Inducible Rescue of N-methyl-d-Aspartate Receptor Deficiency to Study Brain Plasticity

by

Catharine Mielnik

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Pharmacology and Toxicology
University of Toronto

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Abstract

Although brain plasticity is thought to be highest in the developing brain, evidence has shown that the adult brain is capable of a number of mechanisms that underlie developmental plasticity. This is important for the treatment of neurodevelopmental disorders, where insults occur during plastic and vulnerable developmental windows, ultimately leading to symptoms that continue into adulthood. We investigated the plasticity of the brain by disrupting the glutamate system, and determined whether intervention during adulthood could ameliorate neurodevelopmental symptoms. We asked whether the adult brain is sufficiently plastic to allow for rewiring and amelioration of behavioural abnormalities. To address this, a mouse model was generated. The GluN1-inducible rescue (GluN1-IR) mouse model develops in a state of global N-methyl-d-aspartate (NMDA) receptor hypofunction that can then be “rescued”, with NMDA receptor levels being genetically restored. The levels of rescue were quantitatively measured via behavioural, biochemical and molecular means. By restoring NMDA receptor function at two key developmental time points, adolescence and adulthood, we were afforded insight into when the brain may be more plastic and amenable to adaptive changes, potentially identifying key treatment windows for symptoms that arise in a number of neurodevelopmental disorders. However, independent of intervention time or length of recovery, plasticity remained constant.
Even more interestingly, we identified that the degree of plasticity varied depending on brain regions. Cortically-mediated behaviours showed high levels of plasticity, while subcortical behaviours remained resistant to change. This was corroborated with biochemical and molecular outputs. This study suggests that, converse to what was classically believed, plasticity does extend well into adulthood. Limitations in plasticity may not be mediated by developmental age, but may be inherently programmed based on specific circuits for behaviours.
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<tr>
<td>ADHD</td>
<td>Attention Deficit Disorder</td>
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<tr>
<td>AMPAR</td>
<td>α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid Receptor</td>
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<tr>
<td>ASR</td>
<td>Acoustic Startle Response</td>
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<tr>
<td>CaMKII</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase II</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>D1</td>
<td>Dopamine Receptor D1</td>
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<tr>
<td>D2</td>
<td>Dopamine Receptor D2</td>
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<tr>
<td>EAAT</td>
<td>Excitatory Amino Acid Transporter</td>
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<tr>
<td>EPSC</td>
<td>Excitatory Postsynaptic Current</td>
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<td>ESC</td>
<td>Embryonic Stem Cells</td>
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<td>GABA</td>
<td>gamma-Aminobutyric Acid</td>
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<td>GAD</td>
<td>Glutamate Decarboxylase</td>
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<td>GluN1-IR</td>
<td>GluN1-inducible rescue</td>
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<td>GluN1-KD</td>
<td>GluN1-knockdown</td>
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<tr>
<td>GlyT1</td>
<td>Glycine Transporter 1</td>
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<tr>
<td>HDAC</td>
<td>Histone Deacetylase</td>
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<tr>
<td>LTD</td>
<td>Long Term Depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long Term Potentiation</td>
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<tr>
<td>mAChR</td>
<td>Muscarinic Acetylcholine Receptor</td>
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<tr>
<td>MeCP2</td>
<td>Methyl CpG Binding Protein 2</td>
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<tr>
<td>mGluR</td>
<td>Metabotropic Glutamate Receptor</td>
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<tr>
<td>MK-801</td>
<td>Dizocilpine</td>
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<td>NAM</td>
<td>Negative Allosteric Modulator</td>
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<td>NMDAR</td>
<td>N-Methyl-d-Aspartate Receptor</td>
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<tr>
<td>PAM</td>
<td>Positive Allosteric Modulator</td>
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<td>PCP</td>
<td>Phencyclidine</td>
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<td>Polymerase Chain Reaction</td>
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<td>Protein Kinase A</td>
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<td>Post Synaptic Density</td>
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<td>TCAG</td>
<td>The Centre for Applied Genomics</td>
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<td>Toronto Centre for Phenogenomics</td>
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Chapter 1
INTRODUCTION

1 Plasticity of the Brain

Plasticity is a term that describes pliability and malleability, the capacity for a system to respond to either normal or aberrant developmental or environmental changes. This response can result in the adoption of either a new, stable, developmentally appropriate phenotype, or lead to the restoration of old established phenotypes (Dennis et al., 2013). More specifically, plasticity can be defined as the capacity of neurons, and subsequent neural circuits, to change, resulting in a structural and/or functional response to “experience” (Sale et al., 2014). Historically, the term plasticity has been applied to a number of different changes that occur in the brain; from the establishment of neural paths and their resulting “habits” (James, 2013), to the regenerative capacities of the nervous system (Cajal et al., 1991). Today, the term continues to be broadly used to encompass a number of different processes, ranging from molecular, cellular, to even behavioural systems that change and adapt resulting in novel functions (Dennis et al., 2013). In terms of aging and development, plasticity can have contrasting frames of reference; it can refer to the notion that the immature brain has greater functional recovery capabilities (Webb et al., 1996) or conversely to the idea that plasticity, in general, is a property of the brain at all ages (Holtmaat and Svoboda, 2009; Dennis et al., 2013).

In terms of behaviour, and more specifically learning and memory, plasticity is critical. The ability of the brain to change and modify in response to internal or external stimuli is a fundamental property for a number of processes: the adaptability of behaviour, learning and memory, the development of the brain and also in repair following insult. Changes in stimuli lead to experience-mediated modifications that are then translated into patterns of electrical activity within neural circuits. This activity then manifests into functional and structural plasticity via the coordination (across spatial and temporal means) of different cellular and molecular factors. Some of these factors include: altering the efficacy of existing synapses, formation of new synapses, elimination of existing synapses, changes in the arborization of dendrites or axons, or the production of signaling neuromodulators (Sale et al., 2014).
The primary constituent of neural plasticity is synaptic plasticity, a phenomenon that manifests as a response to a pattern of electrical activity at the level of the neuron (Bear and Malenka, 1994). Synaptic plasticity involves both functional and structural changes that arise from a prolonged increase in synaptic activity that is induced by repeated bursts of correlated pre- and post-synaptic activity (Bear and Malenka, 1994; Sale et al., 2014). An increase in synaptic activity leads to long term potentiation (LTP) of the synapse, while a decrease in synaptic activity leads to long term depression (LTD). An increase or decrease in activity is determined by the relative spike timing and firing rate of both the pre- and post-synaptic neuron (Magee and Johnston, 1997; Markram et al., 1997; Sjöström et al., 2001; Sale et al., 2014). Furthermore, determination of the occurrence of LTP versus LTD is heavily influenced by the level of previous activity that the circuit has encountered (Deisseroth et al., 1995). This will be further elaborated on in section 2.

1.1 Classical View of Plasticity

It is believed that the developing, juvenile brain is more plastic in comparison to the adult brain; considered to be completely developed, therefore more fixed and no longer capable of adapting circuit connectivity (Ehninger et al., 2008). It has been widely accepted that the younger the organism, the more plastic the brain is, a concept that has been termed ‘young age plasticity privilege’ (Dennis et al., 2013). However, extended periods of plasticity may not always be beneficial in nature. Plasticity can include mechanisms that produce both adaptive and maladaptive changes. Exposure to adverse events is inevitable. The same mechanisms that underlie development in the immature brain, also make the maintenance of an equilibrium more difficult during adverse events; the highly plastic environment makes it far more difficult to maintain a homeostatic state (Dennis et al., 2013).

Within the already heightened state of plasticity during adolescence, there exist further specific periods within development of even higher plasticity, termed “critical periods”. Critical periods are defined as time points, within development, when specific experiences influence neurogenesis, brain sculpting and learning (Hubel and Wiesel, 1963). So, to further restrict time periods of plasticity, not only is the brain more plastic the younger it is, but certain processes within development also need to occur in a defined time frame, within a “critical period”, to allow for proper wiring and formation of circuits.
Critical periods are numerous, and highly specific. There exist specific critical periods for specific functions, for specific circuits within a single function, and for specific time points in a single circuit; age is not the only driving force behind the onset and offset of critical periods (Michel and Tyler, 2005). For example, thresholds of intracortical inhibition in the visual cortex mediate onset and offset of its corresponding critical period, and these thresholds can be manipulated pharmacologically in the mature brain to reinstate the plasticity of the “critical period” (Hensch, 2005; Spolidoro et al., 2009; Dennis et al., 2013). These kinds of temporal constraints imposed on plasticity pose problems if a timeframe is missed for a certain event, or if an insult occurs during a specific window. If such strong constraints on plasticity exist in the brain, then it would lead one to infer that tightly regulated developmental plasticity is integral. Once missed, it should be impossible, or extremely difficult, to have that window of plasticity re-open.

1.2 Plasticity in the Adult Brain

Despite it being believed that the younger brain is more plastic than the fully-developed, adult brain, recent studies have shown that this notion may not be completely true. The brain, in general, has a great capacity for plasticity, with the ability to adapt, change structure, and connectivity based on the demands it encounters (Lövdén et al., 2013). Furthermore, it has been shown that not all circuits are shaped during critical periods. While some circuits establish connectivity through innate mechanisms, with little contribution from experience, others maintain a high degree of plasticity throughout life. A few examples of these plastic types of circuits are found in the basolateral nucleus of the amygdala, the molecular layer of the cerebellar cortex, or even the CA1 region of the hippocampus (Malenka and Nicoll, 1999; Medina et al., 2002; Knudsen, 2004). In these more plastic circuits, the range of potential stable patterns of connectivity is broad, and continues to remain broad throughout the lifetime of the animal or human. However, it is important to note that most circuits operate somewhere between these two extremes of limited to unlimited plasticity; innate mechanisms may establish the initial pattern of connectivity, but there is still the potential for change based on experience (Knudsen, 2004).

Plasticity occurs in the adult brain. There exist a number of processes that occur in the adult, developed brain that require plasticity to take place. Some examples of plasticity in the adult
brain include the following: learning and memory processes, learning of new motor or perceptual ability, development of fear responses to stimuli, etc. (Sale et al., 2014). Furthermore, adult plasticity has been shown in a number of sensory cortices, even outside the classic “critical developmental window”. For example, the adult cortex has shown the potential for high levels of plasticity (Sawtell et al., 2003), as well as exhibiting a number of features that occur in developmental plasticity, including structural changes in spines, axons and dendrites (Florence, 1998; Trachtenberg et al., 2002; Tailby et al., 2005). Continuing to use the development of the cortex (specifically visual) as an example, studies have revealed that a plastic state, closely resembling one observed during its critical period, can in fact be reactivated via environmental and pharmacological manipulations (Bavelier et al., 2010; Baroncelli et al., 2011; Castrén et al., 2012). A combination of appropriate rehabilitation and targeting of suitable molecular or biochemical candidates, can lead to the recovery of function in abnormally wired networks.

Therefore, by stepping outside of the rigid concept that the adult brain is static and cannot be re-wired, the possibility of targeting adult, innate plasticity mechanisms can be a reality. Primarily, a change in context regarding plasticity can allow for the treatment of a number of disorders stemming from miswired circuits. This highlights the possibility that developmental disorders, which arise due to abnormal early miswiring or insult, can be treated well into adulthood if the correct plasticity mechanisms are targeted (Castrén et al., 2012). Importantly, by being able to “reactivate juvenile-like” plasticity in the adult brain, this could allow for the recovery of abnormal symptoms that are present in a number of neurodevelopmental disorders; disorders that are grouped based on disabilities that involve some form of disruption to brain development. Studies in animal models of neurodevelopmental disorders (primarily single-gene) have shown that cognitive deficits and neurological impairments that are associated with neurodevelopmental disorders can, in fact, be reversed, even in adulthood (Ehninger et al., 2008). This is further discussed in section 7.

1.3 Mechanisms Underlying the Difference Between the Young and Adult Brain

The mechanisms underlying the difference between the young, plastic brain and the aged, static brain are numerous. The decrease in plasticity seen in the aged brain has been hypothesized to stem from the idea of “sensitive periods” (as previously discussed); the aged brain being outside
of these developmental periods. The reduction in plasticity that is associated with the end of these sensitive periods can possibly be explained by the following; endogenous factors that control maturation reduce plasticity, learning leads to self-termination of the sensitive period, or plasticity is not actually reduced but more its constraints become more stable (Johnson, 2005). Furthermore, evidence has shown that subtle changes in neuron morphology, cell-cell interactions and gene expression may contribute to alterations seen during the reduction of plasticity in older animals (Burke and Barnes, 2006). Morphological changes that occur with ageing are not universal across the entire brain, and are region specific. Changes in dendritic branching and synaptic density make up the majority of effects on neuron morphology (Buell and Coleman, 1979; 1981). Despite these morphological changes, most electrical properties actually remain constant during development (Barnes, 1994). To modify a circuit or network, cooperativity between active synapses is necessary, and a reduction in synaptic density (partly due to morphological changes) can be problematic (Burke and Barnes, 2006).

At a genetic level, there are a number of changes in expression that help explain decreased plasticity in the adult brain. It has been shown that genes associated with energy metabolism, biosynthesis and activity-regulated synaptogenesis are basally downregulated in the hippocampus with increasing age. Furthermore, early immediate genes such as Arc and Narp are also downregulated with age (Blalock et al., 2003). Therefore, since synaptic connectivity, and ultimately LTP, require gene expression and de novo protein synthesis to take place, it is reasonable that aged animals show a decrease in plasticity; decreased expression of early immediate genes and other basally expressed genes necessary for synaptogenesis limit synaptic changes (Burke and Barnes, 2006).

Changes to overall plastic properties of circuits can also lie in changes that occur at the level of the receptor. In terms of glutamate signaling, the major excitatory neurotransmitter in the brain, the channel properties of the N-methyl-d-aspartate (NMDA) receptor are based on subunit composition. Briefly, studies have shown that an increase in the speed of the NMDA receptor-EPSC (excitatory postsynaptic current) decay has been associated with a gradual shift in receptor composition: GluN2A subunits replace GluN2B subunits during postnatal development. The GluN2B-containing complexes show longer excitatory postsynaptic potentials (EPSPs) than those tetramers containing GluN2A subunits. During development, with NMDA receptors rich in GluN2B subunits, the receptor is afforded a longer window for the detection of synaptic
coincidence, potentially allowing for an increase in synaptic plasticity (Tang et al., 1999). The developmental switch from GluN2B-rich NMDA receptors, to GluN2A-containing receptors may be an underlying mechanism for the closure of key critical and developmental periods where there is increased plasticity during development.

2 Glutamate Signaling in the Brain

2.1 The Glutamate Synapse (an overview)

Glutamate acts as the primary excitatory neurotransmitter in the mammalian central nervous system (CNS). Following depolarization, the glutamate synapse releases glutamate into the synaptic cleft and binds glutamate receptors. There exist two types of membrane bound glutamate receptors: ionotrophic and metabotropic (Chiocchetti et al., 2014). Within those two classes of receptors, they are further divided into distinct families. The ionotrophic glutamate receptors are ligand-gated ion channels that subdivide into three families, dependent on their selective agonists: α- amino-3-hydroxy-5-methyl-4-isoxazolopropionate (AMPA) receptor, N-methyl-D-aspartate (NMDA) receptors, and kainate receptors (Coyle, 2006; Chiocchetti et al., 2014). One gene family encodes AMPA receptor subunits; GRIA1-4. Three gene families encode NMDA receptors; GRIN1, GRIN2A-D, and GRIN3A-B. Two gene families encode kainate receptors; GRIK1-2 and GRIK3-5 (Chiocchetti et al., 2014). AMPA receptors primarily generate EPSCs that are responsible for triggering action potentials, while NMDA receptors play a crucial role in synaptic plasticity, which is integral for memory and learning, along with contributing to EPSCs (Coyle, 2006).

The metabotropic glutamate receptors (mGluR) are G-protein coupled receptors that, when stimulated, lead to increased intracellular secondary messenger levels, resulting in the regulation of ion channels and stimulation of other pathways. mGluRs are typically divided into three classes, based on pharmacological properties and intracellular signaling transduction mechanisms (Conn and Pin, 1997; Gerber et al., 2007). Eight mGluR subtypes have been identified and divided into three families, dependent on sequence homology and cell signaling activation. Group I mGluRs, found postsynaptically, couple to G_{q/11}, resulting in an increase of Ca^{2+} from intracellular stores where they modulate neuron excitability; mGluR1 and mGluR5 fall under group I (Shigemoto et al., 1997; Dhani and Ferguson, 2006; Ribeiro et al., 2017). Both group II and III mGluRs negatively regulate adenylyl cyclase via coupling with G_{i};
mGluR2 and mGluR3 belong to Group II, and mGluR4, mGluR6, mGluR7 and mGluR8 belong to Group III (Gerber et al., 2007). Group II and Group III mGluRs are primarily localized presynaptically and act as autoreceptors to regulate signaling via inhibition of glutamate or gamma-aminobutyric acid (GABA) release (Figure 1) (Schoepp, 2001).

Physiologically, the glutamate synapse demonstrates a tightly regulated functional relationship between the presynaptic terminal, the postsynaptic spine, and the surrounding astrocyte (Araque et al., 1999; Coyle, 2006). Glutamine is supplied by the astrocyte, which is then synthesized into glutamate (in the presynaptic neuron), and packaged into vesicles, being released during action potentials. Glutamate is released into the synaptic cleft, and then binds to postsynaptic receptors, as described previously (Coyle, 2006). The astrocyte plays an important role in the maintenance and regulation of glutamate transmission; not only does it provide glutamine for glutamate synthesis, lactate for oxidative metabolism, but it also regulates NMDA receptor function. Astrocytes express glutamate transporters (excitatory amino acid transporter 1 and 2, EAAT1 and 2), that are responsible for clearing the synaptic cleft of glutamate, and protecting against excitotoxicity (Schlüter et al., 2002). EAAT3-5 are transporters that are neuron specific. In general, EAATs are located pre-, post-, and peri-synaptically, and regulate glutamate levels in the synapse, allowing for spatio-temporal control of glutamate (Chiocchetti et al., 2014). Furthermore, the glycine transporter 1 (GlyT1) is also expressed on astrocytes and regulates levels of synaptic glycine (Zafra et al., 1995), a co-agonist for the NMDA receptor. It is apparent that astrocytes play an important role in modulating NMDA receptor processes, which can then be further regulated based on regional expression.

