory (LeDoux,

2000). Further experiments in this thesis will focus on the investigating the regulation of these splice variants in fear memory processing. It is also of interest that all the schizophrenia genes of interest that were detected were expressed in the hippocampus and prefrontal cortex as these two regions are commonly smaller in volume in individuals with schizophrenia in comparison to controls (Harrison & Weinberger, 2005). The differential expression patterns for the different splice variants of *Nrg1* and *Dtnbp1* is suggestive of different functional roles for these splice variants. Therefore the findings in this chapter highlight the importance of studying the different splice variants of genes in schizophrenia research and in our long-term memory investigations in the next chapter.

CHAPTER 4

REGULATION OF SCHIZOPHRENIA SUSCEPTIBILITY GENES OF INTEREST IN CONSOLIDATION OF CONTEXTUAL FEAR CONDITIONING

4.1 INTRODUCTION

In the previous chapter probes were designed that detected all *Nrg1* splice variants and *Nrg1* type I, II and III splice variants separately, all *Dtnbp1* splice variants and predicted *Dtnbp1* splice variants starting with exons 1, 5 and 9 separately, *Egr3*, *Nab1* and *Nab2*. The expression of these genes and splice variants were characterized throughout the whole rat brain. As specific labelling was not detected for all *Disc1* splice variants or *Disc1* L and Lv splice variants separately, or for *Nrg1* type IV splice variants, these splice variants will not be investigated further.

4.1.1 Schizophrenia and Memory

Schizophrenia susceptibility genes may contribute to memory impairments present in schizophrenia (as discussed in 1.1.4). In particular, hippocampal-dependent associative long-term memory (LTM) is a type of memory impaired in schizophrenia (Boyer et al., 2007). Contextual fear conditioning (CFC) in rats is a test of hippocampal-dependent associative LTM (Blanchard & Fial, 1968). Information on the neurocircuitry and genes involved in fear conditioning memory obtained from studies in rats can be extended to help understand the mechanisms underlying associative LTM in humans (Delgado et al., 2006). Therefore if any schizophrenia susceptibility genes are regulated after CFC in rats this would provide evidence suggesting a functional role for that schizophrenia susceptibility gene in a core functional domain impaired in schizophrenia.

The fear-conditioning paradigm has been used in many laboratories since 1959 (Baron, 1959) and is well established for studying associative memory (Fanselow, 1980). Contextual fear-conditioning (CFC) occurs when an aversive unconditioned stimulus (US), in this case a footshock, is presented to a rat shortly after it is placed in a neutral experimental context. This context then becomes a conditioned stimulus (CS) capable of eliciting detectable conditioned fear responses (CR), including freezing behaviour, when the rat is re-exposed to it (Fanselow, 1980) (see 1.2.2). In this chapter gene expression is assayed in a group of rats that have been conditioned and in three control groups; a naïve group, a CS-only group and a latent inhibition (LI) group, as used in a previous study (Hall et al., 2000). The CS-only group is exposed to the CS for the same time period as the conditioned group but does receive a US. This determines whether regulation is associated with exposure to a novel context. LI is a long-lasting phenomenon by which non-reinforced pre-exposure to a stimulus retards subsequent conditioning to that stimulus (Lubow & Moore, 1959; McLaurin et al., 1963). The LI group is exposed to the CS for a prolonged period of time before receiving a US. This determines whether regulation is associated with the experience of the footshock (Impey et al., 1998).

CFC can be used to investigate consolidation, reconsolidation and extinction of memory (see 1.2.1). Memory consolidation theory was first proposed in 1900 by Muller and Pilzecker. They suggested that the processes that underlie new memories initially persist in a fragile state and consolidate over time (McGaugh, 2000; Muller & Pilzecker, 1900). Consolidation is the process by which new memories are stored after a novel learning experience. After initial acquisition of a memory, cellular consolidation of the memory has been proposed by different researchers to take different lengths of time before it is complete; ranging from several hours (most commonly researched) to several days (Shedmehr & Holcomb, 1997; Bjordahl et al., 1998; McGaugh, 2000). The consolidation of memory is hypothesised to be mediated by structural changes in the circuitry associated with synaptic plasticity (Bliss & Lomo, 1973; Dudai, 2002). The schizophrenia susceptibility genes of interest, *Nrg1*, *Dtnbp1, Egr3* and *Bdnf*, have each been shown to be involved in synaptic plasticity, a cellular model of memory (Harrison & Weinberger, 2005; see 1.3.2). Other research findings for these genes also suggest that they contribute to memory function.

4.1.2 Schizophrenia Susceptibility Genes of Interest and Memory

NRG1 is involved in the modulation of synaptic plasticity in the hippocampus (Huang et al., 2000; Kwon et al., 2005; Li et al., 2007; Eilam et al., 1998; Roysommuti et al., 2003) and prefrontal cortex (Gu et al., 2005; Stefansson et al., 2002) (see 1.1.3); both regions of the brain required for memory function. Also in these brain regions a *Nrg1* variant has been associated with reduced activity in patients with schizophrenia during a Hayling sentence completion task, a task known to activate frontal and temporal brain regions (Hall et al., 2006). Another fMRI study has shown that schizophrenia risk alleles in *NRG1* are positively correlated with hyperactivation in the cingulate gyrus, left middle frontal gyrus, bilateral fusiform gyrus and the left middle occipital gyrus during encoding of an episodic memory, and in the left middle occipital gyrus during retrieval of an episodic memory (Krug et al., 2009). Mutant mice heterozygous for a partial deletion of the EGF-like domain in Nrg1 ($Nrg1^{+/-}$) showed a significant reduction in freezing behaviour in a LTM test 24 hours following CFC (Ehrlichman et al., 2009). Performance in a task testing short-term and working memory was impaired in heterozygous mutant mice with a targeted disruption for type III Nrg1 (Chen et al., 2008). However there were no apparent working memory deficits in mice with heterozygous deletion of transmembrane-domain NRG1 (O'Tuathaigh et al., 2007).

DTNBP1 has a widespread expression pattern throughout the adult human brain (Weickert et al., 2004) and the adult rat brain (see 3.3.2) including in the hippocampal formation, prefrontal cortex and amygdala, all of which are brain regions involved in memory function. DTNBP1 protein is localized to asymmetric synapses in pyramidal cells in the CA1 suggesting that DTNBP1 may be involved in glutamatergic neurotransmission (Talbot et al., 2006). Two studies have identified a correlation between variants of *Dtnbp1* and cognitive impairments, some of which were in memory function (Burdick et al., 2006; Donohoe et al., 2007). Neuroimaging studies have identified a correlation between different variants of Dtnbp1 and impairments in working and episodic memory in humans, and in some cases these variants of Dtnbp1 were also associated with schizophrenia (Wolf et al., 2010; Markov et al., 2010; Thimm et al., 2010). *Dtnbp1* variants have also been associated with reduced prefrontal brain activity using an electrophysiological method coupled with a cognitive task (Fallgatter et al., 2006). The behavioural characteristics of the *Dtnbp1* transgenic mouse, called the sandy mouse, includes a variety of memory impairments (Feng et al., 2008; Takao et al., 2008; Bhardwaj et al., 2009; Cox et al., 2009; Jentsch et al., 2009). The evidence of an association between *Dtnbp1* and

cognitive function, including various memory impairments, and its localization to glutamatergic synaptic transmission combined with its expression in the hippocampus and prefrontal cortex is suggestive of a role for *Dtnbp1* in memory function.

Egr3 is a transcription factor with a zinc finger motif that is highly homologous to that of the other *Egr* family members (O'Donovan et al., 2000). *Egr3* is expressed in the cerebral cortex, hippocampus, amygdala and basal ganglia. Although *Egr1* is known to have a critical role in the reconsolidation of long-term fear memory in the CA1 of the hippocampus (Lee et al., 2004; Hall et al., 2001), the role for *Egr3* in memory is less well characterized. Context and cued-associative learning and memory, and short-term and long-term object recognition memory are significantly impaired in *Egr3*$^{-/-}$ mice (Li et al., 2007). In contrast another family member, *Egr2*, is not considered essential for learning and memory as conditional *Egr2* knock out mice have no impairments in fear learning, conditioned taste aversion memory or in a spatial navigation memory (Poirier et al., 2007). Hence despite their structural similarity, not all *Egr* family members have similar functions (Poirier et al., 2007). NAB1 and NAB2 are both repressors of EGR3 transcriptional activity in neurones and could therefore be involved in regulating memory consolidation by modulating the *de novo* gene expression resulting from EGR3 upregulation within an activated neuron (Svaren et al., 1998). *Nab2*, but not *Nab1*, is rapidly and transiently regulated in response to cocaine administration in rats (Jouvert et al., 2001) and in response to Nerve Growth Factor (NGF) stimulation in PC12 cells (Svaren et al., 1996) and so may also be regulated during memory consolidation.

BDNF has been shown to be involved in the molecular mechanisms underlying memory in many studies (see Cunha et al., 2010). In particular, BDNF has already been shown to be upregulated during, and necessary for, the consolidation of contextual fear memory (CFM) (Hall et al., 2000; Lee et al., 2004). Therefore the investigation of Bdnf expression in this chapter could act as appositive control.

4.1.3 Outline of Experiments

Experiment 1 determines whether the conditioning procedure produces CFM. Experiment 2 investigated whether *Nrg1* type I, II and III splice variants, all *Dtnbp1* splice variants (pan *Dtnbp1*), *Dtnbp1* splice variants starting with exons 1, 5 and 9 separately, *Egr3*, *Nab1*, *Nab2* and *Bdnf* are regulated 2 hours following CFC. The time-point of 2 hours following CFC was

chosen as it gave the best coverage for when all genes of interest should be upregulated to some degree following neuronal stimulation based on available literature. *Nrg1* upregulation following kainic acid stimulation peaked between 2.5 and 4.5 hours in different brain regions of interest. In the hippocampus *Nrg1* expression peaked at 2 hours following a forced locomotor test and 1 hour following LTP stimulation (Eilam et al., 1998). *Egr3* peak upregulation was observed between 1 and 2 hours following maximal electoconvulsive seizures and was present 30 min and 1 hour following high frequency stimulation in the hippocampus (Yamagata et al., 1994). *Bdnf* is known to be upregulated at 30 min following CFC (Hall et al., 2000) and there is no activity-dependent expression profile information currently known related to *Dtnbp1*. Importantly two hours post-CFC is also within the consolidation period (McGaugh, 2000). As *Bdnf* has been shown to be regulated following CFC it was used in experiment 2 as a positive control (Hall et al., 2000). ISH was used to investigate the expression of the genes of interest in regions of the brain involved in circuitry known to support consolidation of CFC (see 1.3). All genes or splice variants of interest were assayed in the CA1, CA3 and DG of the hippocampus, with the exception of *Nrg1* type II and *Nrg1* type III that were unmeasurable in the CA3 and DG. For *Egr3* expression in the medial prefrontal cortex (cingulate cortex (Cg1), prelimbic cortex (PrL) and infralimbic cortex (IL)), and in the amygdala (dorsolateral nucleus (DLA), lateral nucleus (LA) and basolateral nucleus (BLA)) was also conducted. Finally *Nab2* was also assayed in the CA1 and in the DLA nucleus of the amygdala. These studies tested the hypothesis that regulation of *Egr3*, *Nab1*, *Nab2*, and different splice variants of *Nrg1* and *Dtnbp1* are associated with consolidation of CFM. Experiment 3 investigated *Egr3* regulation in the CA1 in consolidation of CFC using QPCR. Experiment 4 established the time profile of *Egr3* regulation in the CA1 following CFC using ISH. Finally, experiment 5 investigated the effect of intrahippocampal infusions of *Egr3* antisense on CFC, to determine if EGR3 is necessary for consolidation of CFM.

4.2 METHODS

4.2.1 Subjects

The subjects were adult male lister hooded rats (280-350g; Charles River, UK). They were housed in pairs in a holding room maintained at 21°C under a reverse light cycle (12 h light/dark; lights on at 10:00 P.M). All experiments were conducted in the dark period for the

rats. Animals were allowed *ad libitum* access to food and water. All procedures were conducted in accordance with local Cardiff University Ethical Committee approval and the United Kingdom 1986 Animals (Scientific Procedures) Act (Project license PPL 30/2236).

4.2.2 Experiment 1: Conditioning Procedure Produces Long-Term CFM and Establishment of the Control Groups

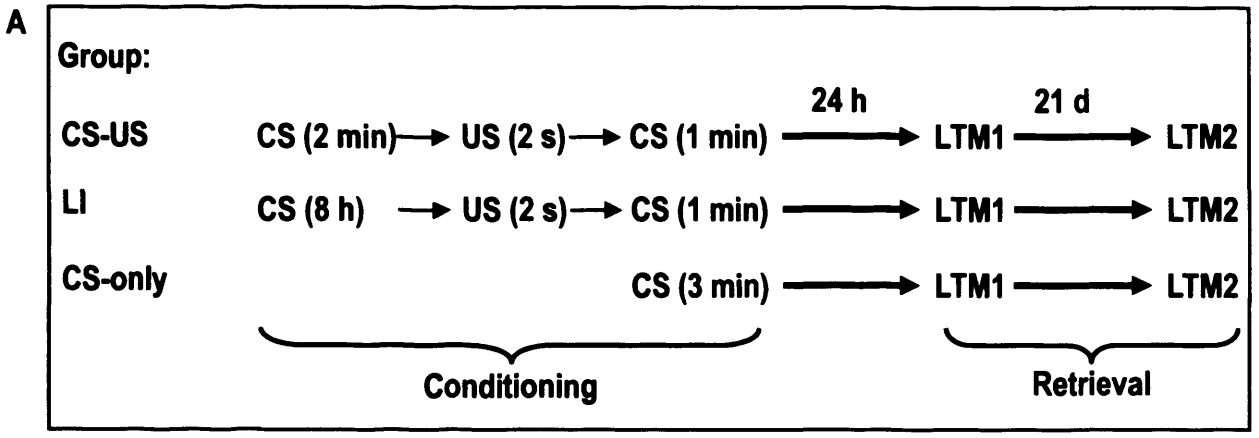
The contextual fear-conditioning chambers described in 2.2.1 were used. Rats were divided into three groups:- (i) CS-US (n=4), (ii) CS-only (n=4), and (iii) latent inhibition (LI) (n=4). Rats were handled for 5 -10 min each, for 3 consecutive days. On day 4 the rats in the CS-US group were fear-conditioned. Conditioning consisted of giving an electric footshock (2 s, 0.5 mA shock; US) after the rat had been in the conditioning chamber (CS) for 2 min. After a further 1 min in the chamber the rats were returned to their home cage. The rats in the CS-only control group were placed in the conditioning chamber for 3 min and then returned to their home cage. Rats in the LI control group were placed in the conditioning chamber for 8 h and then received an electric footshock before being returned to their home cage 1 min later. Both 24 hours and 3 weeks later the rats were returned into the conditioning chamber for 2 min, for LTM1 and LTM2 retrieval tests, respectively.

The behaviour of the rats in the boxes was digitally recorded. The freezing behaviour was scored by observation and recording the presence or absence of freezing behaviour every 10 s. Freezing behaviour was scored throughout the 2 min pre-US and 1 min post-US periods for the CS-US and LI group rats and in the equivalent first 2 min and final 1 min period for the CS-only group rats. Freezing behaviour was also recorded for the 2 min LTM1 and LTM2 tests (Fig. 4.1 a).

4.2.3 Experiment 2: Profiling the Expression of Schizophrenia Susceptibility Genes after CFC using *In Situ* Hybridisation

4.2.3.1 Behaviour

Rats were divided into four groups:- (i) CS-US (n = 4), (ii) CS-only (n = 4), (iii) LI (n = 4) and (iv) naive (n = 4). The CS-US, CS-only and LI groups experienced the same conditions described in experiment 1 with the exception that the rats were killed 2 h after conditioning by CO_2



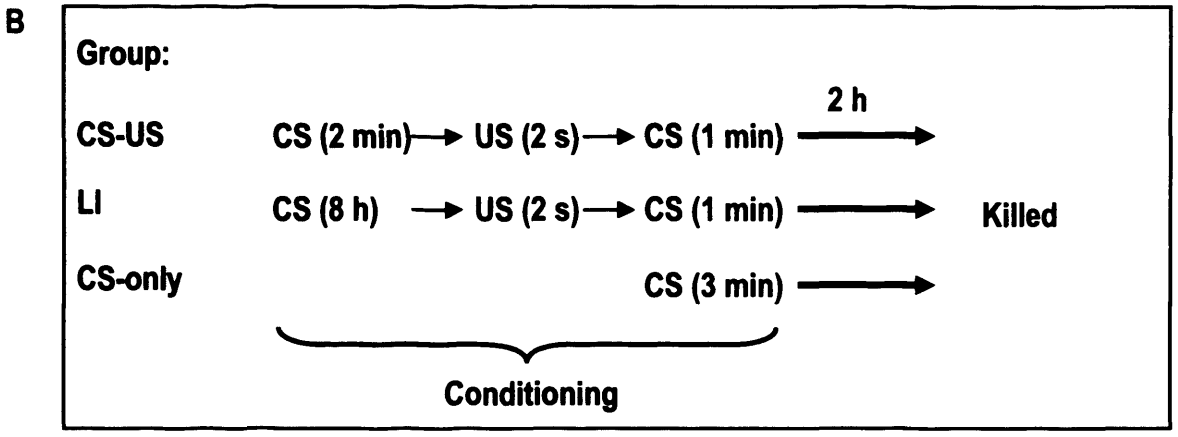


Figure 4.1. Contextual fear conditioning protocol for investigating consolidation of CFM. (A) Schematic showing behavioural procedure used for experiment 1 to test whether the conditioning procedure produces long-term memory and to establish the control groups. (B) Schematic showing behavioural procedure used for experiment 2 to provide tissue to profile the expression of the schizophrenia susceptibility genes of interest after contextual fear conditioning. Conditioned Stimulus (CS), Unconditioned Stimulus (US), Long-Term Memory test (LTM), Latent Inhibition (LI).

asphyxiation (Fig. 4.1 b). Rats in the naïve control group remained in home cages until killed by CO_2 asphyxiation. The brains were excised and rapidly frozen on dry ice before storage at -80°C.

In order to increase the number of rats in each group for the hippocampal regions and to provide amygdala tissue for analysis, the behavioural experiment was repeated with an n = 6 in each group. Quantification of *Nrg1* type I splice variants, *Egr3* and *Nab2* expression in the tissue obtained from the repeated behavioural experiment was performed using ISH.

4.2.3.2 *In Situ* Hybridization (ISH)

ISH was carried out as described in 2.5. The oligonucleotide probes designed in Chapter 3 were used to assay mRNA expression of *Nrg1* type I, II and III splice variants, pan *Dtnbp1*, predicted *Dtnbp1* splice variants starting with exons 1, 5 and 9, *Egr3*, *Nab1* and *Nab2*. A *Bdnf* oligonucleotide probe was designed, complementary to nucleotides 1238-1282 of NM_012513, with the sequence: 5'-AGTTCCAGTGCCTTTTGTCTATGCCCCTGCAGCCTTCCTTCGTGT-3'. All oligonucleotides were 3' end-labelled with [α-^{35}S]dATP using terminal deoxynucleotidyl transferase; the specific activity of the labelled probes are given in Table 4.1. For tissue obtained from the initial behavioural experiment, ISH was carried out on coronal 14 μm sections of the prefrontal cortex (approx. bregma 3.7 mm) and hippocampus (approx. bregma -3.3mm) (3 labelled sections and 2 non-specific labelled sections for each region). For tissue obtained from the repeated behavioural experiment, ISH was carried out on 14 μm sections of the amygdala (approx. bregma -3.1mm) and hippocampus (4 labelled sections and 3 non-specific labelled sections for each region). Hybridised sections with a ^{14}C Microscale (Amersham, UK) were opposed to autoradiographic film for 4 - 17 days (Table 4.1). After obtaining appropriate exposure the films were developed and scanned at 1200dpi and the high resolution images were saved as .tiff files ready for densitometric analysis. Sections hybridised with *Nrg1* type I, II and III splice variants, *Egr3*, *Nab1*, *Nab2* and *Bdnf* from the initial behavioural experiment and with *Nrg1* type I splice variants, *Egr3* and *Nab2* from the repeat behavioural experiment were then dipped in K5 photographic emulsion as described in 2.5.5. They were exposed for 7 - 17 weeks (Table 4.1) at 4°C before development and counterstaining with 0.1% thionin.

Table 4.1. Specific activity of S^{35}-labelled oligonucleotide probes. These probes were used to detect expression of genes of interest in adult rat brain sections 2 hours post-training in the CS only, LI and CS-US groups and in the naïve group of rats. The number of days that the autoradiographic film and the photographic emulsion were exposed to the labelled tissue sections are given.

Gene	Gene subtype	Specific Activity (dpm/µl)	Days on film	Days on emulsion
Nrg1	I	216 000	7	55
	I	148 500	12	104
	II	382 500	11	72
	III	305 100	9	80
Dtnbp1	Exon 1	176 100	14	97
	Exon 5	244 300	14	97
	Exon 8	288 800	10	71
	Exon 9	306 300	10	71
Egr3	Pan	210 500	6	57
Egr3	Pan	227 200	10	75
Nab1	Pan	208 000	7	84
Nab2	Pan	185 300	17	119
Nab2	Pan	249 000	11	83
Bdnf	Pan	252 300	4	55

4.2.3.3 Densitometric Analysis

For densitometric analysis, ImageJ was used to measure the density of selected regions of the brain as described in 2.5.7. Optical density values were converted to nCi/mg by reference to the microscale. Gene expression levels in the CS-US, CS-only and LI groups were standardised as a percentage of the mean level of the naïve group for each region. Densitometric analysis was used to determine the expression of *Nrg1* type I splice variants, all predicted *Dtnbp1* splice variants, *Egr3* and *Bdnf* in the CA1, CA3 and DG regions of the hippocampus. *Egr3* expression was also measured in the Cg1, PrL and IL regions of the prefrontal cortex and in the DLA, LA and BLA nuclei of the amygdala. *Nrg1* type II and III splice variants, Nab1 and *Nab2* expression were measured in the CA1 region of the hippocampus only. *Nab2* was also measured in the DLA nucleus of the amygdala. For each subject, the average non-specific densitometric value for a region was subtracted from the average total densitometric value for that region to provide the final specific densitometric value. Gene expression levels in the CS-US, CS-only and LI groups were standardised as a percentage of the mean expression levels in the naïve group for each region.

4.2.3.4 Silver Grain Image Collection and Analysis

Images of emulsion-dipped sections were obtained on a light microscope, through a 100x magnification lens under oil immersion, with a digital camera (see 2.5.8). The focus was on the silver grains (SG) with cells detectable in the background. Photomicrograph images were collected from the CA1 region in all hippocampal sections for *Nrg1* type I, II and III splice variants, *Egr3*, *Nab1*, *Nab2* and *Bdnf*. The number of SG per cell were counted using ImageJ over sufficient randomly selected counterstained neurones, from each region for each subject, such that the SE of the counts for any region was less than 10% of the population mean (approximately 36 total labelled cells and 24 nonspecific labelled cells). The specific SG count was then calculated for each region by subtracting the nonspecific counts from the total counts for each subject. Gene expression levels in the CS-US, CS-only and LI groups were standardised as a percentage of the mean number of SG in the naïve group for each region. The average number of silver grains per cell for the naïve group was recorded for each gene of interest.

Heavily labelled *Bdnf*-positive cells in the CA1, CA3 and DG regions of the hippocampus were subjectively identified in two ways. Firstly, in photomicrographs as cells that were very densely covered with SG, by observation approximately 80 SG/cell as opposed to approximately 10 SG/cell that was found in the majority of the cells. Secondly, they were subjectively identified through a dark-field microscope as sparsely distributed clusters of bright points. These clusters of bright points were confirmed to represent high levels of *Bdnf* expression in cells by switching to light-field conditions without moving the slide. The number of heavily labelled *Bdnf*-positive cells per hippocampal region per section were counted under dark-field conditions using the light microscope at 10x magnification. The number of SG per cell in the subjectively identified heavily and non-heavily labelled *Bdnf*-positive cells from the randomly taken photomicrographs were counted using ImageJ. These quantifications were determined from the same 3 total and 2 nonspecific *Bdnf*-labelled sections as used for the densitometric analysis.

4.2.4 Experiment 3: Regulation of *Egr3* in Consolidation of CFC using QPCR

4.2.4.1 Behaviour

Rats were divided into three groups:- (i) CS-US (n=6), (ii) LI (n=6) and (iii) naive (n=6). These rats underwent the same behavioural protocol as in experiment 2. Two hours after training the rats were killed by CO_2 asphyxiation, the brains were excised immediately from the rats, the CA1 regions of the hippocampus dissected out and placed in an Eppendorf of RNAlater and stored at -80°C.

4.2.4.2 QPCR

Two QPCR experiments were performed on the same dissected CA1 hippocampal sample (see 2.6.1). RNA was extracted using a Qiagen RNeasy protect mini kit from the CA1 tissue (see 2.6.2). In the initial QPCR experiment the concentrations of the 18 RNA samples from experimental rats were determined (Table 4.2) and used to standardise the amount of RNA that underwent reverse transcription as described in 2.6.3. In the technical replicate experiment, samples were diluted 1:20 in ddH_2O to ensure that the concentration of cDNA was distributed within the concentration range of the standard curve. The concentration of the diluted samples were determined (Table 4.3) and again used to standardise the amount of RNA that underwent reverse transcription across all the samples. A five point standard curve

Table 4.2. Concentration of RNA in CA1 samples 1 to 18 used for QPCR experiment 1. Sample purity and level of contaminants are derived from the ratio of absorbance levels measured at a wavelength of 260 μm, 280 μm and 230 μm. * indicates sample that was used for creating the standard curve.

Sample	Concentration (ng/μl)	Purity (A_{260}/A_{280})	Contaminants (A_{260}/A_{230})
S1	281.20	2.04	2.16
S2	302.30	2.03	2.13
S3	666.60	2.08	2.00
S4	821.20	2.08	2.03
S5 *	488.90	2.00	1.81
S6	480.70	1.99	2.11
S7	484.80	1.98	2.10
S8	363.30	2.02	2.17
S9	519.50	1.98	2.12
S10	460.30	2.03	1.75
S11	371.50	2.05	1.72
S12	542.20	1.96	2.09
S13	443.80	1.99	2.12
S14	639.30	2.07	1.99
S15	536.10	1.99	2.00
S16	314.30	2.03	2.17
S17	652.50	2.08	2.11
S18	566.30	1.96	2.02

Table 4.3. Concentration of RNA in CA1 samples 1 to 18 diluted at 1:20 and undiluted sample 19 used for QPCR experiment 2. Sample purity and level of contaminants are derived from the ratio of absorbance levels measured at a wavelength of 260 μm, 280 μm and 230 μm. * indicates sample that was used for creating the standard curve.

Sample	Concentration (ng/μl)	Purity (A_{260}/A_{280})	Contaminants (A_{260}/A_{230})
S1	13.90	1.80	2.21
S2	14.10	1.89	2.46
S3	35.50	1.86	2.22
S4	44.70	1.83	2.21
S5	28.10	1.81	2.09
S6	25.30	1.94	2.35
S7	26.50	1.89	2.35
S8	16.90	1.91	2.08
S9	27.50	1.90	2.37
S10	25.00	1.86	1.93
S11	18.40	1.91	1.97
S12	28.90	1.94	2.33
S13	22.00	1.91	2.36
S14	34.90	1.76	2.18
S15	32.30	1.81	2.32
S16	14.60	1.91	2.47
S17	38.50	1.80	2.45
S18	32.30	1.81	2.44
S19 *	367.20	2.05	1.76

was generated by three-fold dilutions as described in 2.6.4. In the initial QPCR experiment the standard curve was created using RNA from one of the naïve group CA1 samples. In the technical replicate QPCR experiment, the standard curve was created using RNA from the CA1 of an additional rat under naïve conditions. QPCR was performed in duplicates of each of the 18 CA1 samples, and in triplicate of each standard curve dilution for *Egr3* and the reference genes *Hmbs*, *Sdha* and *Ubc* as described in 2.6.4 and 2.6.5.

4.2.4.3 QPCR Analysis

Any outliers within the triplicates from the standard curve dilutions were removed in order to produce a more accurate standard curve. To meet the exclusion criteria the range of triplicate values must be greater than one Ct value. The value that is furthest from the standard curve is excluded. The standard curve was used to determine the relative amount of cDNA at threshold level for each sample. The relative level of expression of each GOI was quantified using the Ct value and the standard curve as described in 2.6.5. Fold changes in cDNA levels between the LI and CS-US groups relative to the naïve group were calculated as in 2.6.5.

4.2.5 Experiment 4: Time Profile of *Egr3* Regulation following CFC

12 rats underwent conditioning, as described in experiment 1. After conditioning the rats were returned to their home cages. 4 hours, 8 hours and 24 hours after conditioning the rats were killed by CO_2 asphyxiation and processed for ISH as described above (see 4.2.3.2). In addition, two naïve rats were taken from home cages and killed immediately without undergoing any conditioning and were processed. The tissue collected from these rats was combined with tissue from experiment 2. This provided 2 hour post-conditioning time point sections and further naïve sections. Therefore naïve sections and sections collected at 2 h, 4 h, 8 h and 24 h were used to determine the time profile of *Egr3* regulation post-conditioning using ISH.

4.2.6 Experiment 5: Effect of Intrahippocampal Infusions of *Egr3* Antisense on CFC

Two different *Egr3* antisense oligonucleotides (ASO) and one missense oligonucleotide (MSO) (18 nucleotides long) were designed as described in 2.3.1. The sequences for these oligonucleotides are also given in 2.3.1. Rats were divided into three groups:- (i) *Egr3* antisense I oligonucleotides (ASOI) group (n=6), (ii) *Egr3* antisense II oligonucleotides (ASOII)

group (n=6), and (iii) missense oligonucleotides (MSO) group (n=6). All groups underwent surgery to put in place bilateral indwelling hippocampal cannula at AP -3.50, relative to bregma, as described in 2.3.2. A week after surgery the rats received a habituation infusion of 2μl of PBS at 0.125μl/min, and a day later they received an infusion of 2 μl of 1 nmol/μl ASOI, 2 μl of 2 nmol/μl ASOII or 2 μl of 1 nmol/μl MSO in PBS at 0.125μl/min, as described in 2.3.3. 90 min after the infusion the rats were conditioned as in 4.2.1. 24 hours later the rats had a LTM1 test consisting of a 2 min re-exposure to the CS only. Similar LTM2 and LTM3 retrieval tests were performed 14 days and 21 days post-conditioning respectively. The average level of freezing behaviour in the ASOI group, ASOII group and the MSO group for the pre-US period, post-US period, LTM1 test, LTM2 test and LTM3 test was calculated.

After behavioural analysis, rats were killed by CO_2 asphyxiation. The brains were excised and rapidly frozen on dry ice before storage at -80°C. Histological assessment of the cannulae placement was performed by using a cryostat to cut and collect 14μm sections from the dorsal hippocampus that the cannulae targeted, and using thionin staining and a light microscope to identify the cannulae endpoints as described in 2.4.

4.2.7 Behavioural Analysis

The number of 10 s intervals that had freezing behaviour present was divided by the total number of 10 s intervals observed. This was then expressed as the percentage of time spent freezing for each test.

4.2.8 Statistical Analysis

Repeated Measures Analysis of Variance (ANOVA) was used to identify significant differences in the freezing behaviour between the different behavioural groups after CFC as there was more than one measurement observed for each rat. For experiment 5 the ASOI group was compared to the MSO group, and the ASOII group was compared to the MSO group. One-way ANOVA was used to identify significant differences between the pre-US and post-US freezing behaviour in the CS-US group. One-way ANOVA and the Fishers Least Significant Difference (FLSD) statistical tests were used to measure differences between the levels of gene expression in the different behavioural groups or at different time points post-conditioning

determined using ISH. For experiment 2 the standardised ISH results from the initial behavioural experiment (n = 4/group) were combined with the standardised ISH results from the second behavioural experiment (n = 5 or 6/group) to produce ISH results based on 9 or 10 rats per group. One-way ANOVA was performed on the normalised QPCR data to measure differences between the levels of *Egr3* expression in the different groups.

4.3 RESULTS

102 rats were used in this study in total. 12 rats were used in the establishment of the CFC procedure and control groups in experiment 1. 40 rats were used for ISH analysis of gene expression following CFC in experiment 2. One rat from the LI group in experiment 2 was asleep before, during and after the administration of the footshock and so was excluded from further analysis. 20 rats were used for QPCR analysis of *Egr3* expression in experiment 3. 12 rats were used to determine the time profile of *Egr3* expression in experiment 4. 18 underwent surgery for behavioural analysis. Histological analysis showed that cannulae terminated bilaterally in the dorsal hippocampus in all of the operated rats and none showed signs of gross damage to the hippocampus (Fig. 2.1).

4.3.1 Experiment 1: Conditioning Procedure Produces Long-Term CFM

There was an effect of group on freezing behaviour during conditioning and in retrieval tests ($F_{(5.356, 24.103)\ \varepsilon = 0.893} = 17.019$, $p = 0.000$, repeated measures ANOVA). This was manifested by an increase in freezing behaviour in the CS-US group selectively following the CS-US pairing ($F_{(3, 12)} = 21.909$, $p = 0.000$, ANOVA) (Fig. 4.2 a). Notably, the LI group demonstrated significantly attenuated conditioning as there was no freezing behaviour present in the retrieval tests.

The increase in freezing behaviour in the LTM tests in the CS-US group was an index that CFM lasted at least 14 days. The data shows that no CFM was formed in the CS-only or LI control groups. Thus, this conditioning procedure does produce long-term CFM while the CS-only and LI control groups do not.

