Molecular pathways responsible for NMDA receptor-mediated behavioural plasticity

by

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Abstract

Neurodevelopmental disorders occur from genetic and environmental risk factors that impair the formation of networks involved in learning and memory. Impaired NMDA receptor signaling has been implicated in the pathobiology of schizophrenia and autism. A mouse model of NMDA receptor knockdown and inducible rescue was used to determine the reversibility of neurodevelopmental insults on hippocampal function. Mice were studied for their ability to learn and remember using the fear-conditioning paradigm, which engages the hippocampus in the formation and recall of contextual memory. In addition, studies were performed to determine whether immediate-early genes were induced in the hippocampus of NMDA receptor deficient mice. The findings of this study show that NMDA receptor deficient mice lack the ability to form fear memories, and that this can be partially rescued by increasing the levels of NMDA receptors in adult mice. Furthermore, several immediate early genes are induced without a resulting formation of fear memory.

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Abbreviations

5-HT	5-hydroxytryptamine
AKAP75	A-kinase anchoring protein 75
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP1	Activator protein 1
AP-5	2R-amino-5-phosphonovaleric acid
Arc	Activity regulated cytoskeleton associated protein
ATD	Amino terminal domain
Bdnf	Brain derived neurotrophic factor
CA1/2/3	Cornu ammonis 1/2/3
CaM	Calcium modulated protein
CaMKIIα	Ca ²⁺ /calmodulin dependent protein kinase II alpha
cAMP	Cyclic adenosine monophosphate
CREB	cAMP response element binding protein
CS	Conditioned stimulus
DEPC water	Diethylpyrocarbonate treated water
DTT	Dithiothreitol
Dusp1	Dual specificity protein phosphatase 1
EDTA	Ethylenediaminetetraacetic acid
Egr1	Early Growth Response 1
EPSC	Excitatory postsynaptic current
EPSP	Excitatory postsynaptic potential
ER	Endoplasmic reticulum
ErbB4	Erb-B2 Receptor Tyrosine Kinase 4
ERK	Extracellular Signal-regulated Kinase-1
Fos	FBJ Murine Osteosarcoma Viral Oncogene Homolog
GABA	Gamma-aminobutyric acid
GDP	Guanosine phosphate
GEF	Guanosine nucleotide exchange factor
GTP	Guanosine triphosphate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HOMER1a	Homer protein homolog 1
IEG	Immediate early genes
IHC	Immunohistochemistry
LBD	Ligand binding domain
LTD	Long term depression
LTP	Long term potentiation
MAGUK	Membrane-associated guanylate kinases
MAP2	Microtubule associated protein 2
MAPK	Mitogen-activated protein kinase
MEK	Mitogen-activated protein kinase
MK801	Dizocilpine
NFKβ	nuclear factor kappa-light-chain-enhancer of activated B cells
NGFI-B	nerve growth factor IB
NMDA	N-methyl-D-aspartate
NR1	NMDA receptor subunit 1

NR1-Cre	NMDA receptor subunit 1 expressing Cre-recombinase
NR1-KD	NMDA receptor subunit 1 knockdown
<i>Nr4a1</i>	Nuclear Receptor Subfamily 4 Group A Member 1
NRF-1	Nuclear respiratory factor 1
NTP	Nucleoside triphosphate
PBS	Phosphate-buffered saline
PCP	Phencyclidine
PKA	Protein kinase A
PLCγ	Phospholipase gamma
PMSF	Phenylmethane sulfonyl fluoride
PP1	Protein phosphatase 1
PPI	Pre-pulse inhibition
PSD	Postsynaptic density
PVDF	Polyvinylidene difluoride
QPCR	Quantitative polymerase chain reaction
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
SAP	Synapse associated protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE	Soluble N-ethylmaleimide-sensitive factor activating protein receptor
SynGAP	Synaptic Ras GTPase-activating protein 1
TBST	Tris-buffered saline with Tween 20
US	Unconditioned stimulus

Chapter 1

INTRODUCTION

1 NMDA receptors

NMDA receptors are critical for brain development, a process necessary to form neural networks permitting cognitive functions such as learning and memory. The role of these receptors in learning and memory has been extensively studied, and continues to be studied today. Signaling cascades from the activation of NMDA receptors, is thought to form the molecular basis of memory. These molecular events include activation of kinases, transcription factors, gene and protein synthesis and structural stability of synapses. From beginning to end, NMDA receptors are required in forming hippocampus dependent learning and memory.

NMDA receptors also play a role in forming activity-dependent connections during development. Because of this, it is likely that developmental loss of NMDA receptor signaling would impair proper connectivity that could have lasting effects in adulthood. To understand the permanency of alterations in cellular connectivity as a result of developmentally impaired NMDA receptor function, we wanted to perform adult rescue of NMDA receptor levels and demonstrate the level of plasticity permissible within the adult brain. I studied the role of NMDA receptors specifically within the hippocampus, a brain region known to retain high levels of plasticity. From the results, I hoped to understand the extent to which hippocampal dependent memory can recover from a developmental insult in NMDA receptor function.

1.1 Structure

N-methyl-D-aspartate receptor (NMDA receptor) family comprises three different subunits. GluN1 (NR1) subunit is encoded by a single gene producing 8 splice variants, GluN2 (NR2A-D) and GluN3 (NR3A and B) subunits are encoded by 4 and 2 genes respectively. NMDA receptors form heterotetramers composed of two obligatory NR1 subunits and two NR2 and/or NR3 subunits (Monyer et al. 1992; Ishii et al. 1993; Sobolevsky et al. 2009). The receptor has 4 domains involving an extracellular amino terminal domain (ATD), a ligand binding domain (LBD), a transmembrane domain and a carboxy-terminal domain (Stephenson et al. 2008). There are three transmembrane regions (M1, M3 and M4) that create the channel pore (Blanke & VanDongen 2009). M3 and M2 are required in channel gating, while M2 is also required in

cation specificity of the pore (Vyklícky et al. 2014). The carboxy-terminal domain interacts with intracellular scaffolding proteins that can be phosphorylated in order to regulate surface expression of receptors (Traynelis et al. 2010a).

NMDA receptors composed of NR1 and NR2 subunits combine in a 1:1 ratio and require NR1 to bind two molecules of glycine and NR2 to bind two molecules of glutamate through their respective LBDs (Miyazaki et al. 1999; Furukawa & Gouaux 2003; Sobolevsky et al. 2009; Vyklícky et al. 2014). NR1 and NR2 subunits create a Ca^{2+} permeable pore with a voltage dependent Mg^{2+} block (Nowak et al. 1984; Chatterton et al. 2002). Upon receptor activation, conformational changes in the LBD causes rotations in M3 causing M2 to move away from the pore, removing the physical pore block (Vyklícky et al. 2014). The M2 membrane domain and negatively charged residues C-terminal to the M3 membrane domain, selectively permits Ca^{2+} entry through the pore (Salter et al. 2009).

Figure 1.1 Schematic diagram of NMDA receptor functional domains. ATD, aminoterminal domain. LBD, ligand binding domain. TMD, transmembrane domain. CTD, carboxy-terminal domain.

1.2 Regulation

Transcriptional regulation of *Grin1* (NR1) and *Grin2* (NR2) are important in mediating surface expression (Ramsey 2009; Jiang & Jia 2009). Upregulation can occur through transcription factor binding of the promotor region, a silencer region in exon1 and a 27bp GC-rich region (Salter et al. 2009). Transcription factors that bind to these regions include Sp1, AP1 complex

(Fos/Jun), NRF-1, Tbr-1, CREB and NF κ B (Salter et al. 2009). Once translated, an ER retention signal prevents NR1 and NR2 from being exported out of the ER without co-expression of both subunits (McIlhinney et al. 1998; Standley et al. 2000). NR1 subunits are expressed in excess of NR2A due to a reduced rate of degradation (Atlason et al. 2007). Stabilization of NR2A expression only occurs after co-expression with NR1 (Atlason et al. 2007). This thesis will further characterize NR1 and NR2A subunit expression at synaptic densities.

1.3 Function

Upon binding of glutamate and glycine, the physical barrier is removed from the pore opening by way of M2 and M3 structural shifts (Vyklicky et al. 2014). In addition, the Mg²⁺ block within the channel pore requires a voltage-dependent release from the channel pore, preventing spontaneous receptor activation (Blanke & VanDongen 2009). Requirement for both receptor ligand and voltage gating mechanism provides coincidence detection. Agonist and co-agonist binding, as well as the removal of the Mg²⁺ block causes influx of Ca²⁺ selectively through the pore. The slow activation and deactivation of the receptor allow for functional and structural plasticity (Lester et al. 1990). Deactivation of the receptor is dependent on receptor subunit composition and the level of post-synaptic activity (Furukawa & Gouaux 2003). In addition, modifications to the receptor such as phosphorylation of the receptor or allosteric modulation can reduce deactivation time (Blanke & VanDongen 2009). In this way, presynaptic activity can be encoded by the extent of NMDA receptors activation and consequent upregulation of downstream effectors.

1.4 Downstream Signaling

NMDA receptor mediated influx of calcium leads to the activation of downstream kinases that propagate and amplify the signal. Activated kinases include calcium/calmodulin (CaM)-dependent protein kinase II (CaMK), protein kinase A (PKA) and mitogen-activated protein kinase (MAPK). Through kinase activity, activated downstream mechanisms potentiate the synapse.

1.4.1 PKA

CaM activation of adenylyl cyclase amplifies the activation signal thorough increases in cAMP, which can further activate PKA. PKA phosphorylates AMPA receptors and opposes the activity

of the phosphatase PP1, increasing receptor cell surface expression (Wang & Peng 2016). In addition, PKA regulates the activity of PP1 by disassociating it from its anchoring proteins spinophilin and neurabin, which can affect spine morphology (Oliver et al. 2002; Nguyen & Woo 2003). PKA can also activate mitogen activated protein kinase kinase (MEK), which is upstream of MAPK activation (Waltereit & Weller 2003). PKA can be localized to post-synaptic densities through association with anchoring proteins such as MAP2 and AKAP75 (Nguyen & Woo 2003). Structural modulation can also be mediated by PKA dependent phosphorylation of SNARE-binding protein complexin, increasing neurotransmitter release (Cho et al. 2015).

1.4.2 MAPK

MAPK activation involves calcium induced GEFs that increase the ratio of Ras-GTP to Ras-GDP, leading to activation of Raf (Thomas & Huganir 2004). Raf induction of MEK causes phosphorylation and activation of MAPK (Rosen et al. 1994). Once activated, MAPK is capable of phosphorylating nuclear transcription factors by translocating to the nucleus once activated (Wang & Peng 2016). MAPK can also increase the AMPA receptor cell surface expression and increase dendritic spines (Wang & Peng 2016). Studies suggest a requirement for MAPK activity in long-term potentiation (LTP) and induction of CREB, described in section 4.2.8 *Duspl* (Atkins et al. 1998).

1.4.3 CaMKII

CaM binding to CaMKII allows auto-phosphorylation and activation of the kinase. Once activated the kinase is capable of directly interacting with NMDA receptors and AMPA receptors to stabilize cell surface expression (Nguyen & Woo 2003; Coultrap & Bayer 2012). In addition, PSD-95 proteins such as α -actinin, PSD-95, synaptic adhesion molecule, densin180 has been shown to interact with CaMKII (Wang & Peng 2016). CaMKII can activate microtubule associated protein 2 and neurofilament L, which can further structurally stabilize receptors to the postsynapse (Wang & Peng 2016). CaMKII may also take part in activation of MAPK by inhibition of SynGAP, a Ras inhibitor (Waltereit & Weller 2003) and activate CREB, as described in section 4.2.7 CaMKII α (Thomas & Huganir 2004). Through these mechanisms, CaMKII activity is required in LTP generation upon NMDA receptor activation (Silva et al. 1992).

1.5 Memory

LTP is a molecular mechanism of memory that can be generated by NMDA receptors through stable structural and functional modifications at activated synapses. Bliss and Collingridge found that a tetanic stimulation increased synaptic potentials over long periods of time, spanning months in animals (Bliss & Collingridge 1993). Further implication of NMDA receptor activity in memory was demonstrated by the use of AP5 causing impairments in both LTP and spatial memory (Morris et al. 1986). Similar results were found in a genetic approach using hippocampus selective NR1 knockout (Tsien et al. 1996).

Memory consolidation is a process by which stabilization of memory occurs through NMDA receptor mediated molecular processes. Initially, memory is susceptible to manipulations by protein synthesis inhibitors, inhibitors of gene transcription or seizures (Nakazawa et al. 2004). The process by which long-term memory is formed, involves the activity of NMDA receptors and its downstream signalling molecules, such as PKA, CaMKII α and MAPK. The activity of these kinases can stabilize the structure and function within activated synapse through both immediate protein synthesis and upregulation of gene expression. Requirement of NMDA receptors activity up to 2h after training and not days following training in a spatial learning task, supported the idea of a time window during which protein and gene expression were required for memory consolidation (Nakazawa et al. 2004).

Once memory has been encoded, NMDA receptors might be specifically required in the retrieval of associative memory. Evidence has shown that recall of simple spatial cues are maintained with NMDA receptor antagonism following training (Nakazawa et al. 2004). However, retrieval of spatial reference memory is impaired in hippocampal NR1 knockout mice due to an inability to activate neural networks that are formed from association of different spatial cues (Nakazawa et al. 2002). In order to generate a place map, associative learning links different cues within the surrounding environment. This creates a network of neurons that are associated with each other, such that firing of one neuron activates the complete network (Nakazawa et al. 2004). Therefore, impaired NMDA receptor signalling can affect recall of different spatial cues that provide a reference of place.

1.6 NMDA receptor hypofunction in schizophrenia

Disorders affecting NMDA receptor function can impair cognitive processes, such as learning, decision making, attention and executive function. Pharmacological agents have been unable to improve cognitive impairments in schizophrenia and mood disorders, highlighting the need for research in this field (Millan et al. 2016). Current research points to the glutamate signalling pathway as a potential target for improving memory.

NMDA receptors dysfunction hypothesis is a central unifying concept behind the pathophysiology of schizophrenia. Earlier PET studies had found reduced tonic and increased phasic dopamine transmission within the prefrontal cortex, and hyperdopaminergia within the striatum (Frohlich & Van Horn 2014). In support of this, pharmacological dopamine receptor antagonists have been used as antipsychotic drugs. Therefore, a dysregulation in the dopamine neurotransmitter system was thought to underlie the etiology of schizophrenia. Further research found that NMDA receptor antagonists, such as phencyclidine and ketamine, produced negative, positive and cognitive symptoms of schizophrenia in healthy patients and worsened symptoms in schizophrenia patients (Krystal et al. 1994; Lahti et al. 1995). Furthermore, similar to PET studies ketamine induce hyperdopaminergic neurotransmission downstream of NMDA receptor dysfunction, suggesting a possible interaction between NMDA receptor dysfunction and dysregulation of the dopamine system (Coyle 2012). In addition, dysfunction of both glutamate and dopamine neurotransmitter systems provided a molecular mechanism for the combination of symptoms present in schizophrenia (Lakhan et al. 2013). Currently, numerous evidence from post-mortem brains, genetic and pharmacological manipulations suggest a pathological consequence for deficits in NMDA receptor signalling in schizophrenia.

1.6.1 Genetic evidence for a role of NMDA receptors in schizophrenia

Genome wide association studies in patients, have identified susceptible genes that are functionally linked to NMDA receptor signalling (Greenwood et al. 2011). From these candidate genes, many genetic models of schizophrenia have been created. DISC1 can acutely increase NMDA receptor and AMPA receptor membrane expression, however chronic knockout of the gene downregulates surface expression of both receptors, in a PKA and CREB dependent manner (Wang & Zhu 2014). Other genetic models include neuregulin1, which is a growth factor that signals through ErbB4 receptors to regulate development of neurons (Harrison & Law

2006). Interestingly, both over expression and under expression of neuregulin1 impairs regulation of NMDA receptors causing reduced cell surface expression (Stefansson et al. 2002; Pitcher et al. 2011). Dysbindin is another candidate gene important in receptor trafficking of NMDA receptors to the cell surface (Tang et al. 2009). Multiple susceptibility genes affecting NMDA receptor signaling (Hovatta et al. 1999) provides strong evidence for the involvement of NMDA receptor hypofunction in schizophrenia.

1.6.2 Environmental factors and schizophrenia

Genetic heritability in schizophrenia accounts for 70-85% of individuals and does not follow mendelian genetics (Lewis & Levitt 2002). In fact, monozygotic twin studies found only a 50% likelihood of both twins developing schizophrenia (Lewis & Levitt 2002). Therefore, schizophrenia is thought to occur from a combination of genetic predisposition and environmental adverse events. The involvement of environmental factors gained attention from studies that found children born from prenatal infections, prenatal malnutrition and obstetrical complications had a greater risk of developing schizophrenia (Lewis & Levitt 2002). Animal models of environmental stress on the development of psychosis has identified maternal immune activation and post-weaning social stress as potential mediators (Jones et al. 2011a). Maternal immune activation showed increased serotonin 5-HT receptors and decreased metabotropic glutamate receptor levels in their offspring, causing cognitive impairments in spatial learning (Holloway et al. 2013). These studies highlight the impact of environmental insults on neurodevelopment, and their potential role in psychosis.

1.6.3 Neurodevelopmental Disorders

Neurodevelopmental disorders have a delayed symptomatic onset due to a progressive genetic or environmental susceptibility throughout brain development. Such disorders are complex in nature involving multiple genes and a variety of environmental insults, which together increase the risk for psychosis (Lewis & Levitt 2002). Autism, schizophrenia, intellectual disability and epilepsy are among those termed neurodevelopmental disorders (Burnashev & Szepietowski 2015). Individuals with schizophrenia can start to exhibit milder forms of social withdrawal and irritability at a younger age, while the complete set of symptoms do not emerge until adolescence (Malmberg et al. 1998). This slow progressive display of symptoms is thought to result from perturbations during critical periods of neurodevelopment during early stage of life, which fully

manifests when circuits have completed maturation (Marenco & Weinberger 2000). In line with these clinical observations, several animal models have been developed that have an adolescent onset of behavioural symptoms. These include models with: genetic impairments in glutamate signaling, prenatal stress, neonatal hippocampal lesions, post-weaning social isolation and perinatal immune activation (Jones et al. 2011b; Holloway et al. 2013; Milenkovic et al. 2014).