2.2 Biochemical Organization of the Glutamate Synapse

The glutamate synapse relies heavily on a highly complex macromolecular assembly embedded at synaptic terminals, as well as precisely aligned synapses via trans-synaptic cell adhesion proteins to mediate signaling of neurons (Pocklington et al., 2014). Presynaptically, the ‘active zone’ of the synapse contains ~200 distinct proteins, each integral for the regulation of docking and priming of synaptic vesicles. The active zone serves to localize calcium channels that are responsible for the coupling of presynaptic excitation with the fusion of primed vesicles to result in neurotransmitter release (Morciano et al., 2009; Pocklington et al., 2014). Excitatory synapses distinguish themselves from inhibitory ones via the prominent thickening of the postsynaptic
Figure 1. **Signaling pathways of metabotropic glutamate receptors (mGluRs).** (A) Schematic overview of the synaptic location of mGluRs. Group I mGluRs (mGluR1 and mGluR5) are mostly found postsynaptically, while group II (mGluR2 and mGluR3) and III (mGluR4, mGluR6, mGluR7, and mGluR8) mGluRs are located presynaptically. (B) Schematic highlighting signaling pathway of Group II and Group III mGluRs; coupled predominantly to G<sub>i/o</sub> proteins. Upon agonist activation, these mGluRs mediate downstream inhibition of adenyl cyclase activity, decreasing the levels of cAMP. (C) Following agonist activation, Group I mGluRs promote uncoupling of G<sub>αq/11</sub>, which stimulates PLC<sub>β1</sub> and the subsequent formation of DAG and IP<sub>3</sub>, promoting release of Ca<sup>2+</sup> from intracellular stores. Taken from Ribeiro et al. (2017).
membrane, the postsynaptic density (PSD). The human PSD family contains over 1400 proteins overall, while an individual synapse is made up of about 50-100 biochemical components (Sheng and Hoogenraad, 2007; Bayés et al., 2011). The proteins affiliated with the PSD include a number of integral players in the regulation of synaptic plasticity, the underlying basis for behavioural learning and memory. Mechanisms at the synaptic cleft that contribute to synaptic plasticity, both pre- and post-synaptically, include the following: (1) alterations in vesicle release probability, (2) structural remodeling of the synapse, (3) protein synthesis, and (4) trafficking of postsynaptic AMPA receptors (Citri and Malenka, 2008; Pocklington et al., 2014).

Whereas activation and signaling via the AMPA receptor tends to be straightforward, the NMDA receptor proves to be far more complex in nature. Depolarization of the postsynaptic cell is driven via activation of AMPA receptors, which have shown to be associated with small functional complexes composed of receptor subunits and a few cytoskeletal and trafficking proteins (Collins et al., 2006). Conversely, the NMDA receptor requires coordinated activation of a number of downstream pathways, with large complexes formed from membrane-associated guanylate kinase family scaffold proteins that couple the receptor to downstream signaling proteins. These complexes are large in nature, about 2-3MDa in size, and bring together machinery that is involved in a number of pathways that underlie plasticity (Husi et al., 2000; Husi and Grant, 2001; Pocklington et al., 2014). A major structural component of the PSD is the protein PSD-95, a scaffolding protein that lies close to the postsynaptic membrane and interacts with a large variety of membrane-spanning molecules. Therefore, experimentally, the isolation of PSD-95 complexes offers the possibility of a ‘horizontal cross-sectional’ view of the PSD, spanning the active zone, including key interaction proteins that associate with the NMDA receptor and regulate its signaling.

### 2.3 Channel Properties of the NMDA Receptor

NMDA receptors are a family of ionotropic glutamate receptors that mediate the slow, Ca\(^{2+}\) permeable component of excitatory synaptic transmission, which is dependent on subunit composition (Hu et al., 2016). The NMDA receptor is a tetramer that is made up of pairs of two subunits (2:2 stoichiometry): (1) two GluN1 subunits, which are required for channel function, and, (2) two GluN2A-D subunits, which mediate the biophysical and pharmacological characteristics of the NMDA receptor (Lynch and Guttmann, 2001; Coyle, 2006). The GluN1
subunit has a glycine binding site (which can also bind D-serine), while the GluN2 (A-D) subunits contain a glutamate binding site (Figure 2). The GluN1 subunit is encoded by a single gene (Grin1), and can undergo alternative splicing that leads to eight GluN1 splice variants (Zukin and Bennett, 1995).

Electrophysiologically, the NMDA receptor is a ligand-gated voltage-dependent channel, where the channel is blocked at resting membrane potentials via a Mg$^{2+}$ block, which can only be removed following depolarization (Mayer, 2016). Within the channel, there are binding sites for dissociative anaesthetics that are use-dependent, non-competitive antagonists (such as ketamine and MK-801) (Coyle, 2006). Following convergent binding of glycine and glutamate/NMDA and depolarization to remove the Mg$^{2+}$ block, the open NMDA receptor allows for a highly permeable influx of Ca$^{2+}$. The influx of Ca$^{2+}$ activates a cascade of enzymes and other messengers that alter local synaptic biochemistry and gene expression (Hong et al., 2004).

The GluN2 subunit is critical for a number of important biophysical and pharmacological properties that dictate function of the NMDA receptor. These include, sensitivity to Zn$^{2+}$, Mg$^{2+}$, protons, polyamines, affinity for glutamate, modulation of glycine, Ca$^{2+}$ current, single channel conductance and channel kinetics (Cull-Candy and Leszkiewicz, 2004; Paoletti, 2011; Ladépêche et al., 2014). Each GluN2 subunit has a characteristic glutamate response decay signature (deactivation time of the NMDA receptor). The average deactivation time of diheteromeric NMDA receptors (GluN1/GluN2) spans a large range, about 50-fold: GluN2A < GluN2B = GluN2C << GluN2D (from fastest to slowest decay constant) (Figure 3A) (Cull-Candy and Leszkiewicz, 2004). GluN2D containing NMDA receptors produce responses that have decay constants of about 4-5s, which represent exceptionally slow deactivation for a ligand-gated receptor. On the other hand, GluN2A containing NMDA receptors have rapid decay constants, and they predominate NMDA receptors that are present in mature synapses (Cull-Candy and Leszkiewicz, 2004). Decay signatures are not the only characteristic that defines the different GluN2 subunits; conductance rates also differ between subunits. GluN2A and GluN2B subunits generate NMDA receptors with high conductance rates, whereas GluN2C and GluN2D containing receptors show low-conductance openings. Conductance of the channel also depends on its sensitivity to the Mg$^{2+}$ block. This is also mediated by the GluN2 subunit. GluN2A and GluN2B subunits with a high conductance display high sensitivity to the Mg$^{2+}$ block, whereas
Figure 2. **NMDA receptor structure.** GluN1 and GluN2 subunits are composed of a number of conserved domains; an N-terminal domain that contains sites which bind to Zn$^{2+}$, S1 and S2 domains that bind glycine (white) on the GluN1 subunit and glutamate (black) on the GluN2 subunit, and a binding site deep in the pore that binds extracellular Mg$^{2+}$. The ion channel is permeable to Ca$^{2+}$, K$^+$, and Na$^+$. The C-terminal tail of the GluN1 and GluN2 subunits interact with synaptic kinases and structural proteins (in grey). Adapted from Cull-Candy and Leszkiewicz (2004), reprinted with permission from AAAS.
Figure 3. **Electrophysiological properties of the NMDA receptor.** Receptor-channel properties of the NMDA receptor are mediated via the GluN2 subunit. The diheteromeric receptors (2GluN1:2GluN2) have an electrophysiological ‘signature’ that is based on the time constants of deactivation and conductance properties of the GluN2 subunit (GluN2A, turquoise; GluN2B, green; GluN2C, orange; GluN2D, blue). (A) The time constants of deactivation in response to a 1-ms pulse of 1mM glutamate are roughly as follows: GluN2A, 100mS; GluN2B, 250mS; GluN2C, 250mS; GluN2D, 4s. (B) GluN2A and GluN2B subunits give rise to channels with a high conductance state, while GluN2C-containing receptors have lower conductance states, and GluN2D-containing receptors have the lowest conductance rates. Adapted from Cull-Candy and Leszkiewicz (2004), reprinted with permission from AAAS.
GluN2C and GluN2D-containing receptors show low sensitivity to Mg$^{2+}$, and low conductance (Figure 3B) (Cull-Candy et al., 2001; Cull-Candy and Leszkiewicz, 2004).

2.4 NMDA Receptor Expression and Glutamate Signaling During Development

Glutamate signaling plays an important role in the development of neuronal circuits. Neuronal circuits in the brain develop and form relatively rapidly, following a precise sequence of events where neurons will migrate, arborize and finally establish synaptic connections. During development, these synaptic connections will either be stabilized and strengthened, or eliminated (maintenance of synapses) (Ben-Ari et al., 1997). To better stabilize the established synaptic connections, it is critical for activity to occur within the “connection” (Constantine-Paton et al., 1990; Goodman and Shatz, 1993; Ben-Ari et al., 1997). To control the formation and maintenance of newly established synapses, a dramatic and tightly regulated pattern of changes involving voltage- and receptor- operated channels takes place, leading to specific neurotransmitter signaling at sequential developmental steps (Cherubini et al., 1991). Glutamatergic synaptic transmission within the mammalian brain has been detected electrophysiologically at late prenatal stages, and even early in postnatal development (Molnár and Isaac, 2002). Interestingly, in early development, glutamatergic synaptic transmission is primarily mediated via NMDA receptors, without any significant contribution of AMPA receptors (Durand et al., 1996; Wu et al., 1996; Ben-Ari et al., 1997). Therefore, to allow for these connections to properly form, it is imperative that the expression of the NMDA receptor is tightly regulated and controlled.

Expression patterns of the different NMDA receptor subunits vary greatly across the course of development. The GluN1 subunit is expressed and distributed ubiquitously in the brain and throughout the different stages of neurodevelopment, normally in excess in the endoplasmic reticulum (Ju and Cui, 2016). Meanwhile, the GluN2 subunit and its four subtypes (A-D) have more tightly regulated temporal and spatial expression patterns that differ greatly across development. Prenatally, the GluN2B subunit is expressed at high levels, which decline after birth throughout most of the brain (Akazawa et al., 1994; Luján et al., 2005). At birth, and at an early developmental age, NMDA receptors found in the forebrain are almost exclusively
composed of GluN1 and GluN2B subunits (Molnár and Isaac, 2002). The specific temporal control of GluN2B expression suggests that this subunit may play an important role in brain development, the formation of circuits and even potentially cell migration and differentiation; all processes integral to synaptic plasticity (Cohen and Greenberg, 2008). Around birth, GluN2D mRNA is present mainly in midbrain structures, peaking at about P7. Postnatally, GluN2A and GluN2B are expressed in pyramidal cells, while GluN2C and GluN2D mRNAs are expressed in interneurons and in the cerebellum (Monyer et al., 1994). The observed developmental decrease in the forebrain GluN2B:GluN2A ratio is complete by the third or fourth postnatal week in rodents (Molnár and Isaac, 2002).

In the glutamate signaling system, NMDA receptors are not the only receptors with developmentally-regulated expression; AMPA receptors and mGluRs are also tightly regulated. AMPA receptors are expressed, but not functional, early in development, leading to the postulation of the ‘silent synapse hypothesis’ (Isaac et al., 1995; Molnár and Isaac, 2002). mGluRs also show a complex pattern of expression during development, that continues well into adulthood. This complex expression pattern is thought to provide the molecular foundation for cell- and circuitry-specific differentiation in the glutamatergic signaling system (Tanabe et al., 1993; Conn and Pin, 1997). Overall, mGluR1, mGluR2, and mGluR4 show low levels of mRNA expression and then increase during postnatal development. On the other hand, mGluR3 and mGluR5 are highly expressed at birth, and then level off during maturation to adult levels of expression (Catania et al., 1994). Specifically, for group I receptors, mGluR1 predominates during the developmental stage, while mGluR5 has a peak in expression around the second postnatal week, and then expression patterns even out to those seen in adulthood (Shigemoto et al., 1992; Catania et al., 1994; López-Bendito, 2002; Luján et al., 2005). This further solidifies the notion that orderly expression of glutamate receptor genes is imperative for the proper functioning of complex neuronal networks (Stadler et al., 2005). Furthermore, dysregulation of this tightly regulated expression pattern of glutamate receptors may play a role in neurodevelopmental disorders; dysregulation in signaling during development can lead to symptoms manifesting later in life.
2.5 Glutamate Receptors in Synaptic Plasticity

Glutamate signaling plays an important role in the regulation of synaptic plasticity. As briefly described previously, synaptic plasticity is the ability of a neuron to create connections and then regulate synaptic strength and transmission via reaction to neurotransmitter signals. Specifically, glutamate plays an important role in the regulation of LTP and LTD. Following long-lasting glutamatergic activation via a sustained burst from a single synapse, or via coordinated weaker stimulation from a number of pre-synapses, there is an induction of LTP. Briefly, LTP induction begins with the increased phosphorylation and then integration of additional glutamate receptors, AMPA receptors, into the post-synaptic density (Malenka and Bear, 2004). Following this early phase of LTP, continued activation of both NMDA and AMPA receptors converges and leads to the activation of the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-related kinase) pathways, which then leads to the transcription of LTP-associated genes (Kelleher et al., 2004; Chiocchetti et al., 2014).

Specifically, and in more detail, NMDA receptors have classically been deemed ‘coincidence detectors’ of activity at the pre- and post-synaptic level (Hunt and Castillo, 2012; Sale et al., 2014). Influx of Ca\(^{2+}\) following the opening of NMDA receptors triggers a biochemical cascade that involves a number of intracellular kinases: ERK, cAMP-dependent protein kinase (PKA), and calcium calmodulin kinase type II (CaMKII). Steeper influxes of Ca\(^{2+}\) induce LTP, while low and prolonged Ca\(^{2+}\) influx leads to LTD. To consolidate long-term changes for the efficiency of synaptic transmission, the synthesis of new proteins is required. This can occur at either the translational level (via existing pools of mRNA), or at the transcriptional level via the activation of transcriptional factors (Liu-Yesucevitz et al., 2011; Leslie and Nedivi, 2011; Lyons and West, 2011). The synthesis of new proteins within the activated neuron allows for morphological modifications to take place, such as the formation of novel synaptic connections via dendritic spines. This then leads to the formation of novel circuits, or the re-wiring of already established ones.

3 Neurodevelopmental Disorders

Neurodevelopmental disorders encompass a very broad group of disabilities; manifesting as a group of dysfunctions involving some form of disruption during brain development. Overall, this definition encompasses a wide variety of neurological and psychiatric problems that are both
clinically and causally distinct. These include rare genetic syndromes, cerebral palsy, congenital neural anomalies, schizophrenia, autism, attention deficit disorder (ADHD), and epilepsy (Thapar et al., 2016). Grouping these disorders temporally, it should be appreciated that they cover a broad timeline of onset, encompassing both early periods of development and later, ongoing maturation. From a clinical standpoint, neurodevelopmental disorders can be termed ‘developmental brain dysfunctions’, and may result in broad symptom manifestations. These range from less severe disorders (ex. learning disabilities, language disorders, developmental coordination disorder and ADHD), to more severe, classical neurodevelopmental disabilities (ex. intellectual disability, cerebral palsy, and autism spectrum disorders). There exist even a subset of psychiatric disorders (ex. schizophrenia and possibly major affective disorders) that fall under the neurodevelopmental ‘umbrella’ (Moreno-De-Luca et al., 2013).

As outlined in Figure 4, developmental brain dysfunctions can be caused by a number of different factors, with dysfunctions typically manifesting as impairments in cognition, neuromotor control and neurobehavioural function. The underlying causes for neurodevelopmental disorders span a wide genetic range, and can also include environmental factors (such as infections, immune dysfunction, metabolic dysfunction, trauma, etc.) (Ehninger et al., 2008). Furthermore, recent studies have identified the biological background for a number of genetic syndromes that eventually lead to neurodevelopmental disorders (West and Greenberg, 2011). These studies have shown that a number of the pathways that are affected in neurodevelopmental disorders, and their associated genes, play a role in synaptic function (West and Greenberg, 2011; Zoghbi and Bear, 2012), and even protein synthesis within the synapse (Bhakar et al., 2012). Interestingly, it was also seen that a number of genes underlying neurodevelopmental disorders require very precise regulation, and reduction or enhancement of their expression leads to phenotypes, often with dramatically similar clinical presentations (Ramocki and Zoghbi, 2008). This further highlights the necessity for precise and tight regulation of physiological levels of synaptic regulator proteins. For a long time it was believed that neurodevelopmental disorders, such as autism or schizophrenia, were medically untreatable, due to assumptions that brain dysfunction, hardwired prior to diagnosis, could not be reversed (Silverman et al., 2012). The developmental trajectory of neurodevelopmental disorders posed a number of problems in treatment and overall management strategy.
Figure 4. Schematic outline and model of developmental brain dysfunction, including etiological factors and clinical symptomatic manifestations. Adapted from Moreno-De-Luca et al. (2013).
3.1 Progression of Neurodevelopmental Disorders

The progression of neurodevelopmental disorders can be classified into two categories; early-onset and late-onset. Two clear examples of early- vs. late-onset neurodevelopmental disorders are autism spectrum disorder (early-onset) and schizophrenia (late-onset). Autism and schizophrenia are both considered to be neurodevelopmental disorders, and they share a number of clinical features; robust impairments in sociability or interpersonal relations, and cognitive dysfunction (Goldstein et al., 2002). However, clinically, they are considered two distinct disorders. This distinction is due to the time of onset for symptoms; symptoms of autism first appear during early life, while positive symptoms of schizophrenia (used to formally diagnose the disorder) don’t typically appear until after adolescence (Lewis and Levitt, 2002; Goldstein et al., 2002; McCullumsmith et al., 2004; Rapoport et al., 2005). The hallmark behavioural symptoms of autism, impaired social interaction and communication, occur quite early in life and are typically diagnosed by age three (Caronna et al., 2008). Conversely, hallmark symptoms of schizophrenia (hallucinations, delusions, cognitive and social dysfunction) do not appear until after puberty, with a potential appearance of earlier prodromal symptoms (Goldstein et al., 2002). Due to the stark difference in the age of onset for symptoms, but a comparative symptomology, linking autism and schizophrenia raises the following question: can the underlying neural and cellular dysfunctions found in these disorders be caused by dysfunction of the same cellular processes but at different developmental times? This difference in onset can potentially be explained by the formation and maintenance of synaptic connections via dendritic spines (Copf, 2016).

3.1.1 Dendritic Spine Involvement in the Onset of Neurodevelopmental Disorders

The establishment and maintenance of dendritic spines is a dynamic process, that when disrupted, may lead to neurodevelopmental disorders. Changes in dendritic arbor morphology, in part, mediate the dynamic process of synaptic plasticity. Despite dendritic morphology establishment occurring early in embryonic development, dendritic morphology continues to be a dynamic process throughout development and well into maturation. To retain the gross morphology of dendritic spines, active maintenance mechanisms are employed throughout adult life (Copf, 2016). It is important to note, that irregularities in either the proper establishment
(development phase) or the later regulation (maintenance phase) of dendritic spines can lead to detrimental dysfunction in synaptic plasticity, and therefore, dysfunction in neuronal networks.

As previously mentioned, the onset of neurodevelopmental disorders may stem from the dysfunction of the same cellular process. A candidate of such a cellular process is synaptic connectivity mediated via dendritic spines. Along this line of reasoning, it can be postulated that the onset of autism may coincide with the cellular process of dendritic growth and branching, takes place in early childhood. Meanwhile the onset of schizophrenia may correlate with the later stages of dendritic spine modifications, such as pruning and maintenance, that occur later, well into adolescence (Jan and Jan, 2010; Copf, 2016). Autism has been associated with the phenomenon of synaptic ‘underpruning’, resulting in enlarged regional brain size, hypothesized to be due to the overgrowth or lack of dendritic pruning (Kelleher and Bear, 2008). On the other hand, disorders with a later onset of symptoms, such as schizophrenia, have shown the opposite. Overpruning has been shown in these late-onset disorders, even demonstrating failed maintenance of dendritic spines and synaptic connections. Schizophrenia has been associated with the loss of dendritic spines on cerebral neocortical pyramidal neurons, and a decrease in the density of glutamatergic neurons (Garey, 2010; Dennis et al., 2013). Therefore, schizophrenia empirically shows evidence of categorization as a ‘late-onset’ disorder. These experimental observations correlate with the hypothesis that neurodevelopmental disorders with differing onsets may be due to dysfunction of a common cellular process.