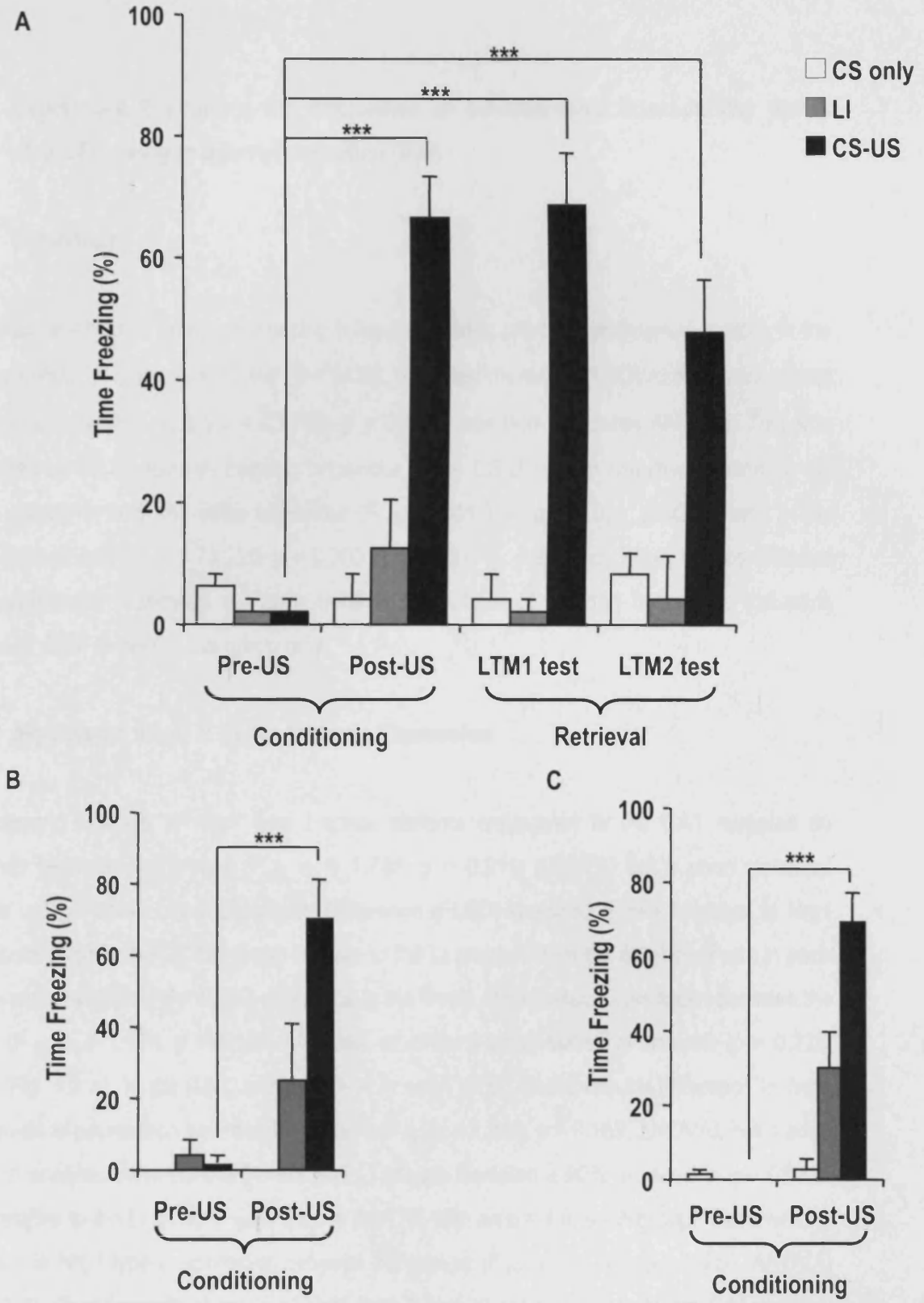


Figure 4.2. Conditioning procedure produces long-term contextual-fear memory, and freezing behaviour during CFC of rats in which gene expression was assayed. (A) Freezing behaviour from experiment 1 during pre-US and post-US phase of conditioning, and LTM1 and LTM2 retrieval tests (n = 4/group). (B) Freezing behaviour from initial behavioural component of experiment 2 during pre-US and post-US phase of conditioning (n = 4/group). (C) Freezing behaviour from increase in n behavioural component of experiment 2 during pre-US and post-US phase of conditioning (n = 6/ group see 4.2.2). *** $p<0.001$, FLSD. Error bars represent SEM. Conditioned Stimulus (CS), Unconditioned Stimulus (US), Long-Term Memory test (LTM), Latent Inhibition (LI).

4.3.2 Experiment 2: Profiling the Expression of Schizophrenia Susceptibility Genes after CFC using *In Situ* Hybridisation (ISH)

4.3.2.1 Behaviour

There was an effect of group on freezing behaviour during pre-US and post-US periods in the initial behaviour ($F_{(2, 9) \varepsilon = 1} = 13.492$, $p = 0.002$, repeated measures ANOVA) and in the repeat behavioural data ($F_{(2, 14) \varepsilon = 1} = 23.768$, $p = 0.000$, repeated measures ANOVA). This was manifested by an increase in freezing behaviour in the CS-US group selectively following the CS-US pairing in both the initial behaviour ($F_{(1, 6)} = 41.354$, $p = 0.001$, ANOVA) and in the repeat behaviour ($F_{(1, 10)} = 76.220$, $p = 0.000$, ANOVA) (Fig. 4.2 b & c). Thus, rats conditioned as in experiment 1 showed a similar behavioural pattern of freezing behaviour, indicating initiation of CFM in the CS-US group only.

4.3.2.2 *Nrg1* type I, II and III Splice Variants Expression

Densitometric analysis of *Nrg1* type I splice variants expression in the CA1 revealed no difference between the groups ($F_{(3, 12)} = 1.748$, $p = 0.211$, ANOVA) but *a priori* statistical analysis using Fishers Least Significant Difference (FLSD) identified a 59% increase in *Nrg1* type I expression in the CS-US group relative to the LI group. When the number of rats in each group was increased to n = 10, no difference in the levels of expression was found between the groups ($F_{(3, 35)} = 1.674$, $p = 0.190$, ANOVA), or in the *a priori* statistical analysis ($p = 0.228$, FLSD) (Fig. 4.3 a). In the CA3, with an n = 4 in each group, there was no difference in *Nrg1* type I levels of expression between the groups ($F_{(3, 12)} = 1.869$, $p = 0.189$, ANOVA), but *a priori* statistical analysis between the CS-US and LI groups revealed a 90% increase in the CS-US group relative to the LI group (Fig. 4.3 b). In the DG, with an n = 4 in each group, there was no difference in *Nrg1* type I expression between the groups ($F_{(3, 12)} = 1.563$, $p = 0.250$, ANOVA) (Fig. 4.3 c). Densitometric analysis of Nrg1 type II and III splice variants in the CA1 was not possible due to the very low levels expressed in this region.

Cellular level analysis on the same *Nrg1* type I splice variants hybridised CA1 region, again showed no difference in expression levels between the groups ($F_{(3, 35)} = 0.273$, $p = 0.844$, ANOVA) (Fig 4.3 d). Cellular level analysis, unlike densitometric analysis, of *Nrg1* type II splice

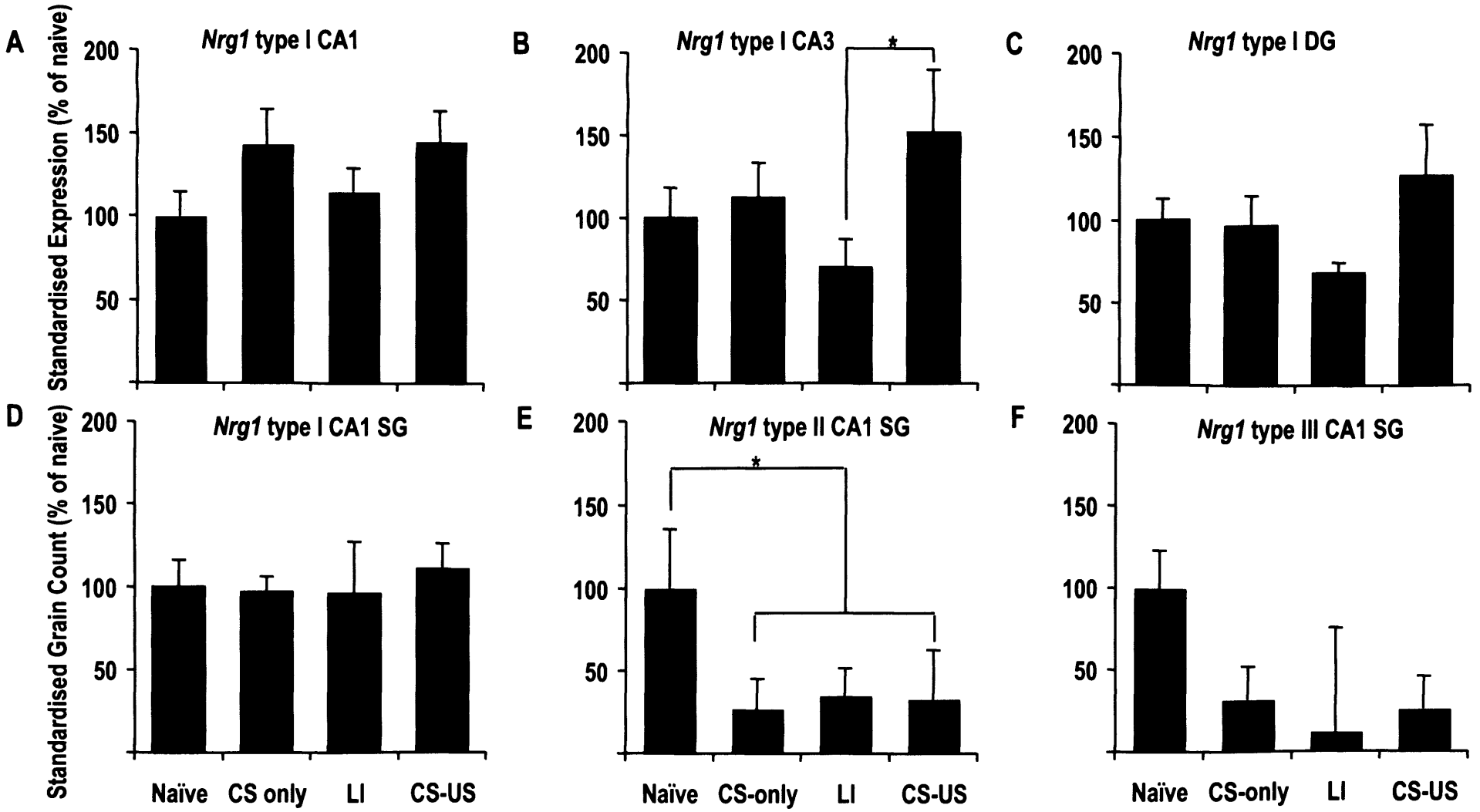


Figure 4.3. *Nrg1* type I, II and III splice variants expression in the hippocampus 2 hours after CFC. ID determined *Nrg1* type I expression in: (A) CA1 (n=10/group), (B) CA3 (n = 4/group) and (C) DG (n = 4/group). SG/cell determined CA1 expression for: (D) *Nrg1* type I (n=10/group), (E) *Nrg1* type II (n = 4/group) and (F) *Nrg1* type III (n = 4/group). Error bars represent SEM. ID, image densitometry; SG, silver grain counting; Nrg1 type I naïve expression (nCi/mg tissue): CA1,18±3 and 20±7; CA3, 16±3; DG, 30±4. Naïve number of SG/cell: Nrg1 type I CA1, 38±4 and 78±21; Nrg1 type II CA1, 4±1.5; Nrg1 type III CA1, 1.6±0.4.

variants expression in the CA1 was possible. While there was no difference between the individual groups ($F_{(3, 12)} = 1.735$, $p = 0.213$, ANOVA), the three behavioural groups combined had decreased levels of *Nrg1* type II expression relative to the naïve group ($F_{(1, 14)} = 5.989$, $p = 0.028$, ANOVA) (Fig. 4.3 e). Cellular level analysis of *Nrg1* type III splice variants expression levels in the CA1 were not different between the groups ($F_{(3, 12)} = 0.923$, $p = 0.459$, ANOVA) (Fig. 4.3 f).

Nrg1 type I splice variants were not regulated in correlation with consolidation of CFM in the CA1 or DG regions of the hippocampus. There was however a significant upregulation of *Nrg1* type I in conditioned rats relative to the LI group of rats in the CA3. *Nrg1* type II and III splice variants were also not regulated in correlation with consolidation of CFM in the CA1, but *Nrg1* type II splice variants were downregulated in all behavioural groups relative to the naïve group.

4.3.2.3 Expression Levels of Probes Designed to *Dtnbp1* Exons 1, 5, 8 and 9

Densitometric analysis in the CA1 revealed that there was no difference in the levels of expression between the groups for *Dtnbp1* exon 1 splice variants ($F_{(3, 12)} = 0.58$, $p = 0.64$, ANOVA), *Dtnbp1* exon 5 splice variants ($F_{(3, 12)} = 0.22$, $p = 0.88$, ANOVA), *Dtnbp1* exon 9 splice variants ($F_{(3, 12)} = 1.38$, $p = 0.30$, ANOVA) and pan *Dtnbp1* (*Dtnbp1* exon 8) ($F_{(3, 12)} = 0.19$, $p = 0.90$, ANOVA) (Fig. 4.4 a, d, g & j). In the CA3 it was revealed that, like the CA1, there was no difference in the levels of expression between the groups for *Dtnbp1* exon 1 splice variants ($F_{(3, 12)} = 0.30$, $p = 0.83$, ANOVA), *Dtnbp1* exon 5 splice variants ($F_{(3, 12)} = 0.06$, $p = 0.98$, ANOVA), Dtnbp1 exon 9 splice variants ($F_{(3, 12)} = 1.17$, $p = 0.36$, ANOVA) and pan *Dtnbp1* (*Dtnbp1* exon 8) ($F_{(3, 12)} = 0.44$, $p = 0.73$, ANOVA) (Fig. 4.4 b, e, h & k). In the DG it was revealed that, like the CA1 and CA3, there was no difference in the levels of expression between the groups for *Dtnbp1* exon 1 splice variants ($F_{(3, 12)} = 0.18$, $p = 0.91$, ANOVA), *Dtnbp1* exon 5 splice variants ($F_{(3, 12)} = 0.24$, $p = 0.87$, ANOVA), *Dtnbp1* exon 9 splice variants ($F_{(3, 12)} = 0.59$, $p = 0.63$, ANOVA) and pan *Dtnbp1* (*Dtnbp1* exon 8) ($F_{(3, 12)} = 0.15$, $p = 0.93$, ANOVA) (Fig. 4.4 c, f, i & l). There were no changes in expression of *Dtnbp1* splice variants in the CA1, CA3 or DG hippocampal regions in correlation with consolidation of CFM 2 hours after CFC.

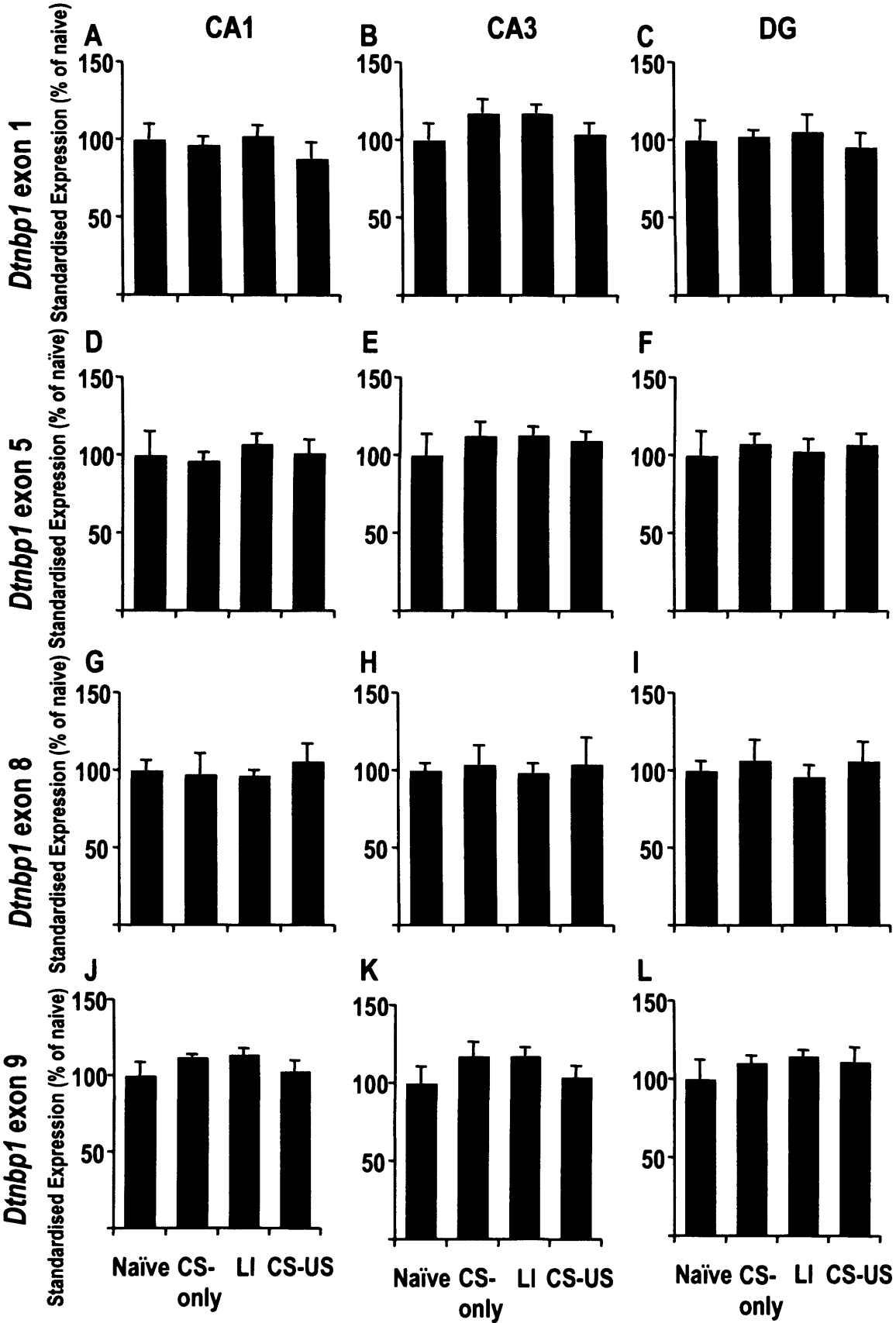


Figure 4.4 *Dtnbp1* splice variants expression in the hippocampus 2 hours after CFC. Expression levels were measured using image densitometry (n = 4/group). *Dtnbp1* exon 1 expression in: (A) CA1, (B) CA3 and (C) DG. *DTNBP1* exon 5 expression in (D) CA1, (E) CA3 and (F) DG. *DTNBP1* exon 8 (pan *DTNBP1*) expression in (G) CA1, (H) CA3 and (I) DG. *DTNBP1* exon 9 expression in (J) CA1, (K) CA3 and (L) DG. Error bars represent SEM. *Dtnbp1* exon 1 naïve expression (nCi/mg tissue): CA1, 30±3; CA3, 16±2; DG, 20±2. *Dtnbp1* exon 5 naïve expression (nCi/mg tissue): CA1, 23±3; CA3, 12±2 ; DG, 14±2. *Dtnbp1* exon 8 naïve expression (nCi/mg tissue): CA1, 21±1; CA3, 15±1; DG, 16±1. *Dtnbp1* exon 9 naïve expression (nCi/mg tissue): CA1, 25±2; CA3, 23±2 ; DG, 24±3.

4.3.2.4 *Egr3* Expression

4.3.2.4.1 Hippocampus

Densitometric analysis of *Egr3* expression in the CA1 revealed that there was a difference in the levels of expression between the groups ($F_{(3, 35)} = 12.426$, $p = 0.000$, ANOVA) (Fig. 4.5 a). *Egr3* was upregulated in the CS-US group relative to the three control groups, with *Egr3* being upregulated by 31% in the CS-US group in comparison to the naive group. These findings suggest that *Egr3* upregulation correlates with consolidation of CFM. In addition, *Egr3* was upregulated in the CS-only group and LI group relative to the naïve group. Cellular level analysis of *Egr3* expression in the CA1 revealed no difference between the groups ($F_{(3, 34)} = 1.947$, $p = 0.141$, ANOVA) (Fig. 4.5 b), but *a priori* statistical analysis revealed an upregulation of *Egr3* expression by 22% in the CS-US group relative to the naïve group. Densitometric analysis of *Egr3* expression in the CA3 and DG revealed that there was no difference in the levels of expression between the groups ($F_{(3, 12)} = 3.002$, $p = 0.073$, ANOVA) and ($F_{(3, 12)} = 3.240$, $p = 0.060$, ANOVA) respectively (Fig. 4.5 c & d). However *a priori* statistical analysis in the CA3 revealed an upregulation of *Egr3* expression by 27% in the CS-US group relative to the naïve group. Also in the DG *Egr3* expression was upregulated in the CS-US group by 21% and 16% relative to the naïve and CS-only groups respectively.

Cellular level analysis of *Nab1* and *Nab2* expression in the CA1 revealed that there was no difference between the groups ($F_{(3, 12)} = 0.149$, $p = 0.928$, ANOVA) and ($F_{(3, 35)} = 1.337$, $p = 0.278$, ANOVA) respectively (Fig. 4.6 a & b). Films obtained from the first *Nab1* and *Nab2* ISH experiment were not suitable for densitometric analysis. As *Nab2* but not *Nab1*has been shown to be transiently upregulated in response to cellular activity (Jouvert et al., 2001), *Nab2* is more likely of the two genes to be regulated following CFC and so densitometric analysis of *Nab2* in the CA1 was performed on a subsequent *Nab2* ISH experiment. The CA1 region was chosen as *Egr3* was regulated in this region. Regional analysis of *Nab2* expression in the CA1 revealed that there was no difference between the groups ($F_{(3, 19)} = 2.500$, $p = 0.090$, ANOVA). However *a priori* statistical analysis revealed that there was an upregulation of *Nab2* by 26% in the LI group relative to the naïve group ($p = 0.019$) (Fig. 4.6 c).

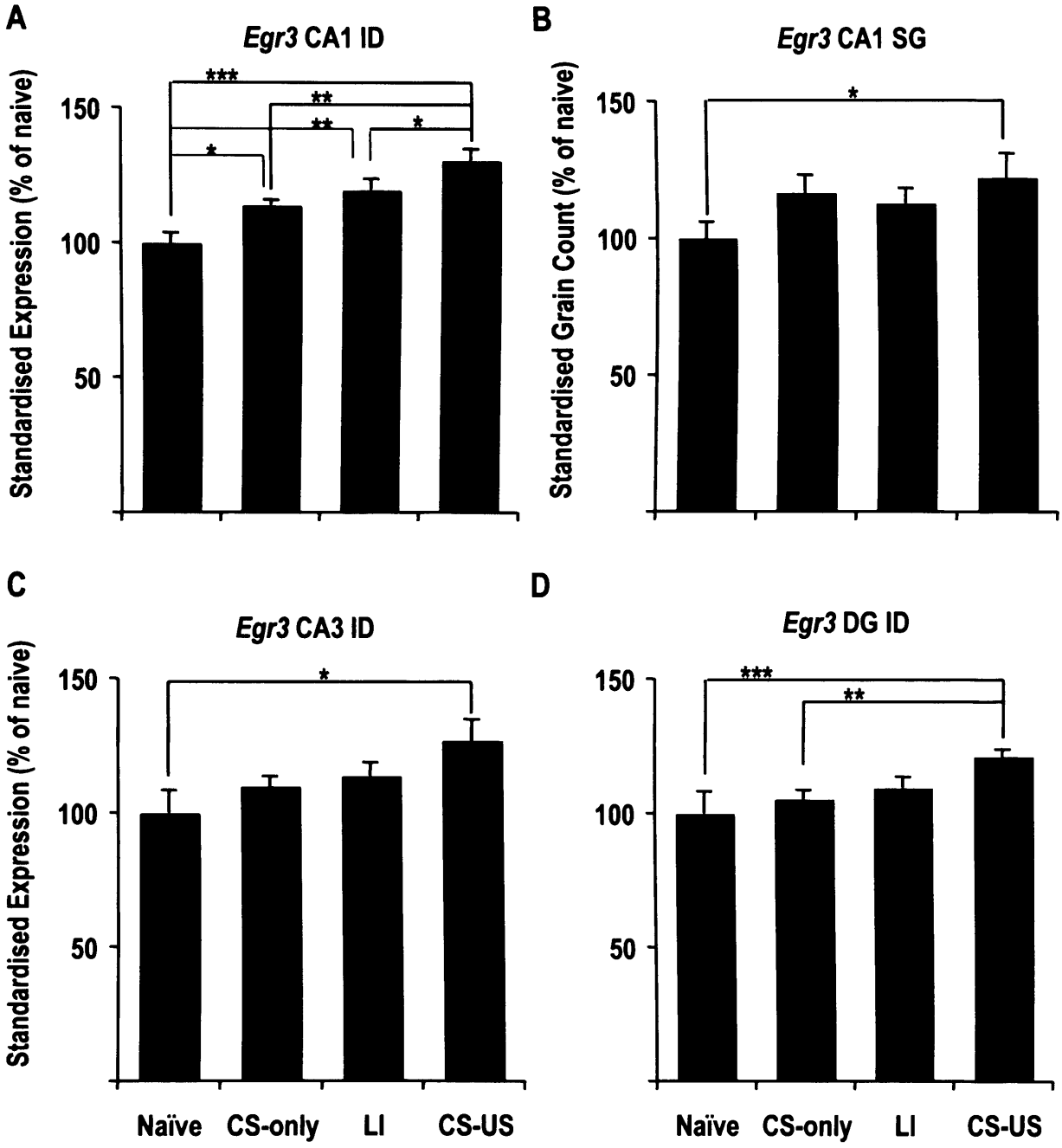


Figure 4.5 *Egr3* expression in the hippocampus 2 hours after CFC. *Egr3* expression in the CA1 field: (A) Regional level measured using image densitometry (n = 10/group), and (B) Cellular level measured using grain counting (n = 10/group). (C) *Egr3* expression in the CA3 region measured using image densitometry (n = 4/group). (D) *Egr3* expression in the DG region measured using image densitometry (n = 4/group). (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$, FLSD). Error bars represent SEM. ID, Image densitometry; SG, Silver grain counting; *Egr3* naïve expression (nCi/mg tissue): CA1, 61±4 & 55±3; CA3, 56±5; DG, 57±5. Naïve number of SG/cell: CA1, 19±3 & 98±5.

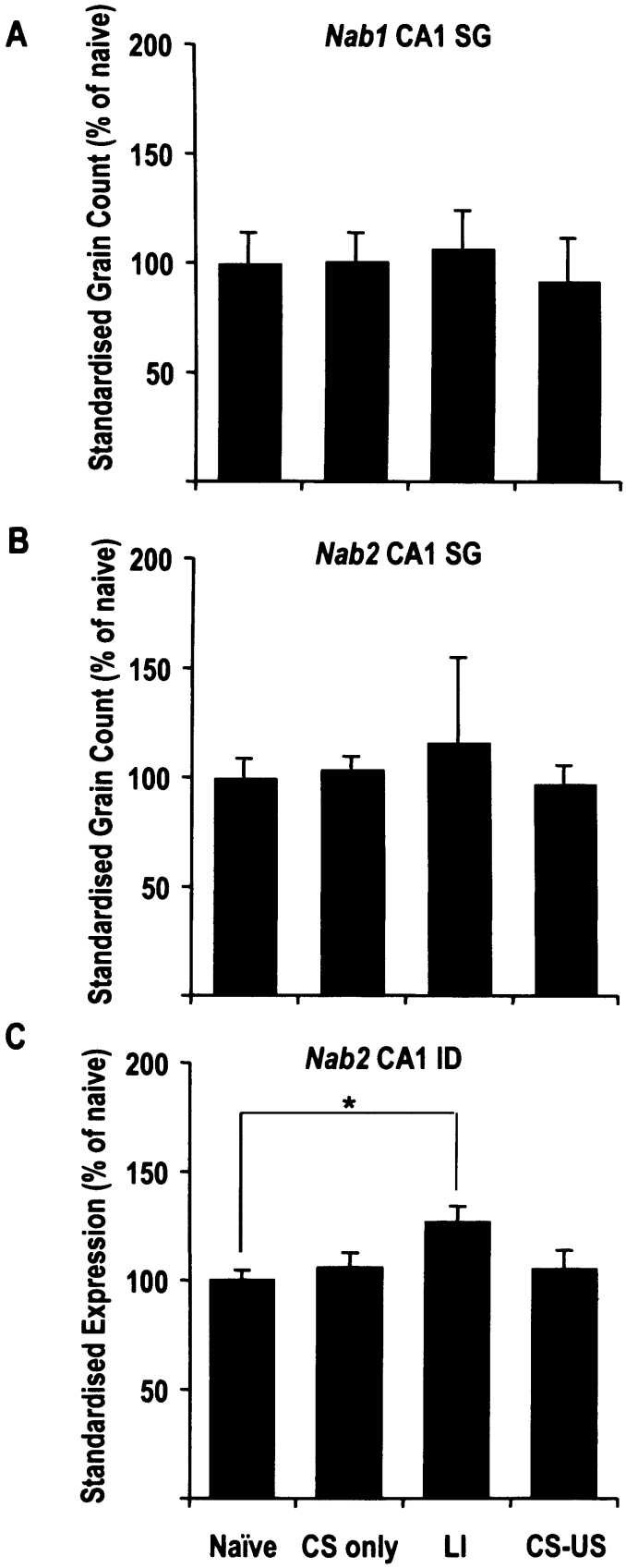


Figure 4.6. *Nab1* and *Nab2* expression 2 hours after CFC. (A) *Nab1* expression in the CA1 measured using SG analysis (n = 4/group). (B) *Nab2* expression in the CA1 measured using SG analysis (n = 10 or 9/group). (C) *Nab2* expression in the CA1 measured using image densitometry analysis (n = 6 or 5/group). (* $p<0.05$) Error bars represent SEM. ID, image densitometry; SG, Silver grain counting. Naïve number of SG/cell: *Nab1* CA1, 10±1; *Nab2* CA1, 20±4 & 47±5. Naïve expression (nCi/mg tissue): CA1, 28±1.

Egr3 expression was upregulated in correlation with consolidation of CFM in the CA1 region of the hippocampus as determined by regional level investigation and supported by findings at the cellular level of analysis. In both the CA3 and DG, *Egr3* expression was upregulated in the CS-US group relative to the naïve group, and also in the CS-US group relative to the CS-only group in the DG. *Nab1* and *Nab2* expression was not regulated in correlation with consolidation of CFM in the CA1, but *Nab2* expression was upregulated in the LI group relative to the naïve group.

4.3.2.4.2 Prefrontal Cortex

Densitometric analysis of *Egr3* expression in the Cg1 cortex revealed that there was a difference in the levels of expression between the groups ($F_{(3, 12)} = 4.050$, $p = 0.033$, ANOVA) (Fig. 4.7 a). *Egr3* was upregulated in the CS-US group by 35% and in the CS-only group by 34% relative to the naïve group. *Egr3* expression levels in the PrL cortex were different between the groups ($F_{(3, 12)} = 9.076$, $p = 0.002$, ANOVA) (Fig. 4.7 b). *Egr3* was upregulated in the CS-US group by 55% and in the CS-only group by 64% relative to the naïve group. Finally, *Egr3* expression levels in the IL cortex were also different between the groups ($F_{(3, 12)} = 5.823$, $p = 0.011$, ANOVA) (Fig. 4.7 c). *Egr3* was upregulated in the CS-US group by 63% and in the CS-only group by 79% relative to the naïve group.

Egr3 was not regulated in correlation with consolidation of CFM in the Cg1, PrL and IL regions of the medial prefrontal cortex. However, *Egr3* was upregulated in the CS-US and CS-only groups relative to the naïve group in these regions of the medial prefrontal cortex.

4.3.2.4.3 Amygdala

Densitometric analysis of *Egr3* expression in the DLA nucleus of the amygdala revealed that there was a difference in the levels of expression between the groups ($F_{(3, 19)} = 3.512$, $p = 0.035$, ANOVA) (Fig. 4.8 a). *Egr3* was upregulated in the CS-US group by 31% relative to the naïve group and by 36% relative to the LI group. While *Egr3* expression in the CS-only group was 77% of that in the CS-US group, *a priori* statistical analysis did not find this difference to be significant. *Egr3* expression in the LA nucleus of the amygdala revealed that there was no difference in the levels of expression between the groups ($F_{(3, 19)} = 1.445$, $p = 0.261$, ANOVA)

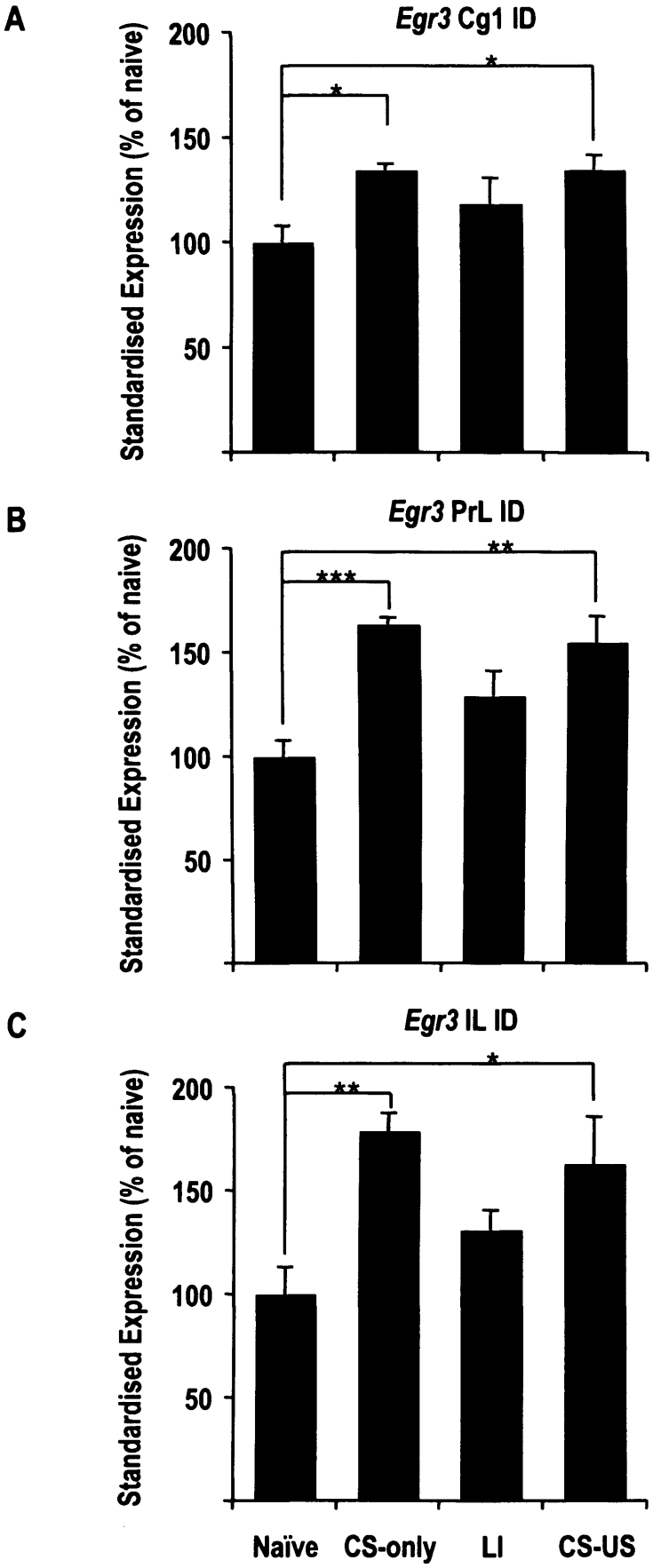


Figure 4.7. *Egr3* expression in the prefrontal cortex 2 hours after CFC. Expression levels were measured using image densitometry (n = 4/group). *Egr3* expression in: (A) the Cg, (B) the PrL, and (C) the IL. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$, FLSD). Error bars represent SEM. Cg1, Cingulate cortex; PrL, Prelimbic cortex; IL, Infralimbic cortex; Naïve expression (nCi/mg tissue): Cg, 67±5; PrL, 61±5; IL, 47±6.