The defining features of neurodevelopmental disorders are prenatal adverse events, the age dependent onset of symptoms, the heterogeneous etiology of these disorders and the potential improvement in symptoms with early treatment (Lewis & Levitt 2002). Early prognosis and treatment has been shown to improve severity in the onset of symptoms (Morrison et al. 2004). This is true not only for treatment with antipsychotic drugs, but also with other types of intervention such as dietary omega-3 supplementation. Interventions that can change the trajectory for the development of psychosis are important areas of research, particularly if the interventions can benefit the development of critical circuits, which current antipsychotic treatments cannot achieve.

2 Transgenic model of NMDA receptor hypofunction

Pharmacological administration of phencyclidine (PCP), ketamine and MK801 have been used to model NMDA receptor hypofunction. However, the limitation of this application is apparent in studying neurodevelopmental disorders with chronic impairments in glutamate neurotransmission. A genetic approach produces similar phenotypes as those seen using pharmacological methods and more importantly can also induce negative, positive and cognitive impairments seen in schizophrenia. Other advantages include less invasive lesions, studies of developmental network function and their implications on other neurotransmitter systems, targeted alterations and use of the natural biological machinery.

2.1 Generation

Null mutations of NR1 were demonstrated to be lethal, therefore subsequent studies used region specific NR1 knockout or global NR1 knockdown (Forrest et al. 1994; Elberger & Deng 2003; Mohn et al. 1999). A genetically engineered NMDA receptor knockdown mouse was used to study glutamate receptor hypofunction. NMDA receptor knockdown was induced by insertion of a 2kb neomycin cassette encoding a polyadenylation sequence at intron 19 of the *Grin1* gene,

producing truncated and non-functional mRNA (Mohn et al. 1999). NR1 knockdown (NR1-KD) mice expressed only 5-10% of basal NR1 mRNA and protein levels (Mohn et al. 1999). In response, NR2A and 2B subunits were also reduced to 10% of basal levels, which translated to an equal deficit in functional NMDA receptors surface expression (Mohn et al. 1999). As previously mentioned, only tetrameric NMDA receptors are released from the ER, therefore an excess of NR2A and 2B is degraded by the 26S proteasome (Ramsey et al. 2008). Brain region specific reductions in expression was proportional to the endogenous NMDA receptor expression patterns, whereby the greatest reductions were in regions with the highest endogenous expression (Ramsey 2009). Compared to controls, NR1-KD mice showed regional [3H] MK801 binding of: 10% in hippocampus, 14% in frontal cortex, 21% in striatum and 40% in brainstem (Ramsey 2009).

A new mouse line generated to allow inducible rescue of NR1-KD was performed through tamoxifen induced Cre-recombinase excision of neomycin floxed loxP sites (Figure 1.2) (Mielnik et al., in preparation). Cre-recombinase is expressed under the ROSA26 promotor, which causes ubiquitous expression throughout the brain (Soriano 1999). Cre-recombinase is fused to an estrogen receptor, which allows it to remain in the cytosol bound to a HSP90 heat shock protein, preventing its activity (Hayashi & McMahon 2002). The estrogen receptor contains a mutant ligand binding domain, allowing it to bind tamoxifen (Hayashi & McMahon 2002). Binding of tamoxifen causes HSP60 to release the estrogen receptor fused Cre-recombinase, allowing translocation into the nucleus where Cre-recombinase excises loxP sites (Hayashi & McMahon 2002). Following tamoxifen administration, the neomycin cassette floxed loxP sites undergo excision by Cre-recombinase. Cre-recombinase activity has been characterized within the hippocampus and other brain regions of NR1-Cre mice (Figure A2). Following neomycin excision, electrophysiological data suggests that cellular membrane potentials are intact and that evoked NMDA receptor potentials recover to WT levels, while NR1-KD mice have perturbed evoked potentials (Figure A3). However, rescue is brain region selective with cortical layer V of the prefrontal cortex having better rescue compared to the striatum (Figure A3). Activity within the hippocampus will be characterized in future experiments.

To generate the NR1 knockdown (NR1-KD) and inducible rescue (NR1-Cre) mouse lines, C57Bl/6 *Grin1*^{neo/+} mice were crossed with C57Bl/6J *ROSA26*^{Cre-ERT2/+} (Mielnik et al., in preparation). The resulting C57Bl/6J *Grin1*^{neo/+}-*ROSA26*^{Cre-ERT2/+} were crossed with 129X1/SvJ

$Grin1^{neo/+}$ to produce the F1 progeny. An F1 background was used to increase hybrid vigor. The F1 progeny predicted Mendelian inheritance of 12.5% for each genotype: $Grin1^{+/+}$ (WT), $Grin1^{+/+}$ - $ROSA26^{Cre-ERT2/+}$ (WT-Cre), $Grin1^{neo/neo}$ (NR1-KD) and $Grin1^{neo/neo}$ - $ROSA26^{Cre-ERT2/+}$ (NR1-Cre). Behavioural and molecular characterization for NR1-Cre mice are currently underway (Mielnik et al., in preparation).

Figure 1.2 Schematic diagram of neomycin insertion mutation. *Grin1* intronic insertion of neomycin cassette creating NR1 knockdown and Cre-induced excision of neomycin. The inserted neomycin cassette causes the RNA polymerase to fall off of the DNA, producing truncated pre-mature mRNA transcripts, which are degraded (Mohn et al. 1999). Occasionally, the RNA polymerase can read through the neomycin cassette producing full transcripts. The administration of tamoxifen allows Cre-recombinase induced excision of loxP sites causing the removal of the neomycin cassette and restoration of full transcript expression in NR1-Cre mice.

2.2 Behavioural Consequences of NMDAR hypofunction

As mentioned previously, there are numerous mouse models of schizophrenia that have been used to study different aspects of the disorder. These models generally exhibit a panel of abnormal behaviours that include: increased activity and stereotypy, impaired sensorimotor gating (PPI), impaired working memory and sociability, and impaired executive function and cognitive flexibility (Jones et al. 2011).

NR1-KD mice were impaired in sociability, PPI, working memory and executive function and showed abnormal anxiety related behaviours (Mohn et al. 1999; Halene et al. 2009; Milenkovic

et al. 2014). Similar to schizophrenia, hyperactivity in NR1-KD mice could be reversed using antipsychotics, such as haloperidol, clozapine, olanzapine and quinpirole (Ramsey 2009). Further molecular analysis found abnormal gamma and theta oscillation in response to auditory and visual cues and as a result impaired performance in PPI (Ramsey 2009). Deficits in sociability was further studied using cFos immunohistochemistry. This study showed that NR1-KD mice had reduced neuron activation in the cingulate cortex and lateral septum following social interaction with a novel mouse (Mielnik et al. 2014). Interestingly, administration of diazepam, an anxiolytic GABA receptor allosteric modulator, improved social interactions and cFos expression in NR1-KD mice (Mielnik et al. 2014).

Indeed, evidence suggests that many of the behavioural abnormalities of NR1-KD may in fact be caused by reduced NMDA receptor mediated GABAergic tone (Gandal et al. 2012). It has been proposed that reduced NMDA receptors activation on GABAergic neurons and the resulting diminished GABAergic tone can cause post-synaptic disinhibition and over excitation, ultimately leading to behavioural abnormalities. Baclofen, a GABA-B receptor agonist improved PPI, locomotor hyperactivity, sociability and spatial learning in NR1-KD mice (Gandal et al. 2012). Further molecular analysis also found reduced expression of parvalbumin expressing GABA neurons in the sensorimotor cortex and prefrontal cortex, as well as abnormal intrinsic cellular activity and gamma oscillations in NR1-KD mice (Gandal et al. 2012). These studies show altered modulatory mechanisms in the brain as a result of NMDA receptor hypofunction. One such modulation might occur through upregulation in kainate receptor expression in NR1 knockdown mice (Duncan et al. 2012). In fact, antagonists for the kainate receptor has been shown to improve PPI acoustic startle response (Duncan et al. 2012). Future studies will be required to uncover the complete adaptations on developmental behaviours and neural circuits in a hypoglutamatergic system.

Developmentally, the behavioural effects of NMDA receptor hypofunction were mainly displayed in adolescent and adult mice, compared to younger mice (Milenkovic et al. 2014). Schizophrenia in humans also has an adolescent onset, with minimal exhibition of behavioural symptoms at younger ages (Malmberg et al. 1998). NR1-KD mice displayed an adolescent (six weeks) onset of behavioural impairments in sociability and working memory (Milenkovic et al. 2014). Mice aged three weeks or younger developed attenuated locomotor, stereotypy and

executive function and PPI deficits, in comparison to adolescents (Moy et al. 2012; Milenkovic et al. 2014).

Recent behavioural characterization of NR1-Cre mice has shown plasticity in the glutamatergic network. Behavioural data showed improvements in sociability, executive function, working memory, locomotor activity, anxiety and PPI in NR1-Cre mice following NMDA receptor expression rescue in ten week adults (Figure A1; Mielnik et al. in preparation). However, the extent of rescue is not similar across all behaviours, implying that varying levels of plasticity exists in the glutamatergic network. Exploration in the plasticity of the glutamate system and its effects on cognitive function will be further characterized in this thesis.

2.3 Molecular Characterization

Behavioural studies on NR1 knockdown showing effects on locomotor activity, may suggest a potential abnormality in the dopaminergic system. Dopamine receptors D1, D2 and D3 radio-ligand binding demonstrated no observed abnormalities in the dopaminergic pathway within the dorsal striatum in NR1KD mice (Ramsey et al. 2008). Further investigation revealed increases in dopamine re-uptake transporter levels at the presynaptic button in the nucleus accumbens and caudate putamen, indicating a hyperdopaminergic state (Ferris et al. 2014). Within the ventral tegmental area, dopaminergic neurons are hyper-excitabile and as a result can produce atypical reward motivated behaviours (Ferris et al. 2014). In support of this, NR1-KD mice showed abnormal reward learning with reduced sensitization to cocaine (Ramsey et al. 2008). As seen in schizophrenia, NMDA receptor deficits produced hyperactivity within the dopamine system.

NMDA receptor hypofunction did not alter the AMPA receptor cell surface expression through modulatory mechanisms (Ramsey et al. 2008). As previously mentioned, NR1-KD mice have reduced GABAergic tone in addition to impaired neural activation evidenced by reduced c-FOS staining (Gandal et al. 2012; Mielnik et al. 2014). Characterization of NR1-Cre mice are currently underway, and will be further explored in this thesis.

3 Hippocampus

3.1 Structural Organization

The hippocampus is made up of cornu ammonis (CA) 1, CA2 and CA3. The dentate gyrus, entorhinal cortex, subiculum, presubiculum and parasubiculum make up the rest of the hippocampal formation (Andersen 2007). Majority of hippocampal inputs are relayed through superficial layer II of the entorhinal cortex, and follows a feed-forward pattern of innervation (Andersen 2007). Axons from the entorhinal cortex synapse onto the dentate gyrus forming the perforant path (O'Keefe & Nadel 1978). The dentate gyrus is made up of three layers consisting of stratum moleculare, stratum granulosum (containing granule cells) and polymorphic layers (hilus) (O'Keefe & Nadel 1978). CA1 and CA3 have five layers in which lies the cell body, the incoming axonal input, the axonal output and dendrites (O'Keefe & Nadel 1978). Granule cells in the dentate gyrus send axons called mossy fibers to the pyramidal cells of the CA3 which in turn project through the Shaffer collateral to CA1. These pyramidal neurons are also known as place cells due to their ability to form firing patterns associated with spatial location (O'Keefe 1979). CA1 forms inputs into deep layers of the entorhinal cortex and the subiculum eventually terminating at the entorhinal cortex. There are also minor inputs from layers II and III of the entorhinal cortex to the CA1, CA3 and subiculum. Among the excitatory cells are interneurons that play a modulatory inhibitory role in the local circuit of the hippocampus. Basket cells of the CA3 and dentate gyrus provide local modulation, while stellate cells form inhibition on distal pyramidal and granule cells (O'Keefe & Nadel 1978).

3.2 Neurocircuitry

Inputs into the entorhinal cortex originate from prefrontal, temporal, parietal, pyriform, olfactory, occipital and cingulate cortices (O'Keefe & Nadel 1978). The amygdala has inputs to the entorhinal cortex and subiculum (O'Keefe & Nadel 1978). Afferents from the brainstem, such as median raphe nucleus and nucleus centralis superior form a major serotonergic input into the CA1 and CA3 regions of the hippocampus (O'Keefe & Nadel 1978). Serotonergic inputs into the hippocampus have an inhibitory action on excitatory pyramidal cells and GABAergic basket cells (Andersen 2007). The locus coeruleus provides noradrenergic inhibitory modulation at CA1 and CA3 regions (O'Keefe & Nadel 1978; Andersen 2007). There are also inputs from the ventral tegmental area into the dentate gyrus providing the dopaminergic input into the

hippocampus (Andersen 2007). Medial septal nucleus innervates the dentate gyrus through both GABAergic and cholinergic inputs, where GABAergic input terminate on hippocampal GABAergic neurons and cholinergic input terminate on granule cells (Andersen 2007). Many modulatory mechanisms mediate hippocampal activity, such that different sensory inputs can either increase or decrease hippocampal activity. This is very evident when studying behaviours in animals, since associative contextual memory encoding requires input of various stimuli into the hippocampus to generate a map of network activation.

Efferents from the hippocampus has been shown to terminate in the septal nucleus, the thalamus, mammillary bodies, amygdala, medial hypothalamus, insular cortex, frontal cortex, perihinal cortex, piriform and occipital cortices (O'Keefe & Nadel 1978; Canto et al. 2008). Many of the regions receiving inputs from the hippocampus, also provide outputs to the hippocampus and thereby allows intercommunication between brain regions.

3.3 Dysfunction

Lesions of the hippocampus has been extensively studied, starting from the first human patient H.M. (Milner 2005). Lesions cause anterograde amnesia and prevent further contextual memory encoding. In addition, pharmacological antagonists of NMDA receptors and genetic models have also shown a glutamate signaling dependent encoding of spatial memory within the hippocampus (Bliss & Collingridge 1993; Tsien et al. 1996). Disorders of the brain have shown functional decreases in hippocampal activity from either a volume reduction or neurotransmitter signaling deficiency causing impaired spatial learning. Early stage Alzheimer's disease patients have neurodegenerative damage to the entorhinal cortex and hippocampus, exhibiting early stages of memory loss (Andersen 2007). Schizophrenia patients have reductions in hippocampal volume from shrinkage and loss of hippocampal cells, abnormal fronto-hippocampal connectivity, reduced post-synaptic density protein and atypical morphology of hippocampal neurons, which suggest a hippocampal contribution to the memory impairments (Fletcher 1998; Harrison 2004). Interestingly, temporal lobe epileptic patients with hippocampal damage show schizophrenia like cognitive deficits (Harrison 2004). Varying levels of alteration in hippocampal activity within different neurological disorders, produces varying degrees of cognitive deficits in contextual learning and memory. Nevertheless, hippocampal dysfunction is a commonality between many disorders.

4 Learning

Scientists have been trying to find the molecular mechanism of memory since the early periods of Ramon-y-Cajal (Korte & Schmitz 2016). Extensive research behind memory encoding within the hippocampus has elucidated some of the molecular mechanisms involved in learning, including the requirement of NMDA receptors (Bliss & Collingridge 1993). At the very beginning, our understanding of how learning forms memory has been extrapolated from studying single cells, however it is becoming clear that memory requires a network (Liu et al. 2014). Continuous effort is being made to understand the influence of the molecular underpinnings of NMDA receptor function and its role in network formation, in order to decipher the processes required in learning and memory.

4.1 Plasticity mediated learning

Plasticity can be defined in the broadest sense as the adaptability of the brain to learned experiences. Hebb defined plasticity as the ability for neurons to strengthen their communication when one neuron caused the subsequent neuron to fire in succession (Muller et al. 2002). Certain forms of learning, such as associative learning model Hebbian plasticity and is currently known as synaptic plasticity (Muller et al. 2002). Synaptic plasticity describes the neurons ability to form and weaken connections at the level of the synapse. A single neuron can have hundreds to thousands of synapses with other neurons, allowing for the neural network to undergo vast modifications (Hawkins & Ahmad 2016). In terms of memory, weakening connections can lead to forgetting and strengthening connections can lead to either short-term memory or long-term memory. In other words, memory is mediated by synaptic plasticity (Bliss & Collingridge 1993). To study memory, we try to define and understand synaptic plasticity at the level of brain regions, neural circuits and cellular processes. On a cellular level, short-term memory and long-term memory are formed through different molecular mechanisms. Short-term memory relies on modifying the presence of pre-existing proteins at the synapse, leading to a short lived increase in the efficiency of communication (Korte & Schmitz 2016). Long-term memory requires gene transcription and translation of proteins to stabilize structural modifications at the synapse and is under strict control by regulatory proteins (Korte & Schmitz 2016). The molecular changes in protein localization, translation and transcription provide a molecular tool to study the mechanisms behind plasticity responsible for learning and long-term memory. It is important to

understand these mechanisms because the brain retains the ability to undergo learning induced plasticity throughout life, and failure to maintain such abilities can result in learning and memory impairments seen in mental disorders (Loeblich & Nedivi 2009).

4.2 Immediate Early Genes (IEGs)

The molecular processes that occur at the cellular level during long-term memory require gene transcription. Gene transcription gives rise to messenger RNA, used to generate proteins that ultimately cause structural modifications at the synapse, allowing for long-term synaptic plasticity. Blockade of transcription, specifically inhibition of the transcription factor CREB prior to learning, prevents long-term memory formation (Kida et al. 2002). Activity dependent increases in Ca^{2+} causes subsequent activation of kinases (CaMKII, PKA, MAPK) responsible for phosphorylating CREB. Phosphatases activated in conjunction with kinases act as homeostatic regulators by de-activating CREB in a time-dependent manner. Once bound to the co-activator, CREB binding protein and phosphorylated CREB can interact with genomic cyclic AMP response elements and transcriptionally activate the expression of learning-induced genes. IEGs are genes that are rapidly expressed for a short period following CREB activation, compared to those transiently expressed (Herdegen & Leah 1998). The function of IEGs are multi-factorial, with the regulation of each IEG being dependent on the activating stimulus and the brain region (Beckmann & Wilce 1997). It is thought that such specificity can arise from brain region specific transcription factor expression, epigenetic marks, presence of activators and repressors surrounding the CREB binding element and the different CREB activation pathways imparting response element binding specificity. Learning induced IEGs encode transcription factors, structural and scaffold proteins and growth factors among other yet to be confirmed effectors (Lanahan & Worley 1998). To date, a few well-validated learning induced IEGs and their regulators have commonly been used as a measure of learning: activity regulated cytoskeleton-associated protein (*Arg3.1/Arc*), brain derived neurotrophic factor (*Bdnf*), Homer scaffold protein 1 (*Homer1a*), nuclear receptor subfamily 4 (*Nr4a1*), early growth response1 (*Egr1*), FBJ murine osteosarcoma cellular oncogene (*c-Fos*), calcium-calmodulin dependent protein kinase II alpha (*CamkIIa*), dual specificity phosphatase 1 (*Dusp1*).