Dendritic spine establishment and maintenance has not only been implicated in autism or schizophrenia, but also in a number of other neurodevelopmental disorders, including, Fragile X Syndrome and Rett Syndrome. These disorders manifest as a similar cluster of clinical symptoms (Copf, 2016). Therefore, it is reasonable to hypothesize that, aside from dysfunctions that occur directly at the level of the synapse, the establishment and maintenance of dendritic spines may also be a commonly disrupted process. Dysfunction in dendritic spine establishment and maintenance, during development, can lead to symptoms associated with neurodevelopmental disorders.

4 Schizophrenia

Schizophrenia is a devastating illness that affects over 50 million people worldwide, and can be classified as a neurodevelopmental disorder. The economic burden of schizophrenia surpasses
most other psychiatric illnesses; more hospital beds are filled by those suffering from schizophrenia than any other medical condition (McCullumsmith et al., 2004). In the broadest sense of the term, schizophrenia can, and most often does, fall under the umbrella of a ‘neurodevelopmental disorder’. The neurodevelopmental basis for the pathology of schizophrenia was first proposed by Weinberger in 1987, stating that schizophrenia is a “neurodevelopmental disorder in which a fixed brain lesion from early in life interacts with certain normal maturational events that occur much later”. This was further elaborated on in later studies, stemming from hypotheses that “a brain lesion can remain clinically silent until normal developmental processes bring the structures affected by the lesion ‘on line’” (Marenco and Weinberger, 2000; Lewis and Levitt, 2002). Schizophrenia can be more specifically characterized as a late onset neurodevelopmental disorder, since symptoms manifest later in life when compared to other neurodevelopmental disorders, such as autism (Copf, 2016). In terms of trajectory, schizophrenia is a life-long mental disorder, of complex nature, involving a number of contributing factors, both genetic and environmental, as well as the interaction between the two (van Os et al., 2008; Demontis et al., 2011).

4.1 Symptoms and Treatment of Schizophrenia

Although the etiology of schizophrenia is unknown, it is characterized by a collection of clinical symptom presentations, most commonly divided into positive and negative symptoms. Positive symptoms include hallucinations (most often auditory), paranoid delusions and psychosis. Far more debilitating are the negative symptoms, which include deficits in normal social behaviours and cognitive impairments that encompass most aspects of cognition including attention, working memory and behavioural flexibility (Moghaddam, 2003). Few individuals suffering from schizophrenia have all of the aforementioned symptoms. The persistence of several characteristic symptoms, such as auditory hallucinations, must be present in order for someone to be diagnosed with schizophrenia. This illness is not a static condition, but a progressive deterioration in the ability of normal function (McCullumsmith et al., 2004). One of the hallmarks of this psychiatric illness is its onset; most commonly post pubertal, correlating with the post-adolescent completion of synapse elimination. Therefore, deficits that occur during the development of the brain only become evident in adulthood, manifesting as behaviours typical of those suffering from schizophrenia (Keshavan, 1999).
The treatment of schizophrenia remains imperfect, with current therapies of schizophrenia treating symptoms, and not the underlying cause of the disease. A number of neurotransmitter systems are implicated in schizophrenia, including dopaminergic, glutamatergic, and serotonergic systems. This only adds to the difficulty in effectively treating the disorder (Steeds et al., 2015; Gogos et al., 2015). The discovery that effective doses of antipsychotic drugs were linked to the blockage of dopamine receptors, more specifically dopamine receptor 2 (D2)-type, ushered in a new era of treatment in schizophrenia (Seeman et al., 1975; Creese et al., 1976). To date, the pharmacological treatment of schizophrenia remains virtually the same. Developed in 1961, clozapine remains one of the most efficacious antipsychotic drugs. Overall, there still remain a considerable number of patients that are resistant or only partially responsive to available antipsychotic medications (Lieberman et al., 2005). Failure in attempts to develop a more effective compound, with fewer adverse effects, is most noticeable in the treatment of negative symptoms and cognitive deficits, key predictors of functional disability (Moghaddam and Javitt, 2012). The field currently finds itself in great need of both alternative medications in the treatment of schizophrenia, but also in need of alternative targets for treatment (Abbott, 2010).

A number of theories exist for the etiology of schizophrenia, and although the dopamine hypothesis (dysregulation of dopamine neurotransmission) has been the prominent theory, the emergence of the glutamate hypothesis can be dated back over 50 years. Dopaminergic models are limited to a small number of brain regions, with secondary dysregulation seen elsewhere in the brain. In contrast, glutamatergic transmission, and therefore glutamatergic models, cover a broader landscape in the brain; all cortical efferents, the majority of cortical afferents and cortico-cortical connections. Therefore, the deficits seen in glutamatergic models are distributed throughout cortical and subcortical regions, involving sensory and higher cortical brain regions (Javitt, 2009).

5 **Glutamate Hypothesis of Schizophrenia**

The glutamate hypothesis for the etiology of schizophrenia suggests that the underlying cause for a number of dysfunctions seen in schizophrenia stems from a deficit in glutamate neurotransmission. As previously mentioned, glutamate is the major excitatory neurotransmitter in the mammalian CNS, specifically in pyramidal cells. Pyramidal cells are integrated into the
efferent and interconnecting pathways within the cerebral cortex and limbic system, brain regions that have been implicated in the pathophysiology of schizophrenia (Tsai and Coyle, 2002; Demontis et al., 2011). Furthermore, glutamate hypofunction is consistent with the hypothesis that schizophrenia is a neurodevelopmental disease, manifesting due to improper circuit formation during brain development (Rapoport et al., 2005).

5.1 NMDA Receptor Implication in Schizophrenia

The NMDA receptor first reached the forefront of the glutamate hypothesis of schizophrenia when studies showed that certain drugs of abuse were capable of recreating symptoms of schizophrenia. Specifically, phencyclidine (PCP) and ketamine were capable of recapitulating psychosis, social withdrawal and working-memory deficits, all symptoms typically seen in schizophrenia (Javitt and Zukin, 1991; Krystal et al., 2002). These drugs’ effects were shown to be mediated by the blockade of the NMDA receptor channel; NMDA receptor antagonists. The hypothesis for NMDA receptor hypofunction underlying the manifestation of schizophrenia is supported by a number of studies: NMDA receptor antagonist studies (Luby et al., 1959; Javitt and Zukin, 1991; Krystal et al., 1994), brain imaging studies (Heckers, 2001; Yamasue et al., 2004), genetic studies (Harrison and Owen, 2003; Kirov, 2005), postmortem studies (Woo et al., 2004), and pharmacological interventions that enhance NMDA receptor function in subjects with schizophrenia (Coyle, 2006). Evidence has also emerged that supports the notion that psychosis, which is not unique to schizophrenia, is secondary to NMDA receptor hypofunction (Kegeles et al., 2000).

The glutamate synapse has emerged as one of the most prominent targets for potential therapeutic intervention in schizophrenia (Javitt, 2004). This is advantageous, as the glutamate synapse is a target-rich environment that contains a large number of presynaptic, postsynaptic and regulatory proteins that represent suitable targets for drug development (Moghaddam, 2003; Marek, 2010). Furthermore, a number of convergent lines of evidence demonstrate that the hypothesis for hypofunction of the NMDA receptor leading to symptoms of schizophrenia might, in fact, reflect some kind of dysregulation at the receptor level, rather than an actual deficit in the number of NMDA receptors (Kantrowitz and Javitt, 2010). Genetic mutations of the NMDA receptor subunits, therefore, could account for the dysregulation observed at the receptor level. A number of studies have found associations between schizophrenia and GRIN1 gene
polymorphisms (Begni et al., 2003; Georgi et al., 2007; Galehdari et al., 2009), GRIN2A variations (Itokawa et al., 2003; Iwayama-Shigeno et al., 2005; Tang et al., 2006) and GRIN2B variations (Qin et al., 2005; Martucci et al., 2006; Demontis et al., 2011).

Glutamatergic dysfunction can account for disrupted signaling in the dopamine system, classically seen in schizophrenia. Although it has been proposed that positive symptoms observed in schizophrenia are due to a hyperactive state of dopamine transmission, they can also be accounted for as a consequence of changes that occur in glutamatergic signaling. Changes seen in dopaminergic function within schizophrenia may actually be a secondary downstream outcome to reduced activity at the glutamate synapse (Laruelle, 2014). Linking the dopamine and glutamate hypotheses, studies have shown that NMDA receptor antagonists, leading to sustained disruption in NMDA receptor transmission, cause changes in dopamine transmission. Both a deficit in cortical dopamine and subcortical hyperactivity of dopamine may be related to persistently disrupted NMDA receptor activity. It is postulated that glutamate can affect dopamine neuron activity in the substantia nigra and the ventral tegmental area. Decreased NMDA receptor transmission leads to diminished prefrontal activity, which then leads to a lowering of mesocortical dopamine transmission. This results in the worsening of cognitive function. Chronic disruption in dopamine can lead to the positive symptoms seen in schizophrenia (Cepeda and Levine, 1998; Yang et al., 1999; Nicola et al., 2000; Konradi and Heckers, 2003; Howes et al., 2009). Furthermore, NMDA receptor hypofunction and dopamine dysfunction are connected as NMDA receptors have been shown to directly interact with dopamine receptors. This interaction manifests as a physical connection between the dopamine receptor 1 (D1) and GluN1 subunits, modifying and regulating D1 receptors and their trafficking (Fiorentini et al., 2003; Pei et al., 2004; Ju and Cui, 2016). Therefore, it is reasonable to link dysregulation in dopamine transmission with a hypofunction of GluN1 subunits, which can account for the abnormal dopamine activity associated with schizophrenia symptoms (Laruelle, 2014; Ju and Cui, 2016).

The glutamate hypothesis can also account for dysfunction and alterations seen in GABAergic signaling in the cortex. Postmortem findings have shown that individuals with schizophrenia have altered levels of expression of the machinery components required for the proper function of GABA, the primary inhibitory neurotransmitter in the brain (Lewis and Moghaddam, 2006; Gordon, 2010). Studies have shown that these changes in the ‘machinery’ of GABAergic
signaling occur primarily in parvalbumin containing fast-spiking interneurons, including a decrease in the expression of glutamate decarboxylase (GAD) 67 and parvalbumin itself (Lewis and Moghaddam, 2006). This suggests that the GABA system is in a hypoactive state, and that the NMDA receptor may act as a monitor of overall excitatory activity in the cortex via its expression on parvalbumin-expressing interneurons (Figure 5) (Gordon, 2010). Therefore, this would physiologically manifest as decreased NMDA receptor signaling being interpreted by the interneuron as not enough activity taking place. This would result in a decrease of interneuron activity, in an effort to restore excitatory/inhibitory balance that is required for correct circuit function (Gordon, 2010). This theory of ‘disinhibition’ further cements the role of the NMDA receptor in the pathology of symptoms seen in schizophrenia.

Aside from dysfunction of the NMDA receptor itself, schizophrenia has been associated with a number of alterations in the regulation of the NMDA receptor; altered levels of NMDA receptor-associated postsynaptic proteins (Beneyto and Meador-Woodruff, 2008) or even altered neuregulin-1 activation of ErbB4 receptors (Hahn et al., 2006). Numerous susceptibility genes have been identified to be involved in the pathogenesis of schizophrenia (Harrison and Weinberger, 2005; Allen et al., 2008), and a number of these variants are related to NMDA receptor-mediated glutamatergic signaling in the brain (Ju and Cui, 2016). Being related to NMDA receptor-mediated signaling, these genes and their variants, are also involved and implicated in neurodevelopment and the formation of brain circuits. This involvement can be thought to underlie symptoms of schizophrenia when disrupted (Harrison and Weinberger, 2005; Ju and Cui, 2016). Therefore, since NMDA receptors play a critical role in the formation, strengthening, and elimination of neural connections (Waites et al., 2005), it is realistic to hypothesize that they play an important role in the neurodevelopmental hypothesis of schizophrenia as well.

6 Neurodevelopmental Hypothesis of Schizophrenia

Schizophrenia can be, and in most cases is, considered a neurodevelopmental disorder. The onset of schizophrenia follows a developmental trajectory that spans from prenatal brain development to adulthood. It has been speculated that if researchers and clinicians approach schizophrenia from a neurodevelopmental standpoint, the identification of discrete disease stages would allow
Figure 5. Disinhibition phenomenon occurring in the glutamate hypothesis of schizophrenia. (A) NMDA receptor signaling, and subsequent excitatory levels, are monitored via inhibitory neurons. In a normal physiological state, inhibitory neurons will monitor and maintain appropriate balance between inhibition and excitation via GABA release from the interneuron. (B) In a state of NMDA receptor hypofunction, as found in schizophrenia, the excitatory signal is disrupted and there is dysfunction in the ‘monitoring’ of balance between excitatory/inhibitory balance. This leads the inhibitory neuron into adjusting GABA levels as if there is not enough excitatory stimulation, causing a decrease in inhibition levels, and a disinhibition of excitatory neurons via inhibitory neurons. Adapted from Gordon (2010).
for the focus to be on disease prevention in a pre-psychosis stage (Insel, 2010; Catts et al., 2013). As previously described by Weinberger et al. (1987), schizophrenia is a consequence of genetic predisposition and the presence of early adverse events that remain latent throughout the first 20 years of life, later manifesting in early adulthood as psychosis (Catts et al., 2013). During prenatal brain development, pathogenic insults (both genetic factors and environmental stressors) can trigger changes in the precise cascade that is required for the proper development of synaptic connections and overall function (Hayashi-Takagi and Sawa, 2010). The onset of symptoms across developmental time points show little to no overt behavioural abnormalities manifesting during childhood. Prodromal symptoms (mild and transient psychosis, social withdrawal and subclinical emotional and cognitive changes) of schizophrenia present themselves as early as 2 years prior to the onset of psychosis. First-episode psychosis occurs in early adulthood, between 15-25 years of age in males and females. Diagnosis and treatment are initiated within the first few years following the onset of psychosis (Hayashi-Takagi and Sawa, 2010).

The neurodevelopmental hypothesis of schizophrenia states that the etiology of schizophrenia may involve pathological processes (as previously described) that begin before the brain approaches its adult anatomical state in adolescence (Rapoport et al., 2005). A combination of understanding the molecular substrates of neuroplasticity, along with elucidating the mechanisms for the pathophysiology of schizophrenia, has led to the hypothesis that schizophrenia is a disorder of neuroplasticity, at both a cellular and molecular level (McCullumsmith et al., 2004). Developing as early as late first or early second trimester, these abnormalities in neurodevelopment have been suggested to lead to the activation of pathological neural circuits during adolescence or young adulthood. These later lead to the manifestation of positive or negative symptoms, or both, in adulthood (Fatemi and Folsom, 2009). As previously mentioned, it has been understood that the developing, juvenile brain is plastic and flexible, capable of changes in circuit connectivity. Conversely, the adult brain was thought to be fixed and inflexible (Ehninger et al., 2008). This would have significant implications for the treatment of schizophrenia under the neurodevelopmental hypothesis; how does one “rewire” the brain to reverse deficits in synaptic connectivity in adulthood if the adult brain is “fixed” and inflexible?

A number of recent studies in animal models of neurodevelopmental disorders have demonstrated that treatment of disrupted molecular and cellular mechanisms, specifically in adults, result in dramatic improvements in function, even cognition (Ehninger et al., 2008).
These cellular mechanisms seem to retain the capacity for change, even well into adulthood. Thus, it is plausible that adult rescue of underlying deficits in neurodevelopmental illnesses, such as schizophrenia, may allow for robust plasticity mechanisms to compensate for, or even correct, specific developmental pathologies (Ehninger et al., 2008).

7 Animal Models of Neurodevelopmental Disorders

7.1 Modeling Neurodevelopmental Disorders and their Rescue in Adult Rodents

A number of studies in rodents have shown that neurodevelopmental disorders can be rescued in adulthood. Neurodevelopmental disorders that have shown a reversal of symptoms, even in adulthood, include the following: (1) rescue of spatial learning deficits in neurofibromatosis I models (Costa et al., 2002; W Li et al., 2005), (2) reversal of learning and memory deficits, long-term potentiation (Fernandez et al., 2007) and spatial learning deficits (Rueda et al., 2008) in Down’s syndrome models, (3) rescue of long-term memory deficits in Rubinstein-Taybi Syndrome (Bourchouladze et al., 2003; Alarcón et al., 2004), (4) reversal of morphological and cognitive symptoms observed in Fragile X Syndrome, including immediate recall, short-term memory (McBride et al., 2005), and dendritic spine density (Dölen et al., 2007), and (5) a rescue of neurological symptoms found in Rett Syndrome manifestations (MECP2 mutations) including hindlimb clasping, irregular breathing, gait, and tremor (Guy et al., 2007). These studies highlight that enhanced expression of a dysfunctional gene, or even amelioration of signaling in an affected pathway, can in some cases reverse symptoms, even after full development of symptoms has occurred and treatment is well into adulthood (Ehninger et al., 2008). Therefore, it is reasonable to infer that the adult brain retains a level of plasticity that is capable of overcoming developmental “miswiring” stemming from genetic insult. Further detail into models of neurodevelopmental disorders and their rescue in adulthood will be elaborated below.

7.1.1 Pharmacological Adult Rescue in a Model of Autism

Neurodevelopmental disorders, such as autism, have long been thought to be pharmacologically untreatable since dysfunctions occur in the developing brain and become hardwired long before diagnosis. Recent studies have highlighted that in a number of cases of autism, the dysfunction in wiring occurs due to mutations in genes that control ongoing formation and maturation of
synapses, challenging the classical view of treatment in neurodevelopmental disorders (Silverman et al., 2012).

A study completed by Silverman, et al. (2012) looked to pharmacologically target the mGluR5 receptor as a potential site for intervention in autism. mGluR5 has been shown to be involved in the pathology of Fragile X Syndrome, and, due to the overlapping symptoms between Fragile X Syndrome and autism, targeting of the mGluR5 was undertaken (Bear et al., 2004; Dölen et al., 2007). Using BTBR mice, an established model of autism, Silverman et al. (2012) showed that GRN-529, an mGluR5 negative allosteric modulator (NAM), reduced repetitive behaviours, spontaneous stereotyped jumping, and partially reversed their deficits in sociability. This study highlights the possibility that a pharmacological intervention, that focuses on a single target, may be beneficial in the alleviation of a number of behavioural symptoms that occur in autism. Or even potentially, other clinically overlapping neurodevelopmental disorders.

### 7.1.2 Adult Genetic Rescue of Rett Syndrome (MeCP2)

Rett syndrome is caused by a mutation, or mosaic expression of mutant copies, of the X-linked MeCP2 gene (coding for a methyl-CpG-binding protein), resulting in severe symptoms that fall along the spectrum of autism-based disorders (Amir et al., 1999; Guy et al., 2007). Using two different mouse models of MeCP2 “dysfunction”, Guy et al. (2007) and Sztainberg et al. (2015) showed that restoration of functional MeCP2 protein to physiological levels resulted in the rescue of symptoms characterized in autism-spectrum disorders. While one study looked at the restoration of normal MeCP2 expression in a monogenic loss-of-function model (Guy et al., 2007), the other looked at the restoration of MeCP2 expression in a duplication model (Sztainberg et al., 2015). This highlighted that a disruption of MeCP2 expression, in either direction, leads to symptomatic presentation. Furthermore, subsequent restoration of MeCP2 to physiological levels, even in adulthood, leads to the amelioration of these symptoms. Both studies used a tamoxifen inducible Cre-recombinase method to genetically reverse phenotypes.