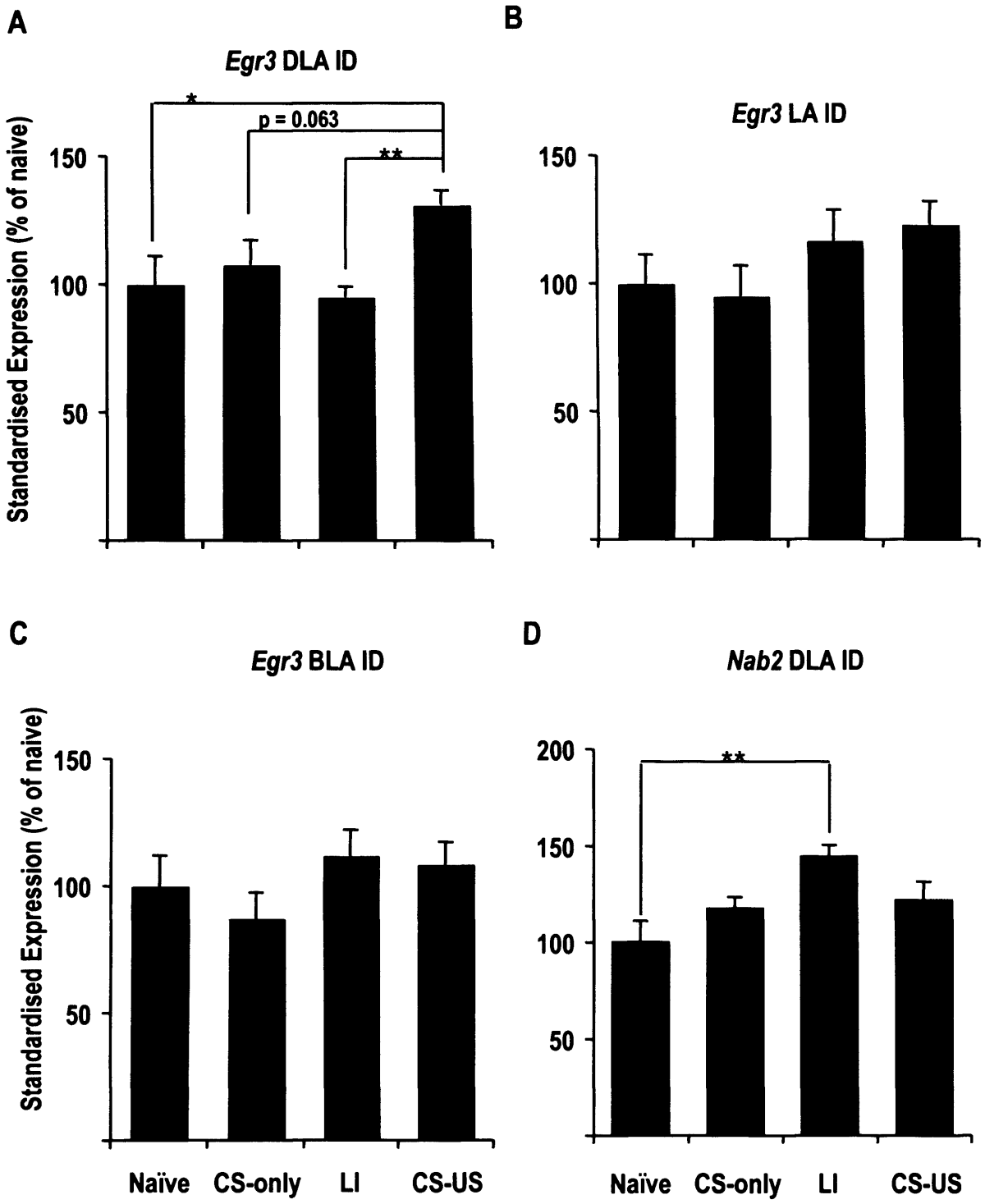


Figure 4.8. *Egr3* expression in the amygdala 2 hours after CFC. Expression levels were measured using image densitometry (ID) (n = 6 or 5/group). *Egr3* expression in the: (A) DLA nucleus, (B) LA nucleus, and (C) BLA nucleus. (D) *Nab2* expression in the DLA nucleus. (* $p<0.05$, ** $p<0.01$). Error bars represent SEM. DLA, Dorsolateral; LA, Lateral; BLA, Basolateral; *Egr3* Naïve expression (nCi/mg tissue): DLA, 32±4; LA, 23±3; BLA, 22±3. *Nab2* Naïve expression (nCi/mg tissue): DLA, 12±1.

(Fig. 4.8 b) or in the BLA nucleus of the amygdala ($F_{(3, 19)} = 1.122$, $p = 0.365$, ANOVA) (Fig. 4.8 c).

Nab2 expression was investigated in the DLA nucleus of the amygdala as *Egr3* was regulated in this region. Densitometric analysis of *Nab2* expression in the DLA nucleus of the amygdala revealed that there was a difference between the groups ($F_{(3, 19)} = 4.075$, $p = 0.022$, ANOVA) (Fig. 4.8 d). *Nab2* expression in the LI group was upregulated by 44% relative to the naïve group.

Egr3 was not regulated in correlation with consolidation of CFM in the LA or BLA nuclei of the amygdala. However, in the DLA nucleus of the amygdala *Egr3* was upregulated in the CS-US group relative to the naïve and LI group and the difference relative to the CS-only group was approaching significance. Also in the DLA nucleus of the amygdala, *Nab2* was upregulated in the LI group relative to the naïve group.

4.3.2.5 *Bdnf* Expression

Densitometric analysis of *Bdnf* expression in the CA1 revealed that there was a difference between the groups ($F_{(3, 12)} = 5.821$, $p = 0.011$, ANOVA) (Fig. 4.9 a). *Bdnf* was upregulated in the CS-US group by 28% relative to the naïve group and was also upregulated in the LI group by 37% relative to the naïve group. In the images taken for *Bdnf* cellular analysis, it was observed in all three regions of the hippocampus that a minority of the cells were substantially more heavily labelled for *Bdnf* than the majority of the cells. In turn, the majority of the cells still had much higher levels of *Bdnf* than the non-specific labelled cells. This observation led to the expression of *Bdnf* being assayed by multiple approaches. Firstly, the number of heavily labelled *Bdnf*-positive cells in the CA1 region of the hippocampus were counted. There was no difference between the groups ($F_{(3, 12)} = 3.333$, $p = 0.056$, ANOVA), however *a priori* statistical analysis revealed that number of heavily labelled *Bdnf*-positive cells was increased by 46% in the LI group relative to the naïve group, and the three behavioural groups combined had an increased number of heavily labelled *Bdnf*-positive cells relative to the naïve group ($F_{(1, 14)} = 8.413$, $p = 0.012$, ANOVA) (Fig. 4.9 b). Secondly, silver grain density in the heavily labelled *Bdnf*-positive cells was counted to identify whether the levels of *Bdnf* expression within the heavily labelled *Bdnf*-positive cells was regulated after CFC. Analysis of *Bdnf* expression in the heavily labelled *Bdnf*-positive cells in the CA1 revealed that there was no difference between

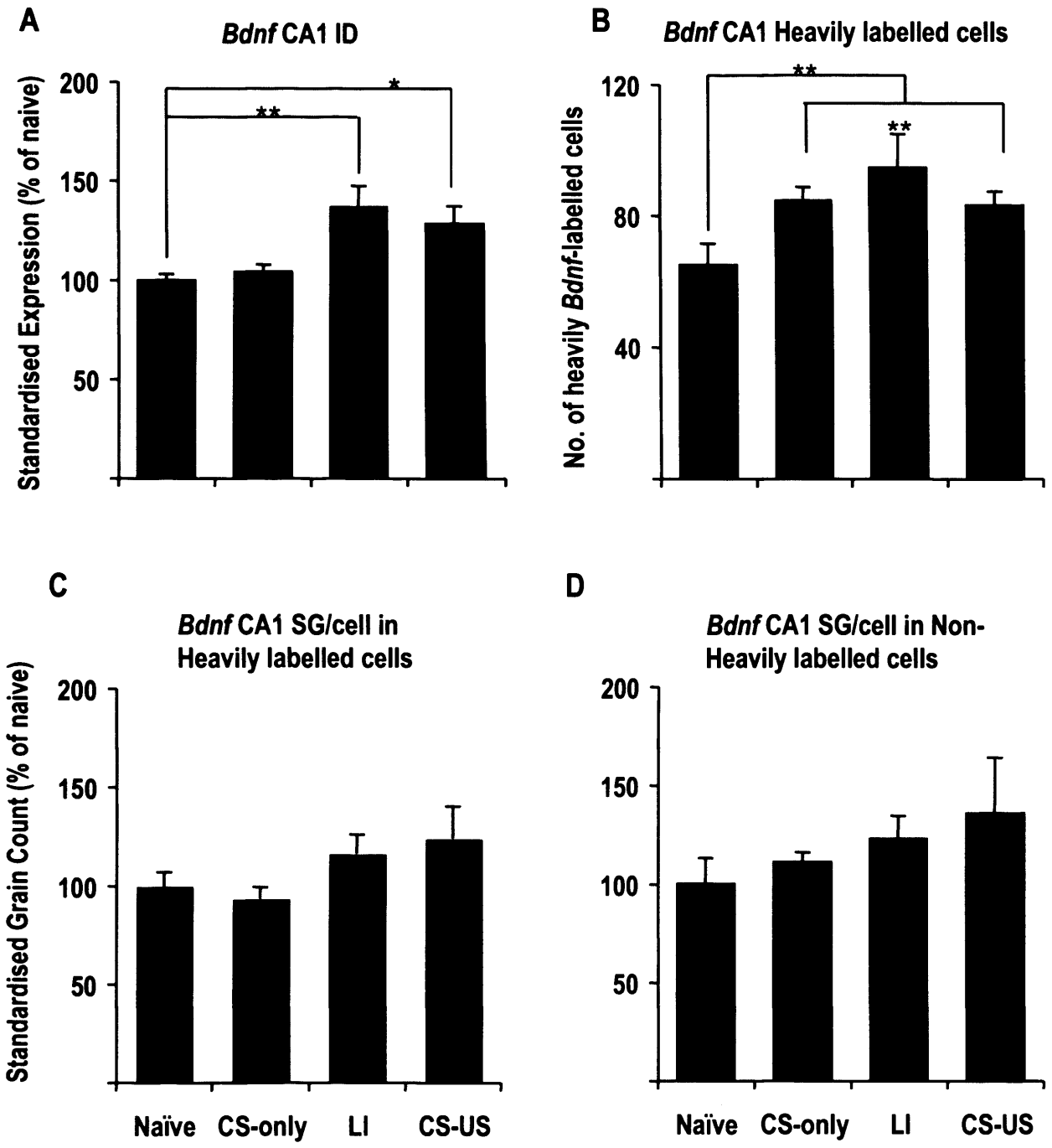


Figure 4.9. *Bdnf* expression in the CA1 region of the hippocampus 2 hours after CFC. (A) *Bdnf* expression measured using image densitometry. (B) Number of heavily *Bdnf*-labelled cells. (C) *Bdnf* expression levels in the heavily *Bdnf*-labelled cells measured using silver grains counting. (D) *Bdnf* expression levels in the nonheavily *Bdnf*-labelled cells measured using silver grains counting. * $p<0.05$, ** $p<0.01$ indicates significance relative to the naïve group. Error bars represent SEM. (n=4/group) *Bdnf* naïve expression (nCi/mg tissue): CA1, 6±0.2. Naive number of heavily labelled *Bdnf*-positive cells: CA1, 65±7. Naïve number of SG/cell: Heavily labelled, 73±5; Non-heavily labelled, 8±1.

the groups ($F_{(3, 12)} = 1.855$, $p = 0.191$, ANOVA) (Fig. 4.9 c). Thirdly, silver grain density in the less heavily labelled *Bdnf*-positive cells were counted. Analysis of *Bdnf* expression in the non-heavily labelled *Bdnf*-positive cells in the CA1 revealed that there was no difference between the groups ($F_{(3, 11)} = 0.936$, $p = 0.456$, ANOVA) (Fig. 4.9 d).

Bdnf was not regulated in correlation with consolidation of CFM in the CA1 region of the hippocampus. This finding is in contrast to the upregulation of *Bdnf* expression detected in a similar previous study (Hall et al., 2000). However this difference could be due to the levels of expression being measured at 2 hours in this study as opposed to 30 mins in the study by Hall and colleagues (2000). Regional level investigation determined that *Bdnf* was upregulated in both the CS-US and LI groups relative to the naïve group. However at the cellular level the number of heavily labelled *Bdnf*-positive cells were increased in all behavioural groups relative to the naïve group. There was no change in the levels of *Bdnf* expression within either the heavily or non- heavily labelled *Bdnf*-positive cells populations in the CA1.

Densitometric analysis of *Bdnf* expression in the CA3 revealed that there was no difference between the groups ($F_{(3, 12)} = 2.556$, $p = 0.104$, ANOVA) (Fig. 4.10 a). *A priori* statistical analysis revealed that *Bdnf* expression in the CS-only group was downregulated by 24% relative to the naïve group. The number of heavily labelled *Bdnf*-positive cells in the CA3 were not different between the groups ($F_{(3, 12)} = 1.620$, $p = 0.237$, ANOVA) (Fig. 4.10 b). *Bdnf* was not regulated in correlation with consolidation of CFM in the CA3 region of the hippocampus but was downregulated in the CS-only group relative to the naïve group.

Densitometric analysis of *Bdnf* expression in the DG revealed that there was a difference between the groups ($F_{(3, 12)} = 13.625$, $p = 0.000$, ANOVA). *Bdnf* expression was downregulated in all groups compared to naïve; there was a 37% decrease in the CS-US group, a 45% decrease in the CS-only group and a 21% decrease in the LI group (Fig. 4.10 c). The number of heavily labelled *Bdnf*-positive cells in the DG were different between the groups ($F_{(3, 12)} = 3.742$, $p = 0.042$, ANOVA). There was an increase in the number of heavily labelled *Bdnf*-positive cells in the CS-only group by 25% and in the LI group by 36% to those in the naïve group. While the number of heavily labelled *Bdnf*-positive cells in the CS-US group was 121% of that in the naïve group, *a priori* statistical analysis did not find this difference to be significant. The three behavioural groups combined had an increased number of heavily

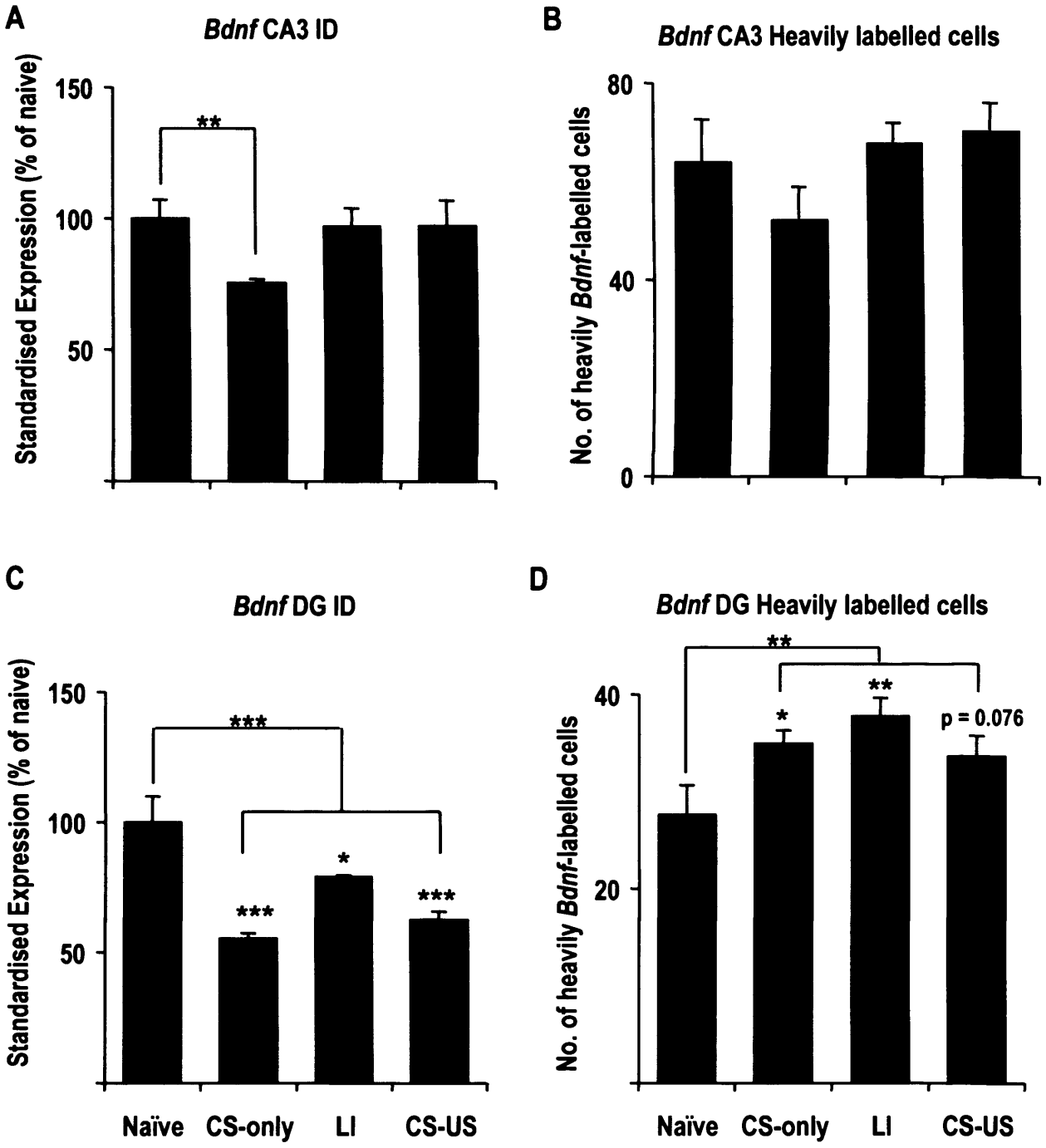


Figure 4.10. *Bdnf* expression in the CA3 and DG regions of the hippocampus 2 hours after CFC. (A) *Bdnf* expression levels in the CA3 measured using image densitometry. (B) Number of heavily *Bdnf*-labelled cells in the CA3. (C) *Bdnf* expression levels in the DG measured using image densitometry. (D) Number of heavily *Bdnf*-labelled cells in the DG. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ indicates significance relative to the naive group. (n = 4/group) Error bars represent SEM. ID, Image densitometry. *Bdnf* naïve expression (nCi/mg tissue): CA3, 22±2; DG, 26±3. Naive number of heavily labelled *Bdnf*-positive cells: CA3, 64±8; DG, 28±3.

labelled *Bdnf*-positive cells relative to the naïve group ($F_{(1, 14)} = 9.591$, $p = 0.008$, ANOVA) (Fig. 4.10 d).

Bdnf was not regulated in correlation with consolidation of CFM in the DG region of the hippocampus. However regional analysis in the DG determined that *Bdnf* was downregulated in the behavioural groups relative to the naïve group, while analysis at the cellular level determined that there was an increase in the number of heavily labelled *Bdnf*-positive cells in the behavioural groups relative to the naïve group.

4.3.3 Experiment 3: Regulation of *Egr3* in Consolidation of CFC using QPCR

To clarify whether *Egr3* is regulated in the CA1 in correlation with CFM, QPCR was used to measure *Egr3* mRNA expression in CS-US, LI and naïve groups. No CS-only group was included due to there being no difference between the levels of *Egr3* expression in the LI and CS-only groups in either level of analysis from the ISH experiment.

4.3.3.1 Behaviour

There was an effect of group on freezing behaviour during pre-US and post-US periods in the initial behaviour ($F_{(1, 10)}\ \varepsilon = 1 = 24.096$, $p = 0.001$, repeated measures ANOVA) (Fig. 4.11 a). The freezing behaviour was selectively increased in the post-US period in the CS-US group ($F_{(1, 10)} = 96.571$, $p = 0.000$, ANOVA). Thus this group underwent CFC as observed in experiments 1 and 2. Consequently it would be predicted that the rats in the CS-US group in this experiment would have formed a CFM had they not been killed 2 hours after conditioning.

4.3.3.2 QPCR

Only two reference genes were used in the creation of the normalisation factor in the initial QPCR experiment as the *Hmbs* standard curve was of poor quality. In both the initial QPCR experiment and in the technical replicate experiment, an outlier was identified amongst the six values within the naive group. As this sample was consistently an outlier despite different RT reactions being used, the sample was considered contaminated and so sample 2 was removed from analysis. In total, 5 out of 120 of the total standard curve values were excluded as outliers

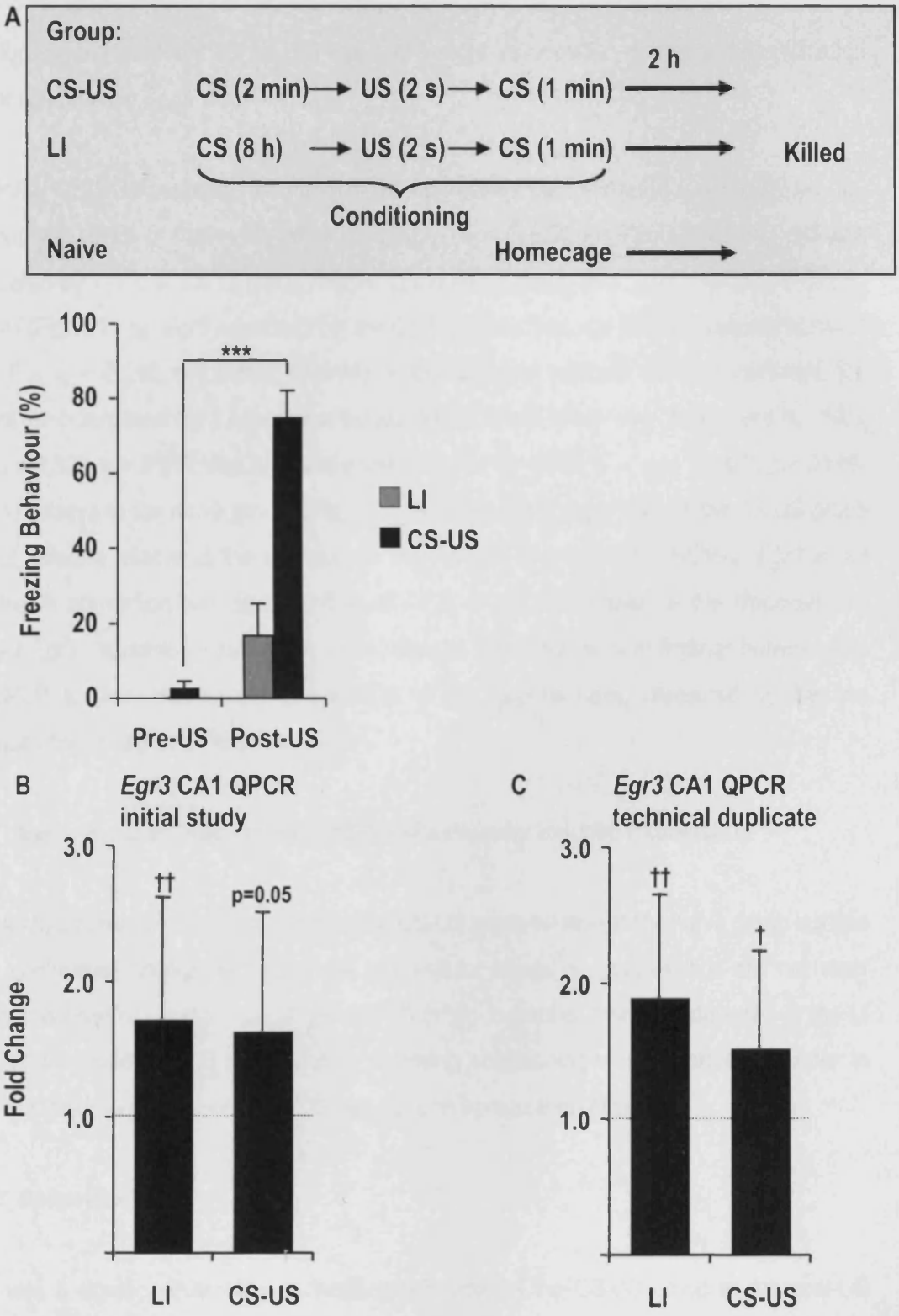


Figure 4.11 Regulation of *Egr3* in Consolidation of CFC using QPCR. (A) Schematic of behavioural protocol used and freezing behaviour observed from experiment 3 for the pre-US and post-US periods (n=5 or 6/group. (B) *Egr3* expression in the CA1 measured using Q-PCR (initial study). (C) *Egr3* expression in the CA1 measured using Q-PCR (technical replicate). *** $p<0.001$, FLSD. † indicates significance relative to the naive group († $p<0.05$, †† <0.01). Error bars represent SEM.

to produce quality standard curves. All standard curves, dissociation curves and amplification plots can be found in Appendix I.

In the initial QPCR experiment, the mean of the normalised *Egr3* expression values in the CS-US group was 164% of that in the naïve group ($F_{(1, 10)} = 5.108$, $p = 0.050$, ANOVA), and was upregulated by 172% in the LI group relative to the naïve group ($F_{(1, 10)} = 13.433$, $p = 0.005$, ANOVA) (Fig. 4.11 b). *Egr3* expression in the CS-US group was not different relative to the LI group ($F_{(1, 10)} = 0.200$, $p = 0.664$, ANOVA). In the technical replicate QPCR experiment, the mean of the normalised *Egr3* expression values in the CS-US group was upregulated by 153% ($F_{(1, 10)} = 8.523$, $p = 0.017$, ANOVA) and in the LI group by 190% ($F_{(1, 10)} = 13.922$, $p = 0.005$, ANOVA) relative to the naïve group (Fig. 4.11 c). Again *Egr3* expression in the CS-US group was not different relative to the LI group ($F_{(1, 10)} = 2.469$, $p = 0.147$, ANOVA). *Egr3* is not regulated in correlation with consolidation of CFM in the CA1 region of the hippocampus. However *Egr3* expression was behaviourally induced. The differences in findings between ISH and QPCR is likely due to the components of the regions being measured by the two techniques being slightly different.

4.3.4 Experiment 4: Time Profile of *Egr3* Behaviourally Induced Expression

Egr3 has been shown to be upregulated in the CS-US group relative to the naïve group but due to the contrasting findings from regional and cellular levels of analysis it is still not clear whether the level of upregulation in the CS-US group is greater than that detected in the LI group. A time profile on *Egr3* expression following conditioning was performed in order to determine the time-point of maximal *Egr3* expression upregulation after CFC.

4.3.4.1 Behaviour

There was a significant increase in freezing behaviour in the CS-US group in the post-US period relative to the pre-US period for the 4 h post-conditioning ($F_{(1, 6)} = 45.632$, $p = 0.001$, ANOVA), 8 h post-conditioning ($F_{(1, 6)} = 37.0$, $p = 0.001$, ANOVA) and 24 h post-conditioning groups ($F_{(1, 6)} = 54.0$, $p = 0.000$, ANOVA) (Fig. 4.12 a).

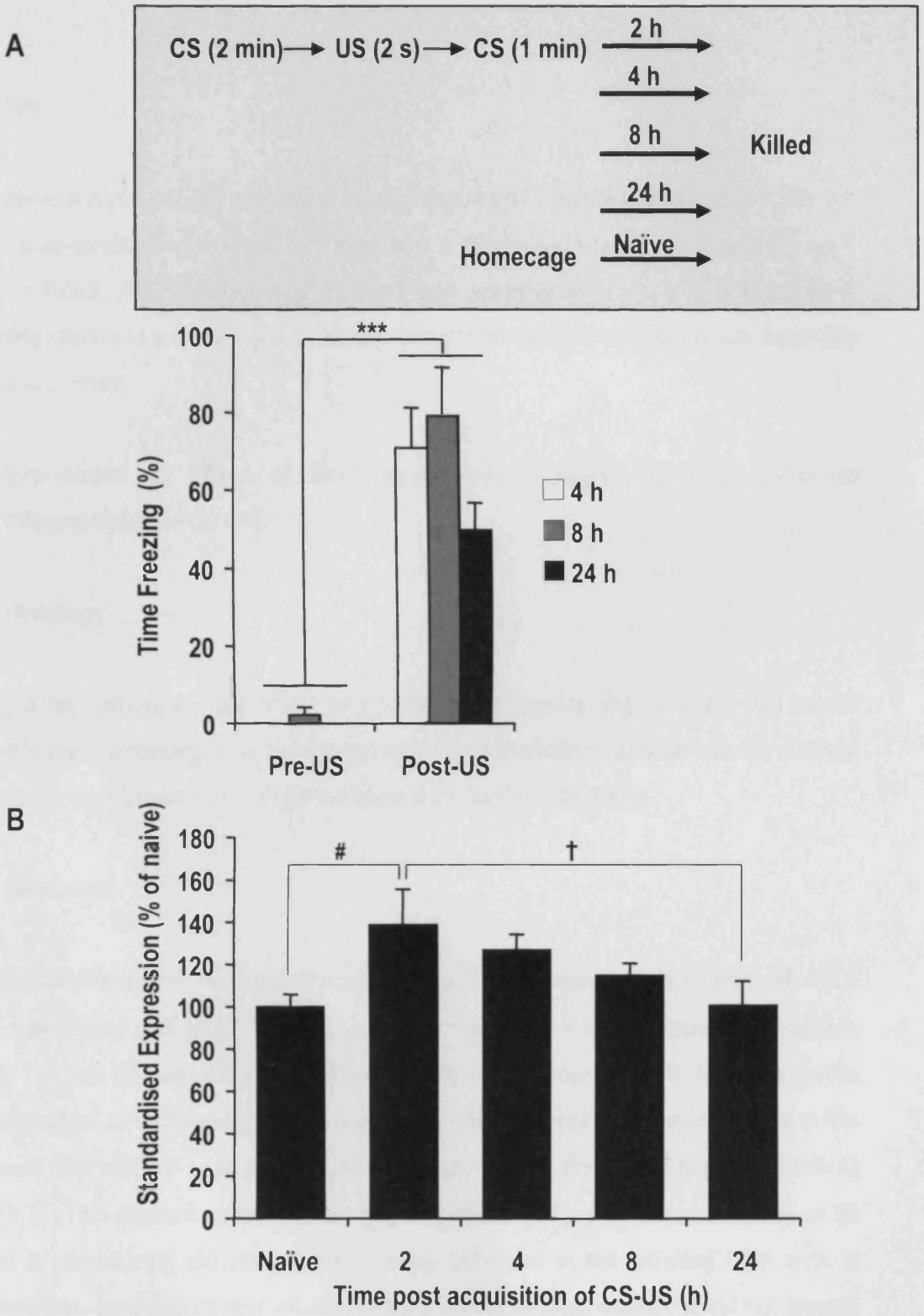


Figure 4.12 Time Profile of *Egr3* Behaviourally Induced Expression in the CA1.
(A) Schematic of behavioural protocol used and the freezing behaviour observed from experiment 4 in which all rats were conditioned. The difference between the groups is the time period after conditioning at which they were killed (4 h, 8 h & 24 h). (B) *Egr3* expression in naïve rats (n=10), 2 hours post aquisition(n=6), 4 hours, 8 hours and 24 hours post aquisition (n=4/group). *** $p<0.001$, # $p<0.01$ and † $p<0.05$. Error bars represent SEM. Naïve expression (nCi/mg tissue): 12.0±1.

4.3.4.2 ISH

Densitometric analysis of *Egr3* expression in naïve tissue and in tissue collected at 2 h, 4 h, 8 h and 24 h post-conditioning revealed that there was a difference between the groups ($F_{(4, 23)}$ = 2.817, p = 0.049, ANOVA) (Fig. 4.12 b). *Egr3* was upregulated by 39% at 2 hours post-conditioning relative to the naïve group. Behaviourally induced *Egr3* expression was maximally regulated at 2 hours.

4.3.5 Experiment 5: Effect of Intrahippocampal Infusions of *Egr3* Antisense Oligonucleotides on CFC

4.3.5.1 Histology

All but one rat had correct placement of the indwelling cannula (Fig. 4.13 a). This animal appeared to have an enlarged ventricle suggesting that the infusion had been into the ventricle as opposed to the hippocampus. It was therefore excluded from the study.