4.2.1 *Arc/Arg3.1*

Arc protein and gene expression is highly correlated with synaptic plasticity induced long-term memory, especially during learning, memory consolidation, reconsolidation and recall of spatial and fear related memories (Ploski et al. 2008; Chia & Otto 2013; Barry et al. 2015; Gusev et al. 2005; Besnard et al. 2014). Inhibition of *Arc* expression using antisense oligonucleotides inhibited the maintenance of long-term synaptic plasticity and spatial long-term memory, without affecting short-term memory (Guzowski et al. 2000). Conversely, AP5 blockade of NMDA receptor signalling prevented transcriptional upregulation of *Arc* prior to learning and impaired memory recall (Chia & Otto 2013). Additionally, neurons recruited to memory traces are correlated with increased basal expression of CREB and *Arc* (Gouty-Colomer et al. 2016; Han et al. 2007; Guzowski et al. 1999). *Arc* provides a molecular marker for behavioural learning, as numerous studies establish its importance during learning and long-term memory formation.

The molecular mechanism behind *Arc* mediated long-term memory formation begins with the activation of glutamatergic signalling through NMDA receptors. NMDA receptors in the hippocampus can mediate LTP (a cellular basis of synaptic plasticity) through protein kinase (MAPK and CaMKII α) dependent CREB transcription of *Arc* (Bliss & Collingridge 1993; Steward & Worley 2001; Waltereit et al. 2001; Besnard et al. 2014; Donai et al. 2003). Following transcription, *Arc* mRNA is rapidly transported to activated synapses from the nucleus, where it is thought to be translated locally (Steward et al. 1998; Link et al. 1995; Lyford et al. 1995; Park et al. 2008). *Arc* protein concentrate at activated synapses by associating with commonly found proteins at active zones such as dynamin, endophilin, NMDA receptors, post-synaptic density 95 and CaMKII β (Chowdhury et al. 2006; Steward et al. 1998; Rial Verde et al. 2006; Donai et al. 2003; Okuno et al. 2012). *Arc* protein interaction with dynamin and endophilin allows for endocytosis and removal of AMPA receptors from activated synapses (Shepherd et al. 2006). Removal of surface AMPA receptors is important in homeostatic scaling, which prevents altering the electrophysiology of a neuron following LTP and depression, while maintaining the relative strength of the synapse (Chowdhury et al. 2006; Shepherd et al. 2006; Turrigiano 2007). LTD can also be induced by removal of AMPA receptors from the activate synapse, however *Arc* associated LTP occurs through regulation of the actin cytoskeleton (Waung et al. 2008; Messaoudi et al. 2007; Rial Verde et al. 2006). *Arc* gene expression is required to maintain its activity during phosphorylation and inactivation of cofilin, an actin

depolymerisation factor that potentiates the loss of filamentous actin at synapses (Messaoudi et al. 2007). The exact mechanism of Arc activity is still being explored due to many missing links, continuous findings on mechanisms of Arc transcriptional activation and its function in long-term memory (Yilmaz-Rastoder et al. 2011; Steward et al. 2014). Still, Arc remains a molecular marker that is consistently used to distinguish neurons undergoing learning induced plasticity.

4.2.2 *Bdnf*

Experiments linking *Bdnf* to LTP suggests that expression of *Bdnf* is necessary for long-term memory. LTP stimulation can increase mRNA expression of *Bdnf* and inhibition of *Bdnf* signalling prevents late phase LTP (Patterson et al. 1992; Kang et al. 1997; Castrén et al. 1993). In addition, activity induced increases in mRNA levels are dependent on NMDA receptors or L-type Ca^{2+} channel mediated Ca^{2+} influx and subsequent CREB activation, processes involved with LTP and long-term memory (Lever et al. 2001; Hartmann et al. 2001; Palomer et al. 2016; Tabuchi et al. 2002). NMDA receptor antagonism with MK801 prevented adenylylate cyclase mediated CREB upregulation of BDNF mRNA expression (Fukuchi et al. 2015). In mice, fear conditioning increased levels of BDNF, while inhibiting *Bdnf* mRNA upregulation prior to training impaired learning in 8-arm radial maze and genetic heterozygotes for *Bdnf* showed impaired learning in Morris water maze and contextual fear conditioning (Linnarsson et al. 1997; Liu et al. 2004; Mizuno et al. 2012; Gonzales et al. 2015). In humans, a VAL99MET mutation in BDNF lead to reduced hippocampal fMRI bold activity and impaired episodic memory (Egan et al. 2003). Mutant VAL99MET mouse models exhibited impaired induction of LTP, reduced NMDA receptors currents and deficits in fear memory (Ninan et al. 2010; Lonsdorf et al. 2015). Numerous evidence suggests a role for BDNF in mediating LTP, particularly in hippocampus related memory functions.

Activation of CREB through NMDA receptor signalling allows for histone modification at *Bdnf* promoters resulting in transcription (Palomer et al. 2016). It has been suggested that once transcribed, *Bdnf* promoters exert synapse specificity by being locally synthesized in potentiated axons so that it is released to activate nearby postsynaptic dendrites (Zhang & Poo 2002). Binding of BDNF dimers to postsynaptic TrkB receptors results in receptor dimerization and autophosphorylation, leading to activation of MAPK and phospholipase- γ (PLC γ) signalling cascades. Downstream of PLC γ , upregulation of intracellular Ca^{2+} activates MAPK and CaMK,

inducing transcription of *Bdnf* through activation of CREB. BDNF can indirectly regulate plasticity through synaptogenesis, destabilization of spines, increased spine density, altered efficiency of neurotransmitter release from axons and enhanced synaptic transmission through surface NMDA receptor expression (Zhang & Poo 2002; Kang & Schuman 1995; Kim et al. 2012; Horch et al. 1999; Sanchez et al. 2006; Ehrlich & Josselyn 2015). Many links have been made between NMDA receptors, CREB and *Bdnf*, however it remains to be seen whether direct manipulations in NMDA receptor expression can alter *Bdnf* transcript levels following induction by fear associated learning.

4.2.3 *Homer1a*

Homer1a and its isoforms are required for cellular growth and homeostatic scaling (Montes-Rodríguez et al. 2013; Sala et al. 2003). Homer1a deficient and overexpressing mice display deficits in spatial and fear memory, suggestive of its role in homeostatic mechanisms (Banerjee et al. 2016; Mahan et al. 2012; Celikel et al. 2007). The Homer family of proteins regulate spine structure and spine maturation, whereby isoform 1b binds Shank and mGluR1/5 to allow for clustering of post-synaptic density proteins, such as NMDA receptors (Ango et al. 2000; Hu et al. 2010; Bertaso et al. 2010). As a feedback mechanism, the activation of NMDA receptors upregulates *Homer1a* gene expression and pharmacological antagonism prevents gene expression (Sato et al. 2001). Homer1a opposes the activity of 1b by encouraging structural reorganization at spines and inhibiting AMPA receptor and NMDA receptor currents, otherwise known as synaptic downscaling (Sala et al. 2003). Upregulation of Homer1a expression following spatial learning and fear conditioning would suggest that synapse remodeling is important for hippocampal based learning (Feldman et al. 2010; Mahan et al. 2012).

Fear learning, CREB and BDNF protein upregulation can induce *Homer1a* mRNA expression (Zhang et al. 2007; Mahan et al. 2012). Neuronal activity dependent upregulation in Homer1a mRNA and protein expression causes a negatively regulation of dendritic spines (Hu et al. 2010). Homer1a has been shown to reduce spine size and prevent clustering of post-synaptic density (PSD) proteins, including NMDA receptors (Sala et al. 2003). Homer1a differently regulates the clustering of proteins due to the absence of the C-term coiled coil domain, which is required for dimerization with other Homer proteins (Brakeman et al. 1997). The N-terminal EVH-1 domain interaction with other PSD proteins and dimerization of Homer proteins allows for clustering of

mGluR1/5, Shank and NMDA receptors (Xiao et al. 2000; Hu et al. 2010; Ango et al. 2000). Therefore, Homer1a competes with its isoforms to prevent clustering of PSD proteins, shrinking spines and regulating their activity (Sala et al. 2003). In the same way, Homer1a prevents mGluR mediated AMPA receptor phosphorylation and maintenance at the synapse leading to reductions in both AMPA receptor and NMDA receptor currents (Hu et al. 2010; Sala et al. 2003; Van Keuren-Jensen & Cline 2006). This provides a mechanism through which induction of Homer1a can lead to homeostatic plasticity, forming a process whereby neurons fine-tune excitatory input through the reduction of noise from neighbouring synapses (Karabanov et al. 2015). Due to the induction properties of Homer1a, many studies have previously used Homer1a as a marker for hippocampus-based learning (Cavallaro et al. 2002; Poplawski et al. 2014).

4.2.4 *Nr4a1*

Nr4a1 is one of three orphan receptors forming a subfamily of Nr4a ligand-independent nuclear receptors. These receptors can be activated by various environmental stimulus, including NMDA receptor activation and fear learning (Keeley et al. 2006). Of the three members of the subfamily of Nr4a receptors, Nr4a1 and Nr4a2 has been shown to be involved in long-term memory of object location (McNulty et al. 2012). Notably, contextual fear learning and spatial discrimination task increased *Nr4a1* gene expression in the hippocampus, which is blocked by impairments in CaMKII signalling (von Herten & Giese 2005; Malkani & Rosen 2000). In addition, pharmacologically blocking NMDA receptor activity prevented upregulation of *Nr4a1* gene expression (Dragunow et al. 1996). *Nr4a1* knockout studies show impaired learning of spatial tasks and prevented transcription dependent LTP (Colón-Cesario et al. 2006; Hawk et al. 2012; Bridi & Abel 2013). Furthermore, missense mutations present in a subset of schizophrenia patients renders Nr4a1 less functional and post-mortem levels of Nr4a1 are reduced in schizophrenics (Buervenich et al. 2000; Xing et al. 2006).

Nr4a1 activity can be induced by kinases involved in memory such as PKA and CaMKII α , both through gene expression and post-translational phosphorylation (Maira et al. 2003; Kovalovsky et al. 2002). Regulation of Nr4a1 gene and protein expression is dependent on NMDA receptor activity and CREB activation, following which Nr4a1 as a transcription factor can bind NGFI- β response elements as monomers or Nur response elements as homo- and hetero-dimers (Yelin Chen et al. 2014; Darragh et al. 2005; Wilson et al. 1992; Philips et al. 1997). Nr4a1 acts as a

transcription factor to regulate expression of those genes involved in memory formation, such as *Bdnf* and *Fosl2* (Hawk et al. 2012; Yelin Chen et al. 2014). Nr4a1-mediated induction of genes within the RhoA/Rac1 pathway regulates spine density and morphology within the CA1 region of the hippocampus, in order to prevent hyper-excitation (Yelin Chen et al. 2014). To this end, NMDA receptor dependent Nr4a1 activity could exert a homeostatic control over the excitability of a synapse, similar to Homer1a and Arc. Disrupting Nr4a1 signalling may impair memory through the described effects. It is still unclear what other regulatory effects Nr4a1 possess due to its transcriptional regulation over various memory related genes. Nevertheless, altered NMDA receptors function should lead to changes in *Nr4a1* mRNA levels, and as a result impact spatial learning and memory.

4.2.5 *Egr1*

Initially discovered to be induced by growth factors, *Egr1* can also be induced by experience dependent LTP through the induction of NMDA receptor-MAPK-CREB pathway (Farina & Commins 2016; Cole et al. 1989; Huckleberry et al. 2015; Guzowski et al. 2001; Jones et al. 2001; Williams et al. 2000; Davis et al. 1992; Besnard et al. 2014). Hippocampal gene expression of EGR1 can specifically be induced by fear conditioning and LTP induction, while blockade of NMDA receptors prevents expression (Keeley et al. 2006; Cole et al. 1989). Transcriptional regulation by CREB and post-translational phosphorylation by MAPK causes *Egr1* to bind EGR and CRE response elements, and transcribe downstream effectors involved in synaptic plasticity, such as Arc (Christy & Nathans 1989; Penke et al. 2011; Baumgärtel et al. 2009; Tyan et al. 2008; Li et al. 2005). Studies have shown that *Egr1* homozygous and heterozygous mutant mice lack spatial memory and have impaired LTP maintenance (Jones et al. 2001). Overexpression of *Egr1* has been shown to increase spatial memory, as well as enhance LTP (Penke et al. 2014). More propelling evidence for its role in memory are findings that schizophrenia patients suffer from decreased cortical *Egr1* expression (Yamada et al. 2007). Altered *Egr1* expression in schizophrenia could exemplify the effect of reduced downstream signaling from NMDA receptors on memory impairments. Although the results to date suggest an important role for *Egr1* in the acquisition of spatial learning, further research is required to determine the downstream molecular mechanism mediating synaptic plasticity. In this study, *Egr1* expression will be used as an indicator for NMDA receptor activity.

4.2.6 *Fos*

The *Fos* gene family comprises of transcription factors, including c-Fos, FosB, Fra-1 and Fra-2 (Milde-Langosch 2005). The family of Fos transcription factors heterodimerizes with the jun family of transcription factors forming the AP-1 complex, which can bind to AP-1 promotor sites to activate downstream gene expression (Chiu et al. 1988; Halazonetis et al. 1988). *Fos* expression is regulated by activation of various kinases (MAPK, ERK and PKC), making it a reliable marker for activated cells (Morgan & Curran 1991). In the same way, *Fos* mRNA or protein can be used to identify neurons that take part in the memory engram (Liu et al. 2012).

Fos expression has been shown to be directly correlated with spatial learning, whereby oligonucleotide mediated inhibition of Fos expression in the hippocampus or cortex can lead to impaired contextual fear memory formation (He et al. 2002; Morrow et al. 1999). Similarly, *Fos* knockout mice also display impaired LTP generation and impaired NR2A specific upregulation following a tetanus, suggesting a role for Fos induced upregulation of NR2A surface expression (Fleischmann et al. 2003). At the same time, in vitro stimulation, pharmacological activation and inhibition of NMDA receptors can respectively activate or prevent *Fos* gene expression (Inada et al. 2007; Bading et al. 1995). In support of the classical NMDA receptor downstream signalling, a 5' and intragenic CRE response element present in the *Fos* gene allows for CREB induced modulation of expression (David D. Ginty et al. 1994; Härtig et al. 1991). Although *Fos* expression can be induced by multiple stimuli activating cell type specific kinases, its expression initiated by NMDA receptors mediated LTP seems to play an important role in plasticity associated memory processes within the hippocampus (Bading et al. 1995; Feinstein et al. 2010). Fear conditioning induces hippocampal expression of Fos mRNA (Keeley et al. 2006). Inhibition of NMDA receptor activity prevents Fos gene expression and leads to memory deficits in avoidance learning (Cammarota, Bevilaqua, et al. 2000). In this study, *Fos* gene expression will be used as a molecular marker of cellular activity following fear conditioning.

4.2.7 *CamkIIα*

Ca²⁺/Calmodulin(CaM)-dependent protein kinase IIA (CaMKIIα) is found primarily in glutamatergic neurons and form a homomeric or heteromeric holoenzyme with CaMKIIb in a 3:1 ratio (Bennett et al. 1983; Coultrap & Bayer 2012). As part of the heteromeric complex, CaMKIIb binds F-actin when intracellular calcium levels are low (Lin & Redmond 2008).

Calcium entry through activated NMDA receptors allows for dissociation from F-actin, CaM activation of CaMKII α and an autophosphorylation event on peptide T286 (Shields et al. 1984; Miller & Kennedy 1986; Dash et al. 1991; Fukunaga et al. 1992; Lamsa et al. 2007).

Autophosphorylation allows for persistent CaMKII α activity even after Ca²⁺ levels decrease, increases affinity for CaM and allows for direct interactions with NMDA receptors and AMPA receptors to increase their activity at potentiated synapses (Hashimoto et al. 1987; Bayer et al. 2001; Lisman et al. 2002; Coultrap et al. 2012). Transcriptional upregulation of *CamkII α* by phosphorylated CREB, can subsequently trigger translocation of the mRNA to activated synapses for local translation, highlighting a potential role for CaMKII α at postsynaptic densities (Mayford, Baranes, et al. 1996; Coultrap & Bayer 2012). Protein phosphatase 1 and 2A act to dephosphorylate and deactivate kinase activity (Lisman et al. 2002). Additional regulation include physical blockade of the catalytic domain by CaMKII inhibitory proteins and autophosphorylation at T301 and T305 preventing CaM binding (Chang et al. 1998; Hashimoto et al. 1987; Coultrap & Bayer 2012).

CaMKII α expression in glutamatergic neurons, mRNA translocation to activated synapse and autonomous activity after activation, as well as its other properties as a kinase makes it a candidate in hippocampal memory formation. Its involvement in memory formation is characterized by mRNA upregulation following fear conditioning and CaMKII α knockout animal models showing impaired LTP generation and impaired spatial learning (Silva et al. 1992; Lepicard et al. 2006; Bachstetter et al. 2014; Goodell et al. 2016). Inhibition of autophosphorylation of T286 in a knock-in mouse model prevented CaMKII kinase activity, caused deficits in spatial learning and reduced stability of hippocampal place cells, however it did not affect contextual memory (Giese et al. 1998; Lisman et al. 2002). Contrarily, overexpression and constitutively active CaMKII α can also lead to impaired memory formation due to a lack of synapse specificity for activated synapses and disrupted hippocampal place fields (Bach et al. 1995; Mayford et al. 1996; Lisman et al. 2002). Disruptions in dendritic translocation of *CamkII α* mRNA impaired synaptic plasticity by reducing LTP and impaired spatial and fear memory (Miller et al. 2002). Since activity of CaMKII α is highly correlated to memory and is dependent on Ca²⁺ entry through NMDA receptors, manipulations in NMDA receptors mediated Ca²⁺ entry should produce similar effects as those seen in knockout models.