Guy et al. (2007) showed that a mouse model of MeCP2 deletion displayed symptoms including hindlimb clasping, irregular breathing and gait, occurrence of tremor and a deficit in LTP. Following genetic re-instatement of MeCP2, adult animals showed amelioration of symptoms to wildtype levels. Similarly, Sztainberg et al. (2015) showed that in their model of MeCP2 duplication, mice exhibited hypolocomotion, increased anxiety-like behaviour, impaired social
behaviour, and enhanced LTP. All symptoms were rescued to wildtype levels once MeCP2 levels were restored to physiological levels in adult mice. Interestingly, this restoration was further corroborated via the use of an antisense oligonucleotide to restore MeCP2 expression levels, showing full restoration of symptoms in adult mice.

7.1.3 Adult Genetic Rescue of Neuregulin 1 Overexpression

Neuregulin has been identified as a susceptibility gene in schizophrenia (Stefansson et al., 2002; Yang et al., 2003), as well as being implicated in the development of the brain (Fazzari et al., 2010; Ting et al., 2011). Yin et al. (2013) used a mouse model of type I NRG1 (neuregulin) overexpression in forebrain regions, including the prefrontal cortex and hippocampus. The overexpressing mice showed behavioural deficits, including hyperactivity, decreased PPI, decreased sociability, and decreased spatial working and reference memory. Furthermore, at the synaptic level, the NRG1-overexpressing mice showed hypofunction of glutamatergic and GABAergic signaling. Following treatment with doxycycline in adulthood, to turn off the expression of the Nrg1 transgene, both behavioural and synaptic deficits were resolved. This study highlights that not only do changes in neuregulin lead to robust behavioural and synaptic dysfunctions, but also that reversal of these symptoms is possible, even in the adult mouse.

7.1.4 Adult Genetic Rescue of SHANK3 Expression

The SHANK3 gene encodes for a postsynaptic scaffold protein (Boeckers et al., 2002) that helps regulate synaptic development (Takeuchi et al., 1997). Mutations in this gene have been shown to associate with cases of autism spectrum disorder (Bozdagi et al., 2010; Shevelkin et al., 2014). It is important to determine if the pathology of the disorder is reversible in adults (Mei et al., 2016). Mei et al. (2016) generated a novel Shank3 conditional knock-in mouse model, whereby, treating with tamoxifen lead to Cre-recombinase induction and temporally-controlled re-expression of the Shank3 gene. In the absence of Cre-recombinase activity, the mice functioned as Shank3 knockout mice, displaying a number of behavioural and synaptic abnormalities: deficits in exploratory behaviour, anxiety, motor coordination, sociability, increased repetitive grooming behaviour, decreased spine density, and impaired neurotransmission in the dorsal striatum. It was shown that restoration of Shank3 expression increased dendritic spine density, restored composition of PSD protein, and improved striatal neurotransmission (Mei et al., 2016). Behavioural rescue proved to be variable as sociability, and repetitive grooming behaviours were
fully rescued following adult intervention, while anxiety and motor coordination could not be recovered. This study highlights the certain degree of continued plasticity that endures well into the adult brain.

7.2 GluN1-KD (NR1-KD) Mouse Model as a Model for Neurodevelopmental Disorders

The GluN1-KD mouse model combines both the glutamate and neurodevelopmental hypotheses for the etiology of neurodevelopmental disorders such as schizophrenia. The GluN1-KD mouse expresses low levels of the GluN1-subunit of the NMDA receptor and is a model of endogenous NMDA receptor hypofunction (Mohn et al., 1999). Based on previous studies using NMDA receptor antagonists PCP and MK-801 to model schizophrenia in animals (Jentsch and Roth, 1999), the GluN1-KD mouse offers “chronic antagonism” throughout development, a concept where pharmacological impairment would be impractical. The GluN1-KD mouse model was the first to be described as a genetic model of sustained NMDA receptor hypofunction (Mohn et al., 1999).

It is important to note that the GluN1-KD mouse model is a knockdown, not a knockout. NMDA receptors are integral for the proper development of the brain as they are involved in the formation and regulation of synapse maturation (Cohen and Greenberg, 2008). GluN1 knockout mice die shortly following birth, indicating that the GluN1 subunit, and subsequent NMDA receptor function, is essential in nerve circuit development (Forrest et al., 1994; Demontis et al., 2011). Due to the lethality of completely knocking out Grin1 (the gene that encodes the essential GluN1 subunit of the NMDA receptor), the GluN1-KD mouse model has a partial loss-of-function, or hypomorphic mutation, of Grin1, making the mouse viable and a useful tool for the study of NMDA receptor hypofunction (Mohn et al., 1999). The hypomorphic mutation is achieved by the targeted insertion of additional foreign DNA into intron 19 of the Grin1 sequence, reducing levels of full-length mRNA. The decreased levels of GluN1 protein lead to decreased levels of functional NMDA receptor channels, impairing glutamate signaling (Mohn et al., 1999).

The GluN1-KD mouse model displays construct validity (Demontis et al., 2011), face validity (PPI and sociability), as well as predictive validity (Mohn et al., 1999; Gandal, Sisti, et al., 2012; Grannan et al., 2016). The GluN1-KD mice display a number of endophenotypes related to
schizophrenia; increased locomotor activity, decreased habituation to a novel environment, increased stereotypic behaviours, decreased performance in spatial and working memory tasks, decreased sensorimotor gating, decreased anxiety-like behaviours, impaired fear-mediated conditioning, and abnormalities in social behaviour (Mohn et al., 1999; Duncan et al., 2004; Moy et al., 2006; Halene et al., 2009; Dzirasa et al., 2009; Moy et al., 2012; Gandal, Sisti, et al., 2012; Milenkovic et al., 2014; Ferris et al., 2014; Mielnik et al., 2014; Grannan et al., 2016). Electrophysiologically, the GluN1-KD mice show a loss in excitatory-inhibitory balance in signaling (Gandal, Sisti, et al., 2012) and a disruption in dopaminergic signaling, with an increase in tonic firing rate (Ferris et al., 2014). These behavioural and electrophysiological endophenotypes, seen in the GluN1-KD mice, are both robust and easily detected.

Pharmacologically, typical and atypical antipsychotics, and even GABA agonists, attenuate hyperactivity, stereotypy, and sociability deficits in GluN1-KD mice, at similar doses as used in pharmacological models of schizophrenia (MK-801 or PCP models) (Mohn et al., 1999; Gandal, Sisti, et al., 2012; Mielnik et al., 2014). Furthermore, the observed hyperlocomotion and deficits in fear-based conditioning seen in the GluN1-KD model are responsive to M1 muscarinic acetylcholine receptor (mACHR) positive allosteric modulators (PAM) (Grannan et al., 2016). Most importantly, the advantage of the GluN1-KD model over pharmacological models remains the impairment of glutamate signaling throughout development, which may more closely mimic the pathophysiology of schizophrenia, especially within the neurodevelopmental hypothesis of the disease.

Therefore, in total, these studies show empirical evidence that disturbances occurring at the NMDA receptor level of glutamatergic signaling, specifically involving the GluN1 subunit, produce endophenotypes that are relevant to neurodevelopmental disorders, including schizophrenia (Ju and Cui, 2016). The GluN1-KD mouse model has the advantage of reproducing both the chronic and developmental nature of NMDA receptor hypofunction that is theorized to occur in neurodevelopmental disorders. The GluN1-KD model exhibits robust and standard behavioural endophenotypes that allow for consistent readouts across studies.
8 GluN1-Inducible Rescue (GluN1-IR) Project

8.1 Rationale

The aim of the GluN1-IR study is to determine if the brain can “rewire” and overcome neurodevelopmental deficits. During childhood and adolescence, there exists an extended period of brain development where synaptic connections are formed and refined. This development occurs prior to the emergence of symptoms in those suffering from neurodevelopmental disorders, such as schizophrenia. This period of plasticity may potentially offer us a therapeutic window and the opportunity to reverse the developmental deficits that lead to these neurodevelopmental disorders.

In terms of schizophrenia, perhaps one possible reason for the failure of antipsychotic treatment in so many patients may stem from the administration of these treatments outside of the critical period of plasticity. The deficits seen in schizophrenia patients may be a result of low glutamatergic signaling that manifests as decreased synaptic communication, as seen in the previously described GluN1-KD mouse model (Mohn et al., 1999). The premise of the GluN1-KD mouse model affords a unique opportunity to perform inducible genetic rescue. Following the genetic basis of the GluN1-KD model, a new floxed version of the insertion mutation could in theory be excised, at any developmental time point, by the inducible action of Cre-recombinase. The generation of a new mouse model, the GluN1-inducible rescue mouse (GluN1-IR), will be required. Therefore, the GluN1-IR mouse model affords the reversal and normalization of the developmental NMDA receptor deficiency during specific developmental time points: adolescence or adulthood (Figure 6).

The onset of schizophrenia follows a developmental trajectory: there exist mild to no behavioural impairments in childhood, followed by nonspecific behavioural changes in adolescence (post-puberty), with the onset of symptoms in early adulthood (Lewis and Lieberman, 2000). In a mouse, these timelines translate to the following ages; 6-weeks is the average age that mice attain puberty, while 10-weeks is the age at which sexual maturity (defined as biological adulthood) is attained in mice (Dutta and Sengupta, 2016). Therefore, to address whether intervention prior vs. after the onset of symptoms would result in a better rescue intervention time points were set at 6-weeks (prior to onset of symptoms) and 10-weeks (after onset of symptoms).
This study, using the novel GluN1-IR mouse model, will provide insight into the brain’s capacity to overcome prenatal and postnatal changes in synaptic communication and wiring. This study proposes to rescue NMDA receptor deficiency at 6-weeks of age (adolescence) and compare it to rescue at 10-weeks of age (adulthood), measuring phenotype reversal through behavioural, biochemical, and molecular studies.

8.2 Hypothesis of Research

We hypothesize that early intervention (6-weeks of age; adolescence) will result in greater rescue of behavioural, biochemical and molecular phenotypes when compared to adult intervention (10-weeks of age).

8.3 Objective of Research

To determine the extent to which developmental NMDA receptor deficiency, and subsequent disturbed glutamatergic signaling, can be overcome via the genetic restoration of NMDA receptors in either adolescence or later into adulthood. This study could potentially infer the intrinsic plasticity levels within the adolescent and adult brain.

8.3.1 Specific Aim 1

The generation of a novel transgenic mouse model is required. The model develops in a state of NMDA receptor hypofunction, but allows for the inducible genetic rescue of NMDA receptor deficiency (GluN1-IR mouse model) in a temporal fashion via the inducible action of Cre-recombinase.

8.3.2 Specific Aim 2

The characterization of the GluN1-IR mouse model following restoration of NMDA receptors, in either adolescence or adulthood. Characterization of the consequences of restored NMDA receptor signaling in mice with chronic developmental deficits in NMDA receptors will be completed via behavioural, biochemical and molecular means including measures of: excision rates of the insertion mutation, GluN1 subunit protein levels, NMDA receptor levels, locomotor, stereotypy, working memory and executive function, anxiety, sociability, RNAseq analysis of changes at the transcript level.
Figure 6. Hypomorphic mutation of *Grin1* reduces NMDA receptor levels in GluN1-IR mice, which is rescued following tamoxifen treatment. In wildtype mice, *Grin1* (coding for the GluN1 subunit of the NMDA receptor) is transcribed and alternately spliced. In GluN1-IR mice, insertion of *neo* (in green) into intron 19 of *Grin1* leads to the production of truncated transcripts due to the polyadenylation signal of the *neo* gene. A percentage of transcripts are transcribed in full (without truncation), where proper splicing of the mRNA removes *neo* and produces functional GluN1 subunits. GluN1 levels are reduced, and since GluN1 subunits are essential in the formation of NMDA receptors, this reduction limits the amount of functional ligand-gated, voltage-dependent ion channels formed (Mohn *et al.*, 1999; Ramsey *et al.*, 2011). Rescue is observed in the GluN1Cre mice once the insertion mutation is excised (via Cre-loxP) following tamoxifen treatment. GluN1 subunit levels are returned to wildtype.
Chapter 2
MATERIALS AND METHODS

9 Generation of GluN1-Inducible Rescue (GluN1-IR) Mouse Model

9.1 Molecular Cloning to Generate GluN1-IR Construct

*Grin1*^{flneo/flneo} mice were generated in house, based on the previously described GluN1-KD mouse (Mohn et al., 1999). Identical to the GluN1-KD model, the *Grin1* gene was modified via homologous recombination with an intervening sequence (neomycin cassette), and targeted into intron 19, but this time flanked by *loxP* sites, so the insertion mutation could be excised following Cre-recombination (see Figure 6). Concisely, the vector (pXena construct; gift from Beverly Koller, UNC Chapel Hill, USA) contained a floxed neomycin resistant gene (‘insertion mutation’; contained STOP codon as well as polyA sequence). Homologous arms (upstream – 5’ and downstream – 3’) of intron 19 of the *Grin1* gene were cloned into the vector, flanking the floxed neomycin cassette (see Figure 7). Specifically, to clone intron 19 of the *Grin1* gene into the pXena vector, the 5’ arm was obtained via digestion of an in-house plasmid, containing the cloned and sequence verified desired genomic fragment, with XmaI and PvuI restriction enzymes. The 5’ arm was then inserted into pBluescript (Addgene) via XmaI digestion and sticky end ligation. To insert the 5’ arm into the pXena vector, the vector was digested with NotI and MfeI, while the pBluescript vector was digested with NotI and EcoRI. The 5’ arm was then ligated into the pXena vector. The 3’ arm (downstream) of intron 19 of the *Grin1* gene was PCR-amplified from mouse genomic DNA and cloned into the pXena vector; both the PCR product and pXena vector (with the 5’ arm already inserted; see previous step) were digested with EcoRI and XhoI and then ligated together. The final product was intron 19 of the *Grin1* gene cloned into the vector flanking the floxed neomycin cassette; GluN1-IR construct (see Figure 7). All restriction enzymes and T4 DNA ligases were from NE Biolabs. The construct was sequenced to confirm molecular cloning.
Figure 7. **Schematic of GluN1-IR construct.** Taking advantage of the inducible Cre/loxP system, NMDA receptor deficiency (as previously generated in the GluN1-KD mouse) can be rescued at varying developmental timepoints. The generation of a new mouse line was required. Sequential *Grin1* genetic material (intron 19) was ligated into the pXena vector on opposing sides of the loxP (shown in blue) flanked pgk-neo (shown in green). This allows for homologous recombination of the foreign DNA (loxP flanked neo) into intron 19 of the *Grin1* gene.
10 Generation of GluN1-IR Chimeras

10.1 Electroporation of ES Cells

Electroporation of the GluN1-IR construct into R1 mouse ES cells (129/Sv strain) was completed by The Centre for Phenogenomics (TCP, Toronto, ON). ES cells underwent positive selection with G418 (BioShop Canada) to select for cells with integration events of the targeting construct. Clonal cell lines were established and were stored at -80°C until PCR and Southern blot assays were performed.

10.2 Screening of GluN1-IR ES Cell Clones

10.2.1 PCR Screening

Drug resistant clones were screened by PCR. Targeted clones were identified using the following primers; wildtype forward 5’-ATG CTG TGT CTT GCT T - TGA T - 3’, mutant forward 5’-ATC GCC TTC TA - T CG C CTT CT - 3’, common reverse 5’-GCC CAC TTT CCC TGG TCT - TCT-3’.

10.2.2 Southern Blot Screening

Targeting events (homologous recombination at the appropriate genomic site) were further confirmed in drug resistant and PCR-confirmed positive clones via Southern blot analysis (in house). Briefly, six ES cell clones were tested for correct homologous recombination of the insertion mutation; IVF4, IVF1, IVE7, VE2, IIID5, and IVB4. Two WT samples of genomic DNA (from in-house mouse colony) were included as negative controls for homologous recombination. 10µg of genomic DNA (from each clone) was digested with HindIII and run on a 0.8% agarose gel. The gel was then placed in Solution A (1.5M NaCl, 0.5M NaOH) and left to shake for 30min. This step was repeated once more with fresh Solution A. The gel was then placed in Solution B (2M NH₄Ac, 0.4M NaOH) and left to shake for 30-min. This step was repeated once more with fresh Solution B. The gel was then placed on a metal platform on a Pyrex dish filled with Solution B in the following configuration; metal platform, Whatman paper ‘wick sheets’ (long sheet spanning the metal platform with ends resting in Solution B in the Pyrex dish), Whatman paper (size of gel), gel (DNA side up), nylon membrane (already pre-soaked in Solution B), pre-soaked Whatman paper (Solution B), small stack of dry Whatman paper, and a larger stack of dry paper towels (all cut to the corresponding dimension of the gel). Stacked configuration was then weighed down and left overnight at room temperature. The
membrane was then briefly rinsed in 2xSSC (0.3M NaCl, 30mM NaCitrate) and then UV-crosslinked and placed into hybridization tubes.

The probe was designed to target sequence located outside of the insertion mutation. More specifically, it was designed 1kb downstream of the 3’-short arm of the generated construct. Therefore, the probe presented an 11kb fragment when no integration of the insertion mutation occurred, or a 13kb fragment with a homologous recombination event and correct integration (Figure 11A). The radioactive (\(^{32}\)P) probe was generated via PCR amplification and labeled using the Amersham Ready-To-Go DNA Labelling Beads (incubation at 37ºC for one hour). The membrane was pre-hybridized with QuikHyb (Agilent Technologies) at 68ºC for 30-min. The denatured probe and sonicated/sheared salmon sperm were added to the membrane (in QuikHyb solution), and allowed to incubate (rotating) at 68ºC for 2 hours. The membrane was then washed with 2xSSC + 0.01% SDS (twice, 15-min/each) at room temperature, and finally washed with 0.1xSSC (15mM NaCl, 1.5mM NaCitrate) for 30-min at 60ºC. The membrane was visualized on film (incubation of one week at -80ºC).

10.3 Diploid Aggregation (via TCP)

Correctly targeted ES cell clones were injected into albino blastocysts (via diploid aggregation completed by TCP) to generate chimeras. Chimeras were then bred to CD1 female mice to obtain ES cell germline transmitted offspring, determined by black eye colour and PCR screening (primers listed in section 10.2.1).

10.4 Breeding of Chimeras to Generate GluN1-IR Mouse Line

Chimeras (male) were generated. Coat colour was used as a subjective reflection of overall chimerism. All chimeras were bred with CD1 females. Germ line transmission was initially determined via eye colour (black), and subsequently confirmed through genotyping (PCR, same strategy as used to genotype the original GluN1KD mouse line, (Mohn et al., 1999)). Genotyping was accomplished with the following primers; wildtype forward 5’-TGA GGG GAA GCT CTT CCT GT-3’, mutant forward 5’-GCT TCC TCG TGC TTT ACG GTA T-3’, common reverse 5’-AAG CGA TTA GAC AAC TAA GGG T-3’. Following germline transmission, GluN1-IR heterozygotes were backcrossed into the C57Bl/6J background, and continuously backcrossed for at least 6 generations prior to starting experimentation. Due to the fact that the original ES
cells were on a 129/Sv background, identified founder chimeras were bred directly to 129/Sv females, resulting in all germline-transmitted progeny from this breeding being homozygous on the 129/Sv background.