4.3.5.2 Behaviour

There was no difference in the levels of freezing behaviour between the rats infused with ASOI and the rats infused with MSO ($F_{(2.69, 24.212)}$ ε= 0.673 = 0.664, p = 0.566, Repeated Measures ANOVA). The rats infused with a different sequence complementary to *Egr3*, ASOII, at double the concentration also showed no difference in the levels of freezing behaviour relative to the rats infused with MSO ($F_{(3.568, 32.11)}$ ε= 0.892 = 1.41, p = 0.255, Repeated Measures ANOVA) (Fig. 4.13 b). Two different concentrations and sequences of *Egr3* antisense infusions at 90 min prior to conditioning did not prevent freezing behaviour in the following LTM tests in conditioned rats. Intrahippocampal infusion of *Egr3* antisense oligonucleotides did not prevent the consolidation of CFM.

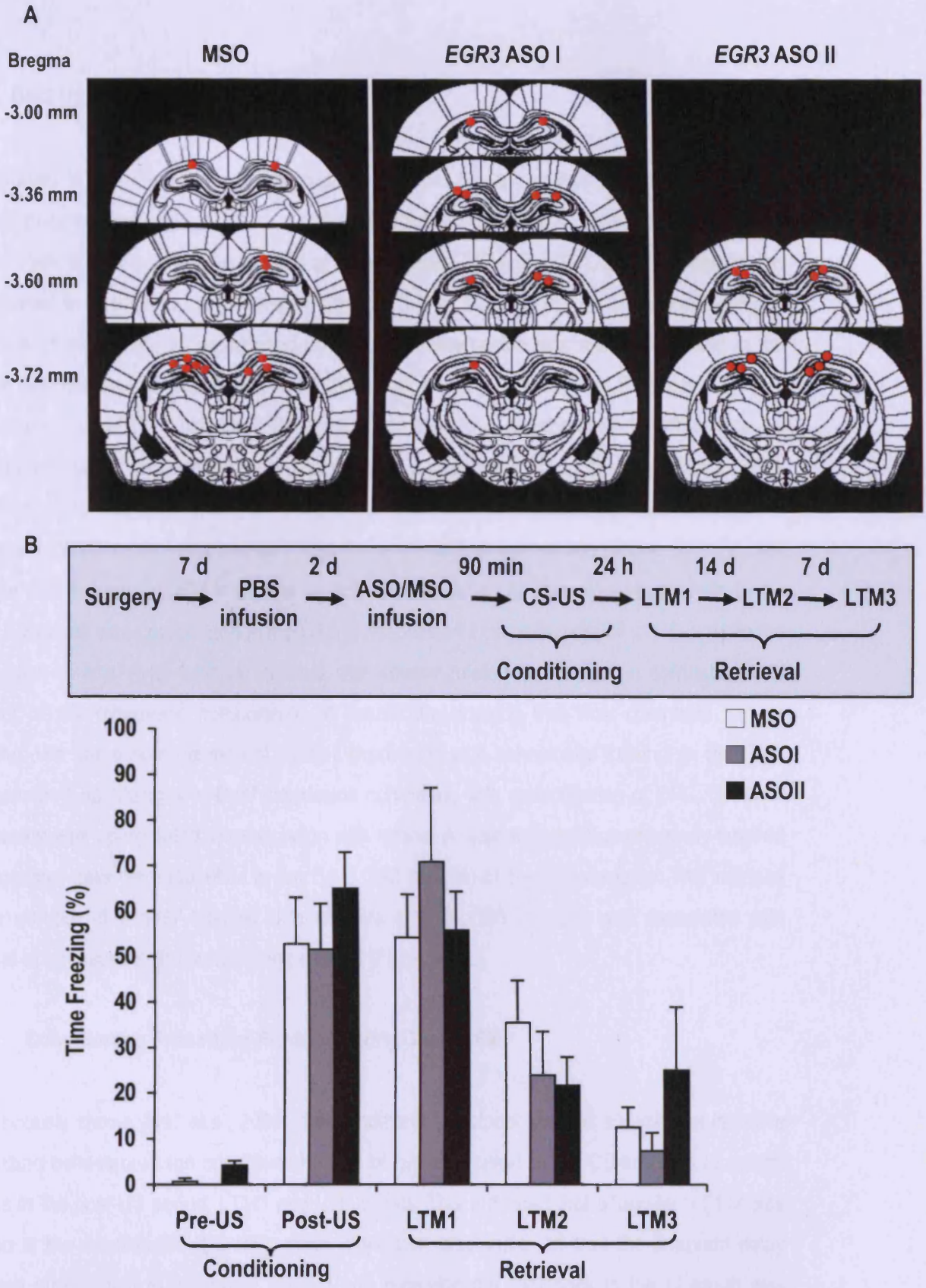


Figure 4.13. Effect of Intrahippocampal Infusions of *EGR3* Antisense on CFC. (A) Schematic indicating the site of infusion of the antisense (ASO) and missense (MSO). (B) Schematic of behavioural and infusion protocol used and the freezing behaviour of rats during CFC and in the following LTM tests for infusions of MSO (1ng/μl) (n = 6), ASOI (1ng/μl) (n = 6) and ASOII (2ng/μl) (n =6). Error bars represent SEM.

4.4 DISCUSSION

The studies in this chapter have investigated whether the expression of the schizophrenia susceptibility genes, *Nrg1*, *Dtnbp1*, *Egr3* and *Bdnf*, is regulated 2 hours after CFC. The upregulation of *Egr3* expression peaked at 2 hours after CFC in the CA1. *Egr3* expression was upregulated in association with consolidation of CFC in the CA1 region of the hippocampus and DLA of the amygdala determined by ISH. *Egr3* expression was also upregulated in the CA1 in association with arousal. The expression of *Nab2*, a repressor of EGR3 activity, was upregulated in the CA1 and DLA following latent inhibition, a procedure in which rats are conditioned but do not learn. These data suggest a role for EGR3 in the formation of CFM. However, a subsequent functional study showed that EGR3 in the hippocampus was not necessary for the consolidation of CFC. *Egr3* expression was upregulated in the Cg1, PrL and IL regions of the medial PFC in correlation with novelty detection. The expression of *Nrg1* type I splice variants was upregulated in the CA3 in association with consolidation of CFC, while the expression of *Nrg1* type II splice variants was downregulated in the CA1 in correlation with arousal; a non-mnemonic components of behavioural training that may contribute to fear learning. We found no regulation of *Dtnbp1* expression with behavioural training. In the CA1, we measured no changes in *Bdnf* expression correlating with consolidation of CFC, but *Bdnf* expression was upregulated in association with stress. A sparse population of heavily labelled *Bdnf*-positive cells were identified in the CA1, CA3 and DG of the hippocampus. The increase in the number of heavily labelled *Bdnf*-positive cells in CA1 and DG was associated with arousal or exposure to the conditioning context (Fig. 4.14).

4.4.1 Conditioning Procedure Produces Long-Term CFM

As previously shown (Hall et al, 2000), the conditioning protocol showed a significant increase in freezing behaviour in the conditioned group of rats compared to the CS-only and LI control groups in the post-US period, LTM1 and LTM2 tests. This indicated that a long-term CFM was formed in the conditioned (CS-US) group. This data also indicated that the temporal delay between introduction to the novel context and receiving the footshock in the LI group was appropriate to prevent formation of a long-term CFM.

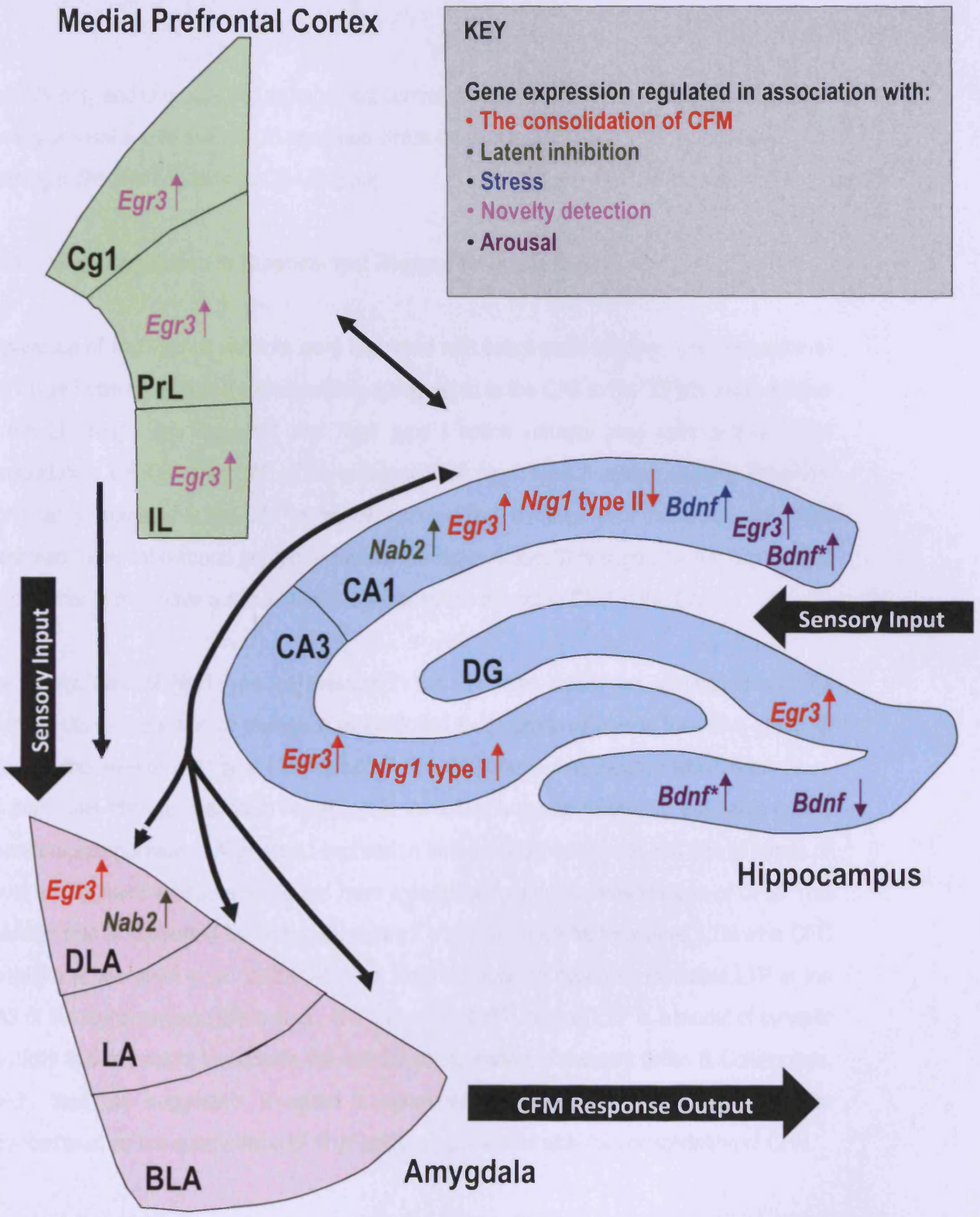


Figure 4.14. Schematic showing genes regulated by behaviour in regions of the medial prefrontal cortex, hippocampus and amygdala. Block arrows, information input and output following CFC; thin black arrows, information flow between brain regions; small arrows, up- or down-regulation of gene expression; *, heavily labelled *Bdnf*-positive cells (other *Bdnf* expression was measured at the regional level of analysis). Medial prefrontal cortex regions: cingulate cortex (Cg1), prelimbic cortex (PrL) & infralimbic cortex (IL). Hippocampal regions: cornus ammonis 1 (CA1), cornus ammonis 3 (CA3) & dentate gyrus (DG). Amygdala regions: dorsolateral nucleus (DLA), lateral nucleus (LA) & basolateral nucleus (BLA); Contextual fear memory (CFM). Not drawn to scale.

The CS–only and LI groups act as important control groups to dissociate context CS-mediated novelty/arousal and footshock US-mediated stress effects, respectively, from associative learning in the fear conditioned CS-US group.

4.4.2 *Nrg1* Regulation in Hippocampal Regions following CFC

Expression of *Nrg1* splice variants were regulated with behavioural training. The expression of *Nrg1* type I splice variants were selectively upregulated in the CA3 in the CS-US group relative to the LI group. This suggests that *Nrg1* type I splice variants may have a role in the consolidation CFM in the CA3. The expression of *Nrg1* type II splice variants were not significantly regulated in the CA1 by behavioural training, but they were downregulated in the combined three behavioural groups relative to the naïve group. This suggests that *Nrg1* type II splice variants may have a role in arousal and/or stress but not in CFM in the CA1.

The upregulation of *Nrg1* type I expression in the CA3 was clearly not associated with the aversive US as there was no change in regulation in the LI group relative to the naïve group. In addition, the level of *Nrg1* type I expression in the CA3 was not associated with CS exposure as there was also no change in regulation in the CS-only group relative to the naïve group. Therefore upregulation of *Nrg1* type I expression in the CS-US group was not due to stress or novel environment exposure and could have a potential role in the consolidation of CFM. This potential role is supported by findings that *Nrg1* $^{+/-}$ mutant mice had impaired LTM in a CFC paradigm (Ehrlichman et al., 2009). Also, as *Nrg1* is known to positively modulate LTP in the CA3 of the hippocampus (Eilam et al., 1998; Li et al., 2007), and as LTP is a model of synaptic plasticity that is thought to underlie the cellular consolidation of memory (Bliss & Collingridge, 1993), then our suggestion is again in agreement with a known role of *Nrg1* in the hippocampus, as the upregulation of *Nrg1* positively correlates with the consolidation of CFM.

The regulation of *Nrg1* type II expression in association with arousal suggests that the *Nrg1* type II splice variants in the CA1 may be involved in responding to stimuli in the environment. However the numbers of silver grains per cell representing expression of *Nrg1* type II splice variants was very low in the control naïve rats (4 grains/cell, see also 3.3.2.3). Therefore, a more accurate measure of the changes in expression of this variant with behavioural training

may require a technique that can amplify the differences between the groups such as RT-qPCR.

Although we have shown that there are regionally selective changes in the hippocampus for specific *Nrg1* splice variants 2 hours after behavioural training, future work determining the individual temporal profile of their expression in the regions of interest are required to determine whether they are regulated at any other time-point following CFC.

4.4.3 *Dtnbp1* Regulation in Hippocampal Regions following CFC

There is no difference in expression levels between any of the behavioural groups in the CA1, CA3 and DG regions of the hippocampus for any of the *Dtnbp1* probes. These results suggest that there is no regulation of *Dtnbp1* expression associated with training in the CFC protocol. This finding is in agreement with a previous study on *Dtnbp1* mutant mice that showed no difference in freezing behaviour in a LTM test following CFC (Bhardwaj et al., 2009). Together these data suggest that there is no role for *Dtnbp1* in hippocampal dependent LTM. However, two other studies in *Dtnbp1* mutant mice find deficits in LTM on a Barnes circular maze test (Takao et al., 2008) and enhanced amygdala-related fear memory in the cued-fear conditioning test (Bhardwaj et al., 2009). It is therefore likely that *Dtnbp1* is required for or contributes to memory acquisition in tasks other than contextual fear long-term memory.

4.4.4 *Egr3* Regulation in Hippocampal Regions following CFC

We showed that *Egr3* expression was upregulated in the CA1 after CFC, with levels maximal at 2 hours, declined by 8 hours, and by 24 hours *Egr3* expression had returned to basal levels. This profile is similar to *Egr3* expression following MECS stimulation that was determined in a previous study (Yamagata et al., 1994).

In the CA1, *Egr3* expression was measured using ISH and analysed at the densitometric and cellular levels, and by RT-QPCR. We showed that *Egr3* expression was upregulated in all three experimental behavioural groups compared to naïve. This suggests that *Egr3* expression was dynamically regulated by context CS-mediated arousal and footshock US mediated stress. Furthermore, there were increases in *Egr3* expression in the fear conditioned CS-US group compared to all other groups when assessed at the densitometric level. This suggests that

Egr3 expression was specifically correlated with formation of the fear memory. These changes in *Egr3* expression are similar to those observed in a previous study for *Bdnf* (Hall et al., 2000). BDNF activity in the hippocampus was subsequently shown to be required for the consolidation of CFM (Lee et al. 2004). Similarly, EGR3 may play a role in the consolidation process. The different sensitivity of each method of analysis to measure small changes in gene expression may underlie why the upregulation of *Egr3* expression was not consistently observed in the CS-US group compared to the other behavioural groups. Nevertheless, our results agree with a previous study in *Egr3* $^{-/-}$ mice that showed impaired LTM in a CFC retrieval test (Li et al., 2007).

Egr3 was also upregulated in CA3 and DG in the CS-US group relative to the naive group. In addition, *Egr3* expression was upregulated in the CS-US group relative to the CS-only group in the DG but not in the CA3. This indicates that behavioural training regulates *Egr3* expression. There were only four rats in each group and the resulting high variability (large SEM) in measuring the expression of *Egr3* may mask true differences between the groups. Future work using an increased number of rats in each group would determine whether *Egr3* expression is specifically associated with the formation of CFM.

Previous work has shown that another *Egr* gene family member, *Egr1,* is also regulated by training in this behavioural protocol but that regulation was seen only in CA1 and in all three experimental groups relative to naïve (Hall et al., 2000). This suggested that EGR1 expression was not correlated with acquisition of CFM. Indeed, infusion of antisense oligonucleotide into the hippocampus to prevent local translation of *Egr1* mRNA and reduce protein levels before CFC did not prevent the consolidation of CFM (Lee et al. 2004). Comparison of our findings with the findings of Hall and colleagues (2000) suggest that *Egr3* and *Egr1* are differentially regulated in the three sub-regions of the hippocampus by behavioural experience and further indicates they may play different roles in CFC.

The CA1, CA3 and DG all have different functions within the hippocampal network. All three regions receive inputs from the entorhinal cortex, this enables mismatch of information detection in the CA3 and CA1. Mossy fibres from the DG act as pattern separators as they make strong and sparse synapses onto the CA3 cells. DG stimulated cells in the CA3 are strengthened by autoassociative recurrent networks resulting in a subset of active cells. This process is thought to be involved in the decorrelation of similar experiences (Treves et al.,

2008). This subset of active cells in the CA3 then activate cells in the CA1 that is considered to be the main output structure of the hippocampal network (Daumas et al., 2005). The CA1, CA3 and DG have distinct but complementary functions in contextual memory consolidation (Vazdarjanova & Guzowski, 2004; Lee & Kesner, 2004). The CA3 is involved in forming fast configural representations (Daumas et al., 2005; Lee & Kesner, 2004). The CA1 is suggested to be important for temporal information processing (Daumas et al., 2005; Lee & Kesner, 2004; Leutgeb & Leutgeb, 2007). While *Egr1* does not appear to be involved in processes in the DG, *Egr3* regulation does. *Egr3* and *Egr1* regulation both appear to be involved in processes in the CA3 and CA1. These findings suggest that *Egr3* and *Egr1* have both similarities and differences in their behavioural related functions in the different sub-regions of the hippocampus.

To determine whether EGR3 in the hippocampus plays a causal role in the formation of CFM, we infused antisense oligonucleotides to prevent local translation of *Egr3* mRNA and therefore reduce EGR3 protein levels in the dorsal hippocampus prior to CFC. Rats infused with *Egr3* antisense oligonucleotides showed no difference in levels of freezing behaviour, during the conditioning period or in the three LTM retrieval tests following CFC in comparison to rats that had received an infusion of missense oligonucleotide. There was also no effect of the *Egr3* antisense oligonucleotides on the persistence of the memory because the rate of extinction in the antisense group did not differ from the missense infused group. These results were found for two different antisense oligonucleotides designed to target *Egr3* and one at double the concentration of the other. Potential reasons for a lack of behavioural effect following the administration of antisense oligodeoxynucleotides (ASO) targeting *Egr3* mRNA could include that:

• Very high levels of *Egr3* expression in the hippocampus may require a greater concentration of ASO to knock down enough *Egr3* mRNA, and thus EGR3 protein, to have an effect on behaviour .

• The tertiary structure of the ASO designed for these experiments may have hindered the complementarity of the ASO to the target *Egr3* mRNA and hence may have prevented or reduced the level of EGR3 knock down.

• The *Egr3* ASO may have only labelled some of the target *Egr3* mRNA for degradation but not enough may have been knocked down to have an effect on behavior. ASO does not normally result in 100% knocked down but instead approximately 60-80% depending on the ASO and

the gene. *Egr1* ASO infused into the same region knocked down EGR1 expression by approximately 70% (Lee et al., 2004).

• The half life of EGR3 protein is unknown and so it is possible the 8 hour presence of ASO may not be enough to prevent EGR3 being expressed and performing its functional role from six and a half hours post-acquisition onwards.

• It is possible that in the absence of EGR3 in the hippocampus that hippocampal involvement in CFC was disrupted and that activity in another brain region may have compensated for the disrupted hippocampal activity. This would be similar to the suggestion by some that in the absence of a functioning hippocampus, fear conditioning in a context can in some cases still take place but it is thought that instead of associating the context with the footshock that the rat may associate one aspect within the context with the footshock leading to an alternative method of learning the association (similar or the same as cued fear conditioning) (Maren et al., 1997; see 1.2.2.1).

• The upregulation of *Egr3* in association with CFC may only be part of the role of that gene in CFC. In addition to *de novo Egr3* expression, the EGR3 protein present in the neuron at the time of CFC may undergo post-translation modification as part of the molecular cascade leading to the modulation of the *de novo* gene expression. Thus the EGR3 protein present at the time of CFC, as opposed to the de novo *Egr3* expressed following CFC, may be necessary for the learning of that particular association memory.

EGR3 protein knockdown by the ASO designed and used in this study remains to be confirmed. This would be done by performing a Western blot or immunhistochemistry on rat brain tissue in the region of brain in which ASO was infused 4 hours post-infusion (Lee et al., 2004). In addition, a post-retrieval short-term memory test could be included in future experiments to rule out the possibility that antisense infusions targeting EGR3 mRNA may have a non-specific effect on hippocampal activity associated with the acquisition of fear memory. Our findings here suggest that EGR3 expression in the hippocampus, and perhaps the CA1 specifically, may not be required for consolidation of CFC. This is similar to previous studies that show that EGR1 is not necessary for consolidation of CFC (Lee et al., 2004).

However, it is possible that *Egr3* may have a role in the stress response that may enhance the memory consolidation process, similar to *Egr1* and *Egr2* (Vreugdenhil et al., 2001; Revest et al., 2005), Indeed we showed increased mRNA levels in CA1 in the LI rats, who received footshock in the context but did not form a CFM. In addition, we also showed that *Egr3*

expression may be regulated by CS-mediated arousal and/or novelty detection, or formation of a context representation memory. Previous behavioural experiments on *Egr3*$^{-/-}$ mice have shown impairments in a selection of tests of the response to stress and for novelty detection (Gallitano-Mendel et al., 2007) For example, *Egr3*$^{-/-}$ mice were hypersensitive to handling stress as measured by behavioural observations concomitant with an increased corticosterone (a stress hormone) response. They also displayed heightened reactivity to novelty stress determined using an open-field activity test, and reduced ability to locate food reward in a novel environment compared to in a known environment. Furthermore, they failed to habituate to the acoustic startle response, and made fewer spontaneous alternations than WT mice in a Y-maze. All these responses were suggestive of hippocampus-based memory deficits. Studies on other IEGs show a correlation between expression and exposure to a novel environment. For example, *c-Fos* and *c-Jun* are upregulated in the hippocampus two hours following a 10 min exposure to a novel environment (Papa et al., 1993).

As the hippocampus is necessary for the integration of spatial and foreground/background features of the environment acquired through exploratory behaviour (Eichenbaum, 1996), *EGR3* in this region could function in novelty detection and/or formation of a context representation memory. Context representations are thought to be stored either as feature representations or conjunctive/configural representations (Rudy & O'Reilly, 2001). Conjunctive representations are the result of separate features becoming bound into a unitary representation, and require interaction between the cortex and hippocampus (Nadel & Willner, 1980). It has been proposed that the hippocampus is essential for the normal functioning of acquisition, storage and retrieval of conjunctive associations (Rudy & Sutherland, 1989). It is therefore possible that the upregulation of *Egr3* in the behavioural groups in the CA1 of the hippocampus could be involved in the consolidation of a context memory. However, based on the *Egr3* expression time profile it is unlikely that the increased *Egr3* expression detected in the LI group is related to the acquisition of a context memory or novelty detection because these rats were killed 10 hours after exposure to the novel context. Rather, the changes in expression of *Egr3* in the LI group are specifically associated with the footshock stressor.

There was no functional assessment of *Nrg1* type I, II, III and pan *Nrg1*, *Dtnbp1* exon 1, exon 5, exon 9 and exon 10, *Nab1* or *Nab2*, as the *Egr3* mRNA expression findings were the most suggestive of the gene being associated with LTM (the level of *Egr3* expression in the test group was significantly upregulated in comparison to all three control groups. However the

gene profiling did also provide evidence that would support further investigation of the genes *Nrg1* type I in the CA3 and *Nab2* in the DLA and CA1 and this would be interesting future work. There was no functional assessment of *Nrg1* type IV or *Disc1* L, Lv or pan as mRNA levels could not be detected for these genes using the probes designed. Therefore there was no indication of regions of interest for functional study. Importantly the absence of regulated gene expression does not mean that those genes are not involved in LTM. For example, MAPK and CREB mRNA levels of expression do not vary during LTM but post-translational modification (phosphorylation) of the MAPK and CREB proteins are essential for the initiation of transcriptional response following activation of a synapse (Atkins et al., 1998; Silva et al., 1998).

4.4.5 *Egr3* Upregulated in DLA but not LA or BLA following CFC

Egr3 was upregulated in association CFC specifically in the DLA of the amygdala. In this subregion of the LA, *Egr3* expression was upregulated in the CS-US group relative to the LI and naïve groups and the difference in expression levels between the CS-US group and CS-only group was approaching significance. Future work repeating our experiments with an increased number of rats in each group would determine whether *Egr3* expression in the DLA is also correlated with exposure to a novel environment. A previous study showed that there was no change in the levels of *Egr3* expression in the DLA of the amygdala after CFC (Malkani & Rosen, 2000). However, this discrepancy may be due to the different time-points at which expression levels were measured. Knock down of EGR3 protein with targeted infusion of antisense oligonucleotides would show whether *Egr3* in the DLA plays a causal role in the consolidation of CFC. The LA of the amygdala is a region of the brain well established to be required for the consolidation of CFC (e.g. LeDoux, 2000). It receives input of sensory information related to the US footshock and contextual information relayed from the hippocampus. The LA is a region in which synaptic plasticity enables association of incoming fear conditioning-related information (Clugnet & Ledoux, 1990; Phillips & Ledoux, 1992; Romanski et al., 1993). EGR3 may play a role in the associative plasticity processes underlying the consolidation of CFM. Furthermore, our results may also suggest a role for EGR3 in fear memory in this specific region of the LA.

We showed no differences in *Egr3* expression in the LA and the BLA between the different groups following CFC. This is in agreement with another study that found no change in *Egr3*

expression up to 1 hour following CFC (Malkani & Rosen, 2000). The regulation of *Egr3* in the DLA, but not in the LA or BLA, of the amygdala is an example of *Egr3* being expressed in a sub-regionally specific manner within a structure. In a similar study, *Egr1* was upregulated in the LA of the amygdala in association with arousal (Hall et al., 2000). This suggests differences in the behaviourally related functions of *Egr3* and *Egr1* in the LA of the amygdala.

4.4.6 The control of EGR3 activity by NAB2 may play a role in the formation of CFM

Interestingly, we observed that *Nab2* expression was upregulated in the CA1 and DLA in the LI group only after behavioural training. NAB2 acts as a repressor of EGR3 activity (Svaren et al., 1998). Therefore, regulation of EGR3 activity by NAB2 may be a potential mechanism that prevents the formation of an association between the CS and US after extended exposure to the CS in the LI group that results in no conditioned freezing behaviour (See Fig. 4.14). A way of testing this hypothesis would be to knockdown NAB2 in the CA1 region of the hippocampus or DLA region of the amygdala using antisense oligonucleotides in the LI group, and then compare the levels of freezing behaviour in the LTM retrieval tests after contextual fear conditioning to those observed in a LI group that was infused with a control missense oligonucleotide. If NAB2 does inhibit formation of CS-US memory, then NAB2 knockdown in the LI group may reveal freezing behaviour at test. This would suggest that Nab2 has a role in the modulating the expression of CFM.

4.4.7 *Egr3* Regulation in Medial Prefrontal Cortex in Correlation with Novelty Detection

Egr3 expression was upregulated in Cg1, PrL and IL regions of the medial PFC in the CS-only and CS-US groups relative to the naïve group. This suggests that *Egr3* expression was regulated in medial PFC by novelty or context memory formation. Lesion studies in the rat show that the medial PFC is required for novelty detection (Dias & Honey, 2002). Furthermore, activation of these regions occurs in response to exposure to a novel environment as show by induction of the activity regulated IEG, cFos (Handa et al., 1993) and increases in dopamine release (Feenstra & Botterblom, 1996). Future work to determine whether the regulation *of* EGR3 *activity* is specifically involved in novelty detection would be to assay *Egr3* expression in the medial PFC regions following an object recognition memory task (Pawlak et al., 2008).

Comparison of *Egr3* regulation patterns in the hippocampus, amygdala and medial prefrontal cortex demonstrate that *Egr3* is expressed in a structure specific manner during CFC.

4.4.8 *Bdnf* Regulation in Hippocampal Regions following CFC

We measured the expression of *Bdnf* in the CA1 as a positive marker of gene expression associated with CFC (Hall et al., 2000). However, at the regional level of analysis measured by densitometry, we found that there was no correlation between *Bdnf* expression and CFC. More specifically, we measured an increase in *Bdnf* expression in the LI and CS-US groups relative to the naïve and CS-only groups, while Hall and colleagues (2000) measured an upregulation of *Bdnf* expression in the CS-US group relative to all control groups. The findings of Hall and colleagues suggested a role for BDNF activity in the hippocampus in the formation of CFM. This role was confirmed in a subsequent study (Lee et al. 2004). However, the disparity between our results and those of Hall and colleagues (2000) are probably due to gene expression being assayed at two different time-points, 2 hours and 30 min, respectively. This suggests that *Bdnf* expression has reached its peak and is at a reduced level of expression when measured here at 2 hours post-training. Future work determining a time profile of *Bdnf* expression regulation in the CA1 following CFC would test this suggestion.

However, LI and CS-US groups both show increased levels of *Bdnf* mRNA in CA1 2 hours post-training. Since both groups received a footshock, it is possible that the stress associated with the aversive US regulated *Bdnf* expression. If the stress–induced regulation of *Bdnf* expression was large, then sufficient levels may be measured 2 hours later, even if levels of *Bdnf* mRNA were declining back to baseline. Other studies have shown that *Bdnf* expression is regulated in response to stress. *Bdnf* expression was increased 2.2-fold in the hippocampus 3 hours following five electroconvulsive shock sessions (Sartorius et al., 2009) and upregulated in the CA1 immediately following 15 min immobilization stress (Marmigere et al., 2003). In contrast, *Bdnf* mRNA levels were decreased in the CA1 region after corticosterone injections (Hansson et al., 2000). This disparity could be because changes in corticosterone levels are only a component of the conditions experienced following a footshock. There is a precedent for plasticity-regulated genes to be regulated in the CA1 in association with stress. Both Egr1 and *Egr2* are upregulated in response to stress or corticosterone administration in CA1 (Vreugdenhil et al., 2001; Revest et al., 2005). A dynamic relationship between corticosterone, learning and hippocampal BDNF has been suggested (Scaccianoce et al., 2003), and this

complex relationship may account for *Bdnf* expression correlating with learning and stress at different time-points, and *Bdnf* being regulated in different directions depending on the cause of the stress response.

When *Bdnf* expression was measured at the cellular level by measuring the density of silver grains per cell, we identified heavily labelled *Bdnf*-positive cells in the CA1, CA3 and DG of the hippocampus. In the CA1 there was an increase in the number of heavily labelled *Bdnf*-positive cells in all behavioural groups suggestive of a role in arousal. Heavily labelled *Bdnf*-positive cells have been identified in the CA1 in a previous study that investigated BDNF protein expression after CFC (Chen et al., 2007). Multiple time-points post-acquisition (2.5 h, 4 h, 6 h, 24h) were studied, and an increase in the number of heavily labelled BDNF-positive cells in the CS-US group relative to the control groups was found that was most apparent at 4 hours (Chen et al., 2007). Our findings conflict with the findings of Chen and colleagues (2007) in two ways. Firstly, we did not see an increase in the number of heavily labelled *Bdnf*-positive cells selectively associated with CFC. Secondly, we measured an increase in the number of heavily labelled *Bdnf*-positive cells in all behavioural groups relative to the naïve group. These differences could reflect that Chen and colleagues (2007) measured protein levels after training while we assessed *Bdnf* expression at the mRNA level. The increased subpopulation of heavily labelled *Bdnf*-positive cells in the behavioural groups could represent a network of cells activated by exposure to the context. The CA1 has been shown to be involved in forming representations of context (context memory) (Frankland et al., 1998; Komorowski et al., 2009). David Marr (Marr, 1971) first proposed that a subpopulation of cells in the hippocampus were activated to be part of a network supporting a single memory, or engram. Experimental support of this idea was shown when the expression of *Arc*, a gene regulated by BDNF, was detected in a subpopulation of activated cells in the CA1 following exposure to a novel context (Vazdarjanova & Guzowski, 2004). This subpopulation of cells was shown to be associated with exposure to a unique environment because when the rats were exposed to a different novel context a different cohort of cells within the CA1 were activated. Crucially when these same animals were re-exposed to the initial environment only the original subpopulation of cells were activated.

There was no difference between the densities of silver grains in the two populations of *Bdnf*-positive cells in the experimental groups in the CA1. Therefore, an increase in the number of

heavily labelled *Bdnf*-positive cells is likely to underlie the increase in *Bdnf*-expression in CA1 measured at the regional level by densitometry.

In the CA3, *Bdnf expression* was downregulated in the CS-only group. This regulation is unlikely to be due to novel context exposure because there was no downregulation in the CS-US group also exposed to a novel environment. In addition, Hall and colleagues (2000) found no change in *Bdnf* expression levels between any of the groups in the CA3 at 30 minutes post-training (Hall et al., 2000). Therefore this is likely to be a false positive Type II error.