4.2.8 *Dusp1*

Mitogen activated protein kinase (MAPK) comprise of three major classes of kinases including ERK, p38 and JNK, of which ERK plays a crucial role in hippocampus based learning (Besnard et al. 2014). Similar to CaMKII α , MAPK acts downstream of NMDA receptor activation to regulate CREB phosphorylation (Xia et al. 1996; Davis et al. 2000). The pathway of MAPK activation involves calcium influx through NMDA receptor channels or voltage-gated calcium channels causing GEFs to increase the ratio of Ras-GTP to Ras-GDP, leading to activation of Raf followed by activation of MEK, a kinase that phosphorylates and activates MAPK (Rosen et al. 1994; Thomas & Huganir 2004). Activation of MAPK is necessary in CREB induction, PKA acts as a converging point of kinase activation pathway by synergistically increasing MAPK activation leading to further CREB induction (Impey et al. 1998). MEK inhibitors impaired LTP in the hippocampus and impaired fear conditioning, suggesting that MAPK can affect memory formation (Atkins et al. 1998; Ohno et al. 2001). Further implications were found when NMDA receptors antagonists prevented MAPK activity, subsequent activation of CREB and *Egr1* gene induction (Camarota, Bevilacqua, et al. 2000; Davis et al. 2000). MAPK activation of CREB initiates a self-regulatory process involving the expression and direct phosphorylation and activation of *Dusp1* (Brondello et al. 1999; Hutter et al. 2000; Rumora et al. 2004; Guo et al. 2012). *Dusp1* gene expression in response to fear conditioning within the hippocampus suggests that *Dusp1* acts as a negative feedback for MAPK activation, during memory encoding processes (Keeley et al. 2006; Sirri et al. 2010). Ketamine antagonism of NMDA receptor activity prevents the upregulation in *Dusp1* gene expression following LTP induction (Qian et al. 2004). Therefore, NMDA receptor activation of MAPK should regulate the expression of *Dusp1* downstream of CREB and provide an indirect measure of learning induced MAPK activity within the hippocampus.

4.3 Plasticity within the adult brain

The extent of plasticity varies throughout development. Previous findings support that the adult brain is capable of a large extent of plasticity allowing for cellular processes involved with learning (Takacs & Hamori 1990; Bower 1990). Specifically, hippocampal based learning has been shown to retain much of their adaptable properties as neurons at juvenile or post-natal stages, including rapid structural remodeling of dendritic spines, neurogenesis and pruning (Altman 1962; Bonhoeffer & Yuste 2002; Dayer et al. 2003; Espósito et al. 2005). Yet, many

genetic factors affecting neonatal integration of neurons into networks required for learning cannot be recovered in adults, suggesting that there is a limit to the extent of plasticity in later stages of life (Bilbo et al. 2006; Kosten et al. 2007). These limits might be imposed by the level of diverging arborization from the dentate gyrus to other areas of the hippocampus and their neuronal regeneration rates (Hastings et al. 2002; He & Crews 2007). These factors highlight differences in the regenerative potential within the adult and post-natal hippocampus. Further investigation is required in order to fully understand the limits of plasticity in re-establishing learning associated networks in adults.

4.4 Fear Memory

Processing of fear memories initiate at the sensory input from the spinal cord into the thalamus. External cues related to the fear response converge onto the amygdala in order to integrate all the cues associated with the fear memory (Maren 2001). Glutamatergic inputs from the dorsal hippocampus to the basolateral amygdala provides contextual information related to the fear memory (Maren & Fanselow 1995; Phelps 2004). Hippocampal lesions disrupt contextual fear memory without affecting tone associated fear memory suggestive of separate fear circuitry (Kim & Fanselow 1992; Fendt 2001). Acoustic sensory input processed by the thalamic medial geniculate nucleus and auditory cortex, and visual sensory information encoded by visual cortex are relayed through the perirhinal cortex converging onto the basolateral amygdala via glutamatergic input (Edeline & Weinberger 1992; LeDoux et al. 1986; Rosen et al. 1992). Lesioning the basolateral amygdala has shown impairments in fear acquisition and response (Weiskrantz 1956; Blanchard & Blanchard 1972; Cousens & Otto 1998). Without conscious processing of sensory stimulus, the thalamo-amygdalar pathway and magnocellular visual input to the amygdala produces the initial fast response to a potential threat and is considered the primary, shorter and cruder perception to a fearful external stimulus (LeDoux et al. 1986; Quirk et al. 1997; Fendt & Fanselow 1999; Méndez-Bértolo et al. 2016). A longer more conscious processing of external cues takes places when stimulus processing occurs at the level of the cortex and hippocampus, providing feedback to the amygdala to appropriate the fearful response (Quirk et al. 1997).

The basolateral amygdala also communicates with the central nucleus of the amygdala sending efferents through the stria terminalis to the hypothalamus and ventral periaqueductal gray,

resulting in freezing response (LeDoux et al. 1988; Wilensky et al. 2006). In addition, alterations in breathing, startle response, arousal, stress hormone release are initiated by the amygdala through outputs to the parabrachial nucleus, caudal pontine nucleus and ventral tegmental area (Kapp et al. 1979; Ottersen 1981; Cechetto et al. 1983; Fendt et al. 1997; Pezze & Feldon 2004; Bourgeois et al. 2001). The network connecting different regions of the brain with the amygdala is not solely a feed forward input, there are efferents from the amygdala projecting to the prefrontal cortex, nucleus accumbens and the hippocampus in order to modulate the fear response (Maren & Fanselow 1995; Ammassari-Teule et al. 2000; Pezze & Feldon 2004; LeDoux 2012; Correia et al. 2016). Amygdalar projections to the nucleus basalis also increases arousal affecting the memory encoding of external cues (Holland 2007; Fendt & Fanselow 1999). Emotion based learning can enhance a memory and in the case of fear conditioning, emotion or fear enhances memory of all the other associated cues (Cahill & McGaugh 1996; Lee et al. 2008). A fear memory network or engram is formed each time particular external sensory cues are interpreted to be fearful. Should any of those external sensory cues present themselves again, the previously formed network elicits a fearful response, characteristic of Pavlovian learning.

Figure 1.3 Simplified diagram of circuits involved in fear conditioning.

Adapted from thebrain.mcgill.ca

4.4.1 Contextual Memory

The encoding, processing and storage of contextual information occurs in various parts of the brain. In particular, the hippocampus is a region of the brain required in processing contextual cues, whereby lesions of either the hippocampus or dorsal hippocampus following fear

conditioning causes contextual learning deficits (Sutherland & McDonald 1990; Maren et al. 1997; Frankland et al. 1998). The involvement of the hippocampus is however more complex, where post-training rather than pre-training hippocampal lesions produce deficits in contextual memory (Phillips & LeDoux 1994; Maren et al. 1997). Further analysis also found that post-training lesions of the hippocampus that were performed 100 days after training maintained contextual memories (Maren et al. 1997; Anagnostaras et al. 1999). These differences had suggested that remote contextual memories were stored elsewhere in the cortex and that there may be a distinction in the way contextual memories are encoded with and without the involvement of the hippocampus (Maren et al. 1997; Anagnostaras et al. 1999; Anagnostaras et al. 2001; Burwell et al. 2004). That is to say the hippocampus allows for a holistic or unified representation of the context, whereas hippocampal lesions causes other brain regions to form elemental representations of the context, such as olfactory or environmental noise as cues (Frankland et al. 1998; Anagnostaras et al. 2001). This unified representation of the context becomes important during spatial tasks where spatial representation is required (O'Keefe & Dostrovsky 1971; Young et al. 1994; Hartley et al. 2014; Keene et al. 2016). Context association with fear is thought to occur within the amygdala (Phillips & LeDoux 1992). Therefore, contextual information processing can occur at multiple sites within the brain. However, integration of all the different cues to create a unified representation of the context occurs in the hippocampus and is remotely stored in the cortex, allowing for discrimination between similar contexts.

Remote contextual information is stored in the prefrontal cortex, which is retrieved by the hippocampus to form discriminative or complex goal oriented spatial learning (Navawongse & Eichenbaum 2013; Place et al. 2016). Contextual memory retrieval seems to work in a similar process as encoding. Hippocampal lesions allow for recall of elemental representations of a simple context (Maren & Holt 2000). However, when rats with hippocampal lesions are put to a test requiring discrimination between similar contextual cues in a T-maze for a food reward, they have a lower number of correct responses for the reward arm of the T-maze (Hirsh 1980). In a separate study, rats were pre-exposed to two different contexts prior to training where one of the two contexts were the same as that used during training day (Maren & Holt 2000). Rats pre-exposed to the context where training took place froze more than those that were pre-exposed to a different context to that used during training (Maren & Holt 2000). The study found, that

pharmacologically lesioning the dorsal hippocampus resulted in non-preferential freezing, suggesting a deficit in discrimination of the context.

5 Study Outline

Neurodevelopmental disorders result from genetic and environmental insults preventing critical periods of brain development during early life, increasing the likelihood for the development of psychosis at a later age. The late onset of symptomatic expression in neurodevelopmental disorders, such as schizophrenia and epilepsy, poses challenges in advances for treatment (Marenco & Weinberger 2000). Cognitive dysfunction proves to be the most challenging due to the gap in knowledge about the complete neural network required in cognitive processes and their ability to change with experience as they adapt to a state of normalcy or disorder. It has become increasingly clear that even within a state of disorder, neurons are capable of recovering normal function following targeted rescue within a dysregulated pathway (Duffney et al. 2015). An area of the brain known to retain such capabilities include the hippocampus (Zhou et al. 2016). The hippocampus is required in learning about the environment and its contextual cues. Learning is a process that requires a high degree of plasticity, and therefore it is understandable that the hippocampus has the ability to undergo neurogenesis, modify dendritic complexity and alter synaptic strength specifically at those neural networks involved in learning (Altman 1962; Leuner & Shors 2004). With this level of regenerative capability, one would expect that the hippocampus may be capable of recovering from developmental insults, and with it recover processes required in learning. To test this, we studied plasticity of the hippocampus and the associated circuits involved in learning using adult rescue of developmental glutamatergic deficiency, as model for neurodevelopmental disorders. Current literature demonstrates the extensive ability for the hippocampus to undergo learning mediated plasticity. Therefore, **we hypothesize that neurodevelopmental insults, such as NMDA receptor knockdown and deficiency in glutamatergic signaling, can be overcome to allow for recovery of hippocampus based learning.**

5.1 AIMS

AIM1: Measure fear learning in NR1 knockdown mice and NR1-Cre rescued mice to characterize plasticity of the hippocampal dependent fear circuit.

In this study, we wanted to assess whether there were deficits in long-term memory in NMDA receptor knockdown mice, and whether rescue in glutamate signalling could recover long-term memory. Previous studies using CA1 specific NMDA receptor knockout and NMDA receptor antagonist animal models, found impairments in fear mediated long-term memory (Cui et al. 2004; Tsien et al. 1996). **It can be expected that mice lacking hippocampal NMDA receptor expression will experience deficits in long-term contextual memory.** Although the knockdown in NMDA receptor is global, we place emphasis on the function of the hippocampus and molecular signalling events in this region.

Fear based learning requires the hippocampus to associate context with fear. Such forms of learning are classical forms of Pavlovian learning, using emotionality as a strong associative factor in contextual learning. Fear is the most instinctive emotion with stress inducing flight or fight response in animals. Therefore, we wanted to take advantage of a behaviour in which higher order cognitive functions, such as foresight in anxiety, would not be required. Fear based learning strategically allowed strong associative learning without the requirement for more complex forethought. Therefore, we used a fear paradigm to assess hippocampal function.

AIM2: Measurement of NR1-KD and NR1-Cre basal hippocampal gene expression of NMDA receptor associated plasticity genes: *Arc*, *Fos*, *CamkIIa*, *Egr1*, *Nr4a1*, *Bdnf*, *Dusp1*, *Homer1a* and *Grin1*.

NMDA receptor function can be determined by its downstream activation of kinases and CREB mediated gene expression. Impaired basal activity of the NMDA receptor in the hippocampus by application of AP5, can reduce the expression of CREB mediated gene expression, including levels of *Dusp1* and *Bdnf* (Y. Chen et al. 2014). We wondered whether knockdown and rescue in NMDA receptor expression could alter basal NMDA receptor activity due to the necessities of remodeling in neural networks in order to establish proper hippocampal function. By using gene expression as an indirect measure of receptor activity, we hypothesized that **NMDA receptor knockdown would prevent basal expression of genes, and that rescue in receptor expression would partially or fully restore basal gene expression levels.** In addition, altered regulation of genes from manipulations in NMDA receptor expression would allow for interpretations about the types of remodelling that were taking place, since each gene mediates different aspects of neuronal plasticity, detailed in section 4.2 IEGs. The basal expression levels within each

genotype could also be used to compare to induction by fear associated learning (AIM 3) as a molecular read out of hippocampal plasticity.

AIM3: Identify hippocampal gene expression patterns in NR1-Cre and NR1-KD mice following induction of NMDA receptor dependent contextual fear learning.

NMDA receptor activity lead downstream activation of genes through CREB activation is required in hippocampus based learning. Inhibition of NMDA receptor activity has been shown to downregulate CREB mediated gene expression and impair spatial learning (Morris et al. 1986; Czerniawski et al. 2011). Alternatively, NMDA receptor activity can lead to CREB activation and gene expression (Cammara, Bevilacqua, et al. 2000). Overexpressing NMDA receptors has been shown to potentiate learning (Tsien et al. 1999). Inhibitors of gene transcription and inhibitors of CREB, applied either before or right after training can impair learning (Squire & Barondes 1970; Bourtschuladze et al. 1994). These studies provide evidence for the requirement of NMDA receptor and CREB induction to mediate learning. In addition, provides evidence for the idea that acquisition of learned behaviours should correlate to levels of NMDA receptor activation and to induction of CREB mediated gene expression. As a result, we expect **levels of learning-induced genes should be reduced in an NMDA receptor hypofunction state and upregulated upon rescue of NMDA receptor expression**. Gene expression therefore provides a molecular measure of learning as a consequence of alterations in NMDA receptor function. Inference can also be made about the level of hippocampal plasticity based on the extent of induction in learning associated genes, following rescue of NMDA receptor expression.

Chapter 2 METHODS

1 Animals

All procedures were conducted in accordance with the University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee and with CCAC guidelines for the care and use of animals.

1.1 Housing

Mice were housed 1-4 per cage with a 12h light-dark cycle (7:00-19:00) with ad libitum access to food (Teklad 2018, Envigo, IN, USA) and water. Mice were tested during the light cycle (9:00-18:00) without access to food or water for the duration of the testing period. Mice were naïve to all tests performed.

1.2 Transgenic Mice

To generate the NR1 knockdown (NR1-KD) and inducible rescue (NR1-Cre) mouse lines, C57Bl/6 $\text{Grin1}^{\text{neo/+}}$ mice were crossed with C57Bl/6J $\text{ROSA26}^{\text{Cre-ERT2/+}}$. The resulting C57Bl/6J $\text{Grin1}^{\text{neo/+}}$ - $\text{ROSA26}^{\text{Cre-ERT2/+}}$ were crossed with 129X1/SvJ $\text{Grin1}^{\text{neo/+}}$ to produce the F1 progeny. A F1 background was used to increase hybrid vigor. The F1 progeny predicted Mendelian inheritance of 12.5% for each genotype: $\text{Grin1}^{+/+}$ (WT), $\text{Grin1}^{+/+}$ - $\text{ROSA26}^{\text{Cre-ERT2/+}}$ (WT-Cre), $\text{Grin1}^{\text{neo/neo}}$ (NR1-KD) and $\text{Grin1}^{\text{neo/neo}}$ - $\text{ROSA26}^{\text{Cre-ERT2/+}}$ (NR1-Cre).

1.3 Tamoxifen

Tamoxifen was administered to induce Cre-recombinase mediated excision of neomycin. At 10wks of age, mice housed 1-4 per cage were moved into cages housing 2-3 per cage. Male mice that were weaned together, remained together during tamoxifen treatment to avoid aggression towards intruders. Female mice that were weaned together were sometimes separated prior to tamoxifen treatment. Tamoxifen (Sigma-Aldrich T5648, MO, USA) was dissolved in corn oil by heating to 62°C in a water bath, with intermittent shaking for 1h. Tamoxifen was administered orally using a 22G oral gavage needle, given at a concentration of 20mg/ml, totaling 6mg/mouse. At the start of tamoxifen treatment, a single bolus was orally administered concomitantly with

tamoxifen chow (Envigo TD130857, IN, USA), at a 500mg/kg formulation. Tamoxifen chow (28 pellets/mouse) was used to maintain tamoxifen on board for 2 weeks. Three tamoxifen pellets per mouse were soaked in water, put inside of a cup and placed on the floor of the cage for easier access to food. Wet pellets were given again 1 week after the start of treatment. After 2 weeks, mice were placed in new cages with regular chow (Teklad 2018, Envigo, IN, USA). Three days following conversion to regular chow, mice were placed in new cages with regular chow to be considered tamoxifen-free and non-toxic to handlers.

2 Behavioural Procedures

Male and female mice aged 14 weeks were used for all behaviours. All procedures have been approved by the University of Toronto Animal Care Committee. Mice were naïve to all behaviour tests.

2.1 Locomotor Activity

Mice were placed in a Plexiglass chamber (20×20×45cm³) for 2h. Total distance travelled was measured for 2h, segmented into 5 minute bins by infrared beam breaks using VersaMax activity monitors with X-Y sensors (Omnitech Electronics, Columbus, OH, USA). Activity was measured on the last day of habituation for fear conditioning.

2.2 Fear Conditioning

Freezing was defined as a lack of movement for 2s, except that required for breathing. A threshold for percent freezing was determined by the experimenter based on the activity of each individual mouse, as it varied.

2.2.1 Apparatus

The fear training and contextual testing were conducted in a conditioning chamber (12"W×10"D×12"H) with grid shock floors, a drop pan under the grid floor, ambient light, speakers, clear acrylic back and front walls and metal sidewalls (Coulbourn Instruments, PA, USA). The speaker was connected to a Sony sound system, used to generate a 95dB tone stimulus. A ceiling mounted camera was used to digitally record mouse activity and freezing was measured using an automated scoring system on the Freeze Frame3 software (Actimetrics, IL, USA). The conditioning chamber was housed inside an isolation cabinet (23"W×17.74"D×24"H)

with a fan in the center of the back wall, producing 65dB background noise. ACT-711 USB Interface (Actimetrics, IL, USA) was used to control shock and tone stimulus output, as well as camera input to the FreezeFrame3 software. Shock intensity was manually set to 0.5mA and applied through the FreezeFrame3 software using a Precision Animal Shocker unit (Coulbourn Instruments, PA, USA).