11 Tamoxifen-Inducible GluN1-IR Mouse Colony

11.1 Animals

Animal housing and experimentation were carried out in accordance with the Canadian Council in Animal Care (CCAC) guidelines for the care and use of animals. Mice were group housed with littermates on a 12-h light-dark cycle (0700 to 1900h) and were given access to food (2018 Teklad Global 18% Protein Rodent Diet, Envigo, www.envigo.com) ad libitum, unless otherwise specified. Mice were tail clipped and had their toes tattooed at P11 (± 3 days) for genotyping and weaned at P21. Toe tattooing was used to identify all experimental mice.

11.1.1 Tamoxifen-Inducible Cre Mouse Line (ROSA26\textsuperscript{CreERT2})

\textit{ROSA26}\textsuperscript{CreERT2} (tamoxifen inducible) mice were obtained from Jackson Laboratory (008463; B6.129-Gt(Rosa)26Sor\textsuperscript{tm1(cre/ERT2)Tji/J}), and were previously described (Ventura et al., 2007). The Cre gene was identified using the following primers: common forward 5’- AAA GTC GCT CTG AGT TGT TAT-3’, wildtype reverse 5’- GGA GCG GGA GAA ATG GAT ATG-3’, mutant reverse 5’- CCT GAT CCT GGC AAT TTC G-3’.

11.1.2 Cre-Reporter Mouse Line (ROSA26\textsuperscript{tdTomato})

The Cre-reporter mouse line used, \textit{ROSA26}\textsuperscript{tdTomato}, was obtained from Jackson Laboratory (007914; B6.Cg-Gt(Rosa)26Sor\textsuperscript{tm14(CAG-tdTomato)Hze/J}) and was crossed with the \textit{ROSA26}\textsuperscript{CreERT2} line. The mouse line expresses tdTomato following Cre-mediated recombination. The mouse line was used to ensure ubiquitous expression of Cre following tamoxifen administration as was previously described (Madisen et al., 2010). The reporter gene was identified using the following primers: wildtype forward 5’- AAG GGA GCT GCA GTG GAG TA-3’, wildtype reverse 5’- CCG AAA ATC TGT GGG AAG TC-3’, mutant forward 5’- GGC ATT AAA GCA GCG TAT CC-3’, mutant reverse 5’- CTG TTC CTG TAC GGC ATG G-3’.
11.1.3  Tamoxifen-Inducible GluN1 Rescue Mouse Line (\textit{Grin1}^{\text{flneo/Cre}})

All experimental animals were F1 progeny of \textit{Grin1}^{\text{flneo/flneo}} heterozygotes; congenic for the C57Bl/6J and 129/Sv background. As the ES cells were on a 129/Sv background, identified founder chimeras were crossed directly with 129/Sv mice to create the \textit{Grin1}^{\text{flneo/flneo}} (129/Sv) line. For the C57Bl/6J \textit{Grin1}^{\text{flneo/flneo}} line, mice were backcrossed for at least 6 generations. The floxed insertion mutation (neo) was identified using the following primers: wildtype forward 5’-TGA GGG GAA GCT CTT CCT GT-3’, mutant forward 5’-GCT TCC TCG TGC TTT ACG GTA T-3’, common reverse 5’-AAG CGA TTA GAC AAC TAA GGG T-3’.

\textit{Grin1}^{\text{flneo/Cre}} mice (\textit{Grin1}^{\text{flneo/flneo}} x \textit{ROSA26}^{\text{CreERT2}}) were generated by having the \textit{ROSA26}^{\text{CreERT2}} mice (C57Bl/6J) bred to heterozygous \textit{Grin1}^{\text{flneo/flneo}} mice on the C57Bl/6J background (Figure 8). The compound heterozygotes (C57Bl/6J) were bred back to the heterozygous \textit{Grin1}^{\text{flneo/flneo}} mice (129/Sv) resulting in F1 progeny at the expected Mendelian frequency for all proposed studies; 12.5% WT, 12.5%WTCre, 12.5% GluN1 and 12.5% GluN1Cre. Primers mentioned above were used to identify the \textit{Grin1} insertion mutation, and primers described in section 11.1.1 were used to identify the Cre gene.

12  Tamoxifen Administration

Tamoxifen was administered to all mice tested, in all genotypes (WT, WTCre, GluN1, GluN1Cre) of \textit{Grin1}^{\text{flneo/Cre}} mice. Optimization of the tamoxifen administration was undertaken (see Appendix A). The optimized dosing regimen was as follows: tamoxifen (T5648, Sigma-Aldrich) was administered via oral gavage (6mg, 20mg/ml dissolved in 100% corn oil at 65°C for 1 hour) on day 1 of treatment. Mice were given access to tamoxifen chow (TD.140425, 500mg/kg, Envigo) \textit{ad libitum} for 14 days. Treatment with tamoxifen started at either 6- or 10-weeks, depending on treatment group (see Figure 9A-C). Following tamoxifen administration, nails were trimmed every two weeks on all mice tested to help prevent confounding factors of repetitive scratching behaviour. Mice did not display any health or behaviour changes while on the tamoxifen diet.

13  Behavioural Testing

F1 male and female (balanced n for sex in each behaviour) \textit{Grin1}^{\text{flneo/Cre}} mice were used for all behavioural testing. Testing was done at either 14-weeks (tamoxifen at 6- or 10-weeks) or 18-
Figure 8. Breeding strategy for inducible rescue of NMDA receptor deficiency in GluN1-IR mice. GluN1-IR +/- CRE-ERT2 mice, expressing the tamoxifen-inducible Cre-ERT2 fusion protein in all cells, will be bred to heterozygous GluN1-IR (GluN1-IR +/-) mice, to achieve inducible rescue of GluN1 expression. This breeding pair will yield the experimental animals required for further investigation; WT, WTCre, GluN1 and GluN1Cre. Controls for the presence of the Cre transgene and tamoxifen treatment will be in the WT and WTCre mice, with an expected phenotype of normal behaviour and normal NMDA receptor activity before and after administration of tamoxifen. GluN1 mice are expected to have decreased NMDAR levels before and after administration of tamoxifen due to a lack of the Cre transgene. GluN1Cre mice are expected to have reduced NMDAR levels prior to administration of tamoxifen, but restored (rescued) levels once the neo is removed via the induction of Cre-recombinase activity following tamoxifen administration.
weeks (tamoxifen at 10-weeks), see Figure 9A-C. WT mice not expressing the Cre gene, but treated with tamoxifen, were used as littermate controls. All behavioural tests were completed between 0900 and 1500h. All mice were tested for locomotor activity and stereotypic behaviour on the first day of behavioural assessment, a control for tamoxifen consumption. Mice were then assigned to one of two groups for subsequent behavioural tests that spanned three days (Figure 9D,E). Mice were assigned to one of two groups to ensure that all mice 1) were tested for the same total number of days, 2) sacrificed on the same day and, 3) were not tested for too long a period to minimize confounding stress effects. Days 2 through 4 saw group A tested in the puzzle box assay, while group B was tested in the elevated plus maze, social affiliative paradigm, and then pre-pulse inhibition.

13.1.1 Open Field Test

All experimental animals had locomotor activity, stereotypy, and center time measured on day 1 of behavioural testing, using digital activity monitors (Omnitech Electronics, Columbus, OH, USA), as previously described by Milenkovic et al. (2014). Naïve mice were placed in novel Plexiglas arenas (20 x 20 x 45 cm) and their locomotor and stereotypic activity were recorded over a 120-min period in dim light (15-16 lux). Activity was tracked via infrared light beam sensors; total distance traveled, stereotypic movements, and time spent in the center zone were collected in 5-min bins.

13.1.2 Puzzle Box Assay

For all intervention time points in group A, mice were run on the Puzzle Box Assay on days 2-4 of behavioural testing to assess cognition and executive function, adapted from Ben Abdallah et al. (2011), as previously described by (Milenkovic et al., 2014). Consisting of two compartments, the puzzle box contains a start area (58 x 28 x 27.5 cm) in bright light (250lux), and a goal zone (14 x 28 x 27.5 cm) in dim light (5lux). The two areas are separated by a black Plexiglas divider, but are connected via an underpass large enough for mice to pass through easily. Mice were placed in the start box facing away from the divider and the time to move to the goal zone (through the underpass, with both hind legs in the goal zone) was manually scored and recorded. Mice were tested over three days, 3 trials/day (except 1 trial on day 3), with each day consisting of increasingly difficult obstacles in the underpass connecting the start area and
the goal zone. 2-min were given between each trial on a given day, with a max 300-sec allowed for the completion of each trial.

The trials (and obstacles) for the puzzle box were as follows;

*Day 1:* T1 (training) open door and unblocked underpass, T2 and T3 (challenge, then learning) door closed and underpass open

*Day 2:* T4 (explicit memory) identical to T2 and T3, T5 and T6 (challenge, then learning) underpass filled with bedding, same as found in home cage

*Day 3:* T7 (explicit memory) identical to T5 and T6

### 13.1.3 Elevated Plus Maze

For group B, anxiety behaviour was assessed on day 2 of testing via elevated plus maze (Moy *et al.*, 2007). The elevated plus maze was composed of 4 opaque-white arms (2 opposite arms closed, 2 opposite arms open), arranged in a plus shape, with an open center. The dimensions were as follows; maze elevation (38.7cm), open arm (L:30.5cm, W:5cm, H:0cm), closed arm (L:30.5cm, W:5cm, H:15.2cm) and center (5cm x 5cm). The experimental mouse was placed in the center of the maze, and allowed to freely explore the maze for 8-min in dim light (15-16 lux), while being tracked with an overhead camera. Open and closed arm times were recorded and collected by Biobserve Viewer3 software. The percentage of time spent in the open arms as compared to closed arms and center time was calculated and expressed as a percent of time spent in the open arms of the maze. Elevated plus maze trials were conducted by Marija Milenkovic, a research associate in the Ramsey research group.

### 13.1.4 Social Affiliative Paradigm

For group B, social affiliative behaviour was assessed on day 3 of testing, as previously described by Mielnik *et al.* (2014) and Milenkovic *et al.* (2014). Sociability was measured via video recording motion and exploration of the experimental mouse tracked using Biobserve Viewer (version 2) software (center body – reference point). Experimental mice were allowed to explore the open area (opaque white walls, 62 x 42 x 22 cm) for 10-min in dim lighting (15-16 lux). The area contained two inverted wire cups, one containing a stimulus mouse (‘social’) and the other cup empty (‘non-social’). Time spent in each zone (3cm zone around the cup) was recorded via the Biobserve software. Mice used as a social stimulus were novel, wildtype, inbred C57Bl/6J mice that were age- and sex-matched to the test mouse.
Figure 9. Experimental outline for the characterization of behavioural, biochemical and molecular consequences of restored NMDA receptor signaling in mice with chronic developmental deficits in NMDA receptors. GluN1Cre mice with chronic, genetic disruption in NMDA receptor signaling will be rescued by tamoxifen induction of Cre-recombinase at two developmental stages; (A) adolescence (age 6-weeks) and (B and C) adulthood (age 10-weeks). Analysis of behavioural, biochemical and molecular phenotypes of these mice will be performed once they reach adult age at 14-weeks (A and B) or 18-weeks (C). (D&E) Schematic outlining the 5-day behavioural testing scheme. (D) Group A will complete the Open Field test and Puzzle Box, while, (E) Group B will complete the Open Field test, Elevated Plus Maze, Social Affiliative Paradigm and Pre-Pulse Inhibition.
13.1.5 Pre-Pulse Inhibition/Startle Reflex

For group B, pre-pulse inhibition (PPI) of the acoustic startle response was measured on day 4, via SR-LAB equipment and software from San Diego Instruments (San Diego, CA, USA). Accelerometers were calibrated to 700±5 mV and output voltages were amplified and analyzed for voltage changes using SR Analysis (San Diego Instruments), and exported as an excel file. Background white noise was maintained at 65dB. PPI was measured in a 30-min test with 80 randomized trials of: (1) 10 trials pulse alone (2) 10 trials pre-pulse alone (for each pre-pulse; 4dB, 8dB, and 16dB), (3) 10 trials pre-pulse plus pulse (for each pre-pulse; 4dB, 8dB, and 16dB)), and (4) 10 trials no pulse. 5 pulse alone trials were performed sequentially before and after the randomized 80 trials, totaling 90 trials per run. The pre-pulse (4dB, 8dB, or 16dB) was presented 100ms prior to the startle pulse (165dB). The inter-stimulus interval (ISI) was randomized between 5 and 20s. Experimental mice were placed in a cylindrical tube on a platform in a soundproof chamber. Mice were allowed to acclimatize in the chamber and to the background noise for 300s, followed by 5 consecutive pulse alone trials, then by 80 randomized trials (as previously described) and then 5 consecutive pulse alone trials. Pre-pulse inhibition was measured as a decrease in the amplitude of startle response to a 165dB acoustic startle pulse, following each pre-pulse (4dB, 8dB and 16dB). Pre-pulse inhibition trials were conducted by Rehnuma Islam, a graduate student in the Ramsey research group.

14 Harvesting Whole Brain Tissue

On Day 5, following all behavioural testing, mice were sacrificed via live cervical dislocation. Brains were removed and frozen in ice cold isopentane (on dry ice). Brains were then put into 5ml eppendorf tubes and stored at -80°C until further use.

15 Immunohistochemistry

15.1 Cre Reporter (ROSA26<sup>tdTomato</sup> x ROSA26<sup>CreERT2</sup>)

Mice were treated with tamoxifen for 2 weeks (as previously described) and anaesthetized (250mg/kg Avertin; 2,2,2-tribromoethanol (Sigma Aldrich) dissolved in 2-methyl-2-butanol (Sigma Aldrich), 2.5% v/v) 3 days following last day of treatment. Whole brains were perfused with 4% paraformaldehyde (PFA), post-fixed in PFA for 3 hours at 4°C and then transferred to 30% sucrose for 3 days at 4°C. Fixed brains were sectioned at 40µm coronal sections (Bregma
1.78mm, 1.10mm, -1.94mm, -6.00mm). Sections were mounted with Vectashield (with DAPI) and visualized using Nikon Elements software (NIS-Elements Basic Research, version 3.1).

16 Quantitative Polymerase Chain Reaction

16.1 Excision Levels of Insertion Mutation (neomycin)

Genomic DNA was isolated from tissue via isopropanol precipitation. Genomic DNA was further rid of contaminants using chloroform. The amount of insertion mutation present in the genome was assessed via quantitative PCR (Figure 14A). The insertion mutation, and its excision, were identified using the following primers; forward 5’-GTC TAG CTT GGC TGG ACG TAA-3’, reverse 5’-GAG GTT AAC CCC GAT CAT ATT CAA-3’. The reference gene used was for the transferrin receptor (TFRC); forward 5’-CCA GGT AAA GAC CTG CTT TGT A-3’, reverse 5’-CAA GCT AGC ATC CTT CTC CTT C-3’.

17 RNA Isolation, Sequencing and Analysis

17.1 RNA Isolation

Total RNA for RNA sequencing (RNAseq) was isolated from homogenized cortical tissue frozen in TriReagent (BioShop Canada); cortical tissue was used from male mice treated at 10-weeks with tamoxifen (see section 12: ‘Tamoxifen Administration’) and sacrificed at 14-weeks. Four mice were included for each genotype (WT, GluN1 and GluN1Cre). RNA was isolated with TriReagent (BioShop Canada) followed by purity verification via OD260/OD280 ratios taken by an Epoch Microplate Spectrophotometer (BioTek, VT, USA). 2-6µg of RNA was aliquoted for each sample and sent to The Centre for Applied Genomics (TCAG; Toronto, Canada) for RNAseq, RNA integrity analysis, and the generation of raw sequence reads using the Illumina HiSeq 2500 protocol (Illumina, CA, USA). Poly-A isolation, library generation, amplification and sequencing were performed by TCAG.

17.2 RNA Sequencing and Analysis

RNAseq data was analyzed with the tuxedo protocol, as originally described (Trapnell et al., 2012), using the main public online Galaxy platform (Afgan et al., 2016). Briefly, RNAseq reads for each sample were first quality checked with FastQC (version 0.65), followed by preparation for use on Galaxy with FASTQ Groomer (Version 1.0.4) and GRCh38/mm10 mouse genome
alignment with TopHat (Version 2.1.0). The aligned reads were then assembled into transcripts with Cufflinks (Version 2.2.1.0) using a mouse RefSeq annotation from the National Center for Biotechnology Information (NCBI) as an annotation guide. The transcripts assembled for each sample were then combined with Cuffmerge (Version 2.2.1.0). The resulting genome annotation was used to quantify and compare WT vs GluN1 vs GluN1Cre gene expression in Cuffdiff (Version 2.2.1.3) via three pairwise comparisons due to limitations of computing resources. Significance thresholds were set at p and q (false discovery rate) values of 0.05 and 0.1, respectively.

Significant gene differential comparison results from all three pairwise RNAseq runs were combined and aligned to match gene name, loci, and transcription start sites as much as possible. Unrecognized entries were checked again for an identifier using their loci with the Refseq database from the University of California, Santa Cruz genome table browser (Kent et al., 2002; Karolchik et al., 2004). Identified genetic elements were then put through ConsensusPathDB’s gene set over-representation analysis to determine enriched biological processes in level 5 gene ontology categories (Kamburov et al., 2011). Significantly enriched biological processes related specifically to the nervous system or cognitive behaviour were reported. The genes belonging to the neuron differentiation biological process were visualized using STRINGdb (Szklarczyk et al., 2015).

RNAseq analysis was conducted by Yuxiao Chen, a graduate student in the Ramsey research group.

18 Immunoblotting

Cortical and hippocampal tissue were dissected from brains frozen in cold isopentane (over dry ice, previously described in section 14). Tissue homogenates were prepared according to the previously described synaptoneurosome preparation (Li et al., 2010). Tissue was homogenized in homogenization solution (0.32M sucrose, 20mM HEPES pH 7.4, 1mM EDTA) for 30-sec with a hand-held motorized pestle. At 4°C, homogenate was spun at 1000G for 10-min, and then supernatant spun at 10000G 10-min Pellet was resuspended in lysis buffer (50mM Tris HCl pH7.5, 150mM NaCl, 1% Triton X-100, 0.1% SDS, 2mM EDTA). Protein concentration was measured using BCA assay (Thermo Scientific). 30µg of protein were resolved on 4% stacking and 8% separating gels and transferred to PVDF membranes (Pall Life Sciences, NY, USA) via 1 hour transfer at 100V. Total protein was stained for using REVERT™ Total Protein Stain Kit
Membranes were blocked in 5% milk in TBS-T (TBS + 0.1% (v/v) Tween-20) for 30-min and then incubated in primary antibodies (5% milk in TBS-T) overnight at 4°C. Primary antibodies for GluN1 (NR1) and GluN2A (NR1) proteins were as follows:

NR1 – 1:250, mouse IgG, Upstate (Millipore) Cat No: 05-432, Lot: 2538
NR2A – 1:1000, rabbit IgG, Upstate (Millipore) Cat No: 07-632, Lot: 32704

Blots were washed in TBS-T, incubated with anti-mouse IRDye 680 (1:10000, LI-COR) and anti-rabbit IRDye 800 (1:10000, LI-COR). Blots were visualized and densitometry was analyzed using the LI-COR Odyssey system and software.