In the DG, measured densiometrically *Bdnf* expression was downregulated in all behavioural groups relative to the naïve group. But assessing *Bdnf* expression at the cellular level revealed that the number of heavily labelled *Bdnf*-positive cells increased in all behavioural groups relative to the naïve group, suggesting that *Bdnf* expression is differentially regulated by arousal in two populations of DG cells. Candidate populations include the granule cells, neurogenic cells, basket cell interneurons or glial cells, and changes in transcriptional response to neuronal activity have been measured in them all (Treves et al., 2008; Parpura & Zorec, 2009; Ohba et al., 2005). The heavily labelled *Bdnf*-positive cells are unlikely to be neurogenic cells, because neurogenic cells are located in the sub-granular zone of the DG (Kuhn et al., 1996) while we observed that the heavily labelled *Bdnf*-positive cells were sparsely distributed all over the DG. The heavily labelled *Bdnf*-positive cells could be granule cells activated in response to context exposure which may contribute to the context memory engram, as discussed above. In order to investigate whether the heavily labelled *Bdnf*-positive cells were interneurons or glial cells the behavioural experiment could be repeated and double labelling ISH could be performed with the probe for *Bdnf* combined with either a probe for *Gad 65* to identify any co-localization with GABA containing interneurons (Benson et al., 1994), or probe for *Gfap* (a marker of astrocytes) or *Cd11b* (a marker for microglia) to identify any co-localization with glial cells (Pixley et al., 1984; Akiyama & McGeer, 1990).

There is a possibility that *Bdnf* expression regulation in the LI and CS-US groups could be associated with stress. However different studies have found *Bdnf* expression to be downregulated (Rasmusson et al., 2002; Gronli et al., 2006; Bland et al., 2007), unchanged (Allaman et al., 2008; Hall et al., 2000) or upregulated (Marmigere et al., 2003) in the DG in association with stress. This range in findings is likely due to different stressors being used and *Bdnf* expression being assayed at different time-points after the stress was experienced. In

addition, different transcripts of *Bdnf* can be regulated in different directions in the DG in response to the same type of stress (Nair et al., 2007). However as *Bdnf* was also regulated in the CS-only group, that did not receive any aversive US stress, the regulation in this experiment is unlikely to be due to stress alone.

4.4.9 Conclusion

We suggested that schizophrenia susceptibility genes may have a role in the consolidation of CFM and measured the regulation of these genes after contextual fear conditioning. Here we show differential expression of schizophrenia susceptibility genes with behavioural training (Fig. 4.14). Egr3 expression regulation in the CA1 and DLA and *Nrg1* type I expression regulation in the CA1 only, were associated with CFM. Regulation of *Nab2* expression in the CA1 and DLA correlated with latent inhibition, while regulation of *Nrg1 type II*, *Egr3* and *Bdnf* expression in the CA1, *Egr3* in the medial PFC, and *Bdnf* in the DG was associated with arousal, stress and/or context representation memory. Therefore mutated variants of these genes may contribute to the cognitive impairments of schizophrenia through disruption to any of these behavioural responses. Further work using designed behavioural procedures are required to fully investigate these potential roles. Notably, we showed in this Chapter that while increases in *Egr3* expression in the CA1 was correlated with CFM, we showed that EGR3 activity in the hippocampus may not be necessary for consolidation of CFC. This is analogous to the role that EGR1 plays in the in consolidation of CFM (Hall et al. 2000, Lee et al. 2004). However, EGR1 was necessary for the reconsolidation (restabilisation after labilisation) of CFM after recall (Lee et al. 2004). In the next Chapter, we investigated whether EGR3 plays a functional role in the reconsolidation of hippocampal-dependent fear memory.

CHAPTER 5

REGULATION OF *EGR3*, *EGR1* AND *NAB2* IN RECONSOLIDATION AND EXTINCTION OF CONTEXTUAL FEAR CONDITIONING

5.1 INTRODUCTION

In the previous chapter *Egr3* expression was shown to be upregulated in association with consolidation of contextual fear conditioning (CFC) in the CA1 region of the hippocampus and in the DLA nucleus of the amygdala. However EGR3 was not found to be necessary for consolidation of CFC in the hippocampus. *Nab2* expression was upregulated in association with latent inhibition in the same two regions. A mechanism was proposed suggesting that NAB2 may act as a modulator of EGR3 activity in the consolidation of contextual fear memory (CFM) such that consolidation of CFM only proceeds upon presentation of a US when the CS is novel and this involves *Nab2* not being upregulated. In this chapter *Egr3* and *Nab2* expression and additionally *Egr1* expression is investigated to identify if any of these genes are regulated in correlation with reconsolidation or extinction of CFC. EGR1 is from the EGR family, and like EGR3, has a NAB2 binding site (O'Donovan et al., 1999; Svaren et al., 1996). EGR1 has already been shown to be necessary for reconsolidation of CFM (Lee et al., 2004).

5.1.1 Reconsolidation and Extinction

Reconsolidation and extinction are processes that can occur in response to retrieval of a memory. Retrieval of a previously established memory is thought to render the memory labile and susceptible to change (Misanin et al., 1968). Reconsolidation is the process by which labile memories are stabilized after retrieval (Nader, 2003). Extinction is the loss of a learned performance that occurs when a Pavlovian signal (CS) is repeatedly presented without its reinforcer (US). Extinction does not reflect destruction of the original learning, but is currently thought to involve formation of a CS-no US memory (Bouton, 2002). Different conditions of

recall favour reconsolidation or extinction (Debiec et al., 2002; Nader, 2003; Eisenberg et al., 2003; Pedreira & Maldonado, 2003; Suzuki et al., 2004; Lee et al., 2006). In rodents having undergone CFC this is observed by the presence or absence of freezing behaviour in a retention test after different length recall exposures (Suzuki et al., 2004; Barnes and Thomas, 2008). The neural circuitry of reconsolidation may include the hippocampus, entorhinal cortex, amygdala, medial prefrontal cortex (mPFC) and nucleus accumbens (Tronson and Taylor, 2007; see 1.2.3.2). The neural circuitry of extinction is known to include the hippocampus, mPFC and amygdala (Quirk and Mueller, 2008; see 1.2.4.2).

5.1.2 *Egr1*, *Egr3* and *Nab2*

Egr3 has the same nine-nucleotide DNA binding domain as the transcription factor *Egr1* (*zif268*/*Ngfi-a*/Krox-*24*). This could lead to both genes promoting transcription of the same set of target genes in response to activity. However, differences in the rest of their protein structure could partially or totally segregate the type or temporal profile of the target genes expressed (Poirier et al., 2008). Both EGR3 and EGR1 contain an R1 repression domain that binds NGFI-A-binding proteins 1 and 2 (NAB1 and NAB2). NAB1 and NAB2 most commonly act as co-repressors of EGR transcriptional activity (Russo et al., 1995; Svaren et al., 1996), but NAB2 enhancement of EGR activity has been identified in Schwann cells, therefore the function of NABs has been suggested to depend on cell type (Desmazieres et al., 2008). *Nab2*, in addition to being constitutively expressed like *Nab1*, can be expressed in an activity-dependent manner (Svaren et al., 1996; Jouvert et al., 2002).

Egr3 and *Egr1* have similar developmental and anatomical patterns of expression, similar anatomical activity-dependent expression patterns, and similar, but not identical, activity-dependent expression time profiles in response to maximal electroconvulsive seizure (MECS) in the hippocampus (Yamagata et al., 1994; Beckmann & Wilce, 1997). EGR1 activity in the hippocampus is necessary for reconsolidation (Lee et al., 2004). Upregulation of *Egr1* in regions of the hippocampus, amygdala, mPFC and nucleus accumbens correlating with retrieval of hippocampal-dependent LTM could be indicative of roles for *Egr1* in other regions of the brain in reconsolidation or extinction (Hall et al., 2001; Thomas et al., 2002; Malkani and Rosen, 2000; Herry and Mons, 2004). No studies have looked at *Nab2* expression in LTM yet. The similarities and differences between *Egr3* and *Egr1*, and the possible interactions with *Nab2*, make *Egr1* and *Nab2* interesting candidates to investigate for regulation in

reconsolidation and extinction in different brain regions. *Egr1* has been shown to be upregulated after retrieval (Hall et al., 2001) and EGR1 has been shown to be necessary for reconsolidation of CFM (Lee et al., 2004), therefore if *Egr1* is upregulated following short recall session exposure in this study then this will act as a positive control for the ISH results.

5.1.3 Outline of Experiments

Experiment 1 was performed to establish whether a short recall period of 2 min, 2 days after CFC, resulted in reconsolidation of the CFM. Furthermore, to establish whether a long recall period of 10 min, 2 days after CFC, will result in extinction of the CFM. Experiment 2 investigated the levels of expression of *Egr3*, *Egr1* and *Nab2* in brain regions that contribute to the neuronal circuitry supporting reconsolidation and extinction of CFM. This is assayed by performing ISH on brain tissue obtained 30 minutes after exposure to a short or long recall of CFM test that lead either to reconsolidation or extinction of that memory. The regions investigated include the CA1, CA3 and DG of the hippocampus, the LA and BLA nuclei of the amygdala, and the Cg1 and PrL/IL regions of the mPFC. Experiment 3 investigated the effect of intrahippocampal infusions of *Egr3* antisense on reconsolidation of CFC, to determine if EGR3 is necessary for reconsolidation of CFM.

5.2 METHODS

5.2.1 Subjects

Forty-five male Listar hooded rats (280-300g; Charles River, UK) were housed in pairs and kept in a holding room at 21°C under reverse light-dark conditions (lights off at 10 am). Animals were allowed *ad libitum* access to food and water. All rats were handled for 5 min on three consecutive days before conditioning.

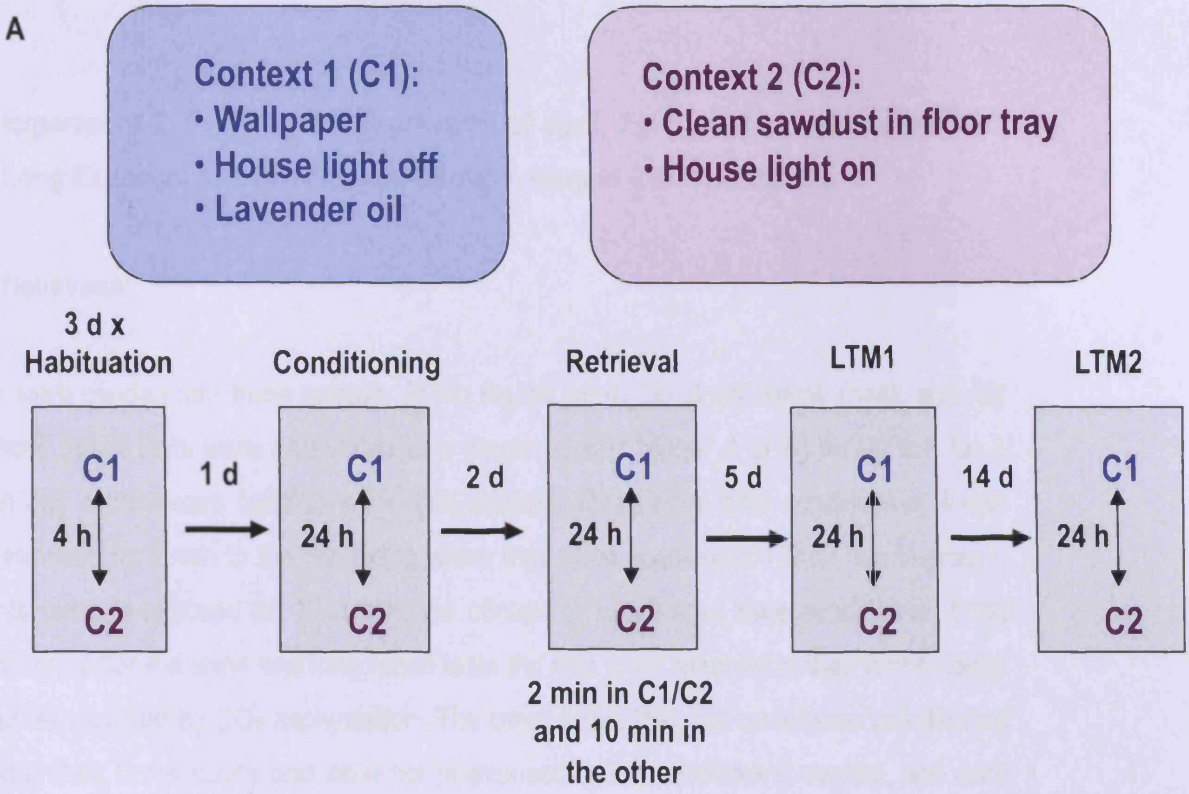
5.2.2 Experiment 1: Investigation of the Effect of Different Length Recall Tests following CFC on CFM

5.2.2.1 Behavioural Apparatus

As described in 2.2.2, two contextual fear-conditioning chambers were used, but they were designed to differ in a number of features including size, spatial location, odor and lighting. Exposure to each context was separated by a minimum of 4 hours. Context A had wallpaper, no house light on and lavender oil, while context B had clean sawdust in the floor tray and the house light on. The experiments were performed during the lights off period for the rats.

5.2.2.2 Behavioural Paradigm

Twelve rats were habituated to the contexts A and B for 20 min for 3 days. On day 4 half the rats were conditioned to context A and the other half to context B (Fig. 5.1). On day 5 rats were conditioned in the context that they had not already been conditioned in. Conditioning consisted of receiving a footshock (2 s, 0.5mA shock; the unconditioned stimulus [US]) 2 min after being placed in the conditioning chamber (the conditioned stimulus [CS]). After a further 1 min in the chamber the rats were returned to their home cages. Two days after conditioning rats were re-exposed to one of the contexts that they had been conditioned to, half for 2 min and the other half for 10 min. Three days after conditioning rats were re-exposed to the other context that they had been conditioned to, again half for 2 min and the other half for 10 min. The order of the contexts that the rats were exposed to during the recall tests was the same as during conditioning. The first long-term memory test (LTM1) was performed 4 days later and a second test (LTM2) performed 14 days later. The LTM tests consisted of placing the rats in one of the conditioned contexts for 2 min followed the next day by 2 min in the other conditioned context. The order to which each rat was exposed to the two contexts in the LTM tests was the same as in the conditioning training (Fig. 5.1 a).



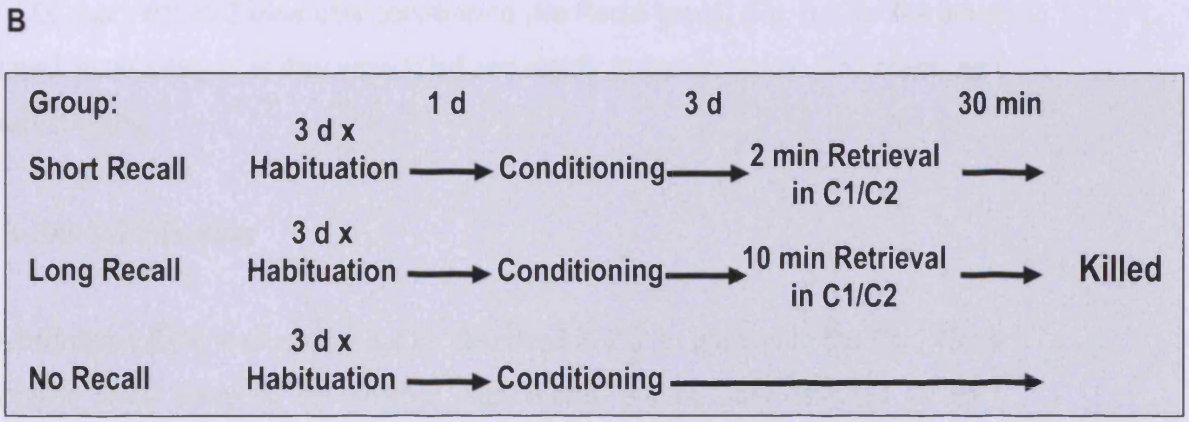


Figure 5.1. Schematic of behavioural paradigm used to investigate the effects on LTM of 2 min and 10 min retrieval exposures to a context already associated with CFM. (A) Schematic of behavioral paradigm used to investigate whether 2 min short recall of a CFM lead to reconsolidation of the CFM, and whether a 10 min long recall of a CFM leads to extinction of a CFM. Each rat formed two CFM, one associated with each context (C1 and C2). Each rat had 2 min exposure to one of the contexts and a 10 min exposure to the other context 2 days after being conditioned. Long-term memory (LTM) tests were performed to investigate if the different time length of retrieval periods effected LTM of that specific memory. (B) Schematic of the behavioural paradigm used to investigate gene expression following Short Recall and Long Recall of a CFM. d, day; h, hours; LTM, long-term memory.

5.2.3 Experiment 2: Profiling the Expression of *Egr3*, *Egr1* and *Nab2* after Short and Long Exposure to a Conditioned Context using *In Situ* Hybridisation

5.2.3.1 Behaviour

The rats were divided into three groups: (i) No Recall (n=4), (ii) Short Recall (n=4), and (iii) Long Recall (n=4). Rats were habituated to a single context (either A or B) for 20 min for 3 days. On day 4 they were conditioned in that context. Three days after conditioning, 4 rats were re-exposed for 2 min to the context to which they were conditioned (Short Recall group), and 4 rats were re-exposed for 10 min to the context to which they were conditioned (Long Recall group). After the short and long recall tests the rats were returned to their home cages and killed 30 min later by CO_2 asphyxiation. The other 4 rats that had undergone conditioning remained in their home cages and were not re-exposed to the conditioning context, and were killed by CO_2 asphyxiation 3 days after conditioning (No Recall group) (Fig. 5.1 b). The brains were excised immediately after they were killed and rapidly frozen on dry ice. The brains were then stored at - 80°C.

5.2.3.2 *In Situ* hybridisation

In situ hybridisation (ISH) was carried out as described in 2.5. In addition to the *Egr3* 45mer oligonucleotide probe designed for previous experiments, probes were designed for *Egr1*, complementary to nucleotides 460-505 of the *Egr1* gene (Milbrandt, 1987), and *Nab2*, complementary to nucleotides 891-935 from transcript XM_235224 (NCBI). All oligonucleotides were 3' end-labelled with [α-^{35}S] dATP using terminal deoxynucleotidyl transferase. The specific activity of the labelled probes are given in Table 5.1. All labelled probes were used to assay mRNA expression. ISH was carried out on coronal 14 μm sections of the mPFC (3 labelled sections and 2 non-specific labelled sections), hippocampus (4 labelled sections and 2 non-specific labelled sections) and amygdala (3 labelled sections and 2 non-specific labelled sections). Hybridised sections were opposed to autoradiographic film for 3 to 7 days to check that the probes detected labelling specific to gene expression (Table 5.1). Hybridised sections were then dipped in K5 photographic nuclear emulsion as described in 2.5.5. They were

Table 5.1. Specific activity of S^{35}-labelled oligonucleotide probes. These probes were used to detect expression of genes of interest in adult rat brain sections 30 minutes post-training in the Recall and No Recall groups and in the naïve group of rats. The number of days that the labelled tissue sections were exposed to photographic emulsion are given.

Gene	Specific Activity (dpm/µl)	Days on emulsion
Egr1	152 900	21
Egr3	138 100	49
Nab2	207 400	49

exposed for 21 to 49 days (Table 5.1) at 4°C before being developed and then counterstained with 0.1% thionin.

5.2.3.3 Silver Grain Image Collection and Analysis

Images of emulsion-dipped sections were obtained on a light microscope, through a 100x magnification lens under oil immersion, with a digital camera. The focus was on the silver grains (SG) with thionin stained cells detectable in the background. Photomicrograph images were collected from the Cg1 and PrL/IL regions in all medial PFC sections, from the CA1, CA3 and DG regions in all hippocampal sections, and from the DLA, LA and BLA nuclei in all amygdala sections for *Egr3*, *Egr1* and *Nab2*. The number of SG per cell were counted using ImageJ over sufficient randomly selected counterstained neurons, from each region, bilaterally, for each subject, such that the SE of the counts for any region was less than 10% of the population mean. Approximately 24 total labelled cells (from the hybridised sections) and 18 non-specific labelled cells (from the control sections hybridised with labelled probe and 100x excess unlabelled probe) were measured to meet this criteria. The specific SG count was then calculated for each region by subtracting the non-specific counts from the total counts for each subject. Gene expression levels in the Short Recall and Long Recall groups were standardised as a percentage of the mean number of SG in the control No Recall group for each region.

Heavily labelled *Egr3*- and *Egr1*-positive cells in the DG region of the hippocampus were subjectively identified through a dark-field microscope as sparsely distributed clusters of bright points. These clusters of bright points were confirmed to represent high levels of *Egr3* and *Egr1* expression in cells by switching to light-field conditions without moving the slide. The number of heavily labelled *Egr3*- and *Egr1*-positive cells was counted under dark-field conditions using the light microscope at 10x magnification. This analysis was determined from the same *Egr3*- and *Egr1*-labelled sections as used for the silver grain counting analysis.

5.2.4 Experiment 3: Effect of Intrahippocampal Infusions of *Egr3* Antisense on Short Exposure to a Conditioned Context

The *Egr3* antisense oligodeoxynucleotides (ODNs), ASOII, and the missense ODNs (MSO) used in chapter 4 were used to investigate whether EGR3 is required for reconsolidation of CFM. This was done through hippocampal infusions of the *Egr3* antisense prior to a CFM short

retrieval test and analysing freezing behaviour in following LTM tests. The sequences for the ODNs are given in Table 2.1. All rats underwent surgery and were divided into two groups:- (i) *Egr3* ASOII group (n=9), and (ii) MSO group (n=12). The surgery involved putting in place bilateral indwelling hippocampal cannula at AP -3.50, relative to bregma, as described in 2.3.2. A week after surgery the rats received a habituation infusion of 2 μl of PBS at 0.125 μl/min and a day later they underwent CFC. Three days later they received an infusion of 2 μl of 2 nmol/μl ASOII or 2 μl of 1 nmol/μl MSO in PBS at 0.125μl/min, as described in 2.3.3. The rats were placed back in the conditioning chamber for 2 min, 90 min after the infusion, for a Post-Retrieval (PR) test. The rats were placed in the conditioning chamber 3 hours later for a post-retrieval short-term memory (PR-STM) test and 24 hours later for a LTM1 test. Both these tests consisted of a 2 min re-exposure to the CS only. Similar LTM2 and LTM3 retrieval tests were performed 14 days and 18 days following the LTM1 test respectively. The average level of freezing behaviour in the ASOII group and the MSO group for the pre-US period, post-US period, PR test, PR-STM test, LTM1 test, LTM2 test and LTM3 test was calculated.

After behavioural analysis, rats were killed by CO_2 asphyxiation. The brains were excised and rapidly frozen on dry ice and stored at - 80°C. Histological assessment of the cannulae placement was performed by using a cryostat to cut and collect 14μm sections from the dorsal hippocampus that the cannulae targeted, and using thionin staining and a light microscope to identify the cannulae endpoints as described in 2.4.

5.2.5 Behavioural Analysis

The number of 10 s intervals that had freezing behaviour present was divided by the total number of 10 s intervals observed. This was then expressed as the percentage of time spent freezing for each test.

5.2.6 Statistical Analysis

Repeated measures Analysis of Variance (ANOVA) was used to identify significant differences in the freezing behaviour between the different behavioural groups prior to and following CFC for Experiments 1, 2 and 3. For analysis of the ISH results in Experiment 2 one-way ANOVA and the Fishers Least Significant Difference (FLSD) statistical tests were used to measure

differences between the levels of gene expression in the different behavioural groups. *A priori* planned comparisons comparing the level of gene expression in the Recall groups relative to the No Recall group were performed using the FLSD test.

5.3 RESULTS

5.3.1 Experiment 1: Investigation of the Effect of Different Length Recall Tests following CFC on CFM

The effect of fear conditioning and short and long recall tests in two contexts is shown in Figure 5.2 a. There were significant effects of training and test phases on freezing behaviour ($F_{(4.225, 46.475)}$ $\varepsilon = 0.469$ = 11.518, p = 0.000, repeated measures ANOVA). Rats showed significant levels of freezing behaviour in the post-US session compared to the pre-US session in both the C1 and the C2 conditioning trials. Freezing behaviour in both the Short Recall and the first 2 min of the Long Recall groups was no different from that in the post-US sessions showing that rats in these groups had formed a CS-US memory. There was no significant difference between the freezing behaviour in the short recall session copmpared to the LTM1 and LTM2 retention tests in the Short Recall group. In contrast, there was a significant decrease in the freezing behaviour in the first 2 min of the long recall session compared to the LTM1 and LTM2 retention tests in the Long Recall group. The freezing behaviour in the final 2 min of the long recall session was half that observed in the first two min indicating that there was within-session extinction present in the Long Recall group ($F_{(2.681, 29.487)}$ $\varepsilon = 0.670$ = 8.257, p = 0.001, repeated measures ANOVA) (Fig. 5.2 c).

5.3.2 Experiment 2: Profiling the Expression of *Egr3*, *Egr1* and *Nab2* after Short and Long Exposure to a Conditioned Context using *In Situ* Hybridisation

5.3.2.1 Behaviour

The behaviour that the rats underwent prior to ISH showed an effect of fear conditioning (Fig. 5.2 b). There were significant effects of training and test phases on freezing behaviour ($F_{(1.353, 9.471)}$ $\varepsilon = 0.677$ = 16.711, p = 0.001, repeated measures ANOVA). Rats showed significant levels of

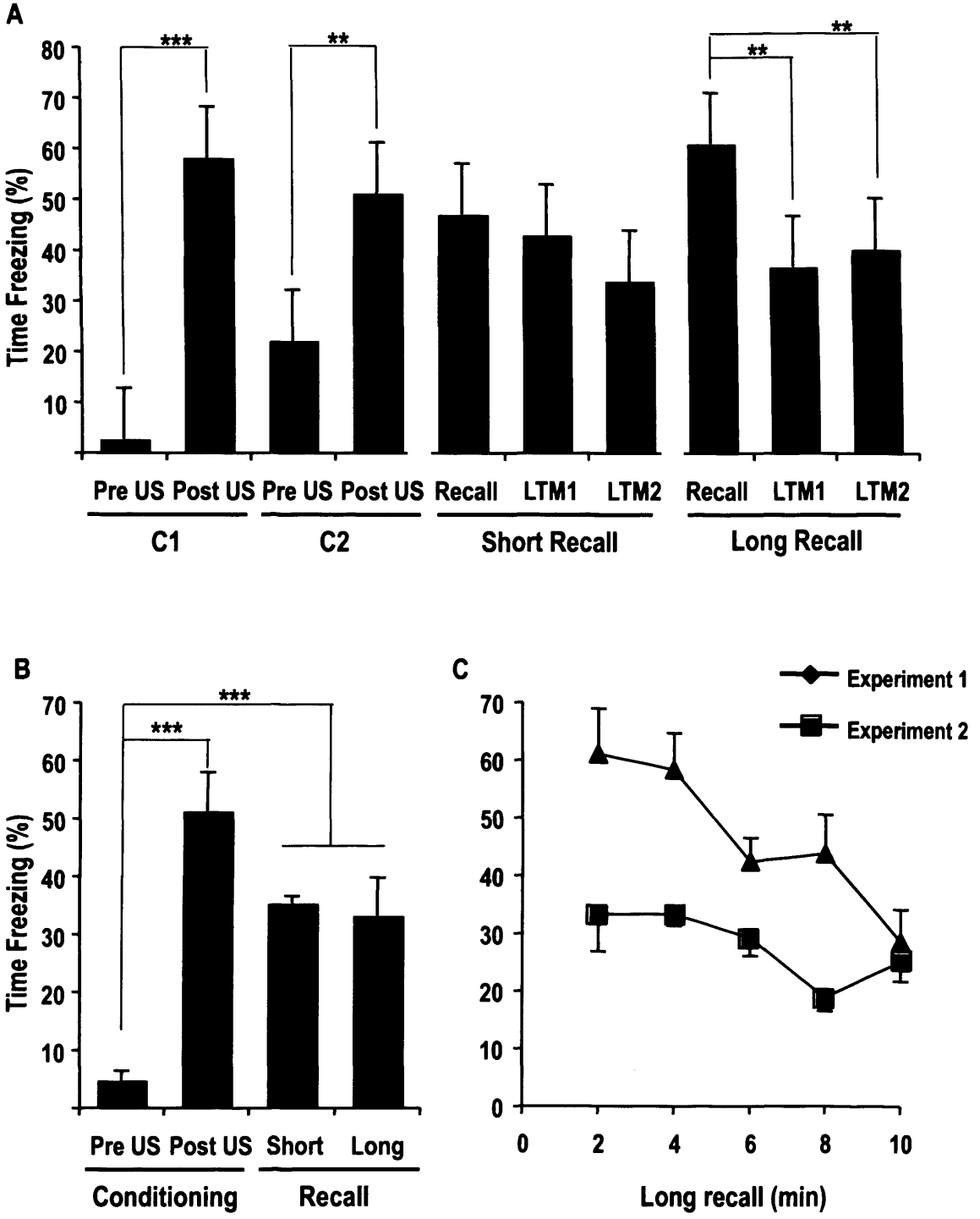


Figure 5.2. Short (2 min) and long (10 min) exposure to a conditioned context. (A) Freezing behaviour following short and long exposure to a conditioned context showing no change in freezing behaviour in LTM tests following Short Recall but significant reduction in freezing behaviour in LTM tests following Long Recall. (B) Freezing behaviour of rats during conditioning, Short Recall and the first 2 min of Long Recall, for rats that were killed for Experiment 2. (C) Freezing behaviour in 2 min intervals throughout Long Recall sessions from both Experiment 1 (triangle) and Experiment 2 (square). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

freezing behaviour in the post-US session compared to the pre-US session in the conditioning trial. Freezing behaviour in both the Short Recall group and the first 2 min of the Long Recall group was significantly increased compared to pre-US session in the conditioning showing that rats in these groups had formed a CS-US memory. There was no significant difference in the levels of freezing behaviour between the Short Recall group and the first 2 min of the Long Recall group. The freezing behaviour in the final 2 min was not significantly reduced compared that observed in the first two min of the extinction session for the Long Recall group indicating that there was no significant within-session extinction present in the Long Recall group (Fig. 5.2 c).

5.3.2.2 Hippocampal Expression of *Egr1*, *Egr3* and *Nab2* after Short and Long Exposure to a Conditioned Context

The levels of *Egr1*, *Egr3* and *Nab2* expression measured in the hippocampal CA1, CA3 and DG regions in the Short Recall, Long Recall and No Recall groups are presented in Figure 5.3. While there was no effect of group on *Egr1* expression in the CA1 region of the hippocampus ($F_{(2,9)} = 3.928$, $p = 0.059$, ANOVA), planned comparison between both of the Recall groups and the No Recall group showed that *Egr1* expression was upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group (Fig. 5.3 a). *Egr1* expression was also upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group in the CA3 region of the hippocampus ($F_{(2, 9)} = 8.470$, $p = 0.009$, ANOVA) (Fig. 5.3 b). There was no difference in expression levels of *Egr1* between any of the groups in the DG region of the hippocampus (Fig. 5.3 c). *Egr3* was upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group in the CA1 ($F_{(2, 9)} = 9.634$, $p = 0.006$, ANOVA) and CA3 ($F_{(2, 9)} = 15.280$, $p = 0.001$, ANOVA) regions of the hippocampus. There was no change in levels of expression in the DG region of the hippocampus (Fig. 5.3 d - f). *Nab2* was upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group in the CA1 ($F_{(2, 9)} = 25.535$, $p = 0.000$, ANOVA), CA3 ($F_{(2, 9)} = 13.105$, $p = 0.002$, ANOVA) and DG ($F_{(2, 9)} = 19.660$, $p = 0.001$, ANOVA) regions of the hippocampus (Fig. 5.3 g - i). Thus, *Egr1*, *Egr3* and *Nab2* are upregulated by both short and long exposure to a conditioned context in the CA1 and CA3, and *Nab2* was also upregulated in the DG under both recall conditions.

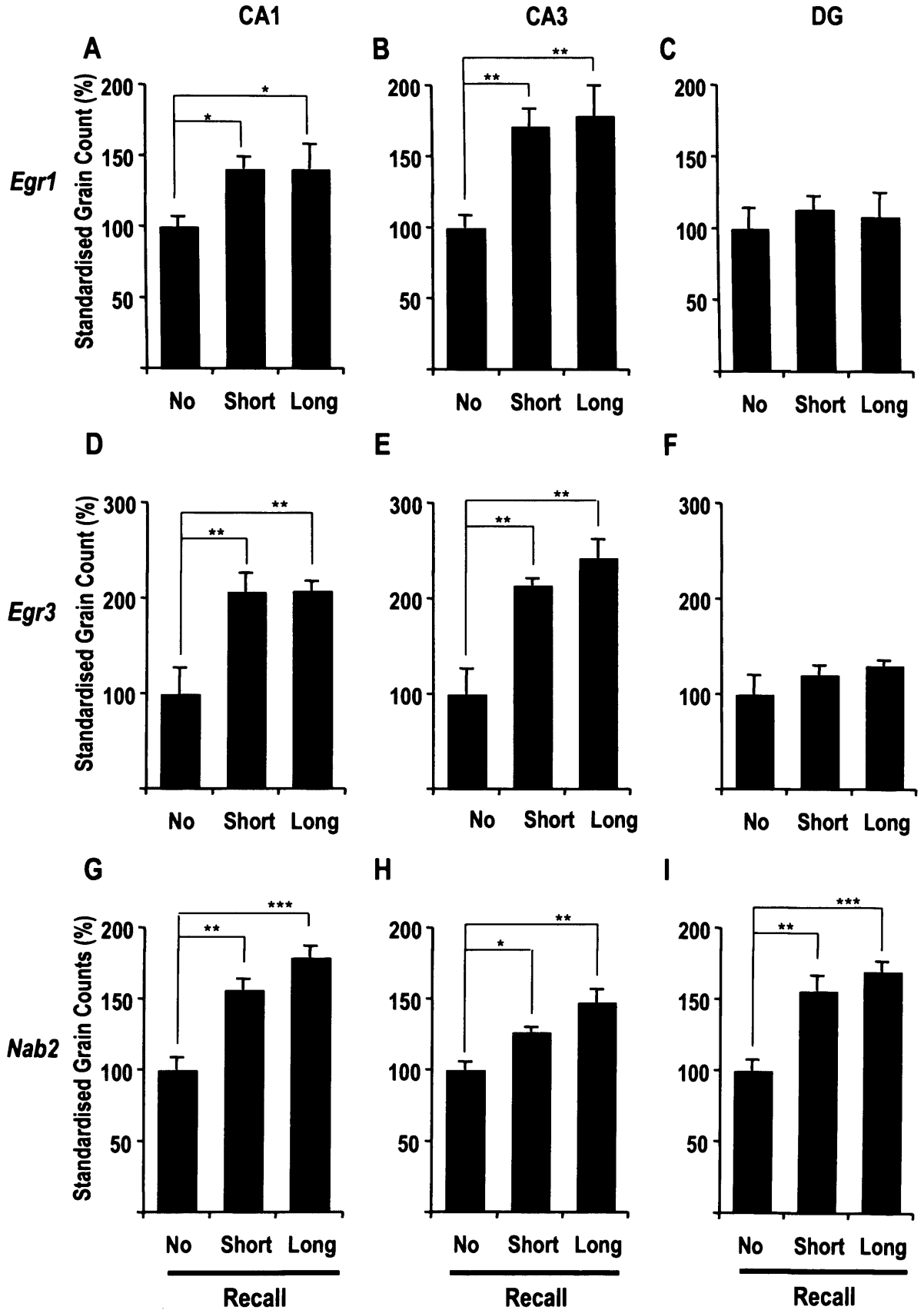


Figure 5.3. *Egr1*, *Egr3* & *Nab2* expression 30 min after Short (2 min) and Long (10 min) Recall in the CA1, CA3 and DG regions of the hippocampus. *Egr1* expression in the CA1 field (A), CA3 field (B), DG (C). *Egr3* expression in the CA1 field (D), CA3 field (E), DG (F). *Nab2* expression in the CA1 field (G), CA3 field (H), DG (I). * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ Error bars represent SEM. No Recall SG/cell for *Egr1*: CA1, 202±14; CA3, 65±6; DG, 53±7. No Recall SG/cell for *Egr3*: CA1, 39±10; CA3, 33±9; DG, 41±8. No Recall SG/cell for *Nab2*: CA1, 38±3; CA3, 47±3; DG, 35±3.