Testing for cue associated learning was conducted in a novel context with white acrylic floors and walls creating a 3D isosceles triangle (9"Wx8"Dx7"H, two angles at 61°). A removable clear acrylic was placed on top of the novel context during the test, to prevent mice climbing out of the box.

2.2.2 Training

Mice were habituated to the experimenter for 3min every day for 5 days prior to training and testing. Habituation was conducted in a different room from that used for training and testing. The day after habituation, mice were trained in a conditioning chamber cleaned with Virox disinfectant. Mice were first acclimated to the conditioning chamber for the first 2min. Subsequently, 5 successive tones (CS) (15s, 1.0kHz, 95dB pure tone) co-terminated with 5 foot shocks (US) (1s, 0.5mA), with a 60s inter-trial interval. To minimize basement affect in NR1-KD freezing behaviour, 5 shocks were administered to produce maximal freezing response. After training, mice were returned to their home cage and left in an adjacent room for 30min to prevent further cues from being associated with the training. All freezing behaviour was measured and analyzed using Freeze Frame3 (Actimetrics, IL, USA).

Figure 2.1 Tone (CS) and shock (US) stimulus used during training for fear conditioning.

2.2.3 Re-training

A separate cohort of NR1-KD mice were re-trained 24h after training, prior to being tested on contextual and cue fear memory tests. Re-training followed the same conditioned stimulus procedure as training day. Virox disinfectant was used for cleaning.

2.2.4 Context Associated Fear Memory Test

Mice were placed inside the same conditioning chamber 24h after training or re-training. Freezing was measured for 5min. Virox disinfectant was used to prevent altering olfactory cues.

2.2.5 Cue Associated Fear Memory Test

Mice were placed inside a novel context with white acrylic walls, cleaned with 4% acetic acid. Similar to training, mice acclimated for 2min, followed by 5 successive tone stimuli (95dB) without shocks, and 60s inter-trial intervals. Freezing was measured during each 15s tone stimulus.

2.2.6 Suppression Activity

Baseline activity (cm) was measured for 2min during habituation on training day, prior to tone-shock pairings. Test activity (cm) was measured during 2min of contextual fear testing, on day2 of fear conditioning. MOV video files were exported from FreezeFrame3, converted to AVI and analyzed for distance traveled through automated tracking using Biobserve (Biobserve GmbH, Bonn, Germany). The suppression activity was calculated using the equation, $[(\text{activity during test})/(\text{baseline activity} + \text{activity during test})]$ (Anagnostaras et al. 2000).

2.2.7 Burst Activity

Baseline activity was measured for 2s prior to the first shock on training day. Shock response activity was measured for 1s during the first shock on training day. Burst activity served as a measure of pain sensitivity (Anagnostaras et al. 2000). AVI video files were analyzed for average velocity (cm/s) over 1s, through automated tracking with Biobserve (Biobserve GmbH, Bonn, Germany).

3 Molecular Procedures

3.1 Sample Collection

All brains were dissected on dissection blocks on ice and separated into cortex, hippocampus, amygdala, cerebellum and striatum. Thirty minutes after fear conditioning, mice were cervically dislocated and dissected brain regions for qPCR were collected and homogenized using a hand-held homogenizer and RNase free homogenization tips, in tubes containing 0.4ml Tri-reagent

(Molecular Research Center INC., OH, USA). Between each dissection, dissection block, blades and tongs were cleaned sequentially with diethylpyrocarbonate (DEPC) treated water, 75% ethanol, RNAase out and DEPC water. Samples were kept on ice, topped up with 0.6ml of Tri-reagent, split into two aliquots of 0.5ml and then topped up with another 0.5ml of Tri-reagent for a final volume of 1ml per tube. QPCR samples were stored at -80°C until all samples were collected. Mice were cervically dislocated and brains used for NR1 westerns were collected 24h following cued memory test. Dissected brains for westerns were frozen in liquid N₂ and stored at -80°C, until all samples required were collected. For immunohistochemistry (IHC) procedures, mice were anaesthetized with an injection of 250mg/kg Avertin, 35-40min after fear conditioning. Brains were collected 40-50min after fear conditioning, intracardially punctured using a 27G needle and perfused with phosphate buffered saline (PBS) using a peristaltic pump (P1, GE Healthcare Life Sciences, IL, USA), for the first 3min at a pump flowrate of 8 and followed by 4% paraformaldehyde for 10min at a pump flowrate of 3. Brains for IHC were kept in 4% paraformaldehyde for a minimum of 7h, after which were transferred and stored in 30% sucrose until all samples required were collected.

3.2 RNA Isolation

All procedures were performed in a fumehood to prevent intoxication from chloroform use. Hippocampal samples in TRI-reagent were thawed to room temperature for 5min. Samples were subsequently centrifuged at 12 000 relative centrifugal force (rcf) for 10min at 4°C. Subsequently, the supernatant was transferred into tubes containing 0.2ml chloroform. Tubes were incubated at room temperature (RT) for 10min, followed by centrifugation at 12000rcf for 10min at 4°C. Greater or equal to 200µl of the aqueous layer containing the RNA was carefully extracted and placed in a tube containing 2µl of glycogen (RNA grade, ThermoFisher, MA, USA). The tube was mixed by agitation to increase precipitation of RNA, followed by addition of 1ml of isopropanol. Tubes were centrifuged at 12000rcf for 8min. at 4°C, after which the supernatant was discarded. Non-presence of a pellet resulted in a second centrifugation at 12000rcf for 8min. prior to removal of supernatant. The pellet was washed twice with 1ml of 75% ethanol, each time followed by centrifugation at 7500rcf for 5min. at 4°C and discarding of supernatant. Following the last wash, the ethanol was discarded and the pellet was allowed to dry for 30min at RT. Dried pellets were re-suspended in DEPC water, placed on ice for another 30min. in order for the pellet to dissolve. RNA concentration was measured using Epoch

spectrophotometer (BioTek, VT, USA) and expressed as ng/ μ l units in a Take3 session on the Gen5 software (BioTek, VT, USA). RNA was further qualitatively verified by the 260/280 absorbance ratios from the Biosensor for purity and agarose gel electrophoresis to identify bands for the 26S and 28S ribosome for quality measures. RNA samples with ≥ 1.8 , 260:280 ratios and distinct 26S and 28S ribosomal bands were used for further qPCR analysis.

3.3 RNA Processing

RNA isolated samples were synthesized into cDNA using SuperScript III (Invitrogen 18080-051, USA). Each RNA sample was diluted into two wells of a 96-well microcentrifuge plate, where one sample served as a positive control and the other a negative control for the SuperScript III reverse transcriptase. Both wells contained 1 μ l of random hexamers (ThermoFisher, MA, USA), 1 μ l of dNTP mix (10mM each) (Vivantis, CA, USA) and 13 μ l of 500ng of RNA sample (diluted with DEPC water). The plate was sealed with film, briefly spun down and heated in a heating block to 65°C for 5min and quick chilled on ice. A master mix was produced separately for the positive and negative control, containing per well: 4 μ l of 5X First Strand Buffer, 1 μ l 0.1M DTT and 1 μ l RNaseOUT™ (40units/ μ l) (ThermoFisher, MA, USA). The master mix for the positive control also contained 1 μ l SuperScript III reverse transcriptase per reaction well. The master mix for the negative control contained 1 μ l of DEPC water instead of the reverse transcriptase. Two additional wells contained DEPC water with and without reverse transcriptase, as a control for water contamination. A total reaction volume of 20 μ l was achieved prior to PCR reaction. The plates were sealed with film, slightly agitated to mix well contents and spun down in a microcentrifuge. The PCR cycle for cDNA synthesis involved: 60min incubation at 50°C followed by inactivation of reverse transcriptase at 70°C for 15min. cDNA was diluted 1:100 with DEPC water, prior to quality assurance for both the positive and negative control wells, using PCR amplification of cDNA with a previously validated primer (Table 1). The PCR product was run on an agarose gel electrophoresis system to identify single bands of PCR product for the positive controls, and a lack of a band for the negative controls and for the wells containing water. Once quality of cDNA had been verified, the samples were used for qPCR analysis.

3.4 DNA Isolation

DNA isolation was conducted immediately following RNA isolation. From the tube containing the homogenized sample in Tri-reagent, the aqueous layer containing RNA was discarded, 300µl of 100% ethanol was added and mixed by inversion. Samples were incubated at RT for 2-3min and centrifuged at 12000 revolutions per minute (rpm) for 5min at 4°C. The supernatant was removed and DNA pellet was washed twice with 1ml of 0.1M sodium citrate in 10% ethanol. For each wash step, samples in 0.1M sodium citrate were gently vortexed, then incubated for 30min. with periodic mixing and centrifugation at 12000rpm for 5min at 4°C. After the final wash, the sodium citrate was discarded and 1.5ml of 75% ethanol was added, followed by periodic mixing and centrifugation at 12000rpm for 5min at 4°C, for a total of 10-20min. As the final step, the supernatant was discarded and the DNA pellet dried at RT for 30min. Once the pellet was completely dried and invisible to the naked eye, the pellet was re-suspended in 300µl of 10mM Tris, pH8.8. The DNA was left overnight at RT to better dissolve, after which the samples were gently vortexed and centrifuged at 12000rpm for 10min at 4°C. The supernatant was transferred into a new tube and the sample 260:280 ratio and concentration (ng/ul) was read using the Epoch spectrophotometer (BioTek, VT, USA). Samples with a 260:280 ratio of ≥ 1.4 were diluted with DEPC water to concentrations of 500ng/µl.

3.5 QPCR analysis

Primers specific to the genes of interest were selected and ordered through Integrated DNA Technologies. Primers were diluted to 100µM using DEPC water. Different sets of forward and reverse primer pairs were run on PCR reactions requiring per reaction (total volume 20µl): 1mM dNTP, 0.25µM forward primer, 0.25µM reverse primer, 10% DMSO, 0.3mg/ml BSA and 0.0625units/ul Taq polymerase (ThermoFisher, MA, USA) (cycles listed in Table1). The resulting products were run on agarose gel electrophoresis and verified for a single band PCR product. Selected primer sets were used for qPCR of *Arc*, *Fos*, *CamkIIa*, *Egr1*, *Nr4a1*, *Bdnf*, *Dusp1*, *Homer1a* and *Grin1* (Table 2). QPCR components required per reaction well, included (total reaction volume of 20µl): 2µl of cDNA or DNA (previously diluted 1:100 with DEPC water), 6.8µl of DEPC water, 0.6µl of each forward and reverse primers and 10µl of SensiFAST™ SYBR green (Bioline USA Inc., MA, USA) (cycles listed in Table 1). Samples were loaded in triplicates for each gene of interest and for a housekeeping gene in a clear 96-well

plate. Negative controls for reverse transcriptase was also loaded onto plates, in order to determine the cut-off cycle for each gene amplification.

Detection of SYBR logarithmic increase in signal was detected and recorded by CFX96 Touch Real-time PCR detection system (Biorad, PA, USA). BioCFX Manager3.1 (Biorad, PA, USA) was used to generate an excel output of the Cq values for each sample, their standard deviations from triplicates and melt curves as a qualitative measure of primer efficiency. Single sample values from triplicates were excluded and means recalculated, if the value was outside standard deviations. Quantification for qPCR were performed by normalizing each sample to a housekeeping gene, *Pgk1*, producing ΔCq values for each sample. The samples are further normalized to a single sample within a single 96-well plate, resulting in $\Delta\Delta Cq$ values. The $\Delta\Delta Cq$ values were converted to fold changes, using the equation $2^{-(\Delta\Delta Cq)}$. The fold changes from each gene was normalized and represented as fold changes relative to naïve WT controls. Each genotype contained a sample size of 5-6 mice brains.

Table 1. PCR and qPCR amplification cycles used to amplify primer binding regions.

Temperatures of 94-95°C was used to denature the DNA/cDNA, 60-62°C was used for primer annealing and 72°C allowed for primer extension. PCR and qPCR runs of 40 cycles were used to sufficiently detect low levels of expression (Peake 1989). The extended 7min PCR amplification was used to complete extension of all PCR products.

PCR amplification cycles		QPCR amplification cycles	
Number of Cycles		Number of Cycles	
1	94°C for 2min	1	95°C for 2min
40	94°C for 30sec 60°C for 30sec 72°C for 30sec	40	95°C for 5sec 62°C for 10sec 72°C for 20sec
1	72°C for 7min	1	65°C for 5sec
1	12°C hold	1	95°C for 5sec

Table 2. Forward and reverse primers used for qPCR. Amplicon size ranged between 70-110 base pairs. Products with 50-60 GC rich regions were chosen to increase efficiency of primer-to-product disassociation. Melting curves were verified for primer specificity to qPCR product. Primer efficiencies between 90-105% were verified using dilution curves, as per MIQE guidelines (Bustin et al. 2009).

Gene name	Forward primer	Reverse primer
<i>Arc/Arg3.1</i>	GCT CCC TTC CGG CTT CT	CCA GCT CCA TCT GGT CGT
<i>Fos</i>	CTT CGA CCA TGA TGT TCT CG	TGG TAG GAA AGG CTG T
<i>Bdnf</i>	TTA CCT TCC TGC ATC TGT TGG	GTC ATC ACT CTT CTC ACC TGG
<i>Egr1</i>	ACC TGA CCA CAG AGT CCT T	AGT CGT TTG GCT GGG ATA AC
<i>CamkIIα</i>	CAG CCA GAG ATC ACC AGA AG	GTC ATG GAG TCG GAC GAT ATT
<i>Dusp1</i>	CCC ATT AGA TGA GAC CCT ATC CT	GCT GGA AGT TGG GTG TAG ATG
<i>Homer1a</i>	GAA GTC GCA GGA GAA GAT G	TGA TTG CTG AAT TGA ATG TGT ACC
<i>Nr4a1</i>	GTC CGC TCT GGT CCT CAT	ATG CGA TTC TGC AGC TCT TC
<i>Grin1</i>	GTC CTC CAA AGA CAC GAG CA	GGC CCT CCC TCT CAA TA
<i>Neomycin</i>	GTC TAG CTT GGC TGG ACG TAA	GAG GTT AAC CCC GAT CAT ATT CAA

3.6 NR1 Western Blot

3.6.1 Synaptosomal Preparation

Dissected brain samples frozen in N₂ and stored in -80°C, were thawed on ice. All procedures were performed on ice or at 4°C. Samples were homogenized in a homogenization solution (0.32M sucrose, 20mM HEPES pH7.4, 1mM EDTA in H₂O, pH7.4) also containing protease inhibitors (1.5µg/mL aprotinin, 10µg/mL leupeptin, 10µg/mL pepstatin A, 0.1mg/mL benzamidine and 0.1mM phenylmethanesulfonylfluoride (PMSF)). The homogenizing solution was added to samples and homogenized using a hand-held homogenizer and clean homogenizer tips. Samples were centrifuged at 1000rcf for 10min and the supernatant was extracted into a separate tube. Consequently, samples were centrifuged at 10000rcf for 10min and the supernatant was discarded. The pellet was re-suspended in 50ul of protein lysis buffer (50mM Tris HCL pH7.5, 150mM NaCl, 1% TritonX-100, 0.1% sodium dodecyl sulfate (SDS) and 2mM EDTA in H₂O) containing protease inhibitors (1.5µg/mL aprotinin, 10µg/mL leupeptin, 10µg/mL pepstatin A, 0.1mg/mL benzamidine and 0.1mM phenylmethanesulfonylfluoride (PMSF)). Samples concentrations were determined using a BCA protein assay (Pierce, USA), stored at -80°C and later used in immunostaining.

3.6.2 Immunostaining

BCA concentrations for crude synaptosomal protein samples were used to dilute proteins to 2µg/µl with protein lysis buffer. Diluted samples were combined with 5% 2-mercaptoethanol and 25% 4X sample buffer pH6.8 (0.25M Tris Base, 277mM SDS, 40% glycerol, 119mM bromophenol blue in H₂O), for a final loading volume of 20µl per well. Prepped samples were placed in a 55°C heat block for 10min to denature proteins and stored at -20°C. Samples were thawed, vortexed and 20ul was loaded on a 4% SDS-PAGE stacking gel and separated using a 7.5% SDS-PAGE separating gel running at 130mV at RT for 2h, in running buffer pH8.1-8.3 (25mM Tris base, 192mM glycine, 3.5mM SDS in H₂O). A wet transfer was performed onto PVDF membranes at 100mV at RT for 1h in transfer buffer (25mM Tris base, 192mM glycine, 20% methanol, 0.025% SDS in H₂O). Following transfer, the PVDF membrane was re-activated in methanol, stained using REVERT total protein stain, washed with REVERT wash solution and imaged using Odyssey (LI-COR, NE, USA). The membrane was de-stained using REVERT reversal solution and blocked for 1h using 5% milk in tris-buffered saline with 0.1% Tween20 (TBS-T). Membranes were incubated with NR1 (1:500) (Millipore 05-432, MA, USA) and NR2A (1:800) (Millipore 07-632, MA, USA) primary antibody diluted in 5% milk in TBST, kept overnight at 4°C on a rotating orbital shaker. The following day, membranes were washed three times and stained with goat anti-mouse AlexaFluor680 (1:5000) (ThermoFisher, MA, USA) for NR1 and goat-anti rabbit AlexaFluor680 (1:5000) (ThermoFisher, MA, USA) for NR2A secondary antibody diluted in 5% milk in TBST, and kept for 1h at RT in a light sensitive container. The membrane was washed three times in a light sensitive container prior to imaging using Odyssey (LI-COR, NE, USA). Blot quantifications were performed using Image Studio Software (LICOR, NE, USA).

3.7 Egr1 Immunohistochemistry

Perfused brains were frozen at -20°C in TissueTek optimal cutting temperature (OCT) compound (Sakura Finetek USA INC., USA). Brains were mounted onto chucks and cut at 40µm sections using a cryostat set at -20°C. Floating sections were washed once in PBS for 10min and concomitantly blocked with 500µl of 4% NGS, 0.5% TritonX-100 in PBS, for 1h on a rotating orbital shaker. Blocking solution was removed and incubated overnight with 200µl of Egr1 primary antibody (SantaCruz SC-189, USA) diluted 1:5000 in blocking solution. Sections were washed three times with PBS and incubated with goat anti-rabbit AlexaFluor680

(ThermoFisher, MA, USA) secondary antibody diluted 1:15000 in blocking solution in a light sensitive container, for 1h on a rotating orbital shaker. Sections were washed three times, mounted onto slides and left to dry in a dark chamber. Sections were mounted with Aqua-Mount mounting media (Polysciences INC., PA, USA) and cover slipped. Images were taken using Odyssey (LI-COR, NE, USA) with the same intensity for all genotypes. Quantification of images was performed using Image Studio Software (LI-COR, NE, USA).