19 [3H]MK-801 Saturation Binding Assay

Cortical, striatal, hippocampal and cerebellar tissue was dissected from whole frozen brains (see section 14). A sufficient amount of protein was needed for total radioligand binding (total 400µg of protein needed/brain region), each brain region was pooled from two separate animals (ex: 2 cortical regions from two separate mice were pooled for a single n, within each genotype).

To prepare membranes, tissue was homogenized in binding buffer (20mM HEPES pH 7.4, 1mM EDTA pH 8.0, 0.1mM glycine, 0.1mM glutamate, 0.1mM spermidine, Aprotinin (1000x), Leupeptin (1000x), Pepstatin A (500x), Benazamidine (1000x) and PMSF (2500x)) using a standing homogenizer and glass Teflon homogenizer tubes. Homogenate was transferred to 12ml round bottom tubes and further homogenized for 30-sec (Polytron, PT-1200-E). Homogenate was cleared via centrifugation at 600G in a Sorvall SM-24 rotor for 10-min at 4°C. Supernatant was transferred to thick walled Sorvall tubes and cleared via centrifugation at 40000G in a Sorvall SM-24 rotor for 15-min at 4°C. Pellet was washed with binding buffer (without protease inhibitors) and re-cleared at 40000G in Sorvall SM-24 rotor for 15-min at 4°C. Pellets were resuspended in binding buffer (without protease inhibitors). Protein concentration was measured using BCA assay (Thermo Scientific). Membranes were diluted to a 1.6µg/µl working concentration and stored at -80°C.

For the [3H]MK-801 saturation protein binding assay, working solutions of [3H]MK-801 and cold MK-801 were prepared. [3H]MK-801 (Perkin Elmer) was diluted to a working concentration of 120nM (final concentration 40nM) in binding buffer. Cold MK-801 (Sigma...
Aldrich) was prepared to a 1200nM working solution (final concentration 400nM; 10x [³H]MK-801) in binding buffer. Binding assays were performed with the NMDA receptor antagonist MK-801 (50µl hot and/or cold), mouse brain membranes (80µg in 50 µl) and binding buffer (50µl; total binding vs. non-specific binding), with a final assay volume of 150µl. Assays were carried out at 32°C for 3 hours in a shaking water bath before termination via the addition of ice-cold wash buffer (20mM HEPES pH 7.4, 1mM EDTA pH 8.0). Vacuum filtration was completed using a 24-well sampling manifold (Brandel Cell Harvester) and Whatman GF/B glass-fibre filters (Brandel, MD, USA) that had been soaked in 0.05% polyethylenimine for 30-min. Each reaction was washed 5 times with wash buffer. Filters were placed in 5mL scintillation fluid (Ultima Gold XR, Perkin Elmer) and allowed to incubate overnight. Radioactivity was quantified via liquid scintillation spectrometry.

20 Quantification and Statistical Analysis

Statistical parameters, including the exact value of n, the definition of measures and statistical significance are reported in the figures and the figure legends. Data are represented as mean ± SEM, as indicated in figure legends. Sample number (n), indicating independent biological samples (balanced for sex), are indicated in each figure and figure legend. Data were analyzed either using a one-way or two-way ANOVA (repeated measures) where indicated, with multiple comparisons and post-hoc Bonferroni’s test, unless otherwise specified. Data analysis was not blinded. Differences in means were considered statistically significant at p<0.05. Significance levels are as follows; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns – not significant. All data analyses were performed using the Graphpad Prism 6.0 software and/or IBM SPSS 23.0 Software.
Chapter 3
RESULTS

21 Generation of the GluN1-IR mouse line

21.1 GluN1-IR targeting construct generation and validation

To investigate whether the brain is capable of “rewiring” circuits disrupted due to neurodevelopmental deficits, the generation of a new mouse line was required. Building on the already established GluN1-KD mouse model (Mohn et al., 1999), the GluN1-IR mouse line was generated to allow for inducible rescue of developmental hypofunction of the NMDA receptor. The GluN1-IR construct was generated and sequenced fully (Figure 7), with the insertion mutation (neomycin cassette) flanked by lox sites. With sequential Grin1 genetic material (intron 19) ligated into the vector on opposing sides of the neomycin cassette, this allowed for homologous recombination of the construct, with inserted foreign DNA (neo), to take place at intron 19 of the Grin1 gene. This construct allowed for the close mimic of the previously generated GluN1-KD mouse model, where insertion of additional foreign DNA (neo) into intron 19 lead to a reduction in full-length mRNA levels (Figure 6).

Electroporation of R1 ES cells, 129 background, with the GluN1-IR construct was completed by Toronto Centre for Phenogenomics (TCP) to generate a knock-in ES cell line. Taking advantage of markers present in the GluN1-IR construct, ES cell lines underwent positive selection using G418. Colonies were picked and expanded. To validate that the insertion mutation was targeted to the correct genomic location of intron 19 in the Grin1 gene, ES cell clones were screened using a PCR strategy (Figure 10). 303 ES cell clones were screened via PCR, and 47 ES cell clones showed targeting events (targeting incidence 1:6). Further confirmation of targeted ES cell lines was achieved via Southern Blot analysis (Figure 11). Taking advantage of a unique HindIII digestion pattern, homologous recombination yielded a 13kb fragment, while the wildtype allele yielded an 11kb probed fragment. Targeted lines were submitted to the TCP for the generation of chimeras; 6 targeted lines were identified, 4 were confirmed via Southern blot, and 2 targeted lines were submitted for the generation of chimeras.
Figure 10. **Screening for gene targeting event in ES Cells (PCR strategy).** (A) Schematic illustrating the PCR (genotyping) strategy for the identification of targeting events in ES cells. The endogenous locus (in grey) is modified through homologous recombination with the GluN1-IR construct (blue) to produce a targeted locus with the introduction of foreign DNA (neo in green). The schematic highlights the location of PCR primers used to amplify the endogenous locus (orange primers; WT forward and common reverse) and the targeted locus (green arrow, mutant forward; orange arrow, common reverse). All samples were run in two separate reactions; GluN1-IR PCR and wildtype PCR. Both reactions yield PCR products of the same size (~2kb) to control for PCR conditions along with DNA quality. (B) Examples of targeted events in ES cells as seen with PCR strategy (on agarose gel). Lanes 2 and 4 represent targeted ES cell lines, while lanes 1 and 3 are not targeted.
Figure 11. Screening for gene targeting in ES Cells (Southern strategy). (A) Schematic illustrating the Southern strategy for the identification of targeting events in ES cells. The endogenous locus is modified through homologous recombination with the GluN1-IR construct (as previously described). The schematic highlights the location of the 1kb probe (red) just downstream of the inserted DNA (blue) used to visualize the digested fragments (with HindIII). (B) Targeted events in ES cell lines as seen with Southern strategy. Four targeted ES cell lines confirmed; IVB4, IIID5, VE2 and IVE7. Wildtype (not targeted) ES cell lines yield 11kb fragments, while targeted ES cell lines yield 13kb and 11kb fragments (heterozygous for targeted allele).
22 GluN1-IR mouse line generated

Eleven male chimeras were created that had >25% ES cell coat colour (agouti), with coat colour used as a subjective reflection of overall chimerism. All chimeras were bred with CD1 females to determine whether the targeted ES cells had contributed to germ cells in the testes. Two chimeras (87a and 88b) displayed germline transmission that was evident by the eye colour of progeny (Figure 12). Germ line transmission was determined initially by eye colour (black), and subsequently confirmed through PCR genotyping. The resulting heterozygous mice were used to generate inbred mouse lines on the 129/Sv and C57Bl/6J genetic backgrounds as described in sections 10.4 and 11.1.3.

23 Tamoxifen robustly induces Cre-recombinase activity in the GluN1 rescue mouse line (Grin1flneo/Cre)

23.1 Tamoxifen dosing regimen was optimized to ensure robust induction of Cre-recombinase activity

To ensure robust induction of Cre-recombinase activity (ROSA26CreERT2; Cre-recombinase), a thorough optimization of dosing and regimen was performed. Both behavioural and biochemical outputs were quantified. This optimization is summarized in Appendix A. We determined that a tamoxifen regimen combining both oral gavage and a high-dose tamoxifen diet (see section 12 for detailed regimen) would result in the greatest Cre-recombinase induction, and mitigate the confounds of stress induced by the route of administration and drug. Further confirmation of robust Cre-recombinase induction was completed using a Cre reporter line (ROSA26tdTomato). The Cre reporter line has a loxP-flanked STOP cassette that prevents the transcription of tdTomato (red fluorescent protein variant). Once bred to Cre-recombinase expressing mice, the STOP cassette can be deleted in the Cre-recombinase expressing tissue of the offspring. This results in the expression of tdTomato, visualized as red fluorescent staining.

We observed that following administration of tamoxifen, there was robust, global induction of Cre-recombinase in the mouse brain; including the cortex, striatum, hippocampus and cerebellum (Figure 13). The tight regulation of the inducible Cre-recombinase under the ROSA26 promotor is evident when comparing animals treated with tamoxifen (right panels; Figure 13).
Figure 12. **Generation of GluN1-IR germline transmitted mouse line via diploid aggregation.** 11 chimeras (male) were created that had >25% ES cell coat colour (brown). (A) Diploid aggregation of GluN1-IR ES cells into pseudo pregnant CD1 female mice was completed by Toronto Centre for Phenogenomics (TCP). (B) Chimera 88b and chimera 87a germline transmitted to allow for generation of the GluN1-IR mouse colony. (C) Example of germline transmitted F1 progeny.
Figure 13. **Tamoxifen administration leads to robust and tightly regulated induction of Cre-recombinase.** Following oral administration of tamoxifen (combination gavage and chow; see Methods), there is robust, global induction of Cre-recombinase in the mouse brain; including cortex, striatum, hippocampus, and cerebellum. Following administration of vehicle (corn oil), there is no induction of Cre-recombinase. Cre-recombinase induction was visualized using ROSA26<sup>tdTomato</sup> reporter mouse line from Jackson Laboratory (B6.Cg-Gt(Rosa)26Sortm14(CAG-tdTomato)Hze/J) and counter-stained with DAPI.
and those treated with vehicle (corn oil) (left panels; Figure 13). DAPI was used as a counter-stain to allow for the visualization of brain region-specific anatomical structure (identification).

24 Excision of the insertion mutation at the genomic level is not dependent on time of intervention, but rather on brain region

To measure the rate of excision of the insertion mutation at the genomic level, a qPCR strategy was designed (Figure 14A). By designing primers that span the junction between the insertion mutation and the genomic DNA of the Grin1 gene, the excision rate could be measured by the loss of the amplification product. The number of amplicons from GluN1Cre mouse DNA was compared to that of GluN1 mice, which have two copies of the targeting sequence (one per allele), and WT mice, which have zero copies of the targeting sequence. This allowed for an estimation of the number of copies of the targeting sequence remaining in GluN1Cre tissue that did not undergo excision by Cre-recombinase. GluN1Cre mice showed similar excision of the insertion mutation across both intervention time points (adolescent and adult) (Figure 14B-F). However, excision levels differed based on brain region. The cortex and hippocampus showed the highest levels of excision rates at 25% (Figure 14B,D). The striatum showed less excision at 15% (Figure 14C), while the cerebellum showed no excision following tamoxifen treatment at either adolescent or adult age (Figure 14E). Levels of excision were measured in the peripheral tissue (ear tissue) as a control, to determine whether the limited excision observed in brain tissue was in fact tissue specific, or mechanism dependent. GluN1Cre mice showed 75% excision of the neomycin cassette in peripheral tissue (Figure 14F). Therefore, it is evident that Cre-mediated excision, specifically at the Grin1 locus, is dependent on brain region, and not developmental age. WT mice showed no copies of the insertion mutation (as expected), while data was normalized to 2 copies of the insertion mutation in the GluN1 mice.

25 Restoration of functional NMDA receptors, is variable, dependent on brain region, and intermediate in nature

A sensitive measure of NMDA receptor rescue at the protein level was required, and to quantify biochemical rescue, [³H]MK-801 saturation binding was used. For MK-801 to bind, a fully
Figure 14. **Excision of the insertion mutation (neomycin cassette) from genomic DNA is not dependent on time of intervention, but rather is brain region specific.** Excision rate is measured via qPCR of genomic DNA from cortex, hippocampus, striatum, cerebellum, and peripheral tissue from male WT, GluN1, and GluN1Cre mice. GluN1Cre mice show no difference in excision rates when comparing between adolescent (6-weeks) and adult (10-weeks) rescue. Difference in excision rates is seen between brain regions; cortex and hippocampus 25%, striatum 15%, cerebellum 0%, and peripheral tissue 75%. Data shown as mean ± SEM. N values are as indicated in the figure for all brain regions and peripheral genomic DNA; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test.
assembled NMDA receptor is required; a complete tetramer is formed from GluN1 and GluN2 subunits (Kovacic and Somanathan, 2010). Therefore, it is reasonable to infer that through the binding of $[^3]$HMK-801, the levels of assembled, fully functional NMDA receptors can be quantified and indirectly estimate GluN1 subunit levels. To quantify NMDA receptor levels in the WT, GluN1, and GluN1Cre mice, membranes were generated from four brain regions; cortex, striatum, hippocampus, and cerebellum. Saturation binding was completed with balanced male and female samples. Saturation binding showed no effect of the presence of Cre-recombinase on levels of NMDA receptor (WT vs. WTCre; Table I).

GluN1 mice showed a marked decrease in NMDA receptor levels in all brain regions tested, similar to what was previously shown (Mohn et al., 1999). Along similar lines to the physiological variability seen in NMDA receptor expression levels across the brain, knockdown levels of the NMDA receptor also varied between brain regions in the GluN1 mice (Table II); the hippocampus saw the greatest decrease with NMDA receptor expression levels at 10-15% of WT, the cortex saw a decrease to 23%, with the striatum at 28-31%, and then cerebellum at 31-40%. The decrease in NMDA receptor levels was varied between brain regions, but was consistent between intervention groups and ages (Table II; no effect of intervention time on knockdown).

Following excision of the insertion mutation, NMDA receptor levels increased (represented by increased $[^3]$HMK-801 binding) in the GluN1Cre mice. Similar to the pattern and brain region-specific nature of the decrease in NMDA receptor expression levels seen in the GluN1 mice, restoration of NMDA receptor levels in the GluN1Cre mice followed a brain-region specific expression pattern (Table II). GluN1Cre mice saw an increase in NMDA receptor expression as follows: the cortex had an increase in NMDA receptor levels to 56-64% of WT levels, the hippocampus to 48-49% of WT, striatum at 41-50%, and cerebellum at 49%. Rescue of NMDA receptor levels varied across brain regions, but was consistent across intervention groups. The biochemical rescue of NMDA receptor expression levels, across all intervention time points and all brain regions, was intermediate in nature, and never reached WT levels. Therefore, the restoration of NMDA receptors, and subsequent glutamate signaling, shows limited intrinsic ability to change, exhibiting only an intermediate level of rescue in terms of NMDA receptor expression. This intermediate rescue is both independent of age of intervention or length of recovery.
Table I. NMDA receptor binding densities of \([3H]MK-801\) (fmol/mg) in brain tissue of WT and WTCre mice.

<table>
<thead>
<tr>
<th>Intervention Time Point</th>
<th>Cortex</th>
<th></th>
<th></th>
<th>Striatum</th>
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<th></th>
<th>Hippocampus</th>
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<tr>
<td></td>
<td>1756.0 ± 115.1</td>
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<td>1679.0 ± 137.2</td>
<td>1708.0 ± 102.9</td>
<td>1380.0 ± 71.1</td>
<td>1477.0 ± 71.1</td>
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<td>904.4 ± 25.3</td>
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<td></td>
<td>1698.0 ± 164.9</td>
<td>1868.0 ± 105.9</td>
<td>2051.0 ± 200.0</td>
<td>1945.0 ± 169.3</td>
<td>1794.0 ± 100.0</td>
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<td>224.0 ± 12.8</td>
<td>203.6 ± 11.8</td>
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</table>

Data expressed as mean ± SEM in fmol/mg of protein. N=5-6/group, combined and equally balanced for sex.

Table II. NMDA receptor binding densities of \([3H]MK-801\) (fmol/mg) in brain tissue of WT, GluN1 and GluN1Cre mice.

<table>
<thead>
<tr>
<th>Intervention Time Point</th>
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<th></th>
<th>Striatum</th>
<th></th>
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<td>Adolesc. + 8weeks</td>
<td>Adult + 4weeks</td>
<td>Adult + 8weeks</td>
<td>Adolesc. + 8weeks</td>
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<tr>
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<td>119.7</td>
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<td>&lt;0.001</td>
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</table>

Data expressed as mean ± SEM in fmol/mg of protein. N=5-6/group, combined and equally balanced for sex. Multiple comparisons two-way ANOVA with Bonferroni’s post-hoc. Red represents greatest decrease, and green represents greatest increase.
26 Restoration of the GluN1 protein is dependent on brain region, and is intermediate in nature.

Targeted insertion of the neomycin cassette into intron 19 of the Grin1 gene leads to decreased levels of GluN1 protein (Figure 15). Protein levels of the GluN1 protein, along with its heterotetramer-forming GluN2A subunit, were measured in the cortex and hippocampus via immunoblotting. It was confirmed that GluN1 mice express 10% or less of WT levels of the GluN1 subunit protein in both the cortex (Figure 15A) and the hippocampus (Figure 15B). Following excision of the insertion mutation, GluN1 protein levels were rescued to 40% of WT in the cortex and 20% of WT in the hippocampus. The same rescue trends were true with GluN2A levels; GluN2A subunit protein levels were 20% of WT levels in GluN1 mice in both the cortex and the hippocampus, with rescued expression levels of 60% and 40% of WT in the cortex and hippocampus of GluN1Cre mice respectively (Figure 15). Protein levels of the GluN1 subunit in the striatum and cerebellum (not shown) were too low for immunoblot detection. Immunoblot samples were prepared from the adult+4 weeks intervention group as radioligand binding did not show an effect of intervention or recovery time. Samples were balanced for male and female mice.

27 Behaviours that show limited plasticity and rescue, independent of age or recovery time

A developmental disruption in glutamate signaling via knockdown of the GluN1 subunit of the NDMA receptor leads to a robust behavioural phenotype, in this study, as well as previously described (Mohn et al., 1999; Duncan et al., 2004; Moy et al., 2006; Halene et al., 2009; Dzirasa et al., 2009; Moy et al., 2012; Gandal, Sisti, et al., 2012; Milenkovic et al., 2014; Ferris et al., 2014; Mielnik et al., 2014; Grannan et al., 2016). We saw that in GluN1 mice, there was an increase in locomotion (Figure 16) and stereotypy (Figure 17), while memory, problem-solving (Figure 18), and anxiety-like behaviours were decreased (Figures 19 and 20) when compared to WT littermates. All behaviours were assessed with a balanced number of male and female mice.
**Figure 15. GluN1 and GluN2A subunit protein levels are decreased in GluN1 mice, increase in GluN1Cre mice following tamoxifen administration.** Immunoblot of 30µg crude synaptoneurosomal protein extract from (A) cortex and (B) hippocampus in male and female WT, GluN1, and GluN1Cre mice aged 14-weeks (tamoxifen intervention at 10-weeks). GluN1 mice show a marked decrease in both brain regions, demonstrating that the insertion event in the *Grin1* gene causes a reduction in the level of GluN1 protein expressed, and subsequently a decrease in GluN2A levels as well (accompanying subunit to form functional NMDA receptor). Glun1Cre mice show an increase in GluN1 and GluN2A subunit protein levels following tamoxifen. Total protein stain was used as loading control. Data shown as mean ± SEM. N values are as indicated in the figure for all brain regions; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, cortex - multiple comparisons one-way ANOVA with post-hoc Tukey’s test; hippocampus - Student’s t-test between GluN1 and GluN1Cre due to high WT variability.
27.1 Hyperlocomotion seen in GluN1 is rescued to intermediate levels in GluN1Cre when compared to WT

Following treatment with tamoxifen, at either adolescence or adulthood, we saw that GluN1Cre mice showed similar levels of change in their locomotor behaviour, following similar directional trends. Specifically, GluN1Cre mice showed that the previously observed hyperlocomotion in the GluN1 mice was ameliorated to an intermediate level of “rescue” following excision of the insertion mutation (Figure 16A,B). There was an effect of genotype across all intervention groups: adolescent+8 weeks – $F[3,160]=227.955$, $p<0.001$; adult+4 weeks – $F[3,164]=176.340$, $p<0.001$; adult+8 weeks – $F[3,168]=339.428$, $p<0.001$. As mice aged (adult + 8 week recovery group), an effect of sex was observed ($F[1,168]=7.063$, $p=0.009$, Figure 16C), but the intermediate nature of rescue in the GluN1Cre mice remained unchanged. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on locomotor behaviour (Figure 16D).