Although, no changes were detected in the level of *Egr1* or *Egr3* expression across the general population of cells in the DG region of the hippocampus in either the Short Recall or Long Recall groups, there were increased numbers of heavily labelled cells in both Recall groups compared to the No Recall group for *Egr1* ($F_{(2, 9)} = 36.652$, $p = 0.000$, ANOVA) and *Egr3* ($F_{(2, 9)} = 16.050$, $p = 0.001$, ANOVA). The number of heavily labelled *Egr1*-positive cells in the Recall groups increased by approximately 1300% relative to the No Recall group, and the number of heavily labelled *Egr3*-positive cells in the Recall groups increased by approximately 700% relative to the No Recall group (Fig. 5.4). The photomicrographs in Figure 5.4 a show heavily labelled *Egr1*-positive cells in the DG in the Long Recall group compared to the No Recall group, and show the more evenly distributed *Egr1* labelling in the CA1 and CA3 regions in these two groups for comparison.

5.3.2.3 Prefrontal Cortex Expression of *Egr1*, *Egr3* and *Nab2* after Short and Long Exposure to a Conditioned Context

Egr1 expression is significantly upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group in the Cg1 ($F_{(2, 9)} = 10.489$, $p = 0.004$, ANOVA), and PrL/IL ($F_{(2, 9)} = 10.718$, $p = 0.004$, ANOVA), regions of the mPFC (Fig. 5.5 a & b). No changes in expression levels of *Egr3* and *Nab2* were detected between either of the Recall groups in comparison to the No Recall groups in either the Cg1 or PrL/IL regions of the mPFC (Fig. 5.5 c - f).

5.3.2.4 Amygdala Expression of *Egr1*, *Egr3* and *Nab2* after Short and Long Exposure to a Conditioned Context

Egr1 expression was upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group in the LA ($F_{(2, 9)} = 5.206$, $p = 0.031$, ANOVA) and BLA ($F_{(2, 9)} = 4.860$, $p = 0.037$, ANOVA) nuclei of the amygdala (Fig. 5.6 a & b). *Egr3* expression was also upregulated 30 min after recall in both the Short Recall and Long Recall groups relative to the No Recall group in the LA ($F_{(2, 9)} = 7.369$, $p = 0.013$, ANOVA) and BLA ($F_{(2, 9)} = 23.125$, $p = 0.000$, ANOVA) nuclei of the amygdala (Fig. 5.6 c & d). In contrast, there was no difference in expression levels of *Nab2* between any of the groups in the LA or BLA nuclei of the amygdala (Fig. 5.6 e & f). Further analysis of *Egr3* expression in the BLA nucleus of the

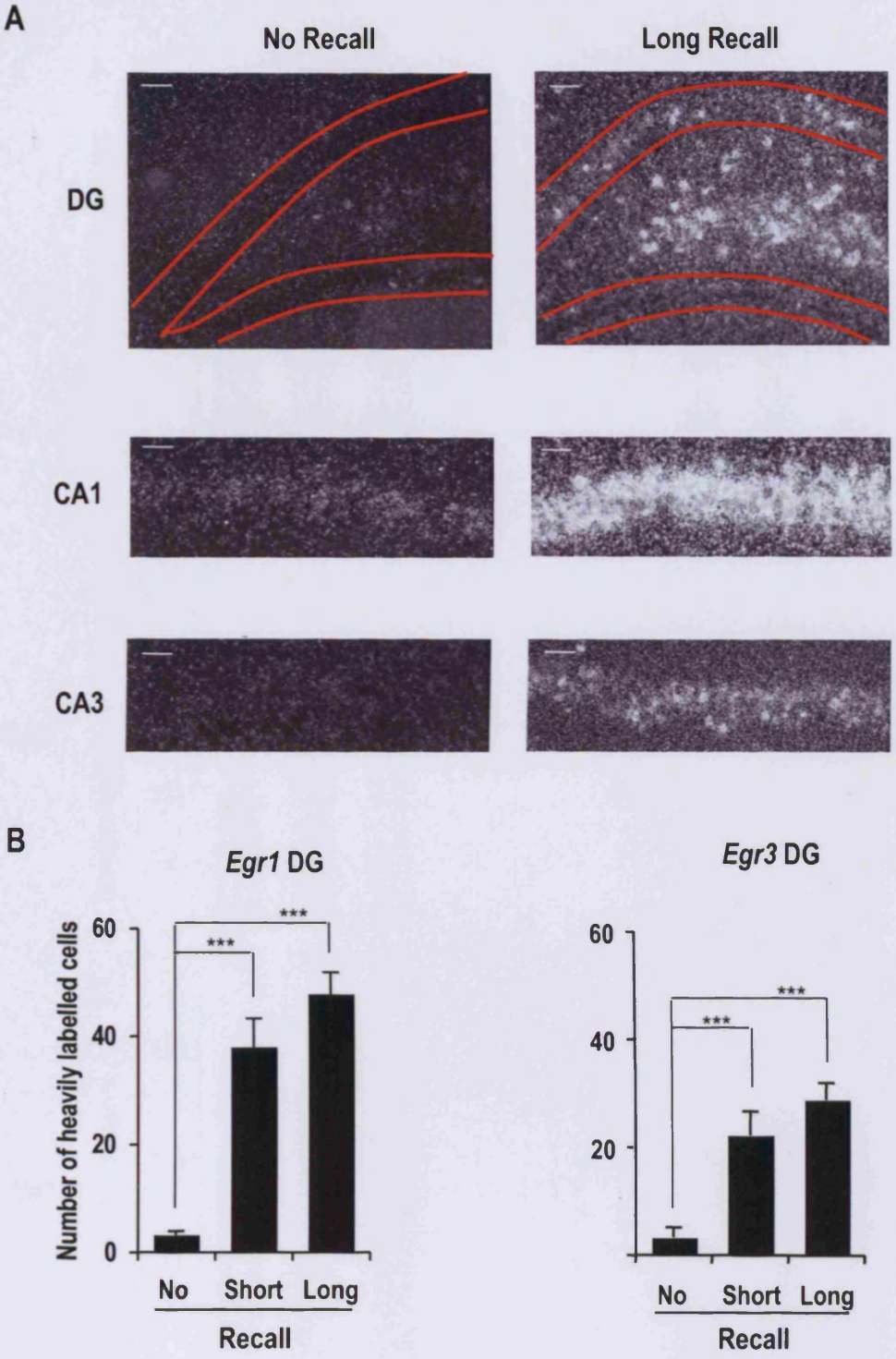


Figure 5.4. Heavily labelled cells for Egr1 and Egr3 mRNA in DG region of hippocampus during short (2 min) and long (10 min) recall. (A) Photomicrographs under darkfield conditions at x10 magnification showing heavily labelled *Egr1*-positive cells present in the dorsal DG for long recall group ,but not in the no recall group. Darkfield photomicrographs of the CA1 and CA3 regions in long recall and no recall groups are provided for comparison. (B) Number of heavily labelled *Egr1*-positive cells and *Egr3*-positive cells in DG for short and long recall groups and the no recall group. *** $p<0.001$ Error bars represent SEM. Scale bar μm.

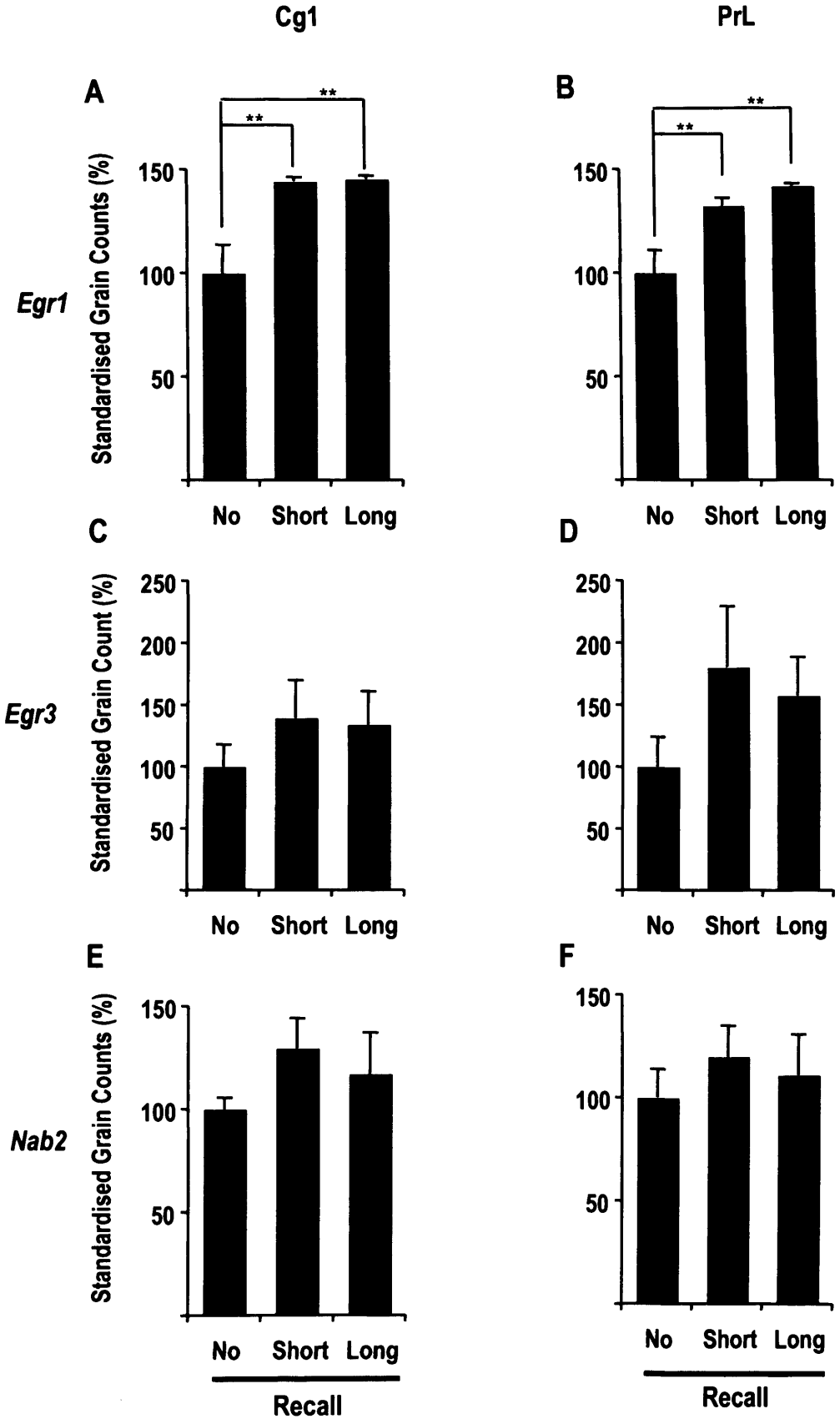


Figure 5.5. *Egr1*, *Egr3* & *Nab2* expression 30 min after short (2 min) and long (10 min) recall in the Cg and PrL regions of the prefrontal cortex. *Egr1* expression in the Cg (A), PrL (B). *Egr3* expression in the Cg (C), PrL (D). *Nab2* expression in the Cg1 (E), PrL (F). ** $p<0.01$ Error bars represent SEM. No recall SG/cell *Egr1*: Cg, 108±15; PrL, 106±11. No recall SG/cell *Egr3*: Cg, 57±10; PrL, 38±9. No recall SG/cell *Nab2*: Cg, 53±3; PrL, 50±7.

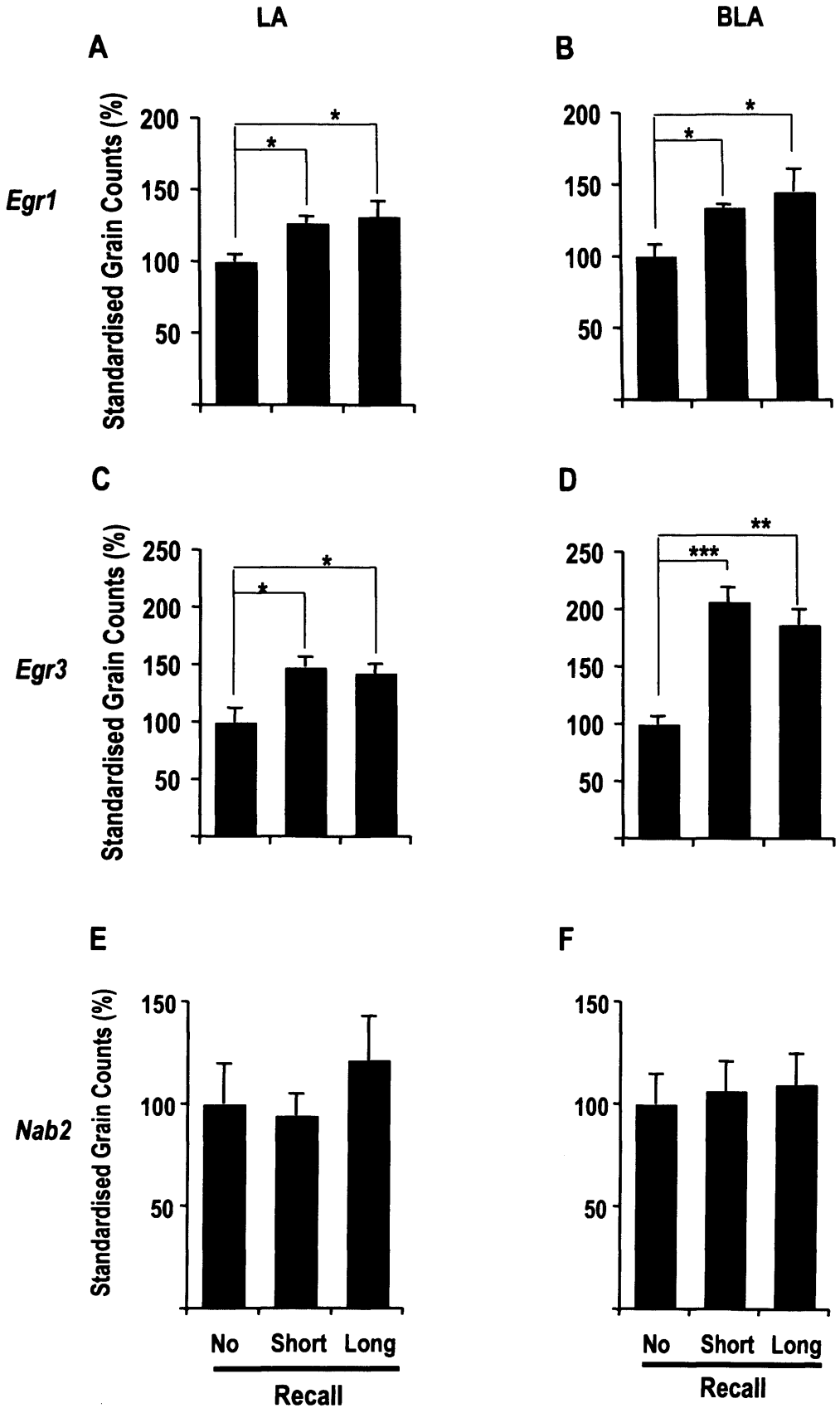


Figure 5.6. *Egr1*, *Egr3* & *Nab2* expression 30 min after short (2 min) and long (10 min) recall in the LA and BLA nuclei of the amygdala. *Egr1* expression in the LA (A) and BLA (B). *Egr3* expression in the LA (C) and BLA (D). *Nab2* expression in the LA (E) and BLA (F). There are no significant differences between any of the groups. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ Error bars represent SEM. No recall SG/cell *Egr1*: LA, 96±5; BLA, 74±6. No recall SG/cell *Egr3*: LA, 33±5; BLA, 20±5. No recall SG/cell *Nab2*: LA, 40±8; BLA, 42±6.

amygdala showed that from the cells counted, 10% of the cells in the No Recall group expressed more than 50 grains per cell, while 27% and 30% of the cells in the Short Recall and Long Recall groups, respectively, expressed more than 50 grains per cell (Fig. 5.7 a - c). This could be indicative of an increase of *Egr3* expression within a subpopulation of the cells.

5.3.2.5 Summary of ISH Results

A summary of *Egr1*, *Egr3* and *Nab2* regulation 30 min after short and long exposure to a conditioned context in the hippocampal regions, mPFC regions and amygdala nuclei is presented in Table 5.2.

5.3.3 Experiment 3: Effect of Intrahippocampal Infusions of *Egr3* Antisense on Short Exposure to a Conditioned Context

Of the 21 rats that underwent surgery, 2 died in the 24 hrs following the practice PBS infusions. Four rats were excluded from the analysis because of blocked or loose (one rat) cannulae. One rat was removed at LTM1 due to illness.

5.3.3.1 Histology

Four rats were identified as having misplaced indwelling cannula. They were therefore excluded from the study. The other 10 rats had correctly placed indwelling cannulae (Fig. 5.8 a).

5.3.3.2 Behaviour

The increased levels of freezing behaviour in both the ASOII and MSO groups in the post-US period during conditioning and in the following retrieval tests indicated that the rats had been conditioned. There was an effect of Test on freezing behaviour ($F_{(3.962, 27.735)\ \varepsilon = 0.660} = 12.974$, $p = 0.000$, repeated measures ANOVA). There was no Test x Group interaction indicating that there was no difference in the levels of freezing behaviour between the rats infused with ASOII and the rats infused with MSO ($F_{(3.962, 27.735)\ \varepsilon = 0.660} = 0.431$, $p = 0.784$, repeated measures

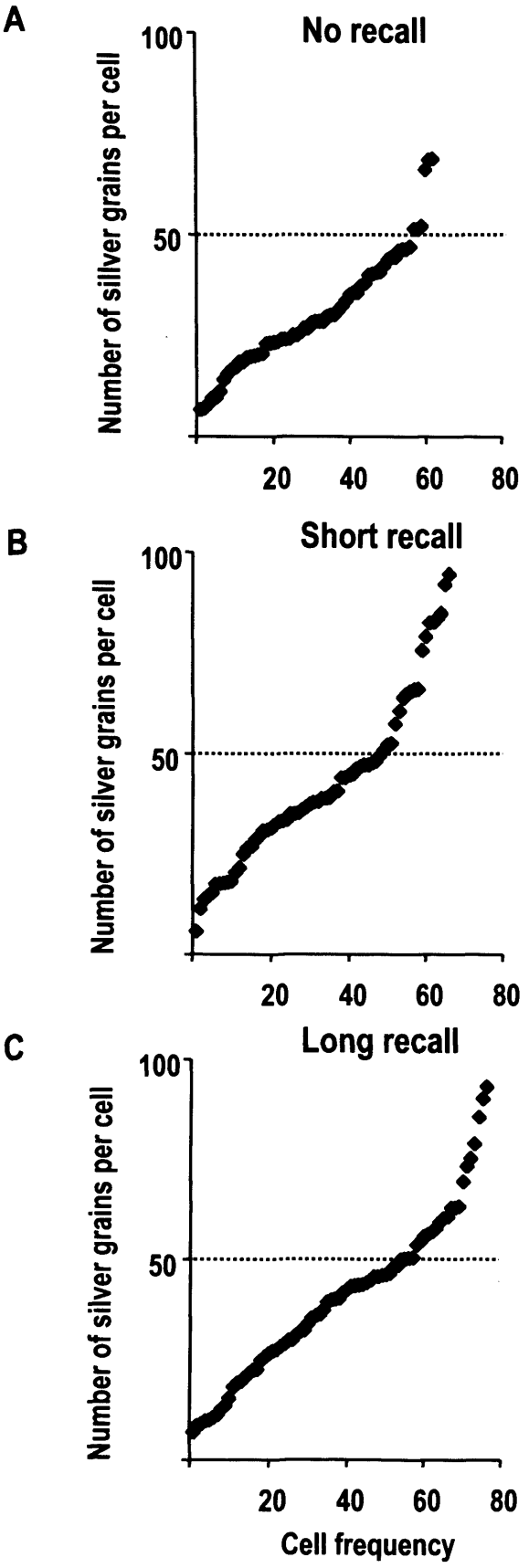


Figure 5.7. The number of cells sampled and the number of *Egr3*-positive silver grains per cell after short recall (2 min) and long recall (10 min) and no recall in the BLA nucleus of the amygdala. 50 or greater silver grains per cell was arbitrarily decided to be the definition of heavily labelled cell. (A) *Egr3* grain no recall group (n = 4). (B) *Egr3* grain short recall groups(n = 4). (C) *Egr3* grain long recall group (n = 4).

Table 5.2. Summary of *Egr1*, *Egr3* and *Nab2* regulation in regions of the hippocampus and prefrontal cortex and nuclei of the amygdala 30 min after both Short Recall and Long Recall of a CFM. ↑, gene expression is upregulated at the cellular level relative to the No Recall group; ↑*, gene expression is upregulated in a small population of cells in the DG relative to the No Recall group; -, no difference in gene expression relative to the No Recall group.

Brain Structure of interest	Region of brain structure	Egr1	Egr3	Nab2
Hippocampus	CA1	↑	↑	↑
	CA3	↑	↑	↑
	DG	↑*	↑*	↑
Amygdala	BLA	↑	↑	-
	LA	↑	↑	-
Prefrontal Cortex	CG	↑	-	-
	PL	↑	-	-

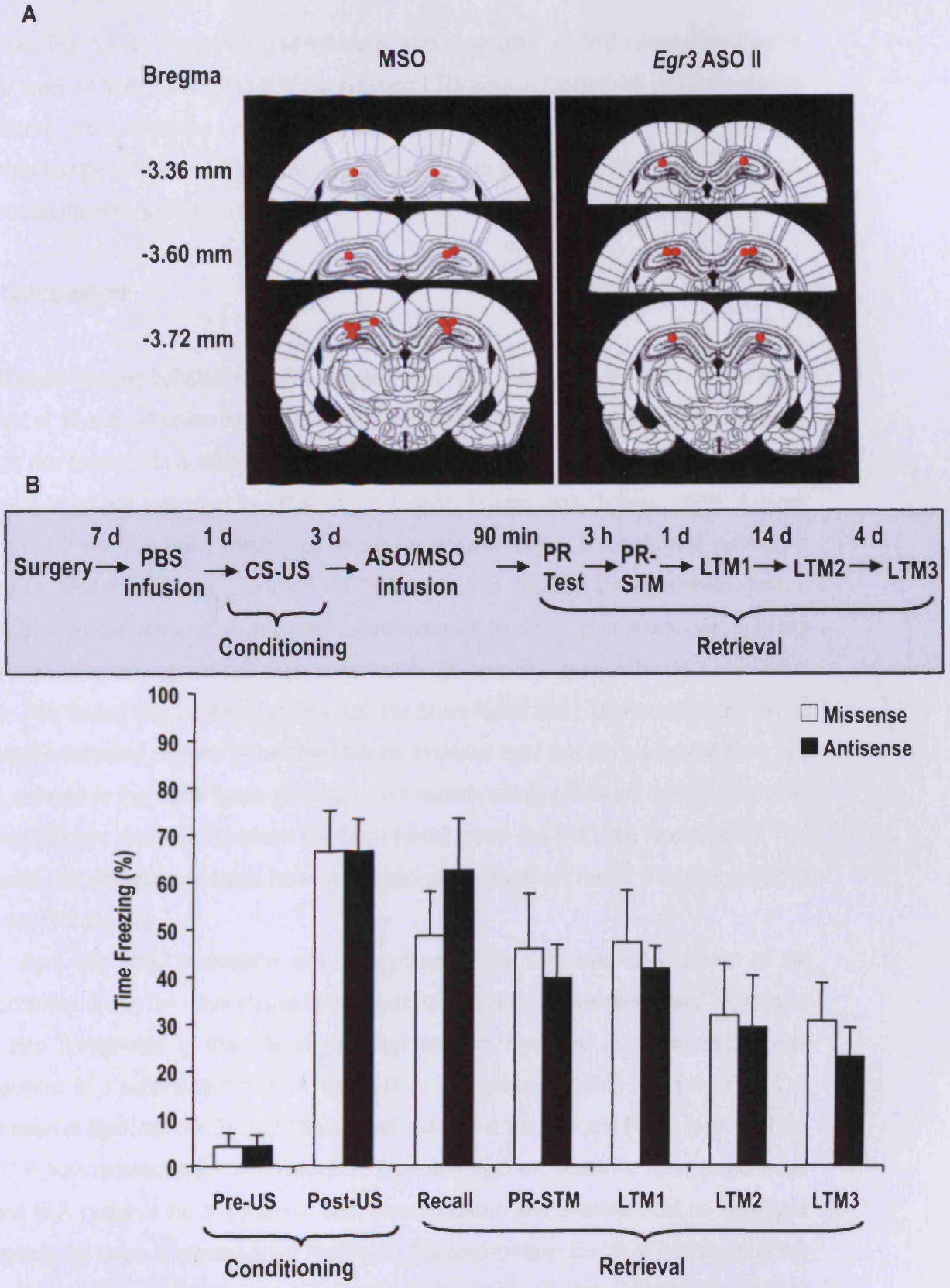


Figure 5.8. Effect of Intrahippocampal Infusions of *Egr3* Antisense on reconsolidation of CFC. (A) Schematic indicating the site of infusion of the antisense (ASO) and missense (MSO). (B) Schematic of behaviour and infusion protocol used and the freezing behaviour of rats during CFC and in the following LTM tests for infusions of MSO (1ng/μl) (n = 6), and ASOII (2ng/μl) (n = 4). Error bars represent SEM.

ANOVA) (Fig. 5.8 b). *Egr3* antisense infusions at 90 min prior to CFM retrieval resulted in similar levels of freezing behaviour in the following LTM tests in conditioned rats compared to conditioned rats receiving missense infusions at 90 min prior to CFM retrieval. Intrahippocampal infusion of *Egr3* antisense ODNs 90 min prior to the short retrieval test did not prevent the reconsolidation of CFM.

5.4 DISCUSSION

Conditioned freezing behaviour in LTM retention tests was reduced in rats exposed to a recall session of 10 min, whereas there was no change in freezing behaviour in the LTM retention tests in rats exposed to a recall session of 2 min. This shows that a recall session of 10 min is sufficient to induce extinction as previously described (Barnes and Thomas, 2008). A recall session of 2 min has been established to induce reconsolidation, in rats having undergone contextual fear conditioning (Lee et al., 2004; Barnes and Thomas, 2008). However for the freezing behaviour observed in rats used to provide brain tissue for gene expression profiling, there was no within-session extinction observed in the extinction session for the Long Recall group. This finding may be interpreted as both the Short Recall and Long Recall groups having undergone the same process (ie reconsolidation). However Bdnf and Arc expression have also been assayed in the same tissue as used in this experiment (unpublished findings) and they showed different responses between the Short Recall group and the Long Recall group. This suggests that different processes have been engaged in the Short Recall group compared to the Long Recall group.

Egr1, *Egr3* and *Nab2* expression was upregulated in the CA1 and CA3 regions of the hippocampus during the initial stages of both reconsolidation and extinction. *Nab2* expression was also upregulated in the DG of the hippocampus. *Egr1* and *Egr3* expression was upregulated in a subpopulation of the DG cells in both reconsolidation and extinction. The expression of *Egr1*, but not *Egr3* or *Nab2*, is upregulated in the Cg1 and PrL/IL regions of the mPFC in both reconsolidation and extinction. *Egr1* and *Egr3* expression is upregulated in the LA and BLA nuclei of the amygdala in both reconsolidation and extinction, but no significant changes in the levels of expression of *Nab2* were detected in either the LA or BLA nuclei of the amygdala in either reconsolidation or extinction. Further study of *Egr3* expression in the BLA nucleus of the amygdala showed that a subpopulation of cells expressed much higher levels of *Egr3* expression compared to the majority of cells. Figure 5.9 summarises all the changes in *Egr1*, *Egr3* and *Nab2* regulation associated with reconsolidation and extinction of CFM. These

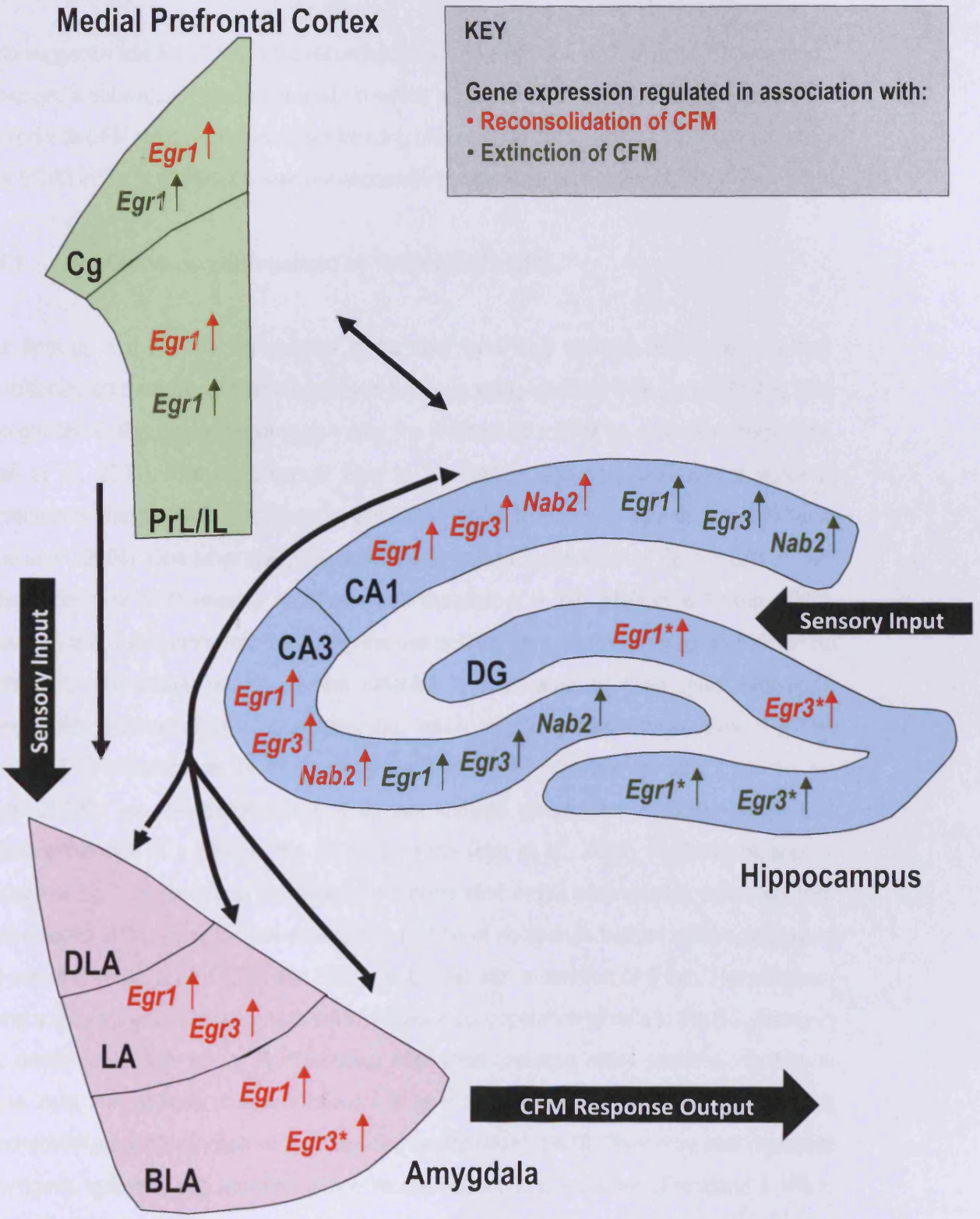


Figure 5.9. Schematic showing *Egr1*, *Egr3* and *Nab2* regulation in association with reconsolidation and extinction in regions of the medial prefrontal cortex, hippocampus and amygdala. Block arrows, information input and output following CFC; thin black arrows, information flow between brain regions; small arrows, up- or down-regulation of gene expression; *, heavily labelled cells (no regulation was detected in the general cell population). Medial prefrontal cortex regions: cingulate cortex (Cg), prelimbic cortex (PrL) & infralimbic cortex (IL). Hippocampal regions: cornus ammonis 1 (CA1), cornus ammonis 3 (CA3) & dentate gyrus (DG). Amygdala regions: dorsolateral nucleus (DLA), lateral nucleus (LA) & basolateral nucleus (BLA); Contextual fear memory (CFM). Not drawn to scale.

data suggest a role for EGR3 in the reconsolidation and extinction of CFM in the hippocampus. However, a subsequent functional study in which hippocampal infusions of *Egr3* antisense 90 min prior to CFM retrieval did not effect freezing behaviour in the following LTM tests, indicating that EGR3 in the hippocampus was not necessary for the reconsolidation of CFC.