4 Statistical Analysis

Statistical analysis was performed using GraphPad Prism5 (GraphPad Software Inc., CA, USA) for univariate one-way and univariate two-way ANOVA with Bonferroni post-hoc. Fear conditioning and behaviour data were analyzed for sex and genotype by multivariate repeated measures or one-way ANOVA with Bonferroni post-hoc, using SPSS (IBM, NY, USA). Suppression ratio was also analyzed using one-sample t-test comparison to a fixed value of 0.5. Comparisons were considered statistically significant at $p \leq 0.05$. Measurements are presented as mean $\pm$ SEM for behavioural tests and mean $\pm$ SD for molecular analysis.

Chapter 3 RESULTS

1 NMDA receptor knockdown and rescue

1.1 Validation of NR1-KD and NR1-Cre mouse models

Initially, naïve NR1-knockdown (NR1-KD) and NR1-Cre mice were analyzed for levels of Cre-recombinase activity, *Grin1* gene expression and NMDA receptor subunit protein levels. A quantifiable measure of the neomycin cassette insertion verified the insertion or excision of the neomycin cassette in NR1-KD and NR1-Cre mouse lines. The data for neomycin expression was normalized to a two-fold change, in order to represent it as two allelic copies within the *Grin1* gene (Figure 4.1A). Primers targeted to the neomycin cassette and to the intron 19 mutagenic site, demonstrated that NR1-KD mice expressed high levels of neomycin copy number in genomic DNA (Figure 4.1A). As expected, there was no expression of neomycin in WT. The neomycin insertion mutation resulted in a 0.84-fold or 84% reduction of *Grin1* mRNA expression levels in NR1-KD compared to wild-type (WT), as seen in Mohn et al., 1999 ($p < 0.001$) (Figure 4.1B). In NR1-Cre mice, the Cre-recombinase mediated excision of neomycin flanked loxP sites resulted in a 60% reduction in neomycin expression relative to NR1-KD ($p < 0.001$) (Figure 4.1A). The *Grin1* gene expression levels were increased by 22% in NR1-Cre compared to NR1-KD ($p < 0.001$) (Figure 4.1B). However, *Grin1* levels in NR1-Cre remained 62% lower than WT ($p < 0.001$) (Figure 4.1B).

Figure 4.1 Rescue from knockdown of *Grin1* expression and Cre-induced excision of neomycin insertion mutation in NR1-Cre. Genomic expression of neomycin cassette and *Grin1* mRNA expression within the hippocampus was measured in NR1-KD and NR1-Cre mice. Genomic DNA (gDNA) and mRNA was collected in 14 week old male mice treated with

tamoxifen. GDNA and mRNA was isolated from the same sample, for qPCR analysis. A. Y-axis is expressed as two copy number for insertion at homozygous alleles. Primers were targeted to the neomycin cassette and a portion of intron 19 for qPCR (Table 3). NR1-KD mice showed higher intronic expression of neomycin cassette compared to NR1-Cre. WT mice lacked neomycin expression. B. *Grin1* mRNA levels in NR1-KD and NR1-Cre were presented as fold changes relative to WT. NR1-Cre showed higher *Grin1* expression compared to NR1-KD. Both NR1-KD and NR1-Cre *Grin1* expression levels were significantly reduced compared to WT. Univariate one-way ANOVA, Bonferroni correction. Comparisons to NR1-KD, ^aP<0.001 and NR1-Cre, ^bP<0.001. N=4-6, mean±SD.

1.2 Hippocampal NR1 and NR2A subunit expression

Following gene expression analysis, functional expression of NR1 and NR2A were quantified to validate the knockdown and rescue of the NR1 subunits. Synaptosomal preparation was used to isolate functional NMDA receptor expression at the pre- and postsynaptic terminals. Previously published results had shown a 90% reduction in NR1 and NR2A subunit levels in NR1-KD mice (^{a,b}p<0.001) (Ramsey et al. 2008). This data was confirmed in the western blot analysis, with NR1-KD mice expressing only 7-15% of NR1 and NR2A of WT levels (^ap<0.001) (Figure 4.2). Relative to NR1-KD, Cre-recombinase mediated rescue increased NR1 and NR2A expression by 14% and 24% respectively (p<0.001, p<0.01) (Figure 4.2).

Figure 4.2 Levels of NR1 and NR2A subunits are decreased with receptor knockdown and increased with Cre-induction. Synaptosomal extraction of proteins was used to enrich for NR1 and NR2A expression. NR1 and NR2A was quantified in the same sample of mice, males and females aged 14 weeks. Western blots were imaged using Odyssey (LICOR) and quantified using Image Studio (LICOR). Representative western blot for NR1 and NR2A subunit protein expression. Total protein stain was used as loading control. NR1 and NR2A protein expression was normalized to total protein and represented as percent of WT. Student's t-test comparison of

NR1-KD to NR1-Cre. Univariate one-way ANOVA, Bonferroni correction. Comparisons to NR1-KD, ^aP<0.001 and NR1-Cre, ^bP<0.001. N=5-6, mean±SD.

1.3 Locomotor Activity

Impaired NMDA receptor activity has been shown to cause increased locomotor activity (Tricklebank et al. 1989). Similarly, previous data had found increased locomotor activity in NR1-KD mice (Mohn et al. 1999). Concurrent work from another graduate student in our laboratory showed that NR1-Cre mice have improved locomotor activity and habituation in a novel context (Figure A1). Therefore, locomotor activity served as a behavioural readout for this study. Hyperactivity was seen in NR1-KD mice with nine times more total distance travelled in a novel context compared to WT (^ap<0.001) (Figure 4.3B). NR1-KD mice also had impaired habituation to a novel context over a 2h run (Figure 4A). Alternatively, NR1-Cre had partial rescue in hyperactivity with attenuated levels of total distance traveled and also showed improved habituation to novel context (Figure 4A,B). NR1-Cre mice travelled four times the total distance of WT and half the total distance of NR1-KD (^bp<0.05, p<0.001) (Figure 4B).

Figure 4.3 Hyperactivity in NR1-KD is partially reduced in NR1-Cre. Male and female mice aged 14 weeks were first habituated to experimenter before testing locomotor activity. Activity was measure using infrared beam breaks, recorded on VersaMax (AccuScan) into 5min bins, which was quantified using VersaDat (AccuScan) as distance (cm) traveled. All genotypes were treated with tamoxifen for 2 weeks as a control for tamoxifen effects on behaviour. A. Data plotted as total activity measured in 5min bins over 2 hours. Activity display a lack of habituation to a novel context in NR1-KD, which was partially recovered in NR1-Cre. WT and WT-Cre did not show differences in habituation to novel context. Statistical significance not shown. B. Distance (cm) travelled over 2h was plotted as total distance. NR1-KD had increased total distance with partial recovery upon Cre-induction. Univariate one-way ANOVA,

Bonferroni correction. Comparisons to NR1-KD, ^aP<0.001; NR1-Cre, ^bP<0.05; WT, ^cP= 1.0. N=10, mean±SEM.

2 Fear Conditioning

Reduced NMDA receptor function within the hippocampus can produce pronounced deficits in contextual memory (Tsien et al. 1996; Frankland et al. 2004; Morris 2013). Deficient glutamatergic signaling in the amygdala has also been shown to prevent encoding of fear associated tone and context memory. Furthermore, knockdown of NMDA receptors causes deficits in short-term memory and anxiety related behaviours, which display impaired higher order processing in NR1-KD mice (Milenkovic et al. 2014) (Figure A1). These behaviours have been shown to be improved in NR1-Cre mice (Figure A1). Improved short-term memory and anxiety in NR1-Cre, lead to further investigations regarding long-term memory functions in fear associated learning. Fear associated learning occurs when an unconditioned stimulus (ie. shock) that elicits an unconditional response (ie. pain and jumping) is paired with a conditioned stimulus (ie. context) originally eliciting a neutral response. The learned association between unconditioned and conditioned stimulus allows the conditioned stimulus to induce a conditioned response (freezing). Recall of these learned associations requires storage into long-term memory.

2.1 Training

NMDA receptor blockade prevents learning in animals (Rezvani 2006). Knockdown of NMDA receptor expression also impaired learning in NR1-KD mice, even with increasing tone-shock pairings (Figure 4.4A). As a result, NR1-KD mice displayed a complete lack of total freezing behaviour (Figure 4.4B). Rescue in NMDA receptor expression improved freezing in NR1-Cre mice, with significant freezing starting at the 4th tone-shock pairing (23±5.4%, p<0.01) whereas WT mice increased freezing response on the 2nd tone shock pairing showing an altered learning curve in NR1-Cre (47±5.2%, p< 0.001) (Figure 4.4A). Also at the 4th tone-shock pairing, freezing behaviour in NR1-Cre was increased relative to NR1-KD (p<0.05) (Figure 4.4A). WT-Cre mice displayed the same freezing patterns as WT mice during the training session (^cp=1.0). Inter-trial interval showed the same freezing patterns as total freezing, which confirmed the learned behaviour (Figure 4.4C). A separate subset of NR1-KD mice were retrained for an additional day to determine whether additional training would induce freezing behaviour. However, retrained NR1-KD mice again displayed a complete lack of freezing in response to

fear conditioning similar to the first training session ($p=1.0$) (Figure 4.4D). There were no sex effects on freezing behaviour ($p>0.4$) (data not shown), therefore freezing behaviour results from both sexes were compiled.

Figure 4.4 Lack of fear response in NR1-KD mice is rescued with NMDA receptor expression. Both males and females, aged 14 weeks were trained for fear conditioning after five days of habituation to experimenter. One day prior to training, mice were tested for locomotor activity in a novel context. All mice tested received tamoxifen, as a control for tamoxifen mediated effects. Training was performed using 5 successive tones (CS) (15s, 1.0kHz, 95dB) which co-terminated with 5 foot shocks (US) (1s, 0.5mA), with a 60s inter-trial interval. Activity was measured for 8min. using Coulbourn Instruments and analyzed using FreezeFrame3 software (Actimetrics). A. Freezing was measured during tone shock pairings (15 sec), numbered from the first to last pairings performed. Background was measured during the first 2min of training, prior to tone or shock and is displayed as average freezing. Increasing tone-shock pairing showed increased freezing over time for WT, WT-Cre and NR1-Cre. WT and NR1-Cre displayed significant freezing relative to baseline and NR1-KD, at the 2nd and 4th tone-shock pairings respectively. B. Total freezing (%) is a measure of freezing over all five tone-shock pairings. NR1-KD showed deficits in freezing. C. Inter-trail interval measured the total freezing between each tone-shock pairing. Across genotypes, percent freezing during inter-trail intervals

are similar to tone-shock pairings. D. NR1-KD mice were re-trained 24h after the first training. Re-trained NR1-KD mice showed no improvement in freezing during training. Multivariate repeated measures ANOVA and univariate one-way ANOVA, Bonferroni correction. Comparisons to NR1-KD, ^aP<0.001; NR1-Cre, ^bP<0.001; WT, ^cP= 1.0. N=16-19, mean±SEM.

2.2 Contextual Memory

NMDA receptors are required in forming contextual memory (Morris et al. 1986). Mice that lack hippocampal NMDA receptor expression display spatial learning and memory deficits (Nakazawa et al. 2004). As such, NR1-KD mice displayed no freezing behaviour ($0.4 \pm 3.8\%$), and thus a complete lack of fear associated contextual memory (Figure 4.5A,B). Retrained NR1-KD mice also failed to freeze within the training context (Figure 4.5C). NR1-Cre mice showed a partial rescue in contextual memory with $30 \pm 5.6\%$ freezing behaviour, equivalent to 40% of WT response ($p < 0.001$, ^b $p < 0.001$) (Figure 4.5B). WT, WT-Cre and NR1-Cre mice displayed relatively consistent percent freezing during the full 5min of testing (Figure 4.5A).

Figure 4.5 Contextual fear memory deficit in NR1-KD mice is partially rescued as a consequence of restored NMDA receptor expression in adult mice. Mice were returned to the context (CS) used for training and recorded for freezing over 5min., 24h after training. A. Percent freezing is presented in 60sec intervals over 5min. NR1-Cre, WT and WT-Cre mice freeze consistently over the 5min test in the trained context (CS). NR1-KD were freezing significantly more than NR1-KD, $^{**}P<0.01$. B. Total freezing (%) is a measure of freezing during the 5min test. NR1-KD failed to freeze as a fear response to context. NR1-Cre showed partial rescue in freezing. C. NR1-KD mice with two training sessions over a period of 2 days had unimproved freezing in context (CS). Univariate one-way ANOVA, Bonferroni correction. Comparisons to NR1-KD, $^aP<0.001$; NR1-Cre, $^bP<0.001$; WT, $^cP=1.0$. $N=16-19$, mean $\pm$ SEM.

2.3 Tone-Associated Memory

Impaired amygdala function is known to cause decreased learning of tone-associated memory in a fear paradigm (Phillips & LeDoux 1992). The amygdala is thought to mediate associations between fearful emotional events with the surrounding cues and allows for a response to fear. NMDA receptors are known to also function in mediating this process within the amygdala, as receptor deficiency impairs tone-associated memory formation (Walker et al. 2005). NR1-KD mice again displayed no freezing behaviour associated with CS tone, with only $4\pm2.5\%$ measurable freezing response (Figure 4.6A,B). Twice re-trained NR1-KD mice also demonstrated a lack of freezing response ($p=1.0$) (Figure 4.6C). Rescue in NMDA receptors recovered freezing behaviour resulting in $51\pm6.0\%$ freezing response, which is 60% of WT response ($p<0.001$, $^b p<0.001$) (Figure 4.6B).

C.

Figure 4.6 Tone associated freezing in NR1-Cre mice was absent in NR1-KD. Mice were placed in a novel context 48h after training. Similar to training, mice acclimated for 2min, followed by 5 successive tone stimuli (95dB) without shocks, and 60s inter-trial intervals. Freezing was measured during 15sec of CS tone. Background was measured during the first 2min prior to CS tone. A. Percent freezing is displayed for each successive tone, numbered 1-5. Multivariate repeated measures B. Total freezing (%) is a measure of freezing during the 5 CS tones. C. NR1-KD mice with two training sessions showed no improvement in freezing due to CS tone. Univariate one-way ANOVA, Bonferroni correction. Comparisons to NR1-KD, ^aP<0.001; NR1-Cre, ^bP<0.001; WT, ^cP= 1.0. N=16-19, mean±SEM.

2.4 Suppression Ratio

Fear response in mice is indicated by freezing behaviour, however mice with hippocampal lesions and NMDA receptor blockade can have an accompanied increase in locomotor activity (Anagnostaras et al. 2000). To mitigate this potential confound, researchers use an alternative measure of fear response by determining whether recall of fear associated contextual memory causes a mouse to reduce its activity relative to baseline. This measure is termed suppression ratio and is calculated by the following equation: (activity during test) / (activity during test + activity during habituation) (Anagnostaras et al. 2000). Mice with contextual fear memory have a reduced suppression ratio, even though freezing behaviour is impaired (Gould & Lewis 2005). NR1-KD mice did not show any freezing behaviour throughout fear conditioning, which prompted our measurements of the suppression ratio. It has been reported that values of suppression ratio lower than 0.5 is the learned response to fear, however a ratio equal to 0.5 displays a complete lack of fear (Annau & Kamin 1961). On the opposite spectrum, values greater than 0.5 suggests learned safety for the context (Annau & Kamin 1961). NR1-KD had a suppression ratio not significantly different from 0.5, exhibiting no fear to the training context

($p=0.2$). NR1-KD suppression ratio was significantly different compared to NR1-Cre and WT ($p<0.05$) (Figure 4.7). NR1-Cre suppression ratio was not significantly different from WT ($p=1.0$). NR1-Cre and WT displayed a suppression ratio less than 0.5 ($p<0.05$).

Figure 4.7 NR1-KD mice have abnormal fear association to context. Baseline activity included the first 2 min prior to tone-shock pairing on training day. Activity in the fear context was measured for an equivalent 2 min. The suppression ratio was determined by (activity during test/(baseline activity + activity during test)). MPEG4 video files were formatted to AVI and used for automated activity monitor on Biobserve, measured as distance travelled (cm). WT-Cre was combined with WT, due to indifference in percent freezing for fear conditioning. Dashed line marks suppression ratio of 0.5. Univariate one-way ANOVA, Tukey's post-hoc. One sample T-test, comparing genotype means to 0.5. * $P<0.05$. $N=3-6$, mean $\pm$ SEM.

2.5 Burst Activity

Measurements in burst activity is used to determine an adequate response to a shock stimulus (Anagnostaras et al. 2000). This measurement is important due to findings that NMDA receptor hypofunction can lead to differences in pain sensitivity (Eisenberg et al. 1993). To determine whether NR1-KD and NR1-Cre mice exhibited normal pain sensitivity to shock, shock response expressed as burst activity was measured as velocity (cm/s) during the first 1s shock stimulus applied during training. Baseline velocity was recorded 2s prior to administration of shock. The results found no genotypic differences in burst activity, measured to be $>80\pm 18$ cm/s or in baseline response measured as $24-30\pm 13$ cm/s across genotypes ($p=0.17$) (Figure 4.8). Burst activity was increased compared to baseline activity within all genotypes ($p<0.05$).

Figure 4.8 Pain sensitivity is conserved across all genotypes. Burst activity was measured during 1sec shock during the first tone-shock pairing on training day. Baseline activity was measured for 2s, prior to shock and during the first CS tone. Data is presented as velocity (cm/s) over 1s. A. Burst activity is the same across all genotypes. Univariate two-way ANOVA, Bonferroni post-hoc. N=4-7, mean±SEM.

3 Basal and Induced Immediate Early Gene Expression

Changes in immediate-early gene (IEG) regulation has been shown to occur both basally and during memory formation from NMDA receptor activity (Y. Chen et al. 2014; Alberini 2009). Measuring basal gene expression levels would give an indication of cellular adaptations to NMDA receptor knockdown and rescue. Learning induced alterations in gene expression would provide a measure of NMDA receptor activation in NR1-KD and NR1-Cre mice. Using qPCR, analysis was performed on 8 genes: *CamkIIa*, *Dusp1*, *Nr4a1*, *Egr1*, *Fos*, *Bdnf*, *Arc* and *Homer1a*. Genes were chosen based on their functional properties in NMDA receptor mediated signaling during memory formation and their induction requiring NMDA receptor activity following fear conditioning. All qPCR analysis were performed with hippocampal samples from male mice, due to previous studies which demonstrated sex differences in gene expression (Berchtold et al. 2008; Kight & McCarthy 2017).