27.2 Increased stereotypic behaviour seen in GluN1 is rescued to intermediate levels in GluN1Cre when compared to WT

Following treatment with tamoxifen, at either adolescence or adulthood, we saw that GluN1Cre mice showed similar levels of change in their stereotypy, following similar directional trends. GluN1Cre mice showed a decrease in repetitive, stereotypic movements when compared to GluN1 mice, but were still at a higher level than those seen in WT mice (Figure 17A). There was an effect of genotype across all intervention groups: adolescent+8 weeks – $F[3,160]=459.259$, $p<0.001$; adult+4 weeks – $F[3,164]=301.851$, $p<0.001$; adult+8 weeks – $F[3,168]=537.974$, $p<0.001$. In the adult+4 weeks recovery treatment group, there was an effect of sex on stereotypy ($F[1,164]=5.747$, $p=0.018$, Figure 17B), however, it is important to note that the intermediate rescue effect remained unchanged. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on stereotypic behaviour (Figure 17C). Most notably, the intermediate rescue observed in locomotion and stereotypic movements in the GluN1Cre mice was similar across all intervention time points, indicating similar levels of plasticity in these behaviours at either adolescence or adulthood.
Hyperlocomotor behaviour is rescued to intermediate levels in GluN1Cre mice when compared to GluN1 and WT mice, regardless of time of intervention. (A) Time course of distance traveled (cm) over 120-mins in 5-min intervals and (B) total distance traveled by male and female mice with adolescent or adult intervention. GluN1Cre mice show no difference in rescue when comparing across intervention timepoints. There is an effect of genotype across all intervention timepoints. (C) There is an effect of sex in the adult+8wks group only; p=0.009. There is no interaction between genotype and sex. (D) Time course and total distance traveled (cm) over 120-mins in WT and WTCre mice (male and female). There is no difference in locomotor activity in WTCre mice when compared to WT. Data shown as mean ± SEM. ****p<0.0001, multiple comparisons two-way (time course) and one-way (total distance) ANOVA, with post-hoc Bonferroni’s test.

(Adolescent+8wks): Male – WT=22, WTCre=24, GluN1=17, GluN1Cre=23 / Female – WT=23, WTCre=16, GluN1=21, GluN1Cre=22. (Adult+4wks): Male – WT=20, WTCre=23, GluN1=22, GluN1Cre=21 / Female – WT=23, WTCre=20, GluN1=24, GluN1Cre=19. (Adult+8wks): Male – WT=24, WTCre=23, GluN1=20, GluN1Cre=19 / Female – WT=23, WTCre=23, GluN1=21, GluN1Cre=23.
Figure 17. Increased stereotypic behaviour seen in GluN1 mice is intermediately rescued in GluN1Cre mice, regardless of time of intervention. (A) Total stereotypic movements over 120-mins in an open-field environment in male and female mice with adolescent or adult intervention. GluN1Cre mice show no difference in rescue (intermediate) when comparing across intervention timepoints. There is an effect of genotype across all intervention timepoints. (B) There is an effect of sex in the adult+4wks group only; p=0.018. There is no interaction between genotype and sex. (C) Total stereotypic movements in WT and WTCre mice (male and female). There is no difference in stereotypic behaviour in WTCre mice when compared to WT. Data shown as mean ± SEM. ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test. (Adolescent+8wks): Male – WT=22, WTCre=24, GluN1=17, GluN1Cre=23 / Female – WT=23, WTCre=16, GluN1=21, GluN1Cre=22. (Adult+4wks): Male – WT=20, WTCre=23, GluN1=22, GluN1Cre=21 / Female – WT=23, WTCre=20, GluN1=24, GluN1Cre=19. (Adult+8wks): Male – WT=24, WTCre=23, GluN1=20, GluN1Cre=19 / Female – WT=23, WTCre=23, GluN1=21, GluN1Cre=23.
27.3 Impaired executive function seen in GluN1 is rescued to intermediate levels in GluN1Cre when compared to WT

In terms of cognitive flexibility and executive function, the GluN1Cre mice showed an intermediate rescue of behaviour as well. The GluN1 mice showed a strong and robust impairment in the ability to cognitively overcome the obstacle presented to them in the puzzle box. GluN1 mice failed to complete the task of moving from the start area to the goal zone within a 300-sec maximum. However, GluN1Cre mice showed a marked improvement in executive function, manifesting in the ability to complete the task, faster than the GluN1 mice, but still not as quickly as WT littermates (Figure 18A). There was an effect of genotype across all intervention groups: adolescent+8 weeks – F[3,84]=57.829, p<0.001; adult+4 weeks – F[3,82]=53.634, p<0.001; adult+8 weeks – F[3,81]=56.479, p<0.001.

Sex played a strong factor in this behaviour, with females generally performing better than males (faster completion of task, shorter latency time). There was an effect of sex in mice rescued in adolescence (F[1,84]=7.411, p=0.008) and an interaction of sex and genotype in mice rescued in adulthood (adult+4 week: F[3,82]=2.941, p=0.038; adult+8 week: F[3,81]=10.087, p<0.001) (Figure 18B). Specifically, this study showed that females, across all genotypes, perform better in the puzzle box test over males when intervened in adolescence, and that females have a better rescue over males when intervened in adulthood. However, it is important to note that, overall, the rescue of behaviour remained intermediate in nature and never reached WT levels. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on executive function (Figure 18C). In brief, the intermediate phenotype of the GluN1Cre mice was consistent across all three intervention groups, further demonstrating that plasticity in the glutamate system at the NMDA receptor level may not be dependent on age, but specific to certain behaviours.

27.4 Decreased anxiety levels seen in GluN1 are rescued to intermediate levels in GluN1Cre when compared to WT

GluN1Cre mice showed an intermediate phenotype in anxiety-like behaviours, as tested in the elevated plus maze. Anxiety was measured by the amount of time spent exploring open arms vs. closed arms in the elevated plus maze; more time spent in the open arms over the closed arms is indicative of decreased anxiety levels, with the opposite being true for time spent in the closed arms over the open arms (Lister, 1987). Where WT mice spent almost all their time in the closed
A Puzzle Box Assay (total)

<table>
<thead>
<tr>
<th>ADOLESCENT + 8wks recovery</th>
<th>ADULT + 4wks recovery</th>
<th>ADULT + 8wks recovery</th>
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<tbody>
<tr>
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<tr>
<td>Grln1 (n=11)</td>
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B Puzzle Box (male vs. female)

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<tbody>
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MALE

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FEMALE

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C Puzzle Box (WT vs. WTCre)

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</tr>
<tr>
<td>WTCre (n=23)</td>
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</table>
Executive function deficits seen in GluN1 mice are intermediately ameliorated in GluN1Cre mice, with a greater rescue in female GluN1Cre mice following adult intervention. (A) Latency (s) to complete trials in puzzle box, male and female mice with adolescent or adult intervention. GluN1Cre mice show overall amelioration in executive function when comparing across intervention timepoints. There is an effect of genotype across all intervention timepoints. (B) There is an effect of sex in adolescent rescue; adolescent+8wks - p=0.011. There is an interaction between genotype and sex in adult rescue; adult+4wks – p=0.038, adult+8wks – p<0.001. (C) Latency (s) to complete trials in puzzle box in WT and WTCre mice (male and female). There is no difference in latency to goal zone in WTCre mice when compared to WT. Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001, multiple comparisons two-way ANOVA with post-hoc Bonferroni’s test. (Adolescent+8wks): Male – WT=12, WTCre=13, GluN1=10, GluN1Cre=13 / Female – WT=13, WTCre=10, GluN1=11, GluN1Cre=10. (Adult+4wks): Male – WT=10, WTCre=13, GluN1=12, GluN1Cre=11 / Female – WT=11, WTCre=10, GluN1=13, GluN1Cre=10. (Adult+8wks): Male – WT=11, WTCre=13, GluN1=10, GluN1Cre=10 / Female – WT=10, WTCre=12, GluN1=11, GluN1Cre=12.
arms, GluN1 mice behaved completely opposite and spent all their time in the open arms (Figure 19A,B). The GluN1Cre mice showed an intermediate phenotype, spending almost equal amounts of time in the open and closed arms, across all intervention time points (Figure 19A,B). There was an effect of genotype across all intervention groups: adolescent+8 weeks – $F[3,78]=207.461$, $p<0.001$; adult+4 weeks – $F[3,75]=252.955$, $p<0.001$; adult+8 weeks – $F[3,80]=186.135$, $p<0.001$.

There was an effect of sex in the adolescent intervention group ($F[1,78]=4.358$, $p=0.040$), and an interaction of sex and genotype in the adult+4 week intervention group ($F[3,75]=0.006$, $p=0.006$) (Figure 19C). Importantly, the intermediate nature of the rescue seen in the GluN1Cre across all time points remained the same, regardless of any differences due to sex. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on anxiety-like behaviour (Figure 19D). Therefore, where the GluN1 mice showed robustly decreased levels of anxiety-like behaviour, the GluN1Cre mice showed an intermediate phenotype: higher anxiety levels than that of the GluN1, but lower than the WT.

These observations were further confirmed in a second measure of anxiety-like behaviour: time spent in center zone of the open field test. Increased time spent in the center zone is representative of decreased levels of anxiety behaviour. The GluN1 mice showed an increase in time spent in the center zone, displaying decreased anxiety-like behaviours, when compared to WT littermates. This is similar to that seen in the elevated plus maze. Following adult intervention (+4weeks and +8weeks recovery), the GluN1Cre mice showed intermediate rescue of decreased anxiety-like behaviours, but no rescue of behaviour following adolescent intervention (+8weeks recovery) (Figure 20A). There was an effect of genotype across intervention groups: adolescent+8 weeks – $F[3,160]=125.197$, $p<0.001$; adult+4 weeks – $F[3,164]=114.918$, $p<0.001$; adult+8 weeks – $F[3,168]=110.044$, $p<0.001$.

There was an effect of sex across all intervention groups: adolescent+8 weeks – $F[1,160]=12.380$, $p=0.001$; adult+4 weeks – $F[1,164]=15.308$, $p<0.001$; adult+8 weeks – $F[1,168]=18.685$, $p<0.001$. There was an interaction between sex and genotype in the adult+4weeks intervention group ($F[3,164]=3.426$, $p=0.019$) (Figure 20B). Overall, it seems that females spent less time in the center zone when compared to males, regardless of genotype. Furthermore, in the adult+4 weeks recovery group, females showed a greater rescue of this
Figure 19. Anxiety-like behaviour deficits seen in GluN1 mice are intermediately ameliorated in GluN1Cre mice, regardless of time of intervention. (A) Representative traces of elevated plus maze (EPM) from WT, GluN1 and GluN1Cre mice. (B) Percent-time spent in open arms of EPM over 8-min, male and female mice. GluN1Cre mice show intermediate amelioration when comparing across all interventions. Effect of genotype across all interventions. (C) Effect of sex adolescent intervention; p=0.04. Interaction between genotype and sex in adult+4wk intervention; p=0.006. (D) There is no difference in percent-time spent in open arms in WTCre mice when compared to WT. Data shown as mean ± SEM, ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test. (Adolescent+8wks): Male – WT=10, WTCre=11, GluN1=11, GluN1Cre=10 / Female – WT=11, WTCre=11, GluN1=10, GluN1Cre=12. (Adult+4wks): Male – WT=10, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=12, WTCre=10, GluN1=11, GluN1Cre=12. (Adult+8wks): Male – WT=13, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=13, WTCre=11, GluN1=10, GluN1Cre=11.
Figure 20. Increased time spent in center zone seen in GluN1 mice is intermediately ameliorated in adult (10-week) rescue of GluN1Cre mice, but not in adolescent (6-week) rescue. (A) Total center time over 120-mins in open-field environment, male and female mice with adolescent or adult intervention. GluN1Cre show intermediate rescue in adult (10-week) intervention time, with no rescue in the adolescent intervention. There is an effect of genotype in all interventions. (B) There is an effect of sex in all interventions; adolescent+8wks group (p<0.001), adult+4wks (p<0.001), and adult+8wks group (p<0.001). There is an interaction between genotype and sex in the adult+4wks intervention timepoint only; p=0.019. (C) Center time in WT and WTCre mice (male and female). Data shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test. (Adolescent+8wks): Male – WT=22, WTCre=24, GluN1=17, GluN1Cre=23 / Female – WT=23, WTCre=16, GluN1=21, GluN1Cre=22. (Adult+4wks): Male – WT=20, WTCre=23, GluN1=22, GluN1Cre=21 / Female – WT=23, WTCre=20, GluN1=24, GluN1Cre=19. (Adult+8wks): Male – WT=24, WTCre=23, GluN1=20, GluN1Cre=19 / Female – WT=23, WTCre=23, GluN1=21, GluN1Cre=23.
behaviour, back to WT levels, when compared to males. However, this sexual dimorphism evens
out to similar levels of rescue across sexes as the mice continue to age (adult+8 weeks recovery).
Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on time
spent in the center zone (Figure 20C). Therefore, where the GluN1 mice show robustly decreased
levels of anxiety-like behaviour, the GluN1Cre mice show an overall intermediate phenotype:
higher levels of anxiety than that of GluN1, but lower than the WT littermates. This data
indicates that the plasticity in glutamate signaling underlying anxiety-like behaviours may be
limited in nature, but not completely static, and independent of developmental time overall.

28 Behaviours that show full plasticity and rescue, independent of age or recovery time

While some behaviours showed limited plasticity following excision of the insertion mutation,
other behaviours looked to be highly plastic in nature, restoring back to WT levels. GluN1 mice
showed a deficit in social affiliative behaviour (Figure 21) and sensorimotor gating, that
manifested as a decrease in pre-pulse inhibition (Figure 22) when compared to WT littermates.
All behaviours were assessed with a balanced number of male and female mice.

28.1 Decreased sociability behaviour seen in GluN1 is rescued completely in GluN1Cre when compared to WT

To assess social affiliative behaviour, mice were tested in the social affiliative paradigm, a
modified version of the three-chamber social interaction test, as previously described (Mielnik et
al., 2014; Milenkovic et al., 2014). Time spent interacting with the novel social mouse, chosen
over interaction with an empty cup, was measured and scored via an overhead camera tracking
system. While GluN1 mice showed a marked decrease in social affiliative behaviour, GluN1Cre
mice showed a higher level of time spent investigating the novel mouse, with interaction levels
similar to those demonstrated by WT mice (Figure 21A,B). GluN1Cre mice showed similar
levels of change in their social behaviour following excision of the insertion mutation, regardless
of intervention in either adolescence or adulthood, or length of recovery time, 4- or 8-weeks.
GluN1Cre mice showed a complete rescue of social affiliative behaviour, with exploration
patterns less erratic than GluN1 mice and more focused around time spent in the social zone
A Representative Social Traces

B Social Behaviour - time spent in zone

C Social Behaviour - time spent in zone (WT vs. WTCre)

Figure 21. Social deficits observed in GluN1 mice are fully rescued in GluN1Cre mice, back to WT levels, regardless of time of intervention. (A) Representative traces of modified three-chamber sociability assay from WT, GluN1 and GluN1Cre mice. (B) Time (s) spent in social and non-social zones in modified three chamber behaviour paradigm over 10-min, in male and female mice. GluN1Cre mice show complete rescue to WT levels of sociability when comparing across interventions and in both sexes. There is an effect of genotype, but not sex, across all interventions. There is no interaction between genotype and sex across all interventions. (C) There is no difference in amount of time spent in either the social or non-social zone in WTCre mice when compared to WT. Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test. (Adolescent+8wks): Male – WT=10, WTCre=11, GluN1=11, GluN1Cre=10 / Female – WT=11, WTCre=11, GluN1=10, GluN1Cre=12. (Adult+4wks): Male – WT=10, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=12, WTCre=10, GluN1=11, GluN1Cre=12. (Adult+8wks): Male – WT=13, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=13, WTCre=11, GluN1=10, GluN1Cre=11.
investigating the novel mouse, like WT (Figure 21A). Furthermore, the time spent in the social zone of the GluN1Cre mice was returned to WT levels (Figure 21B). There was an effect of genotype across all intervention groups: adolescent+8 weeks – $F[3,78]=6.994, p<0.001$; adult+4 weeks – $F[3,75]=7.140, p<0.001$; adult+8 weeks – $F[3,80]=8.824, p<0.001$. There was no effect of sex, nor any interaction of sex and genotype in social affiliative behaviour across any of the treatment groups. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on time spent in the social zone in the social affiliative paradigm (Figure 21C). Therefore, this data suggests that glutamate signaling mediating social affiliative behaviour is highly plastic in nature, and amenable to change regardless of age of intervention.

28.2 Decreased sensorimotor gating seen in Glun1 is rescued completely in GluN1Cre when compared to WT

In terms of assessing sensorimotor gating, pre-pulse inhibition was measured. The startle response is an unconditioned and reflexive response, and pre-pulse inhibition is the biological phenomenon where a weak pre-stimulus (pre-pulse) can suppress that startle response (Paylor and Crawley, 1997). GluN1 mice showed a deficit in pre-pulse inhibition. Following excision of the insertion mutation, GluN1Cre mice showed pre-pulse inhibition levels that were similar to WT littermates, across all pre-pulse levels and intervention treatment groups (Figure 22A). Where WT mice showed a step-wise increase in the inhibition of their startle response with increasing pre-pulse tones, reaching up to 70% inhibition at the highest decibel pre-pulse, GluN1 mice displayed a much more attenuated response.

A full rescue in pre-pulse inhibition deficits was seen in the GluN1Cre mice, regardless of adolescent or adult intervention (Figure 22A). There was an effect of genotype across all intervention groups; adolescent+8 weeks – (4dB) $F[3,78]=11.236, p<0.001$, (8dB) $F[3,78]=1.119, p=0.347$, (16dB) $F[3,78]=3.160, p=0.029$; adult+4 weeks – (4dB) $F[3,75]=8.805, p<0.001$, (8dB) $F[3,75]=14.594, p<0.001$, (16dB) $F[3,75]=9.571, p<0.001$; adult+8 weeks – (4dB) $F[3,80]=20.029, p<0.001$, (8dB) $F[3,80]=11.327, p<0.001$, (16dB) $F[3,80]=6.034, p=0.001$. There was an effect of sex, only in the older group of mice (adult+8 weeks intervention group; Figure 22B), where $F[1,80]=10.519, p=0.001$ (4dB pre-pulse), $F[1,80]=11.453, p=0.001$ (8dB pre-pulse) and $F[1,80]=6.028, p=0.016$ (16dB pre-pulse). It is important to note, that although there was an effect of sex on pre-pulse inhibition at this intervention time point, the trend
remains the same, and is consistent with the other intervention time points. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on pre-pulse inhibition (Figure 22C).