5.4.1 *Egr1* Expression Upregulated by Retrieval of a CFM

Our findings that *Egr1* is upregulated in the CA1 by 2 and 10 mins exposures to a fear conditioned context are complimented by a previous study showing that *Egr1* mRNA is also upregulated in this hippocampal region after the retrieval of a CFM by an 8 min retrieval trial (Hall et al., 2001). The regulation of *Egr1* in the CA1 is also consistent with a series of experiments that showed that hippocampal EGR1 plays a functional role in reconsolidation (Lee et al., 2004). One other study measured no changes in expression of *Egr1* in CA1 30 min after retrieval of CFM using a recall test with duration of 4 min (Malkani & Rosen, 2000). However, this discrepancy may be due to the use of a different control group to account for US (immediate US instead of LI), or the different level of analysis used (autoradiographic densitometry instead of silver grain counting, which inherently has a lower level of spatial accuracy). The increase in the levels of *Egr1* mRNA in CA3 we observed after 2 and 10 min recall of CFM are consistent with a study that showed upregulation of EGR1 protein 2 h following retrieval of a CFM in the CA3/DG region (Lee et al., 2004). Furthermore, lack of change in *Egr1* expression in the general cell population in DG after recall is consistent with observations in the study by Hall et al (2001) that show no change in *Egr1* mRNA expression 30 min after retrieval of a CFM that had a recall test with a duration of 8 min. Nevertheless, using a different analytical approach we identified a subpopulation of cells in the DG, mainly in the dorsal blade, with heavy *Egr1* labelling after short and long recall sessions. Activity in these cells may indicate their involvement in a sparse network of neurons supporting the reconsolidation and extinction of the CS-US memory (Marr, 1970). They may also represent neurogenic cells that are activated during reconsolidation and extinction (Frankland & Miller, 2008; Kee et al., 2007; Ko et al., 2009). However this argument is problematical, since neurogenesis and transport from the SGZ takes days to weeks and we investigated changes in expression only 30 min after recall.

Our studies have identified upregulation of *Egr1* mRNA expression in the Cg1 and PrL/IL regions of the mPFC following short and long recall of CFM, conditions of recall that we show

correlated to the reconsolidation and extinction of CFM, respectively. Post-retrieval upregulation of *Egr1* mRNA in the Cg1 and the PrL has been observed before (Thomas et al., 2002). The regulation of levels of EGR1 protein in PrL/IL mPFC have been associated with extinction training of CFC, with decreases measured after one extinction training session and increases after a second (Herry & Mons, 2004). However in that study, the first extinction session took place 5 hours after conditioning, a time-point at which consolidation of CFC was not likely to have been completed. In addition, no control No Recall group was used so whether the changes identified in EGR1 expression was related to extinction cannot be determined. In conclusion, our findings agree with previous studies that implicate *Egr1* activity in the mPFC by recall of CFC.

Our data show that *Egr1* expression was upregulated in the general cell population of both the LA and BLA nuclei of the amygdala during reconsolidation or extinction of CFM. These findings are supported by two previous studies. Firstly, upregulation of *Egr1* expression was observed in the BLA nucleus of the amygdala 30 min post-retrieval (Hall et al., 2001). Secondly, increases in EGR1 protein expression have also been identified in the mouse lateral amygdala 2 hours after a second extinction training session, 24 hours after cued fear conditioning (Herry & Mons, 2004).

In summary our findings show that upregulation of *Egr1* expression is correlated with both reconsolidation and extinction of CFM in the CA1, CA3 regions of the hippocampus and a subpopulation of cells in the DG regions of the hippocampus, in the Cg1 and PrL/IL regions of the mPFC and in the LA and BLA nuclei of the amygdala.

5.4.2 *Egr3* Expression Upregulated by Retrieval of a CFM

We measured an increase in the expression of *Egr3* in the CA1, CA3 and the DG after recall of CFM by both a 2 min and 10 min exposure to the context CS. The increase in *Egr3* expression in DG, like that for *Egr1*, was confined to a small population of DG cells. These represent novel data concerning the expression of *Egr3* with recall of CFM. This upregulation of *Egr3* in the pattern described above suggests EGR3 may have a role in both reconsolidation and extinction of CFM. However, intrahippocampal infusion of *Egr3* antisense into the hippocampus 90 min before a short recall exposure to a conditioned context did not lead to altered freezing behaviour in the following retention tests. This suggests that EGR3 activity in the hippocampus

is not necessary for reconsolidation of CFM. However, since EGR3 protein expression was not quantified, the efficacy of the antisense approach used in this study to reduce the translation of *Egr3* mRNA to EGR3 protein was not confirmed. Necessary future work includes performing Western blot analysis to determine whether EGR3 protein is regulated in the CA1 following the recall of a CFM, and whether the levels of EGR3 protein are reduced, or knocked down, following recall of a CFM.

Our data show that *Egr3* expression was upregulated in the general cell population of both the LA and BLA nuclei of the amygdala during reconsolidation or extinction of CFM. There are no previous studies on the expression of this gene in these regions during these processes so these findings are novel. An increase in *Egr3* expression in a subpopulation of cells in the BLA nucleus of the amygdala after recall was observed. Studies have identified a role for a minority subpopulation of cells in the BLA called the intercalated cell mass (ICM) compared to the majority of the BLA cell population in extinction (Quirk & Mueller, 2008). A role for the ICM has not yet been investigated in reconsolidation. This data suggests that different populations of cells within the amygdala may differentially contribute to, or participate in, memory processing after recall. It remains to be determined which subpopulation of cells this high *Egr3* expressing group represents, or whether they play a role in the reconsolidation and extinction of fear memory.

There was no change in the level of *Egr3* expression in the Cg1 and PrL/IL regions of the mPFC. In summary our findings show that upregulation of *Egr3* expression is correlated with both reconsolidation and extinction of CFM in the CA1, CA3 regions of the hippocampus and a subpopulation of cells in the DG regions of the hippocampus, and in the LA and BLA nuclei of the amygdala and in a subpopulation of cells in the BLA nucleus of the amygdala, but not in the Cg1 and PrL/IL regions of the mPFC.

5.4.3 *Nab2* Expression Upregulated by Retrieval of a CFM

We measured an increase in the expression of *Nab2*, a repressor of EGR activity, in the CA1, CA3 and the DG after recall of CFM by both a 2 min and 10 min exposure to the context CS. In contrast, no change in *Nab2* expression was observed in the LA or BLA nuclei of the amygdala or in the Cg1 or PrL/IL regions of the mPFC. These findings represent novel data concerning the expression of *Nab2* with recall of CFM. In summary our findings show that upregulation of

Nab2 expression is correlated with both reconsolidation and extinction of CFM in the CA1, CA3 and DG regions of the hippocampus, but not in the nuclei of the amygdala or regions of the mPFC.

5.4.4 Neurocircuitry Supporting Retrieval of a CFM

Our findings suggest that cells in the CA1, CA3 and DG of the hippocampus, the Cg1 and PrL/IL regions of the mPFC and the LA and BLA nuclei of the amygdala are involved in the neurocircuitry involved in reconsolidation and extinction.

5.4.4.1 Hippocampus

A role for the hippocampus has already been implicated in reconsolidation as gene transcription in the hippocampus is necessary for reconsolidation of CFM (Lee et al., 2004). In addition, protein synthesis in the hippocampus is necessary for reconsolidation of CFM (Debiec et al., 2002; Lee et al. 2004; Mamiya et al., 2009), spatial memory (Rossato et al., 2006b) and object recognition memory (Rossato et al., 2007). Molecular activity in the hippocampus such as upregulation of *Egr1* in the CA1 after short recall of a cued fear memory (Hall et al., 2001), hippocampal EGR1 activity in CFM reconsolidation (Lee et al., 2004), and CREB activity and ARC protein expression in the CA1 and CA3 after short recall of a CFM (Mamiya et al., 2009) further support for a role for the hippocampus in the neurocircuitry underlying reconsolidation. A role for the hippocampus has also been implicated in extinction as gene transcription in the hippocampus is necessary for reconsolidation of inhibitory avoidance (IA) memory (Vianna et al., 2003), and protein synthesis in the hippocampus is necessary for extinction of CFM or IA memory (Thomas & Barnes, 2008; Vianna et al., 2001; Power et al., 2006). Molecular activity in the hippocampus such as NMDA receptor activation (Szapiro et al., 2003), kinase activity including PKA, p38 MAPK, JNK, CDK5, PAK-1, MEK/ERK and SRC tyrosine kinases (Bevilaqua et al., 2003; Bevilaqua et al., 2007; Fischer et al., 2006; Rossato et al., 2006a; Sananbenesi et al., 2007; Szapiro et al., 2003), and actin rearrangement (Fischer et al., 2004) are necessary for or correlate with memory extinction. Thus our data showing an upregulation of the expression of *Egr1*, *Egr3* and *Nab2* mRNA in the hippocampus 30 min after recall in the Short Recall and Long Recall groups but not in the No Recall control group suggests that they are likely candidates genes to be involved in reconsolidation and extinction. However our

finding that EGR3 is not required for reconsolidation suggests that any involvement may not be necessary.

5.4.4.2 Prefrontal Cortex

A role for the mPFC has been implicated in reconsolidation as protein synthesis in the PFC is required for the reconsolidation of object recognition memory (Akirav & Maroun, 2006). Molecular activity in the mPFC such as NMDA receptor activity (Akirav & Maroun, 2006), MEK activity (Maroun & Akirav, 2009) and CREB activity (Kida et al., 2002) are necessary for memory reconsolidation. A role for the mPFC in extinction has also been implicated by many studies. Electrolytic lesions of the ventromedial PFC and dorsomedial PFC showed that both these regions were necessary for extinction of cued fear memory and that they have functionally different roles in extinction (Morgan et al., 1993; Morgan & LeDoux, 1995; Quirk et al., 2000). Cell or afferent-specific pharmacological lesions specific to the IL and PL of the medial PFC also found the mPFC to be necessary in extinction of both CFM and cued appetitive memory (Fernandez-Espejo, 2003; Rhodes & Killcross, 2007). Electrophysiological studies indicate that stimulation of the mPFC pre-extinction, during extinction and post-extinction enhances retrieval of cued fear conditioned extinction memory (Milad & Quirk, 2002; Herry & Garcia, 2002; Farinelli et al., 2006). At the molecular level, protein synthesis in the mPFC is necessary for both cued fear conditioned and conditioned taste aversion extinction memory (Santini et al., 2004; Akirav et al., 2006). Molecular activity in the mPFC such as NMDA, $GABA_A$ and β-adrenergic receptor activity (Suzuki et al., 2004; Akirav et al., 2006; Mueller et al., 2008), MAPK and PKA kinase activity (Hugues et al., 2004; Mueller et al., 2008), CREB activity (Mamiya et al., 2009), and increased *c-Fos* expression (Santini et al., 2004) are necessary for or correlate with memory extinction. Therefore our correlation of *Egr1* mRNA upregulation in the Cg1 and IL/PrL region of the medial PFC 30 min after Short Recall and Long Recall groups compared to the No Recall control group identifies *Egr1* as a candidate gene to be involved in reconsolidation and extinction in a second region of the brain in addition to the hippocampus.

5.4.4.3 Amygdala

A role for the amygdala has been implicated in reconsolidation through multiple studies. An electrophysiological study found that disruption of reconsolidation was correlated with a

reduction of synaptic potentiation in the LA nucleus of the amygdala (Doyere et al., 2007). Protein synthesis has also been shown to be necessary for reconsolidation in the BLA in some studies (Nader et al., 2000; Lee et al., 2005). Molecular activity in the amygdala such as NMDA, CB1 and β-adrenergic receptor activity (Lee et al., 2006; Bucherelli et al., 2006; Debiec & Ledoux, 2004), PKA and ERK kinase activity (Tronson et al., 2006; Duvarci et al., 2005), CREB activity (Mamiya et al., 2009) and EGR1 and C/EBPβ activity (Lee et al., 2005; Tronel et al., 2005) are necessary for or correlate with memory reconsolidation. A role for the amygdala has also been implicated in extinction. An electrophysiology study found neuronal activity in the LA nucleus in extinction (Hobin et al., 2003) and protein synthesis in the amygdala has been shown to be necessary for extinction (Lin et al., 2003; Bahar et al., 2003). Molecular activity in the amygdala such as NMDA and GABA receptor activity (Akirav et al., 2006; Berlau & McGaugh, 2006; Lee et al., 2006), PI-3K and MAPK kinase activity (Lin et al., 2003), CREB activity (Mamiya et al., 2009), and increased expression of Bdnf, c-FOS, EGR1 and calcineurin (Chhatwal et al., 2006; Herry & Mons, 2004; Lin et al., 2003) are necessary for or correlate with memory extinction. Thus our data showing an upregulation of the expression of *Egr1* and *Egr3* in the amygdala 30 min after recall in the Short Recall and Long Recall groups but not in the No Recall control group suggests that they are likely candidates genes to be involved in reconsolidation and extinction. In addition, the increase in *Egr3* expression observed in a subpopulation of cells in the BLA nucleus of the amygdala in this study in the Long Recall group may represent the extinction neurons that have also been observed in the BLA nucleus of the amygdala (Herry et al., 2008). However no such reconsolidation neurons have been observed to explain the presence of a similar subpopulation of high *Egr3* expressing cells in the BLA nucleus of the amygdala in the Short Recall group in this study, but a previous study has shown that ablation of a subpopulation of cells that have increased CREB expression in the LA nucleus of the amygdala following fear conditioning results in impaired subsequent memory recall (Han et al., 2007). This evidence supports our finding of a subpopulation of neurons being activated in the amygdala during a short recall session.

5.4.5 Dissociable Roles for *Egr1* and *Egr3* following Recall of a CFM

Our results show dissociable roles for EGR1 and EGR3 in the reconsolidation of CFM. Although, the expression of *Egr1* and *Egr3* are similarly regulated in the hippocampus by retrieval conditions that favour reconsolidation (a 2min exposure to the context CS), we show

that infusions of *Egr3* ASO prior to recall had no effects on CFM. These results contrast to a similar experiment that showed that *Egr1* ASO interfered with reconsolidation (Lee et al., 2004). Thus it appears that EGR1 but not EGR3 activity in the dorsal hippocampus is required for the reconsolidation of CFM.

It is possible that the increase in *Egr3* expression after 2 min context exposure is related to activity in the hippocampus but unrelated directly to reconsolidation. However, unlike in previous chapters the regulation of *Egr3* cannot be associated with footshock stress, as the rats did not receive a footshock in the recall sessions. Neither can the upregulation be due to exposure to a novel environment as the recall test is not the first time the rats have been in the conditioning chamber. However as our results in the previous chapter suggested *Egr3* could be upregulated in response to arousal, the upregulation in the Short Recall and Long Recall groups in this chapter may also be upregulated in response to arousal. Indeed, we saw a similar increase in hippocampal *Egr3* expression after recall by a 10 min exposure to the conditioned context that induced extinction rather than reconsolidation.

Alternatively, an increase in *Egr3* activity in the hippocampus may be related to extinction processes that may be engaged in parallel with reconsolidation. A study on appetitive learning in the honeybee suggested that reactivation of a CS-US memory with five CS-only presentations induced two memory traces, both a reconsolidated acquisition memory and an extinction memory (Stollhoff et al., 2005). They therefore suggested that these processes induced by memory retrieval take place in parallel rather than in an "all-or-nothing" law (Stollhoff et al., 2005). This same group went on to develop the internal reinforcement hypothesis that interpreted these findings, when combined with literature, to propose that in retrieving a consolidated memory, extinction learning and "reminder learning" take place. This introduces a concept of reminder learning in place of reconsolidation and is only theoretical at present (Eisenhardt & Menzel, 2007). Another finding that supports this possibility that reconsolidation and extinction could occur in parallel is that extinction is not a sufficient condition to inhibit induction of reconsolidation (Duvarci et al., 2006). There is a hypothesis of trace dominance that proposes that reconsolidation and extinction processes compete and the dominant one is the one most affected by protein synthesis inhibition (Nader, 2003). It is thus possible that both processes occur in parallel at a molecular and cellular level but only one process is dominant at the behavioural level due to some so far unknown inhibitory mechanism preventing one network being behaviourally expressed under particular conditions.

In contrast to parallel existence of these two processes, there is evidence from fear memory experiments in crabs that show that reconsolidation or extinction after one non-reinforced trial are mutually exclusive, and are "switched on" in isolation, within seconds after the offset of the CS re-exposure (Pedreira & Maldonado, 2003; Perez-Cuesta et al., 2007). Recent observations in our laboratory support the hypothesis that reconsolidation and extinction are mutually exclusive processes. We show that recombinant BDNF infused into the dorsal hippocampus prior to an extended exposure to conditioned context prevents extinction, and further that conditioned fear is maintained when Zif268 ASO is also co-infused (unpublished observations). This suggests that only extinction and not reconsolidation processes are engaged with Long Recall. In addition, when a protein synthesis inhibitor was administered 30 min before a short CS re-exposure, reduced freezing behaviour in a LTM test 24 hours later, suggested reconsolidation of the memory had been disrupted. However, when protein synthesis inhibitor was administered before a long CS re-exposure, no reduction in freezing behaviour in a LTM test 24 hours later, suggested that extinction of the memory had been disrupted (Barnes and Thomas, 2008). The ability of protein synthesis inhibitors to impair reconsolidation and extinction and result in either the loss or maintenance of the conditioned response under different durations of recall further suggests that the two memory processes are independently engaged after retrieval.

It is possible that the activity of the repressor NAB2 regulates the function of EGR1 and EGR3 after recall. *Nab2* expression in the hippocampus is increased after both short and long recall exposures. We hypothesise that NAB2 may regulate the activity of EGR3 after short recall, permitting EGR1 dependent reconsolidation. It is also possible that NAB2 may modulate EGR1 activity after long CS exposure to allow extinction. This hypothesis could be tested if knockdown of the EGR3 activity at long recall with ASO prevented extinction. It would also predict that reducing NAB2 levels during both short and long recall could interfere with reconsolidation and extinction.

While they both show similar upregulation patterns in the hippocampus, our results indicate a brain region specific regulatory response to recall for *Egr1* in the medial PFC. The differential expression pattern in *Egr1* and *Egr3* expression in this brain region is further evidence of dissociable roles for these EGR proteins in memory processes. Further evidence that EGR proteins do not functionally compensate for each other comes form observations in transgenic

mouse models. For example, *Egr1* and *Egr3* expression levels do not change in *Egr2*$^{-/-}$ mice (Poirier et al., 2007). In addition *Egr1*$^{-/-}$ mice and *Egr3*$^{-/-}$ mice behave differently on the same behavioural tasks (Poirier et al., 2008).

5.4.6 Conclusion

The main findings of these experiments is that the neurocircuitry underlying reconsolidation and extinction of contextual fear conditioning in rats are both likely to include the CA1, CA3 and DG of the hippocampus, the Cg1 and PrL/IL regions of the mPFC and the LA and BLA nuclei of the amygdala. In addition, differential expression patterns of the *Egr* transcription factors in the mPFC was identified, as *Egr1* but not *Egr3* was upregulated in both reconsolidation and extinction in the mPFC. No dissociation between reconsolidation and extinction was observed through investigating the expression of *Egr1*, *Egr3* and *Nab2* in the hippocampus, mPFC and amygdala. Finally EGR3 expression in the hippocampus is not required for reconsolidation of CFC.

CHAPTER 6

GENERAL DISCUSSION

6.1 Summary of Results

The experiments presented in this thesis were designed initially to test the hypothesis that the schizophrenia susceptibility genes *Nrg1*, *Dtnbp1*, *Disc1* and *Egr3* are regulated in correlation with hippocampal-dependent contextual fear long-term memory processes in the adult rat brain. From the experimental findings in this thesis it can be concluded that of the schizophrenia susceptibility genes investigated the expression of *Egr3* was upregulated in correlation with the consolidation, reconsolidation and extinction of contextual fear long-term memory (CFM), the expression of *Nrg1* type I splice variants was upregulated in correlation with the consolidation of CFM, and *Dtnbp1* expression was not regulated in association with the consolidation of CFM. No conclusions could be drawn regarding *Disc1* expression as none of the probes designed could detect specific labelling. Bdnf expression was not upregulated in the CA1 at 2 hours following CFC, as might have been expected considering *Bdnf* had previously been shown to be upregulated at 30 min after CFC (Hall et al., 2000). However a small population of cells within the CA1 and DG had an increased number of cells expressing heavy *Egr3* labelling in association with arousal, and at the regional level *Bdnf* was upregulated in association with footshock stress in the CA1. *Egr1* and *Nab2* expression was upregulated in correlation with the reconsolidation and extinction of CFM. Further experiments were performed in order to test the second hypothesis that the schizophrenia susceptibility genes that were regulated in correlation with LTM processes are causally involved in these processes. Knockdown of EGR3 using antisense oligodeoxynucleotides 90 min prior to CFC or retrieval of a CFM did not prevent consolidation or reconsolidation of the CFM respectively. However these findings come with the caveat that knockdown of EGR3 protein was not confirmed.

Our findings add to the evidence implicating the hippocampus, amygdala and mPFC in the neurocircuitry supporting CFM processes. Cells in the CA1, CA3 and a small sparse population of cells in the DG region of the hippocampus, and cells in the dorsolateral nucleus of the amygdala all have gene expression that is regulated in association with the consolidation of CFM. Cells in the CA1, CA3 and DG regions and a subpopulation of cells within the DG region of the hippocampus, in the Cg1 and PrL/IL regions of the mPFC, and in the LA nucleus, BLA nucleus and a subpopulation of cells within the BLA nucleus of the amygdala all have gene expression that is regulated in correlation with reconsolidation and extinction.

6.2 Schizophrenia Susceptibility Genes and CFM

6.2.1 *Egr1*, *Egr3*, *Nab2* and *Nrg1* type I in consolidation, reconsolidation and extinction of CFM

Combining our findings with previous literature shows that neither *Egr1* nor *Egr3* have a functional role in the consolidation of CFM (Lee et al., 2004) but *Egr3* is upregulated in association with the consolidation of CFM. It is possible that compensatory mechanisms, such as EGR2 or EGR4 activity, may function in the role that EGR3 would normally have in the consolidation of CFM. However there is evidence that EGR proteins do not functionally compensate for each other, as no change in *Egr1* and *Egr3* expression levels are observed in *Egr2*$^{-/-}$ mice (Poirier et al., 2007). In addition knockdown of EGR3 activity could not be compensated for by EGR1 activity as EGR1 has been shown not to be regulated or functional required for the consolidation of CFM (Lee et al., 2004). Alternatively, the upregulation of *Egr3* in this study may be related to a role in the molecular activity underpinning the arousal component associated with CFC. Interestingly, both *Egr1* and *Egr3* are upregulated in association with reconsolidation of CFM while EGR1 but not EGR3 activity is required for reconsolidation of CFM. This highlights that EGR1 and EGR3 do not have identical functional roles. The upregulation of *Egr3* in reconsolidation could again be associated with arousal related to the short retrieval session. *Egr1* and *Egr3* expression were both found to be upregulated in correlation with extinction of CFM in this study and EGR1 protein is upregulated in association with extinction (Herry & Mons, 2004) but no functional studies have yet been performed to see if this upregulation is necessary for extinction of CFM.

Our findings that *Nab2* is upregulated in the LI group in both the CA1 region of the hippocampus and the DLA of the amygdala, regions in which *Egr3* is upregulated in association with the consolidation of CFM but not with LI, lead us to hypothesise that the increased levels of NAB2 in the LI group may repress EGR3 activity and thus could be a potential mechanism that prevents the formation of an association between the CS and US after extended exposure to the CS. In effect NAB2 would be acting as a modulator of EGR1 and EGR3 activity in CFM.

NRG1 type I activation of ERBB receptors and BDNF activation of tyrosine receptor kinase can lead to an increase in transcription of EGR3 via separate second messenger cascades . Once translated this increase in EGR3 can lead to an increase in the transcription of other genes including *Arc* and *Egr3* itself (Guo et al., 2010). It is interesting to note that we found both *Nrg1* type I and *Egr3* expression to be upregulated in correlation with the consolidation of CFM. However no functional link can be implied from our findings as the change in regulation of either or both these genes could be related to homeostatic maintenance or regulation by other proteins.

6.2.2 Novelty Detection, Stress, Arousal and CFM

Part of the process of forming an associative CFM is the identification of novel CS exposure. *Egr3* expression was upregulated in the medial prefrontal cortex in association with novel CS exposure. Novelty detection is a well established role for the medial prefrontal cortex (Dias & Honey, 2002; Handa et al., 1993; Feenstra & Botterblom, 1996). Another part of the process of forming an associative CFM is the identification of the US. In CFM the US is an electric footshock and so evokes stress. *Bdnf* expression is upregulated in the CA1 of the hippocampus in association with stress. The hippocampus is a region of the brain rich in glucocorticoid receptors which are receptive to the stress regulated hormones glucocorticoids and corticosterone (Vreugdenhil et al., 2001; Revest et al., 2005). Thus it is possible that BDNF activity in the CA1 of the hippocampus may be one of the molecular mediators of the stress response. In this way upregulation of *Egr3* and *Bdnf* expression in the medial prefrontal cortex and CA1 region of the hippocampus 2 hours after CFC could indirectly contribute to the formation of the association between the context and the footshock in CFM.

Arousal is the behavioural response to stimuli that have some salience. It is unlikely that a rat experiencing a novel environment or receiving a footshock would not be aroused. When gene expression was regulated in all three behavioural groups but not in the naïve group, we suggest that this change in gene expression, which could not be associated with the novel context alone or the footshock alone, could be correlated with an arousal response. *Bdnf* expression in the CA1 and DG regions of the hippocampus, *Egr3* expression in the CA1 and expression of *Nrg1* type II splice variants in the CA1 region of the hippocampus was regulated in correlation with arousal.

Combining our findings with a previous study, *Bdnf* has been shown to be regulated in association with three different behavioural processes in the CA1 of the hippocampus following CFC. *Bdnf* is upregulated in association with the consolidation of a CFM at 30 min post-conditioning (Hall et al., 2000) and upregulated at 2 h post-conditioning in association with stress (detected at the regional level) and arousal (detected at the cellular level). Whether BDNF is functionally involved in stress response and arousal 2 hours post-conditioning is unknown, however this variation in the pattern of *Bdnf* expression within one and a half hours in one brain region highlights the dynamic nature of *Bdnf* expression and the importance of using different timepoints when investigating the molecular correlates of CFM.

6.3 Justification for and Assessment of the Approach taken in this Study in the Context of Current Schizophrenia Research

6.3.1 Problems with Progressing from Gene Variants Associated with Schizophrenia to Determining the Causal Mechanisms Underlying the Pathophysiology of Schizophrenia in Humans

Through the use of linkage, cytogenetic, gene association and GWAS studies researchers are able to identify variants in the genome that are associated with schizophrenia (Harrison & Weinberger, 2005; O'Donovan et al., 2008). Neuroimaging studies have also been used to correlate gene variants with different aspects of schizophrenia (Hall et al., 2006; McIntosh et al., 2007; Lawrie et al., 2008). These individual findings, while providing a starting place for trying to identify what actually causes schizophrenia are all only correlative findings. Schizophrenia does not have any consistent physiological identifiable markers and the disorder is currently diagnosed based on the reporting and observation of positive, negative and

cognitive symptoms (Barch, 2005). Treatment until recently has been targeted at dopamine receptors based on the findings that drugs that modulate dopamine release produce paranoid psychotic symptoms in healthy individuals (Griffith et al., 1968; Angrist & Gershon, 1970; Bell, 1973). While this shows that dopamine signalling is likely to be involved in the pathophysiology of schizophrenia it does not discern whether dopamine dysregulation is a cause or consequence of the disorder. However it is unlikely that disrupted dopamine signalling is the only molecular activity disrupted in schizophrenia as dopamine receptor antagonist drugs only reduce the severity of symptoms but do not ameliorate all the symptoms of schizophrenia (Toda & Abi-Dargham, 2007). In addition postmortem, pharmacological, neuroimaging and gene association studies suggest that glutamatergic, GABAergic and cholinergic signalling are also likely to contribute to schizophrenia pathophysiology (Lisman et al., 2008; Hall et al., 2009; see 1.1.1).

The next step in determining schizophrenia pathophysiology is to use these correlative findings to determine the causal pathophysiology of schizophrenia. In order to implicate any of the gene variants associated with schizophrenia as having a causal role in schizophrenia pathophysiology it would be necessary to either introduce the gene variant into a healthy human to see whether the individual develops schizophrenia, or to replace that gene variant with a healthy gene in a schizophrenic individual to see whether the symptoms of schizophrenia are alleviated. As it is unethical to introduce a gene variant into an individual that is thought to cause a disorder, the only way to prove that any of the identified schizophrenia susceptibility genes have a causal role in schizophrenia would be by a replacement of the gene variants with the healthy variant. The insertion of exogenous "good" DNA into the human body to replace defective DNA to treat diseases caused by a mutant allele of a gene is known as gene therapy (Friedman & Roblin, 1972). Gene therapy has only been used successfully in a few studies and some of these cases have had unexceptable side-effects such as leukaemia (Abbott, 1992; Morgan et al., 2006; Ott et al., 2006; Levine et al., 2006; Maguire et al., 2008; Kaiser, 2009). Anyhow gene therapy is very unlikely to ever work for a disorder such as schizophrenia as the genetic component contributing to schizophrenia pathogenesis is not based on one gene variant but instead results from the combination of many gene variants of varying effect sizes (Craddock et al., 2007). Therefore if gene therapy could be made to work for one of the schizophrenia susceptibility genes it is very unlikely that this would lead to alleviation of the disorder. As replacement of multiple defective genes by gene therapy is not

feasible at present, the identification of causal gene variants in schizophrenia in humans does not appear to be possible.

6.3.2 Using Animal Models to a Determine the Causal Mechanisms Underlying the Pathophysiology of Schizophrenia

In order to understand the physiological causes of schizophrenia it is necessary to perform research in animals. This however raises other problems, as schizophrenia is believed to be a disorder found only in humans. Researchers have approached this problem by performing a range of experiments in animals. A common approach has included investigating whether different schizophrenia susceptibility genes are required for animals to perform behaviours or tasks that are analogous to schizophrenia endophenotypes. Endophenotypes are specific phenotypes of behaviour that are altered in individuals with schizophrenia. These studies are performed by characterising the behaviour of germline knockout or transgenic mice in a battery of behavioural paradigms and tasks. Such a study may observe that in the absence of the schizophrenia susceptibility gene X in the mouse, behaviours A and B are altered. As behaviours A and B are also altered in schizophrenic individuals then it is possible that variants of the schizophrenia susceptibility gene X are likely to be involved in the pathophysiology underlying schizophrenia. For example, in mice carrying a null mutation in the *Dtnbp1* gene, known as the sandy mouse, impairments in working memory and in long-term memory retention, and social withdrawal have been observed (Jentsch et al., 2009; Feng et al., 2008; Takao et al., 2008; Bhardwaj et al., 2009; Cox et al., 2009). These altered behaviours are similar to some of the endophenotypes of schizophrenia and thus DTNBP1 activity is implicated in schizophrenia pathophysiology.

Another approach used to understand the pathophysiology of schizophrenia using animals includes using pharmacological agents to create a mouse model that displays impaired behaviours similar to those impaired in individuals with schizophrenia. Examples of these types of mouse models include mice treated with NMDA receptor antagonists MK801 (Hitri et al., 1993), phencyclidine or amphetamine (Mandillo et al., 2003) which show behaviour that is thought to model some aspects of schizophrenia and provide for investigation of physiology that may be disrupted in schizophrenia.

In some cases basic research in animals has identified proteins that interact with the protein products of schizophrenia susceptibility genes and identified a cellular mechanism in which the protein product is involved through *in vivo* and/or *in vitro* experiments. By combining these basic research findings with current hypotheses of schizophrenia pathogenesis, a greater understanding of the cellular pathways and mechanisms involved in schizophrenia pathophysiology can be obtained. For example a recent review by Maxwell Bennett discussed how schizophrenia susceptibility genes contribute to the molecular mechanisms controlling synapse formation and regression in the context of the neurodevelopmental hypotheses of schizophrenia (Bennett, 2010).

6.3.3 The Validity of the Approach used in this Study for Determining the Causal Mechanisms Underlying the Pathophysiology of Schizophrenia

The approach taken in this study to identify a causal role for a selection of the identified schizophrenia susceptibility genes in schizophrenia differs to other animal studies as we first studied whether they were regulated in correlation with an endophenotype of schizophenia in healthy rats and then if a gene was regulated it was further investigated to determine whether the protein product of that gene had a causal role in the endophenotype, using knockdown of the expression of the gene of interest restricted to the period of time in which the process under investigation is taking place ie. during the behavioural training. The advantages of this approach in comparison to the other approaches using animals to understand schizophrenia is that more genes can be assayed more quickly than using the knockout or transgenic approaches, and by using *in situ* hybridisation to investigate gene regulation following the behavioural task, regional specificity related to the gene's role in that behaviour can be obtained. In addition temporal and spatially specific causal roles for the genes of interest identified by transient knock down of the expression can be obtained in relation to the behaviour. Temporal and spatially specific knockdown of gene expression can also be obtained in some transgenic models but in germline knockout mice where the gene has been knocked out from the beginning of development, it is difficult to be certain whether any behaviour characterised is directly related to that gene or not For example, the change in behaviour could be due to disruption of a particular stage of development that is necessary to enable the mouse to perform the behaviour that is analysed as an index that a particular cognitive process has occurred but that is not necessarily required for the molecular process underlying the behaviour.

We investigated whether a small selection of schizophrenia susceptibility genes had a role in one of the many cognitive processes known to be abnormal in patients with schizophrenia. The processes underlying associative LTM in rats are believed to be the same as those underlying associative LTM in humans (Delgado et al., 2006). The experiments in this study did not identify a causal role for any of the schizophrenia susceptibility genes in associative LTM but this does not by any means suggest that our approach is redundant. It must be considered that there are hundreds of schizophrenia susceptibility genes and many different impaired cognitive processes in schizophrenia. Therefore it is possible that other schizophrenia susceptibility genes have a causal role in associative LTM and that the schizophrenia susceptibility genes that we have studied here could have a causal role in one or more of the other cognitive processes that are impaired in schizophrenia. In this way it can be seen that it will take a considerable amount of time yet before the causal pathophysiology of schizophrenia will be fully understood.