3.1 Functional Group: Kinase

NMDA receptor activity induces CREB phosphorylation and induction of IEGs, such as *Dusp1* and *CaMKIIa* (Keeley et al. 2006; Wang & Peng 2016). Therefore, gene expression analysis was performed on *Dusp1* and *CaMKIIa*. Surprisingly, WT and NR1-KD mice did not show basal difference in the levels of *CaMKIIa* and *Dusp1* (Figure 4.9A, B). This striking result was also

observed in the basal expression of these genes in NR1-Cre hippocampus. Contrary to our hypothesis, the rescue or absence of receptor did not alter the regulation of kinase and phosphatase expression basally. Fear induction of these IEGs were observed in all genotypes, with a 0.6-fold increase in *CaMKII α* and 3-fold increase in *Dusp1* levels ($p < 0.001$). However, no genotype differences were observed in both *CaMKII α* and *Dusp1* ($p = 0.77$, $p = 0.15$ respectively). These results point towards potential compensatory mechanisms in NR1-KD mice for inducing genes during fear conditioning.

Figure 4.9 Fear conditioning increased gene expression of *CamkII α* and *Dusp1*, a MAPK inhibitor. Male mice aged 14 weeks were collected as naïve or 30min after fear conditioning. All mice had received tamoxifen to control for tamoxifen effects on transcription. All fold changes were made relative to WT naïve. A-B. Basal gene expression patterns showed no differential regulation across genotypes. Fear induced upregulation of *CaMKII α* and *Dusp1* expression. WT, NR1-KD and NR1-Cre mice showed similar levels of gene induction for both *CaMKII α* and *Dusp1* genes. *CaMKII α* was upregulated by a factor of 0.6 and *Dusp1* increased 3 fold in fear conditioned mice. Multivariate two-way ANOVA, Bonferroni post-hoc. Naïve vs Fear Conditioned, *** $P < 0.001$. $N = 4-6$, mean $\pm$ SD.

3.2 Functional Group: Transcription Factors

CREB regulated transcription factor expression provides an amplification of genes that are thought to take part in the second wave of transcription required in memory formation (Alberini 2009). These transcription factors allow for targets outside of CREB to be potentiated after NMDA receptor activation (Alberini 2009). Transcription factors, such as *Nr4a1*, *Egr1* and *Fos* has been shown to be upregulated following fear induced learning due to activity of the NMDA receptor (Wang & Peng 2016). *Bdnf*, which encodes the brain-derived neurotrophic factor, is likewise induced by NMDA receptor activity, causing the expression of other transcription factors. QPCR quantification indicates no differential regulation of these genes between NR1-

KD and WT at basal and fear conditioned states (Figure 4.10A-C). *Bdnf* was not induced following fear conditioning in either genotypes ($p>0.09$) (Figure 4.10D). *Bdnf* was differently expressed in NR1-Cre relative to NR1-KD, and this was only after fear conditioning ($p<0.05$) (Figure 4.10D). NR1-Cre mice have similar levels of basal transcription of these genes as WT ($p=1.0$).

Figure 4.10 Learning-induced genes encoding transcription factors were upregulated in all genotypes, 30min after fear conditioning. Male mice aged 14 weeks were collected as naïve or 30min after fear conditioning. All fold changes were made relative to WT naïve. A-C. IEGs were basally regulated at similar fold expression levels across genotypes. Gene expression of transcription factor, NR4A was increased by ≤ 2.6 fold in all genotypes. Levels of *Egr1* expression showed a 2-3 fold induction by fear conditioning. A 4-6 fold change in *Fos* gene expression was also seen with fear conditioning. D. Although basal levels of *Bdnf* gene expression was unaltered between genotypes, NR1-Cre mice showed slightly elevated levels of *Bdnf* compared to NR1-KD. Multivariate two-way ANOVA, Bonferroni post-hoc. Naïve vs Fear conditioned, *** $P<0.001$, ** $P<0.01$, * $P<0.05$. $N=4-6$, mean $\pm$ SD.

3.3 Functional Group: Structural Modification

Among the IEGs that are upregulated, structural genes are induced by CREB to allow for structural modifications at activated synapses (Middei et al. 2012). Structural modifications are

the functional consequence of learning and LTP generation, giving way to a measurable and long-term change in learned behaviours (Middei et al. 2012). *Arc* and *Homer1a* are proteins that regulate this process of structural modification through de-stabilization mechanisms that allow for subsequent remodeling and rebuilding of synapses (Chowdhury et al. 2006; Sala et al. 2003). *Arc* and *Homer* proteins are required for NMDA receptor mediated memory formation (Guzowski et al. 2000). The basal expression patterns of *Arc* and *Homer1a* were maintained across all genotypes ($p=1.0$). The expected alterations in basal and learning induced genes in NR1-KD and NR1-Cre were not present. Increased *Arc* expression after induction showed a 4-5 fold increase in all genotypes. *Homer1a* was not significantly upregulated upon induction in WT, with a 0.6 fold increase (Figure 4.11A). Interestingly, following fear conditioning NR1-KD mice had a 2-fold elevated expression of *Homer1a* in comparison to both WT and NR1-Cre ($p<0.01$, $p<0.05$ respectively) (Figure 4.11B).

Figure 4.11 Fear conditioning increased expression of synaptic plasticity genes required in structural remodeling. Data obtained from tamoxifen treated male mice, aged 14 weeks. All fold changes were made relative to WT naïve mice. A. Genotype comparisons for basal gene expression showed no significant difference. Following fear conditioning, *Arc* expression levels were increased by 4-5 fold. B. There were no difference in basal gene expression patterns across genotypes. Levels of *Homer1a* were found to be upregulated in NR1-KD, to a greater extent than WT and NR1-Cre as a result of fear conditioning. However, fear conditioning did not significantly induce *Homer1a* in WT mice. Multivariate two-way ANOVA, Bonferroni post-hoc. Naïve vs Fear Conditioned, *** $P<0.001$, ** $P<0.01$, * $P<0.05$. N=4-6, mean $\pm$ SD.

4 Learning Induced *Egr1* expression

The gene expression data gave rise to questions on whether protein expression patterns across genotypes were also maintained. The protein quantification of the IEG *Egr1*, provided observations into the translation of gene expression data into functional protein expression.

Mouse brains were collected 40min after fear conditioning. Brain sections cut into 40 μ m was used to stain for Egr1 and was followed by imaging with Odyssey (LICOR). Using this technique, there was no evidence of Egr1 protein increases within the hippocampus. Instead other areas showed upregulation of Egr1 protein expression after fear conditioning. Nevertheless, the staining pattern from the cortex and amygdala showed the exact same results found with gene expression analysis, where no observational differences between genotypes were present in Egr1 protein expression basally or after induction by fear conditioning (Figure 4.12).

Figure 4.12 Qualitative *Egr1* protein expression showed non-differential induction across genotypes. Male mice aged 14 weeks were fear conditioned and 40min after perfused for immunostaining. Odyssey (LICOR) was used to image fluorescence in sections. Qualitatively, *Egr1* protein expression were increased following fear conditioning. No genotype differences were observed in either the hippocampus, cortex or amygdala.

Chapter 4

DISCUSSION, CONCLUSION and FUTURE DIRECTION

1 Discussion

The plasticity of a synapse allows a neuron to encode the patterns and frequency of communication between neighbouring neurons. Communication between neurons provide a mechanism through which different elements of the environment can take part in a single memory and in producing a response that becomes a learned behaviour (Nakazawa et al. 2004). Increasing the strength in communication produces a stronger association between the environment and the learned behaviour, allowing for an immediate response (Anagnostaras et al. 2000). Without such communication, humans or animals disassociate the environment from mediating a proper response, which can affect learned behaviours and ultimately affect survivability. Therefore, learning is controlled by the extent of plasticity present within the brain.

For many years, the molecular mechanism of plasticity has been and still continues to be researched. Today, many researchers agree that NMDA receptors play a vital role in learning processes. More specifically, NMDA receptors are important in encoding spatial learning within the hippocampus (O'Keefe & Nadel 1978; Tsien et al. 1996). Breakdown in the receptor signalling leads to impaired spatial learning and produces deficits in plasticity of the hippocampus (Bach et al. 1995; Baier et al. 2009).

Aberrant glutamate signalling has been seen in multiple disorders including schizophrenia and autism spectrum disorder (Wesseling et al. 2014). These disorders are thought to follow a neurodevelopmental course by which a pre-natal risk causing receptor dysfunction, lead to later onset of full blown psychosis. This neurodevelopmental hypothesis is built on the premise that although children can display mild neurologic abnormalities in early childhood, the complete set of symptoms present within the disorder is displayed in adolescents and young adults (Malmberg et al. 1998). Matured neural networks required for learning in adolescents and young adults do not share the same level of plasticity found prenatally or in childhood (He & Crews 2007). The damage sustained during development therefore leads to lasting effects on the connectivity of neural networks (Marenco & Weinberger 2000). However, as research progresses it is becoming more clear that the adult brain retains a higher degree of plasticity than previously thought. The hippocampus alone has been shown to maintain neurogenesis and to allow for vast modifications

in memory related network through synaptic plasticity far into adulthood (Leuner & Gould 2010). With this knowledge, my thesis focuses on the ability for hippocampus dependent spatial memory to be recovered from a prenatal neurodevelopmental dysfunctions in NMDA receptor activity, seen in disorders such as schizophrenia.

The study was undertaken by using a mouse model of prenatal NMDA receptor knockdown and subsequent restoration of receptor expression in adults, to determine the malleability of the hippocampus in spatial learning mediated behaviours. To test the hypothesis that the adult hippocampus and its related circuits maintains high levels of plasticity in the adult brain, both a behavioural and a molecular approach was used to quantify neuronal plasticity. Previous studies from our lab suggested that knockdown in NMDA receptors leads to alterations in anxiety and hinted at a lack of short-term memory and long-term memory (Figure A1). To more specifically study the fear and anxiety circuit, as well as to directly test long-term memory in the receptor knockdown and rescue states, fear conditioning was used as a behavioural measure of hippocampal plasticity. Fear conditioning was used further corroborated with qPCR results to determine a molecular mechanism of NMDA receptor mediated alterations in hippocampal circuits.

1.1 Molecular and behavioural validation of NR1-KD and NR1-Cre mice

Initially, we validated the efficiency of Cre-recombinase in excising the neomycin cassette. QPCR amplification of genomic DNA was used to quantify the copy number of neomycin, which should be excised by Cre-recombinase. NR1-Cre mice lost 60% of genomic neomycin copy number within the hippocampus. However, this suggests that 40% of genomic neomycin expression remains in NR1-Cre. This could be due to an inaccessibility of the *Grin1* gene to allow for Cre-recombinase induced excision of neomycin.

The degree of NR1 knockdown was similar to what was reported in the original knockdown mouse (Mohn et al. 1999). *Grin1* mRNA expression was reduced by 93% in NR1-KD, expressing only 7% of WT levels. In NR1-Cre mice, Cre-induction lead to 37% of WT *Grin1* gene expression. These mRNA levels of expression is highly correlated with the protein expression seen in both NR1-KD and NR1-Cre mice. It is however interesting that a 60% excision rate amounts to small increases in mRNA expression within NR1-Cre. A potential

explanation might include post-excision silencing of the *Grin1* gene leading to reduced mRNA expression. In a state where the cell suffers from reduced NMDA receptor signalling, compensatory mechanisms already in place might act against overstimulation of the cell by preventing surface expression of NMDA receptors by silencing *Grin1* expression (Noh et al. 2012). Nevertheless, the genomic and mRNA results confirmed that the tamoxifen treatment protocol used in this study lead to Cre-recombinase activity within the hippocampus.

Further protein and behavioural analysis confirmed rescue of NMDA receptor expression in NR1-Cre mice. Western blot for NR1 and NR2A showed that both subunits were dramatically reduced in NR1-KD, and were partially rescued in NR1-Cre mice. Relative to NR1-KD, the induction of Cre-recombinase increased both NR1 and NR2A subunits by 14% and 20% respectively. To place into perspective the functional significance to the rescue of expression, fear mediated learning has been shown to increase NMDA receptor expression by 30% (Cammarota, de Stein, et al. 2000). NR1 and NR2A subunits co-exist at the postsynaptic density in a 1:1 ratio and their expression can predict an increase in functional NMDA receptor expression with Cre-induced rescue. Our western results validated protein subunit loss and rescue in our mouse model.

Locomotor activity was measured reproduce previous results demonstrating hyperactivity in NR1-KD and a partial rescue in NR1-Cre mice. This was important to study because the primary measure of fear memory is freezing behaviour. Hyperactivity has been noted in mice with hippocampal lesions and NMDA receptor antagonism (Anagnostaras et al. 2000). The NR1-KD mice had a profound increase in locomotor activity, which was partially rescued in NR1-Cre mice.

1.2 Contextual and tone associated learning in NR1-KD and NR1-Cre mice

Fear associated learning has been shown to provide a robust phenotypic response. Using this behavioural phenotype the dynamic nature of the hippocampal and amygdala fear related networks were observed. This study tested whether the level of plasticity in the adult brain was sufficient to recover from a neurodevelopmental insult of global reductions in glutamatergic signalling. Such a model is important in disorders with memory dysfunction that are caused by neurodevelopmental exposure to risk factors, preventing the formation of proper neural networks

required in memory. Our study found that a neurodevelopmental hypofunction of the glutamate system prevents the encoding of both context and cue associated fear memories. This study was not among the first to observe a deficit in learning from NMDA receptor knockdown, rather it supports previous findings (Morris et al. 2013; Bauer et al. 2002). Previous studies have also shown that targeted enhancement of the NMDA receptor signaling pathway can enhance LTP generation and enhance memory in adult mice (Tsien et al. 1999; Kleschevnikov & Routtenberg 2001; Billard & Rouaud 2007). The extent of hippocampal dysfunction in the NR1-KD mice and in other mouse models with antagonism of the NMDA receptor, suggests that the network associated with contextual learning is severely impaired. Similarly, fear associated learning in general has been shown to be abnormal in schizophrenia patients (Gur et al. 2007). The NR1-Cre mice showed that the hippocampus can re-establish network circuitry and recuperate from severe neurodevelopmental insults, following recovery of NMDA receptor expression in the adult brain. Recovery of NMDA receptor expression therefore not only restores synaptic communication, but also conserves the ability to form proper neural circuits involved in learning. However, learning in NR1-Cre mice never reach WT levels. This could be due to incomplete restoration of protein expression within NR1-Cre mice. It could also point towards a limit to reconstruction of the neural network within the adult brain.

The extent of rescue in protein and gene expression within NR1-Cre mice directly affects the ability of the mice to learn and retain information. As seen during training, the NR1-Cre mice displayed a slower acquisition of the learned association compared to WT. NR1-Cre activity became significant only at the 4th tone-shock pairing compared to WT, which showed significant freezing starting at the 2nd pairing. The difference in freezing response of the NR1-Cre can be attributed to the proportional level of rescue seen in *Grin1* gene and NR1 protein expression levels. A lack of freezing response in the NR1-KD mice might result from either hyperactivity or an inability to perceive fear, as measured by suppression ratio. Therefore, it appears that there is a threshold in NMDA receptor expression that is required in order to exhibit learned freezing response. In support of this, retrained NR1-KD mice did not improve learning or memory of the context and cue.

Encoding of spatial information is mediated by the hippocampus through glutamatergic signalling (O'Keefe & Nadel 1978; Tsien et al. 1996). Fear dependent contextual information processing was absent in NR1-KD mice, displaying a complete lack of fear response. One

possible explanation for this outcome is impaired hippocampal function in NR1-KD mice. Hippocampal lesions through pharmacological and genetic approaches have all demonstrated the importance of the hippocampus in encoding spatial cues involved with contextual fear conditioning (Anagnostaras et al. 1999). However, researchers have shown that impaired hippocampal function can be overcome by recurrent training (Wiltgen et al. 2006). Freezing was not observed with two days of training, suggesting that the hippocampus is either completely non-functional or that connections with other brain regions that are required in encoding spatial memory are also affected by NMDA receptor knockdown. The inability for NR1-Cre mice to exhibit similar levels of rescue in contextual memory as cue associated memory, could suggest a dysregulation of the amygdala-hippocampal circuit preventing the association of fear with context. Another explanation might involve disconnections between the hippocampus and the prefrontal cortex for long-term storage and retrieval of memories, both of which can impair freezing response (Frankland & Bontempi 2005). This appears unlikely, as NR1-KD mice lack immediate freezing behaviour to shock, a response that does not require input from the prefrontal cortex. Therefore, the most plausible explanation involves deficiencies in encoding both fear and spatial information at the level of the hippocampus and amygdala.

Rescue in both contextual and tone-associated learning would suggest that the hippocampus and amygdala are highly plastic regions of the brain. Compared to training day, NR1-Cre and WT had 53% and 40% respective increases in total percent freezing within the fear context. The increase of freezing response compared to training day suggests that consolidation of memory improves memory recall and thereby freezing in the fear context. Tone associated learning produced a similar increase in WT freezing response. On the other hand, NR1-Cre showed a 72% increase in tone associated freezing response relative to training day, different from the 40% increase seen in context (Figure 4.4B, 4.6B). Tone associated learning and memory is dependent on the function of the amygdala and is independent of the hippocampus (Quirk et al. 1997; Phillips & LeDoux 1992). Increased freezing in tone associated fear memory would suggest that the level of rescue in NMDA receptor protein expression within the amygdala should also be greater than the hippocampus. Future molecular characterization of the amygdala will provide support for the proposed hypothesis. However, NR1-Cre mice also had slower learning and recall of tone associated memory compared to WT, requiring increasing CS tones in order to observe maximal freezing response (Figure 4.6). Recovery in NMDA receptor

expression in NR1-Cre causes better rescue of tone associated memory compared to contextual memory, yet remains slower in encoding and recall of tone associated memory possibly due to the lack of complete rescue in NR1 protein levels. Another reason for impaired freezing behaviour in NR1-Cre mice may be caused by higher locomotor activity relative to WT mice. In fact, the suppression ratio indicates that both WT and NR1-Cre mice have similar reductions in activity within a fearful context. Therefore, differences in percent freezing within the fear associated context and cue tests may simply be a result of higher locomotor activity in NR1-Cre mice.