6.3.4 Modifications Required to the Approach used in this Study for Researching Schizophrenia Pathophysiology

The chances of identifying a causal role for a schizophrenia susceptibility gene in a schizophrenia endophenotype will increase as its role is investigated in more endophenotypes. Therefore more behavioural paradigms and tasks testing more of the endophenotypes need to be established. Behavioural paradigms other than fear conditioning that have been used to investigate molecular mechanisms of associative LTM include inhibitory avoidance (Bernabeu et al., 1997; Taubenfeld et al., 2001; Izquierdo et al., 2007), object recognition memory (Akirav & Maroun, 2006), spatial learning (Davis et al., 1998; Bonini et al., 2007), appetive (drug) pavlovian instrumental learning (Lee et al., 2005), and startle-response (Lin et al., 2003). Other endophenotypes of schizophrenia for which there are already possible analogous behavioural tests include working memory using the Morris water maze (Morris, 1984), latent inhibition using the conditioned emotional response paradigm (Sotty et al., 1996), and decision-making using the stroop test (Haddon et al., 2008). However the involvement of different schizophrenia susceptibility gene variants in different behaviours is not always going to be related to changes in gene regulation. For example, working memory does not depend on *de novo* gene transcription (McGaugh, 2000). In this case it could be disruption to the functional activity of the protein product that contributes to the altered endophenotype. Therefore it would be more

sensible to design an assay to detect the levels of the activated protein if possible, perhaps by performing a Western blot to identify whether the rats killed immediately following the working memory task eg Morris water maze training, have a different level of post-translationally modified protein compared to behavioural control rats. Alternatively, in some cases drugs could be infused to inactivate the protein during the behavioural training to see if the activated protein is required for that endophenotype.

Another consideration for future use of this approach is that recent research in the field of genomic transcription have identified that micro RNA (miRNA) and long non-coding RNA (ncRNA) are also transcribed from the genome in addition to genes and have a range of cellular roles. These miRNA and ncRNA sequences can be transcribed from DNA sequence that also encodes part of a gene (Mattick, 2009). As it is variation in the genome that has been identified in the gene association and GWAS studies it is now necessary to take into account any miRNAs or ncRNAs that might be disrupted by these genomic variations in addition to genes that might be disrupted by the variation.

6.3.5 The Future of Schizophrenia Research

As the causal role for an increasing number of the schizophrenia susceptibility genes become understood, it is likely that through combining the implicated genes with previous basic research literature, such as the protein-protein interactions of that gene's protein product, that the ability to discover further causal roles of additional schizophrenia susceptibility genes will be more forthcoming. It is not necessary to investigate all schizophrenia susceptibility genes to understand the pathophysiology of schizophrenia, as the schizophrenia susceptibility genes in this approach are only being used as a tool to help uncover the pathophysiology of schizophrenia. As explained previously it is not the identification of gene variants, even if they are causally implicated in schizophrenia, that is going to help improve treatment of schizophrenia but it is the identification of the molecular pathways that are disrupted in schizophrenia that will provide pharmaceutical targets. These targets will hopefully be more specific to the symptoms of schizophrenia and thereby maximise reduction of unwanted symptoms without developing unwanted side-effects.

6.4 Future Work

The observations from the experiments in this thesis have identified findings that are worthy of further investigation to contribute to our understanding of the molecular activity underlying hippocampal-dependent LTM, and the role of schizophrenia susceptibility genes in other schizophrenia endophenotypes.

6.4.1 Further Investigation of the Role of Schizophrenia Susceptibility Genes in LTM

Our findings that hippocampal infusion of *Egr3* antisense to knockdown EGR3 expression 90 min prior to CFC or retrieval did not effect the level of freezing behaviour demonstrated by the rats, suggested that EGR3 was not necessary for the consolidation or reconsolidation of CFM. However as stated previously our findings came with the caveat that we did not confirm that the EGR3 protein expression levels had actually been reduced under these conditions, therefore an important piece of future work would include confirmation of EGR3 knockdown following the antisense infusion. This could be done by assaying EGR3 protein levels using Western blotting following antisense infusion. It would also be important to confirm that EGR3 expression levels were not altered by the infusion of the missense oligodeoxynucleotides.

The functional roles of EGR1 and EGR3 proteins in the consolidation and reconsolidation of CFM have been investigated but the functional roles of these proteins have not been investigated in the extinction process of CFM. *Egr1* and *Egr3* mRNA expression has been shown to be upregulated in correlation with extinction of CFM, therefore it will be interesting to see if they have functional roles in extinction of CFM and if there are similar of different functional roles for these two genes in the extinction of CFM then similarities between either extinction and consolidation or extinction and reconsolidation will be identified. Furthermore, from our findings we suggested a mechanism in which EGR3 activity may be regulated by NAB2 to prevent the formation of an association between the CS and US after prolonged exposure to the CS. As suggested previously this hypothesis could be tested with *Nab2* antisense infusion targeted at the hippocampus or dorsolateral nucleus of the amygdala prior to LI to see if in the absence of NAB2 the rats do show freezing behaviour indicative of conditioning having taken place despite the prolonged CS exposure.

Further investigation of regulation of *Nrg1* type I expression following CFC in the CA3 region of the hippocampus is warranted. Firstly generation of a time profile to identify the point of peak *Nrg1* type I mRNA upregulation would be performed and then antisense knockdown of *Nrg1* type I in the CA3 could be performed to investigate whether the upregulation of *Nrg1* type I has a functional role in the consolidation of CFM.

Finally, considering that no change in *Bdnf* expression was observed at 2 hours post-CFC correlating with the consolidation of CFM in this study, but in a previous study *Bdnf* has been identified to be upregulated at 30 minutes post-CFC in correlation with the consolidation of CFM (Hall et al., 2000), and to have a functional role in the consolidation of CFM (Lee et al., 2004), it may be of interest to determine the time profile of *Bdnf* upregulation in the CA1 region of the hippocampus at 30 min, 60 min, 90 min, 2 hours, 4 hours, 8 hours and 24 hours using ISH. It would be of particular interest to analyse the tissue at 30 min to see if a small sparse population of heavily labelled Bdnf-positive cells are present at this earlier timepoint as has been observed at the 2 hour timepoint in this study.

6.4.2 Investigation of the Role of Schizophrenia Susceptibility Genes in Other Schizophrenia Related Behaviours

Other future work based on our findings that gene regulation is suggested to correlate with other behavioural phenotypes, such as LI, stress, novelty detection and arousal, could be pursued. The involvement of the investigated schizophrenia susceptibility genes in these behavioural phenotypes, may also be of interest to schizophrenia research that is trying to understand the pathophysiology of a disorder that is diagnosed based on observation of abnormal behaviour. For example, to further investigate the finding that *Egr3* is upregulated in association with novelty detection in the mPFC, the expression levels of *Egr3* could be assayed in the same region using ISH following an object recognition task.

APPENDIX

The amplification plots, dissociation curves and standard curves obtained and used in the Q-PCR analysis are provided in the following seven figures.

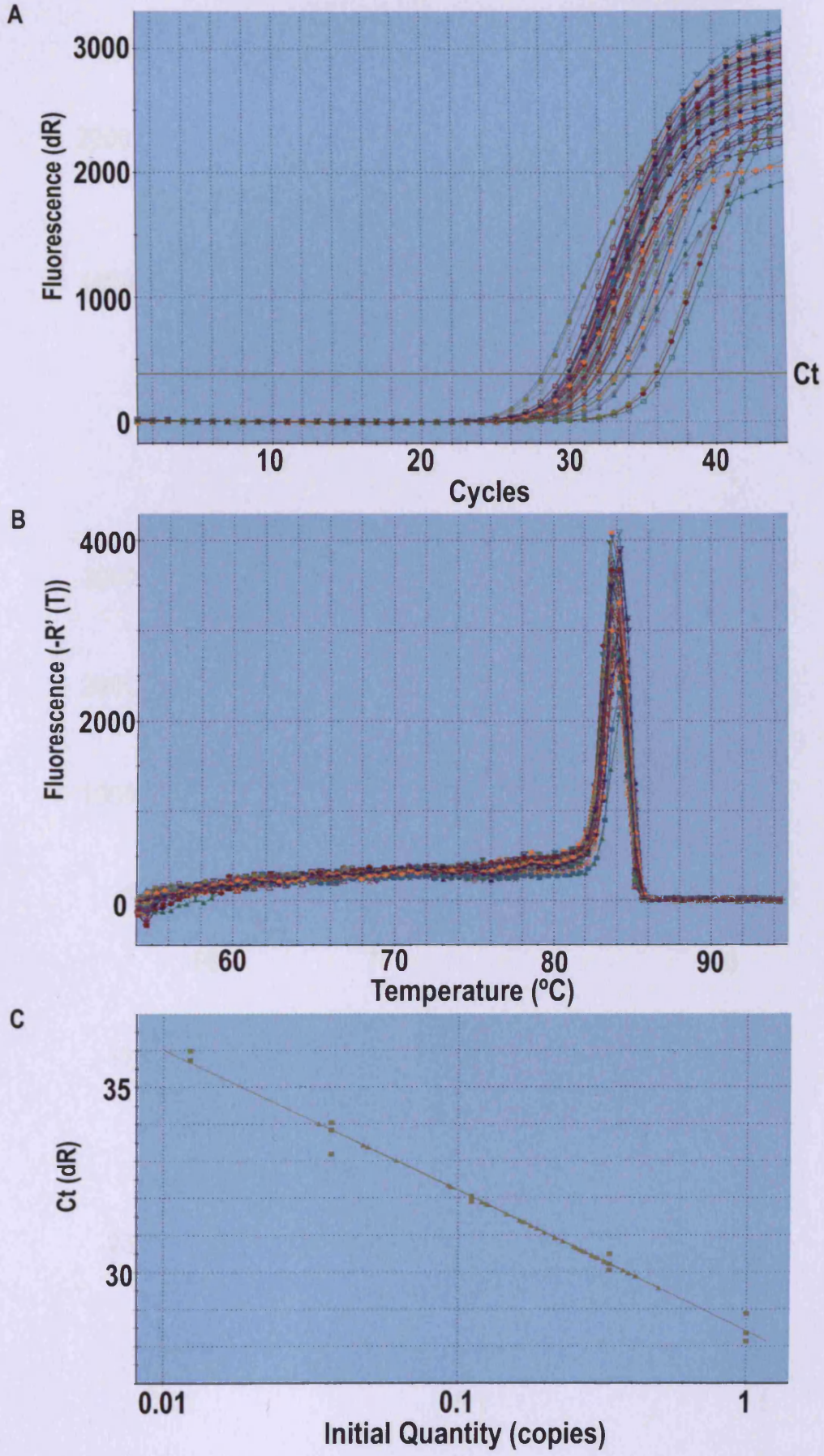


Figure 1. *EGR3* QPCR consolidation experiment 1. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

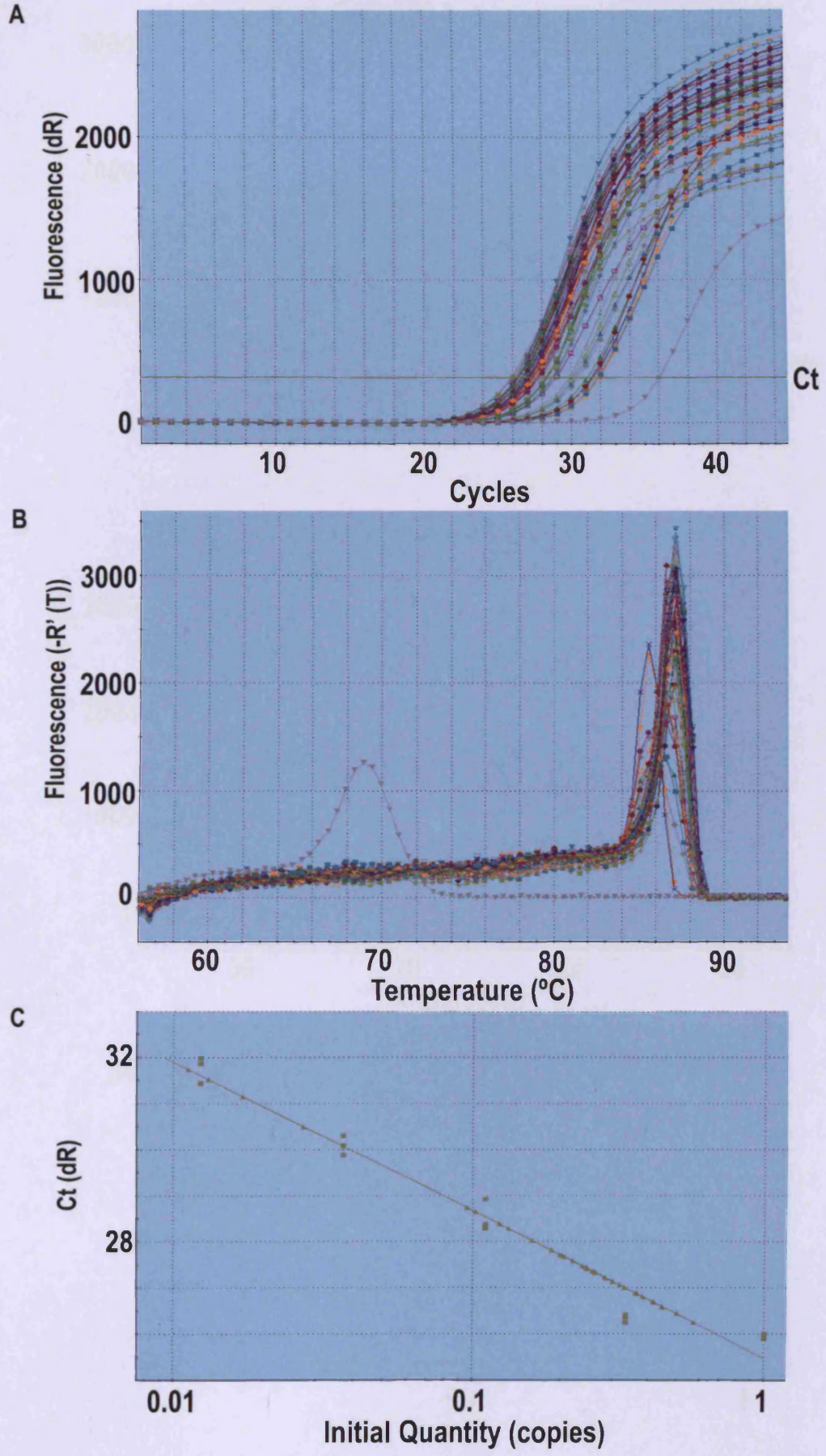


Figure 2. *UBC* QPCR consolidation experiment 1. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

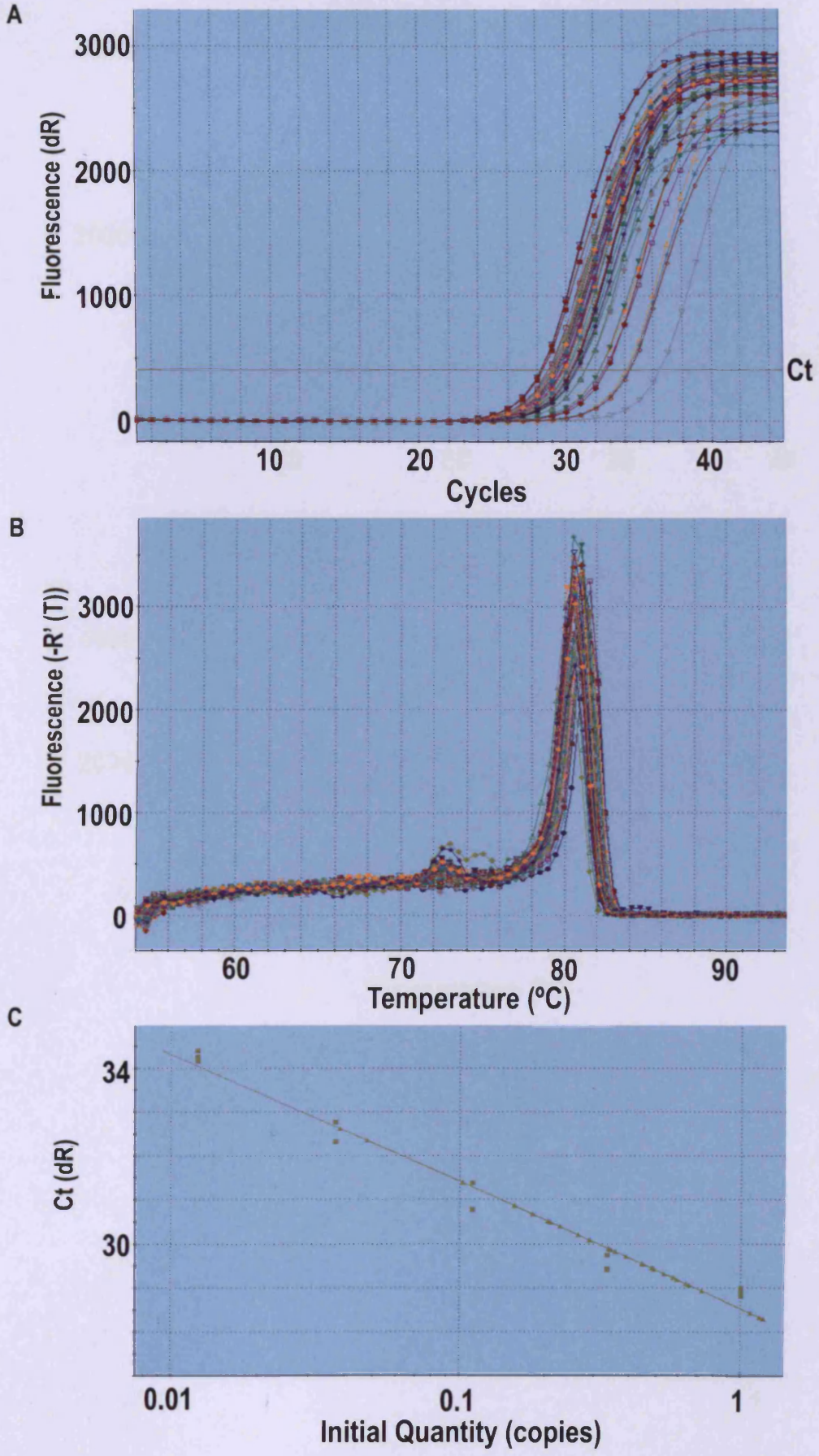


Figure 3. *SDHA* QPCR consolidation experiment 1. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

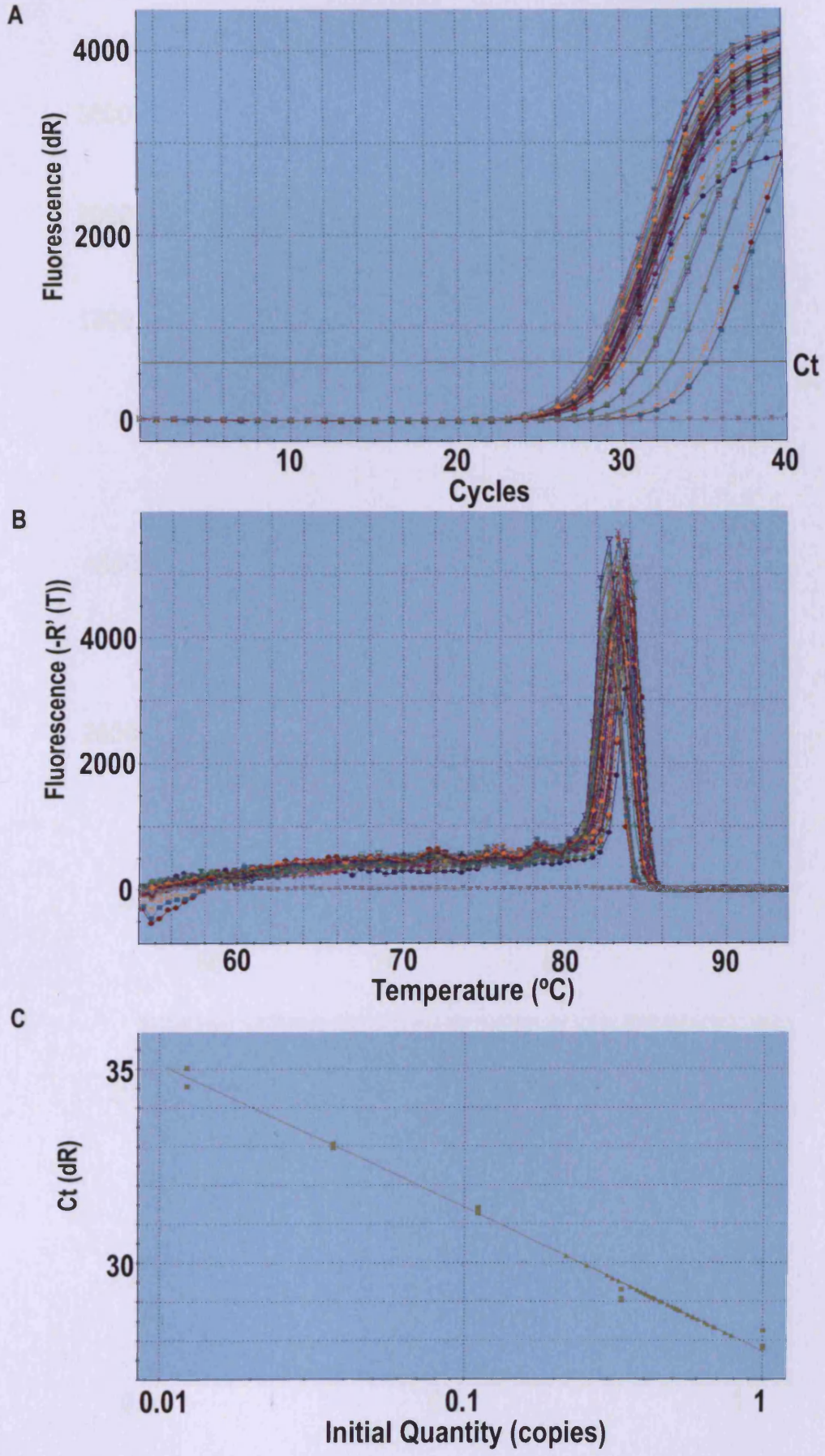


Figure 4. *EGR3* QPCR consolidation experiment 2. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

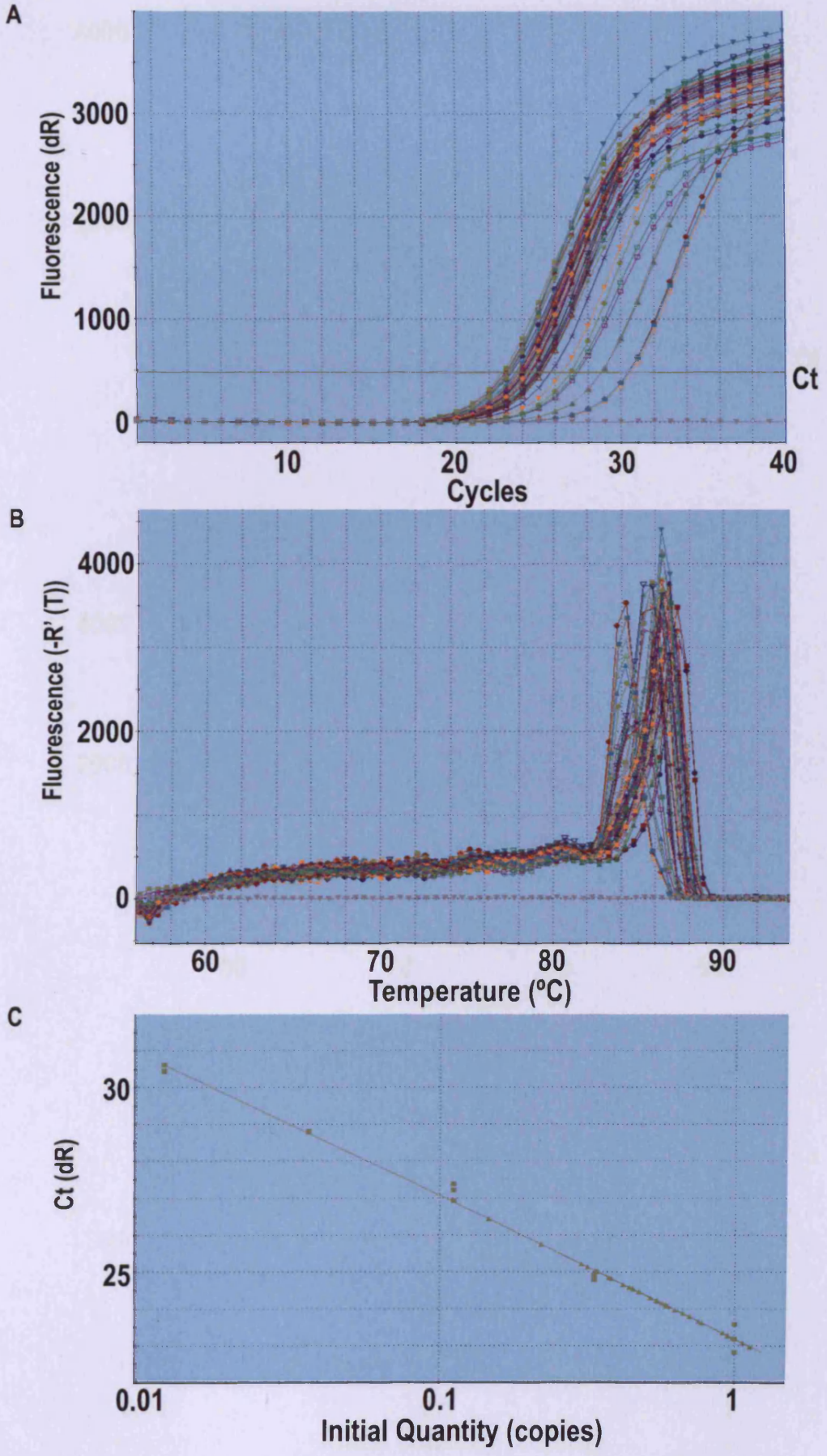


Figure 5. *UBC* QPCR consolidation experiment 2. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

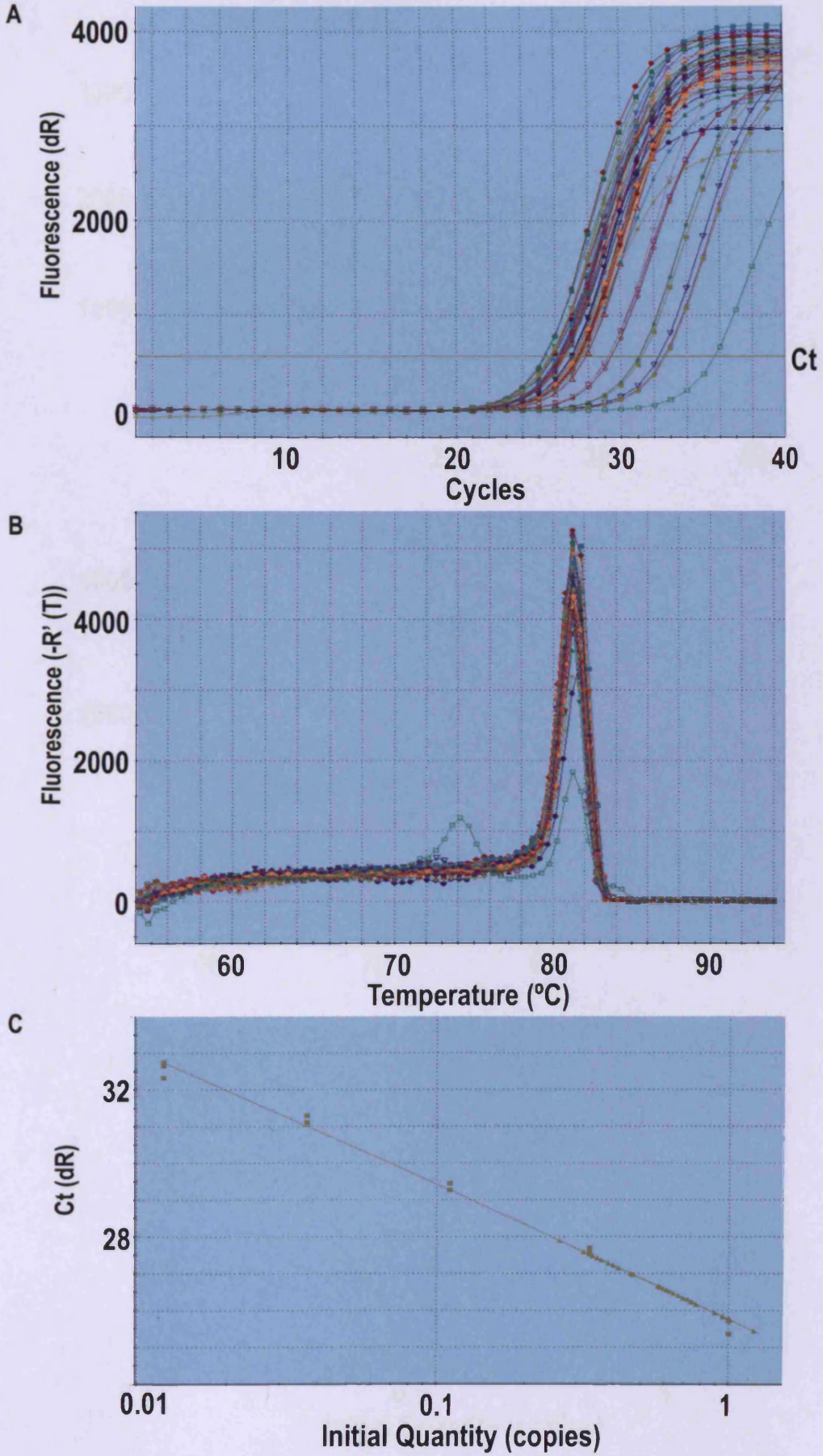


Figure 6. *SDHA* QPCR consolidation experiment 2. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

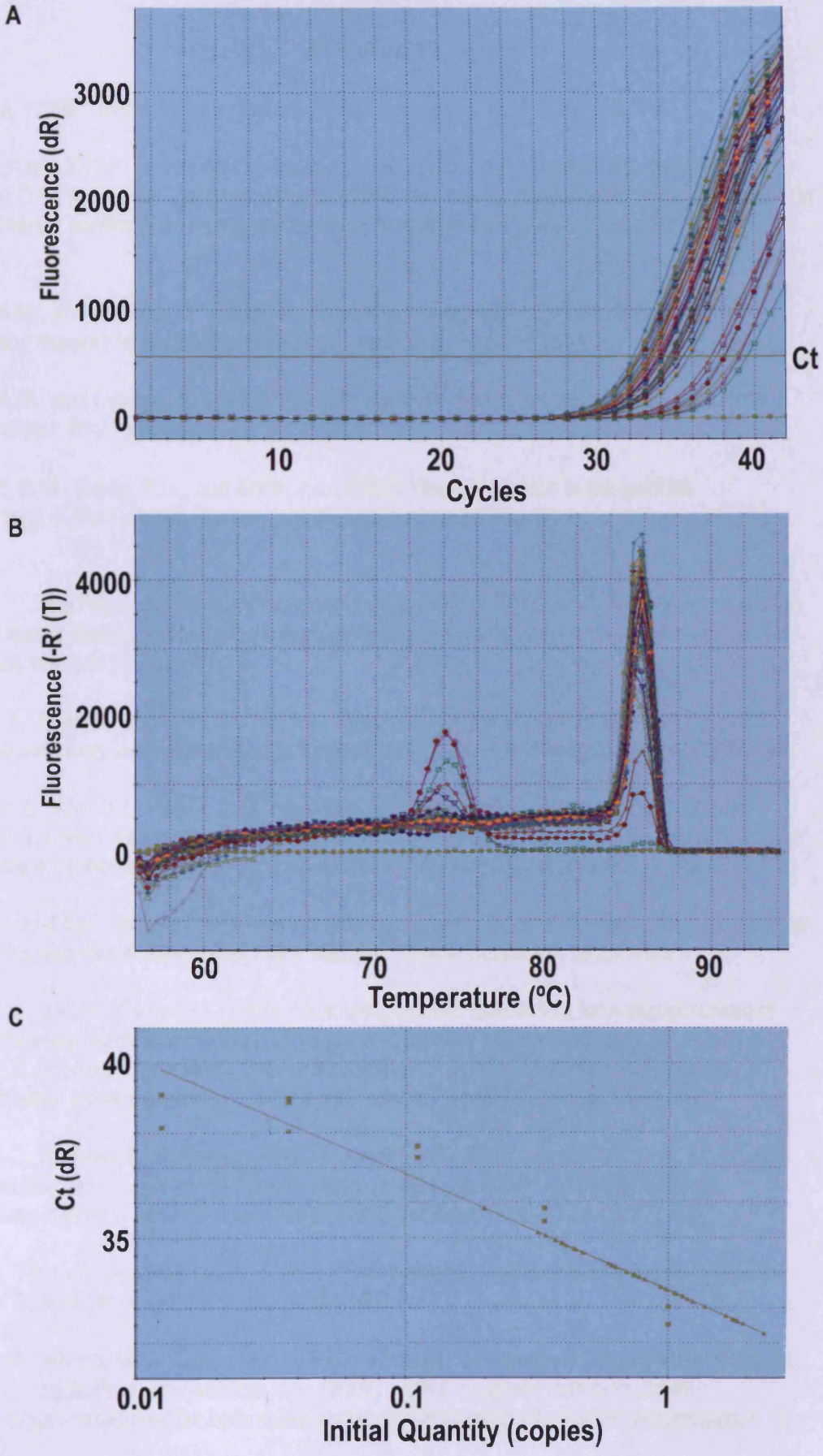


Figure 7. *HMBS* QPCR consolidation experiment 2. (A) Amplification plot. (B) Dissociation curve. (C) Standard curve. Ct, Cycle threshold; dR, magnitude of fluorescence of Reporter dye; -R' (T), negative first-derivative of the melting curve; T, Temperature.

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