Estrogen receptors play a role in hippocampus dependent learning by mediating neurogenesis, increasing spine density, increasing the release of BDNF and by inducing transcription of genes, such as *Fos* (Woolley & McEwen 1994; Wang et al. 2004; Duarte-Guterman et al. 2015). NMDA receptor activity is required in allowing for estrogen mediated increases in spine density (Woolley & McEwen 1994). The loss of both NMDA receptor activity and estrogen can together lead to a greater impairment in learning and memory within NR1-KD mice. Previous studies have shown that tamoxifen inhibition of estrogen receptor signaling lead to impaired learning and memory in female and male humans and animals (Eberling et al. 2004; Talebi et al. 2010; Chen et al. 2002). In particular, reconsolidation and retrieval of fear and spatial memory can be impaired by tamoxifen (Chen et al. 2002; da Silva et al. 2016). There is a possibility that the use of tamoxifen can have cognitive effects, impairing memory formation within mice used to test fear conditioning. We have attempted to control for tamoxifen dependent effects by administering tamoxifen to all mice tested and by giving a two week wash-out period. However, WT and NR1-KD mice without the administration of tamoxifen can be used as an added control in future experiments.

1.3 Fear response in NR1-KD

Hyperactivity from deficits in NMDA receptor function creates challenges when trying to use freezing as a measure of memory. As an alternative measure of memory, researchers have used suppression ratio to observe reductions in locomotor activity from recall of a fearful memory. This provides a reliable way of quantifying fear response, since it also takes into account the baseline activity of each mouse. Using this measure, NR1-KD mice displayed impairments encoding fear, which resulted in the absence of fear response to the context (Anagnostaras et al.

2000). Impaired amygdalar function may prevent the encoding of fear in NR1-KD mice. Contrarily, NR1-Cre and WT mice displayed reduced activity and correlated with percent freezing in the fear context. Further investigation is required to decipher the molecular abnormalities responsible for a lack of fear response in NR1-KD mice.

1.4 Pain sensitivity

Glutamate signalling forms the main excitatory signalling in the brain, and therefore it might be expected that alterations in other sensory circuits may be possible. One such sensory process related to fear learning is the sensitivity to pain required in shock perception. Peripheral expression of NMDA receptors within the spinal cord form the ascending pain signal relaying to the CNS (Petrenko et al. 2003). NMDA receptors play a part in mediating LTP and long term synaptic plasticity within peripheral and central pain circuits. Whereas hyperactivity of NMDA receptors cause neuropathic pain, reduction in expression produces impaired pain sensitivity (Petrenko et al. 2003). However, burst activity in NR1-KD demonstrated that pain sensation was intact. Immediate pain sensation occurs through voltage gated sodium channels at nociceptive fibres, further conveyed by other glutamate signalling systems via the AMPA receptor and kainite receptor (Zhuo 2017). It is possible that the long term changes in pain sensitivity is impaired in NR1-KD mice, however their immediate pain sensitivity was unaltered.

1.5 Basal and Induction of IEGs

The absence of abnormalities in regulation of IEGs as a result of decreased NMDA receptor expression is a surprising finding. According to previous studies, fear conditioning and spatial learning upregulate the expression of the following genes: *CaMKII α* , *Dusp1*, *Nr4a1*, *Egr1*, *Fos*, *Bdnf*, *Arc*, *Homer1a* (Keeley et al. 2006; Lepicard et al. 2006; Czerniawski et al. 2011; Mizuno et al. 2012). As discussed in Section 4.2, the expression of these genes is reported to require NMDA receptor activity. Following fear conditioning, the expected upregulation in IEGs was observed in WT mice. Pharmacological antagonism of NMDA receptor activity prior to LTP induction or fear conditioning has been shown to prevent the upregulation of IEGs (Farina & Commins 2016). Contrary to expectation, NR1-KD mice displayed a similar degree of shock-induced upregulation of IEGs as WT mice. Although there is clear activation of IEGs in NR1-KD mice after shock, it is unknown whether this activation is occurring through an alternate receptor signaling cascade. Interestingly, following fear conditioning *Homer1a* was upregulated

differently in NR1-KD mice. Overexpression of Homer1a can lead to impaired dendritic spine formation, resulting in impaired fear associated learning (Sala et al. 2003). This was the only immediate-early gene that was altered in NR1-KD mice and was normalized in NR1-Cre mice. Therefore, Homer1a represents an interesting target for future study.

Previous pharmacological animal models with glutamatergic signalling deficiencies demonstrated alterations in both basal and fear conditioning induced gene regulation (Y. Chen et al. 2014; Farina & Commins 2016). Within this study, a developmentally chronic deficit in glutamatergic signalling can alter neural networks and cellular mechanisms in such a way that gene expression patterns are different from previous results. Our findings propose that the hippocampus adapts by engaging a NMDA receptor independent molecular mechanism of IEG induction, as a result of neurodevelopmental dysfunction of NMDA receptor signaling. Further investigation will be required to determine the exact pathway leading to IEG induction in a state of NMDA receptor hypofunction.

Furthermore, NMDA receptor rescue maintained basal and induced IEG levels similar to WT. Our results would suggest that restoring NMDA receptor expression in a deficient state, does not lead to changes in gene expression patterns different from WT. It is important to note that tamoxifen induced excision of neomycin may induce immediate alterations in gene expression, which might not be detected two weeks later. In addition, the selected genes within this study may limit the detection of regulation within other genes. In this study, induction of IEG was examined in males, since it was known that alterations in gene expression could be sexually dimorphic (Berchtold et al. 2008; Kight & McCarthy 2017). Future experiments will have to consider the sex, the scope of genes studied and the temporal resolution of gene induction following rescue.

Alterations in NMDA receptor expression can modulate basal levels of IEGs (Chen et al. 2014). In characterizing NR1-KD and NR1-Cre basal gene expression for *Arc*, *Bdnf*, *Fos*, *Dusp1*, *CamkIIa*, *Homer1a*, *Egr1* and *Nr4a1*, we found no difference in basal gene expression relative to WT. The results suggest that there might be compensatory mechanisms in place within NR1-KD mice that maintain basal gene expression patterns within the hippocampus. One such mechanism might involve kainate receptor mediated calcium influx, leading to cAMP dependent PKA activation pathway in CA1 and CA3 (Keller et al. 1992). In NR1-KD mice, behavioural

amelioration with kainate receptor antagonism might point towards hyperactivity of kainate receptor as a compensation for NMDA receptor knockdown (Duncan et al. 2012). L-type calcium channels can also activate transcription factor NF-ATc4 to induce gene expression within the hippocampus (Graef et al. 1999). Basal CREB activity can also be induced by kinases independent of NMDA receptor activity (Lonze & Ginty 2002; Keller et al. 1992). However unlikely, it is possible that 10% of NMDA receptor expression could sufficiently maintain basal gene expression. Furthermore, basal gene expression might not reflect basal protein expression patterns. Preliminary results for basal and fear induced Egr1 protein expression produced no observable difference between genotypes. These results contrast proteomic analysis of the hippocampus that found reductions in CAMKII α and elevations in MAPK (Wesseling et al. 2014). In our study, genes known to be regulated by fear conditioning and NMDA receptor activity were chosen to be analyzed. In future experiments, a complete proteomic analysis and genome wide expression analysis would be beneficial in identifying signaling pathways changed in NR1-KD mice. Nevertheless, our results point towards evidence that there might be compensatory mechanisms that maintain basal gene and protein expression in NR1-KD mice.

IEG induction is critical in the formation of hippocampus based long-term memory (Alberini 2009). The processes that have been described above, can also occur in a learning induced state of hippocampal function. L-type voltage dependent calcium channel blockers can impair long term spatial memory formation (Borroni et al. 2000). The presence of L-type calcium channels at thalamic input to the amygdala and its requirement in LTP, further implicates its role in fear memory (Bauer et al. 2002). It has been proposed that calcium entry through the L-type calcium channels can activate both CaMK and MAPK mediating CREB regulation, similar to NMDA receptor signalling (Bauer et al. 2002). In fact, NMDA receptor and L-type calcium channels are thought to affect similar mechanisms of learning. Kainate receptors have been shown to facilitate LTP generation within the hippocampus giving way to its potential role in memory (Lerma 2003). In fact, kainate subunit GluK4 knockout mice display impaired spatial memory (Lowry et al. 2013). Calcium influx through these and other receptors may convergence on CAMKII and MAPK activation to induce CREB and its associated genes, normally activated by NMDA receptors. CREB induction has in fact been shown to occur through signalling by various growth factors and neurotransmitters. Growth factors, such as nerve growth factor or BDNF can stimulate CREB through TrkA and TrkB receptors giving way to a MAPK dependent activation

of CREB (D D Ginty et al. 1994; Shaywitz & Greenberg 1999). Muscarinic acetylcholine receptors are G-protein coupled receptors that can cause release of Ca^{2+} from intracellular stores, leading to kinase mediated activation of CREB (Shaywitz & Greenberg 1999). The initial response to fear conditioning, molecularly characterized as CREB mediated transcriptional activation, could be maintained through compensatory mechanisms in NR1-KD mice. Further evidence for this was found with *Egr1* immunostaining, although this was not quantifiable. NR1-KD mice produced an observable upregulation in *Egr1* protein expression from fear conditioning that was undistinguishable from WT *Egr1* regulation. These results point towards alternate mechanisms of hippocampal to compensate for the lack of NMDA receptor activity.

There are various ways in which gene expression alone may not be sufficient in the consolidation of memory. NMDA receptors specifically respond to presynaptic activity by way of coincidence detection. Coincidence detection arises from channel opening only when both glutamate release and a depolarization large enough to remove the Mg^{2+} block occur simultaneously, which minimizes activation from weak presynaptic stimulation (Bauer et al. 2002). The receptor activation properties allow synapse specificity between the pre- and post-synapse, and prevents strengthening of generalized neuronal activity. NMDA receptors also allow for communication between the intracellular and extracellular space and mediates strict regulation over neuronal activity. In addition, NMDA receptors directly interact with kinases, post-synaptic density protein forming complexes and receptors to regulate their surface expression (Dalva et al. 2000; Bayer et al. 2001; Bertaso et al. 2010; Bard & Groc 2011). The structural and downstream dynamics in enzymatic activity is controlled by NMDA receptor activity through phosphorylation, similar to *CamkIIa* (Gardoni et al. 2002; Bayer et al. 2001). Likewise, intracellular communication occurs at the surface through phosphorylation and regulation of NMDA receptors (Traynelis et al. 2010b). Therefore, maintenance of long-term synaptic potentiation requires NMDA receptor function. The upregulation in genomic expression alone is not sufficient in mediating long-term memory (Miyashita et al. 2008). Dysfunction in NMDA receptor activity thus impaired consolidation and retrieval of fear memory in NR1-KD mice and rescue recovers these NMDA receptor functions within the cell allowing for partial recovery in fear memory.

2 Conclusion

Neuroplasticity is a mechanism that permits the alteration of cellular memories as a response to learned behaviours. The hippocampus is a brain region with exceptional levels of plasticity that is retained in adulthood, including the ability to regenerate neurons. In agreement with this idea, our study found that adult rescue of NMDA receptor expression within the hippocampus produced a noticeable improvement in learning and memory functions. Thus the adult hippocampus is capable of reorganizing neural networks and forming proper synaptic connections that allow for recovery from a neurodevelopmental insult. The association of fear with contextual and tone cues requires a functional network of neurons that communicate across various regions of the brain. Therefore, it could also be said that the network involved in fear memory is also capable of undergoing vast remodeling. The amygdala is a region of the brain required in fear memory formation, and the recovery in cue associative learning demonstrated the high permissibility for remodelling. The ability for recovery in hippocampal function as well as functions in other related brain regions, suggests that modulators of the glutamate system may be a potential therapeutic target. This statement should be made with caution since overactivation of NMDA receptors can lead to seizures and other cognitive deficits. Instead, allosteric modulators could be used to modify the activation properties of NMDA receptors without affecting activity.

Congenital loss of NMDA receptor expression impaired fear associative learning and memory, which is not surprising given previous genetic and pharmacological studies displayed similar results (Tsien et al. 1996; Langton et al. 2007; Morris 2013). Reproducing the memory deficit demonstrated the importance of NMDA receptor activity as a molecular substrate for memory encoding. NMDA receptors have been shown to be required in mediating the induction of gene expression required in memory encoding. Unexpectedly, our results suggest that there might be alternative mechanisms that are able to compensate for a loss in glutamate signalling at the molecular level, but any compensations that occur are not sufficient to produce fear learning and memory. Proposed mechanisms include the presence of Ca^{2+} permeable kainite receptors and voltage-gated calcium channels, which through the influx of Ca^{2+} can induce similar learning associated genes that are also involved in NMDA receptor activation pathway. However, the induction of genes alone does not mitigate learning processes. NMDA receptors are known to regulate the intracellular and extracellular communication to and from the nucleus of the cell.

The induction and expression of genes form a single, but important factor in encoding memory. NMDA receptors are involved in regulating kinase activity, synapse specificity, surface receptor expression and cytoskeleton modifications through interactions with PSD proteins. These processes are largely mediated by new gene and protein synthesis. However, without specific activity and regulation at synapses through NMDA receptor interaction, those genes and proteins synthesized are not coordinated at potentiated synapses. And as Bliss and Collingridge postulated, potentiation at synapses form the molecular basis of long-term memory within the hippocampus.

This study was an attempt to understand the processes involved in the plasticity of NMDA receptor mediated learning within the hippocampus. A developmental insult from a deficient state of glutamate signalling impairs learning, however the effects are not permanent and in fact can be reversed in adulthood. As the mouse model is further characterized, the molecular basis for the rescue and the exact molecular consequences for NMDA receptor knockdown will be demonstrated in complete detail.

3 Future Directions

Although many of the results confirmed our hypothesis in rescue of hippocampal plasticity mediated learning, there are still behavioural and molecular characterizations that needs to be made in the NR1-KD to develop a complete understanding between the gene and protein expression data in comparison to the behaviour. In an attempt to determine the molecular pathways responsible for behavioural fear response, our results revealed a potential compensation that might be occurring in the NR1-KD. Future studies are required in order to gain evidence for the presence of such mechanisms, in order to explain the lack of altered gene and protein expression patterns within the hippocampus. A potential future experiment might look at the enzymatic activity of CREB, CAMKII α , MAPK and PKA to determine whether there are alterations in activation through one pathway over the other. In addition, determining whether transcription factors different from CREB, are inducing IEGs will provide further information into compensatory mechanisms in NR1-KD mice. Another determinant for compensation are the histone marks present in the genome, predictive of the genes that are transcribed and upregulated. Analysis for genomic regions containing methylation, acetylation and phosphorylation would provide the epigenetic and environmental influences regulating

homeostasis in the NR1-KD mice. Once these mechanisms have been characterized, it would be interesting to determine whether the same pathways are maintained or suppressed in NR1-Cre giving way to a more NMDA receptor dominant signal transduction.

An important distinction must be made between impairments in fear sensation and hyperactivity in NR1-KD mice. In an attempt to decipher those two processes, suppression ratio demonstrated an abnormal perception to fear. However, there is also increased locomotor activity in NR1-KD mice that confound freezing response. To provide further evidence for impairments in fear perception, hypothalamic activation through amygdala innervations can be measured using changes in heart rate, peripheral stress hormone release, immunohistochemical labelling of the amygdala and hypothalamus. Certainly, molecular characterization of the amygdala would be the most direct interpretation of fear perception. Therefore, basal and inducible gene expression analysis should also be conducted within the amygdala, since many of the genes involved in fear associated learning within the hippocampus are also upregulated in the amygdala (Maren 2001). These experiments could be used to determine whether amygdala related circuits are indeed inhibited during fear conditioning in NR1-KD mice, causing a lack of fear perception. Selectively rescuing NMDA receptor levels within the amygdala should lead to improved freezing response in tone associated learning, since the hippocampus do not play a role in encoding tone associations with fear. If amygdala selective rescue or activation does not improve freezing, this might point towards a disconnection between amygdala-periaqueductal grey processes involved in the cessation of locomotor activity, as a response to fear (Kim et al. 2013).

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Appendices

Figure A1. Rescue of hyperactivity, anxiety and executive function in NR1-Cre displays plasticity in NMDA receptor mediated networks. Male and female mice aged 14wk were tested using locomotor activity assay, elevated plus maze and puzzle box to test locomotor activity, anxiety and executive function in NR1-Cre mice. These data were obtained from work by Catharine Mielnik and Marija Milenkovic. (A) NR1-Cre mice have reduced total distance travelled in a novel context, relative to NR1-KD. (B) NR1-KD mice spent more time in the open arm than either WT or NR1-Cre mice, which is a measure of decreased anxiety. NR1-Cre displayed partial rescue of abnormal anxiety related behaviour. (C) Mice were tested over three days for executive function using a goal-oriented puzzle box task. Executive function was measured by the latency to reach a goal box. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. mean $\pm$ SEM. (Mielnik et al., in preparation)

Figure A2. Tamoxifen administration activated Cre-recombinase and induced dTomato reporter expression throughout the brain in NR1-Cre mice. Mice expressing Cre-recombinase and the dTomato reporter were generated in order to observe Cre-recombinase activity within different brain regions. This data was obtained from Catharine Mielnik. (A) Cre-recombinase was expressed under the ROSA26 promoter. Cre-recombinase is retained in the cytosol until tamoxifen allows for translocation into the nucleus. Inside the nucleus, Cre-recombinase excises loxP floxing the transcriptional arrest site, which allows dTomato expression in NR1-Cre mice. (B) Qualitative image of dTomato expression displaying Cre-recombinase dependent excision and nuclear activity across brain regions. (Mielnik et al., in preparation)

A.

B.

Figure A3. NR1-Cre had rescue in NMDA receptor evoked potentials within layer V pyramidal neurons of the prefrontal cortex, but not in medium spiny neurons of the striatum. Intrinsic and evoked potentials were recorded from cortical and striatal slices from three week old mice. This data was obtained from Catharine Mielnik, Mary Binko and Evelyn Lambe. (A) Intrinsic excitability of layer V pyramidal cells in the prefrontal cortex are maintained across genotypes. (B) NMDA agonist evoked potential was perturbed in NR1-KD mice within the prefrontal cortex and striatum. NR1-Cre had rescue of NMDA receptor evoked potential within layer V pyramidal neurons in the prefrontal cortex, similar to WT levels. There was no rescue of evoked potentials in medium spiny neurons of the striatum. (Mielnik et al., in preparation)