Looking more closely at the specifics of the acoustic startle response (ASR) itself, GluN1 mice have an exaggerated ASR when compared to WT littermates (Figure 23A). The ASR itself is highly variable in nature (Falls et al., 1997; Willott et al., 2003). Although pre-pulse inhibition showed full rescue across all intervention groups, it is interesting to note that the ASR did not follow the same trend, and overall, did not show rescue of the exaggerated ASR seen in GluN1 (Figure 23A). In the adolescent+8 weeks recovery group, we can see that the GluN1Cre showed no amelioration of the exaggerated acoustic startle response seen in GluN1 mice, with males exhibiting a higher ASR than females (F[1,78]=8.937, p=0.004). There were some effects of sex, and interactions between sex and genotype, in the ASR (Figure 23B). In the adult+4 weeks recovery group, the sexual dimorphism expanded and male GluN1Cre showed no rescue in the ASR (GluN1 vs. GluN1Cre), but females showed a complete rescue (sex: F[1,75]=6.228, p=0.015; sex* genotype: F[3,75]=12.462, p<0.001). As mice continued to age, (adult+8 week group), the differences between sexes leveled off, and we saw that an intermediate amelioration of the ASR occurred (Figure 23A).

Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on the ASR (Figure 23C). Despite high levels of variability in the ASR, it is important to note that changes in pre-pulse inhibition remain consistent across all intervention (adolescent vs. adult). GluN1Cre show a complete rescue of sensorimotor gating function back to WT levels, a marked increase when compared to GluN1. Consequently, this data further suggests that the glutamatergic signaling system underlying the mechanism of sensorimotor gating may be highly plastic in nature, and although greatly disrupted in GluN1 mice, shows full rescue to WT levels in the GluN1Cre mice.

29 RNAseq: identification of molecular signatures in NMDAR hypofunction and variable recovery in GluN1Cre following re-establishment of NMDAR

Following biochemical and behavioural assessment of rescue after re-establishment of the NMDA receptor, it was important to explore the molecular events that occur as the brain...
Figure 22. Sensorimotor gating deficits seen in GluN1 mice are fully rescued, to WT levels, in GluN1Cre mice, regardless of time of intervention. (A) Sensorimotor gating (pre-pulse inhibition) represented as percent inhibition of acoustic startle response at three pre-pulse tones; 4db, 8db, and 16dB. Male and female mice. GluN1Cre mice show full rescue, to WT levels, when comparing across intervention timepoints. (B) There is an effect of sex in the adult+8wks intervention; 4dB- p=0.002, 8dB-p=0.001, 16dB- p=0.016. There is no interaction between genotype and sex. (C) There is no difference in pre-pulse inhibition in WTCre mice when compared to WT. Data shown as mean ± SEM, **p<0.01, ***p<0.001, ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test, within each pre-pulse decibel. (Adolescent+8wks): Male – WT=10, WTCre=11, GluN1=11, GluN1Cre=10 / Female – WT=11, WTCre=11, GluN1=10, GluN1Cre=12. (Adult+4wks): Male – WT=10, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=12, WTCre=10, GluN1=11, GluN1Cre=12. (Adult+8wks): Male – WT=13, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=13, WTCre=11, GluN1=10, GluN1Cre=11.
Figure 23. Exaggerated acoustic startle response seen in GluN1 mice is further exacerbated in GluN1Cre mice rescued in adolescence, but not changed in mice rescued in adulthood. (A) Acoustic startle response to 165dB startle pulse in pre-pulse inhibition test, male and female mice. Following adolescent intervention, GluN1Cre mice show exacerbation of exaggerated acoustic startle response seen in GluN1 mice (in both male and female), while there is no change in the exaggerated startle response following adult intervention. (B) There is an effect of sex in the adolescent+8wks (p=0.004), and adult+4wks (p=0.015) interventions, with females demonstrating a lower acoustic startle response. There is an interaction between genotype and sex in the adult+4wks intervention (p=0.020), with female GluN1Cre mice showing a full rescue of acoustic startle response, to WT levels. (C) There is no difference in acoustic startle response in WTCre mice when compared to WT. Data shown as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, multiple comparisons one-way ANOVA with post-hoc Bonferroni’s test. (Adolescent+8wks): Male – WT=10, WTCre=11, GluN1=11, GluN1Cre=10 / Female – WT=11, WTCre=11, GluN1=10, GluN1Cre=12. (Adult+4wks): Male – WT=10, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=12, WTCre=10, GluN1=11, GluN1Cre=12. (Adult+8wks): Male – WT=13, WTCre=10, GluN1=10, GluN1Cre=10 / Female – WT=13, WTCre=11, GluN1=10, GluN1Cre=11.
reverses from a state of NMDA receptor hypofunction. Based on previous evaluations via qPCR (section 24), radioligand binding (section 25), immunoblot (section 26), and behavioural studies (sections 27 and 28), there was no difference in rescue based on intervention timepoint. Furthermore, the cortex showed the greatest level of functional recovery. Therefore, the intervention time point of adult+4 weeks recovery was selected, in specifically the cortex, to study gene expression. Males were used for RNAseq analysis due to a lack of sex effects seen in biochemical studies, and similar directional trends observed across sexes in behavioural phenotyping. RNAseq identified varying levels of rescue in gene expression in the cortex.

RNA species from WT, GluN1, and GluN1Cre mice were compared to identify the molecular signatures of NMDA receptor hypofunction and its subsequent rescue. From this comparison, there are 591 genetic elements of interest that were found to be significantly different between at least one pair of genotypes; WT vs. GluN1, WT vs. GluN1Cre and GluN1 vs. GluN1Cre. From these 591 genetic elements, the following were identified; 15 Riken numbers, 3 microRNA, and 85 genetic elements that were not matched to any known or published transcripts.

Comparing GluN1 to WT, 413 genes were identified to be significantly different in their expression in the cortex. The identified genes showed varying levels of rescue in GluN1Cre mice. Furthermore, there were 68 additional genes identified to be uniquely expressed (altered) in GluN1Cre mice when compared to WT or GluN1 mice. This amounted to 481 genes with variable levels of rescue and expression in the GluN1Cre mice following excision of the insertion mutation (Figure 24A). Pairwise comparisons were completed between WT and GluN1 mice, between GluN1 and GluN1Cre mice, and finally between WT and GluN1Cre mice due to limitations in computational resources (see section 17.2).

To better establish levels in rescue of genetic expression within the GluN1Cre, genes were assigned to one of five categories based on the previously mentioned three pairwise comparisons; full rescue, partial rescue, no rescue, unique and worsened (Figure 24A). 49 genes were fully restored to WT levels (‘full rescue’). 288 genes had an intermediate level of rescue, expression levels between WT and GluN1 (‘partial rescue’). 74 genes were resistant to rescue and remained at GluN1 levels (‘no rescue’). 68 genes showed unique expression levels, only altered in GluN1Cre mice, not in WT or GluN1 mice (‘unique’). And finally, 2 genes showed
exacerbated expression levels in the GluN1Cre mice (‘worsened’); Hba-a1 and Hba-a2. The full list of genes, and their rescue status can be found in Table III.

29.1 Genes with the greatest change in expression in GluN1 are attenuated towards WT levels in GluN1Cre

In addition to analyzing genes based on statistically significant changes in expression, genes showing the greatest fold change, as a result of GluN1 knockdown, were also identified (WT vs. GluN1) (Figure 24B). The identified genes had a 2-fold or greater change (either increase or decrease) in expression in the GluN1 mice when compared to WT. Following rescue in the GluN1Cre mice, it was observed that most, if not all, of these identified genes were attenuated back towards WT levels (represented by cells with lighter shading, Figure 24B). Therefore, genes with the greatest perturbation in expression levels were also genes that tended to shift expression levels closer to those seen in WT once expression of the GluN1 subunit was rescued in the GluN1Cre mice.

29.2 Single pathways show variable rescue within their individual gene components

Lastly, to gain further insight into changes and enrichment of critical biological processes resulting from the knockdown of GluN1, and its rescue, pathway analyses were performed on the previously mentioned gene lists (Table III, Figure 24). Focus was put on some of the most significant pathways specific to the nervous system. Among the fully rescued genes, axon regeneration, positive regulation of actin filament bundle assembly, neuron fate commitment and negative regulation of nervous system development were pathways that were significantly over-represented.

It was found that common biological pathways pertaining to the nervous system did not show a uniform level of rescue, but in fact, contained both partially and not rescued genes. Neurogenesis, neuron differentiation, and cognition contained both genes that were partially rescued, as well as genes that were not rescued (Table IV). This is further highlighted in Figure 24C, where a schematic of the genes involved in one example of a common gene ontology level 5 biological process, neuron differentiation, showed varying levels of rescue. Using neuron differentiation as an example, it was found that even within a single pathway, there are varying levels of rescue at the gene expression level; genes that are fully rescued, partially rescued, and
not rescued at all. This further highlights that rescue, or re-establishment of glutamate signaling, is not a uniform process, and is variable within a single brain region, or even a single signaling pathway.
A Rescue of gene expression in the GluN1Cre mice

B Greatest changes of expression in GluN1 mice and its amelioration

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C Neuron Differentiation pathway: variable rescue
Figure 24. Genes show varying levels of rescue following excision of insertion mutation in GluN1Cre mice, manifesting in full, partial and no rescue of gene elements within the cortex. (A) 413 genes were identified with differently regulated expression levels in the cortex of GluN1 mice when compared to WT. This resulted in full (49 genes; green), partial (288 genes; purple), or no rescue (74 genes; red), or worsening (2 genes; navy) following excision of the insertion mutation in the GluN1Cre mice. 68 additional genes were found to be in a unique state of expression in the GluN1Cre mice (yellow). (B) Genes with the greatest change in expression, 2-fold or greater change (either increase or decrease), are listed. Gene expression is represented as a log2 of fold change relative to WT in the GluN1 and GluN1Cre mice. (C) Schematic of genes in a representative common gene ontology level 5 biological process, neuron differentiation, that shows variable rescue of gene expression in a single pathway in the GluN1Cre mice; fully (green), partially (purple), and not rescued (red).
Table III. List of genes from RNAseq analysis that display full, partial, or no rescue in GluN1Cre cortex, and those that are uniquely altered.

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Table IV. Enriched nervous system specific biological processes from RNAseq analysis and their representative genes that display full, partial, or no rescue in GluN1Cre cortex, and those that are uniquely altered (GluN1Cre specific).

<table>
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<tr>
<th>RESCUE STATUS</th>
<th>BIOLOGICAL PROCESS</th>
<th>P value</th>
<th>Q value</th>
<th>COMMON GENES</th>
<th>UNIQUE GENES</th>
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<td><strong>Full Rescue</strong></td>
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<td>Apod</td>
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<td>Positive Regulation of Actin Filament Bundle Assembly</td>
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<td>Myoc Rapgef3</td>
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<td>Neuron Fate Commitment</td>
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Chapter 4  
DISCUSSION

This study compared the plasticity of the adult and adolescent brain. Plasticity encompasses morphological and functional changes in response to environmental inputs or insults (Hummel and Cohen, 2005). Plasticity was thought to be a unique property of developing brains, however evidence has shown that it is also present in the adult brain (Ehninger et al., 2008; Dennis et al., 2013; Lövdén et al., 2013). We set out to address this continued disparity in conventions regarding plasticity, and looked to investigate how plastic the adult brain really is.

To do this, we generated a mouse model, where the brain developed in a state of NMDA receptor hypofunction, disrupting glutamate signaling (Figure 6). This new model was based on the GluN1-KD mouse model that was previously described by Mohn et al. (1999). The GluN1-KD model has a decrease in NMDA receptor function manifesting in the following behaviours: decreased habituation, reduced sociability, locomotor hyperactivity, deficits in sensorimotor gating, cognitive impairments/inflexibility, and aberrant levels of anxiety (Mohn et al., 1999; Duncan et al., 2004; Halene et al., 2009; Ramsey et al., 2011; Gandal, Anderson, et al., 2012; Mielnik et al., 2014; Milenkovic et al., 2014). Taking advantage of the Cre-loxP system, we were able to genetically “rescue” the miswired glutamate system via excision of the original insertion mutation, and restore NMDA receptor function.

Restoration of NMDA receptor function was focused on two key developmental time frames: adolescence and adulthood (Figure 9). Based on previous literature, we hypothesized that rescue in adolescence would yield a greater amelioration of behavioural impairments, however our study disproved this hypothesis. We showed that, independent of when rescue took place (adolescence vs. adulthood), or length of recovery following rescue, the levels of plasticity were constant. Conversely, we were able to identify different levels of plasticity based not on age, but on specific brain regions that mediate certain behaviours.
30 Adolescent vs. Adult Plasticity in the GluN1-IR Model: Experimental Outcomes

30.1 Behavioural Rescue in GluN1Cre Mice

The glutamate system showed limited plasticity in signaling that mediated locomotor behaviour, stereotypy, executive function and anxiety, independent of age or recovery time. Regardless of rescue at adolescence or adulthood, locomotor activity, stereotypy, executive function and anxiety all showed intermediate rescue in GluN1Cre mice, when compared to WT and GluN1 littermates (Figures 16-20). Conversely, sociability and sensorimotor gating behaviours showed robust amelioration, a full rescue, with behaviours returning to WT levels in GluN1Cre mice (Figures 21 and 22). Originally, it was believed that variability in behavioural rescue would be seen across developmental time points, with younger mice showing greater rescue of behavioural deficits than adults. However, in our study, we found that variability in rescue, or “re-wiring”, was not mediated by developmental time points, or even temporal windows of recovery, but rather resolved based on specific brain regions and, inferentially, the circuits contained within. This manifested as varying levels of rescue in different behaviours.

30.2 Biochemical Rescue in GluN1Cre Mice

The differences in the level of behavioural rescue in the GluN1Cre mice was corroborated at the biochemical level; NMDA receptor levels were equally consistently rescued across developmental ages, but varied between brain regions. We investigated functional NMDA receptor levels following excision of the insertion mutation, as well as levels of GluN1 and GluN2A protein. Although expressed throughout the entire brain, NMDA receptor expression levels vary greatly between brain regions. High levels of NMDA receptor expression are seen in the hippocampus and cortex, with lower receptor densities in the striatum and cerebellum (Magnusson, 1995). We focused on these four brain regions for our saturation binding to measure functional NMDA receptors, and we found that levels of NMDA receptor rescue were consistent across all intervention time points.

Regardless of adolescent or adult rescue, the cortex and hippocampus showed the greatest level of recovery at the NMDA receptor level, while the striatum and cerebellum showed modest recovery of functional NMDA receptors (Table II). It is important to note, the greater the initial
knockdown of NMDA receptors, the greater the rescue. While the cortex and hippocampus have a high density of NMDA receptors (around 1500-2000 fmol/mg respectively in WT), the striatum and cerebellum show lower densities (200-1000 fmol/mg). Therefore, while the GluN1 mouse exhibits decreases in NMDA receptor densities to levels that are 10-20% of WT in the cortex and hippocampus, there are more modest decreases seen in the striatum and cerebellum (30-40%). Rescue of NMDA receptor levels in the GluN1Cre mice follows the same trend; around a 30-40% increase seen in the cortex and hippocampus, while the striatum and cerebellum see modest increases of 10-20%. It is reasonable to infer that brain regions with higher densities of NMDA receptors, and therefore more reliant on glutamate signaling, would be more amenable, or more plastic, to change for the restoration of glutamate signaling. This manifested in either intermediate or full rescues in specific behaviours (as described above).

In terms of GluN1, and GluN2A, subunit protein levels, the same trends in rescue were seen as those in saturation binding. Protein levels of the GluN1 protein, and its heterotetramer-forming GluN2A subunit, were reduced in the GluN1 mouse in both the cortex and hippocampus to about 10% of WT levels. GluN1 protein was rescued to 40% of WT in the cortex and 20% of WT in the hippocampus. The same trend of rescue was seen in GluN2A protein levels (Figure 15). Visualization and quantification of protein levels of the GluN1 subunit in the striatum and cerebellum were attempted (not shown), but levels were too low for immunoblot detection. However, it is important to note that the decrease and subsequent rescue in NMDA receptor subunit protein levels mimicked closely the patterns seen in the saturation binding data; brain regions with higher densities of NMDA receptors showed greater knockdown of the GluN1 protein (and subsequent receptor levels), but also showed the greatest level of rescue following excision of the insertion mutation.

**30.3 Molecular Rescue in GluN1Cre Mice**

To determine if variable levels of behavioural and biochemical rescue were due to post-translational mechanisms, the excision rate of the insertion mutation was quantified using qPCR. By looking at the excision rate at the DNA level, this allowed us to determine if the insertion mutation was being excised uniformly across brain regions. If excision was uniform, this would lead us to infer that variability in rescue must be at the post-translational level, leading to varying levels of GluN1 protein, and subsequently varying levels of functional NMDA receptors (as seen
in this study). However, this was not the case. As seen in the excision data (Figure 14), cortex and hippocampus had excision levels of 25%, striatum 15%, and cerebellum did not show any excision. Therefore, we needed to ensure that variability was not due to inconsistent Cre-recombinase activity. Seeing as there were variable levels of rescue, including behavioural, biochemical and at the DNA level, it was important to ensure that the induction of Cre-recombinase activity following tamoxifen administration was uniform in all regions of the brain.

Induction of Cre-recombinase activity was measured using a tdTomato reporter mouse line. Following induction of Cre-recombinase activity, the floxed STOP codon disrupting expression of the tdTomato gene is excised, and results in the expression of red fluorescent protein in the corresponding tissue (Madisen et al., 2010). The Cre-recombinase mouse line that was used for this study (ROSA26\textsuperscript{CreERT2}) showed tightly regulated, and robust induction, of Cre-recombinase activity following tamoxifen administration (Figure 13). There was global induction of Cre-recombinase activity throughout the mouse brain, including the cortex, striatum, hippocampus, and cerebellum. Therefore, the induction of Cre-recombinase activity via tamoxifen administration was not the confounding variable resulting in non-uniform behavioural or biochemical rescue in the GluN1Cre mouse.

Observing that age of rescue, or recovery time, did not influence the level of behavioural amelioration observed in the GluN1Cre mice, focus was placed on adult rescue. Further investigation was completed into molecular mechanisms that could potentially highlight genetic components as useful targets for the increase of plasticity and rewiring of circuits. To do this, RNAseq analysis was performed on cortical tissue from the adult intervention group (+4 weeks intervention group, as 4 weeks should be sufficient time for molecular changes to take place). With such a large data set, interpretation of the results was a large undertaking. For this study, interest in the levels of rescue was foremost, allowing for better focus on potential underlying mechanisms that may explain the behavioural and biochemical results of this study.

As previously described (see section 29), 413 genes were identified to be significantly different in their expression, in the cortex, as a result of GluN1 subunit knockdown (WT vs. GluN1), and subsequently, there were variable levels of rescue within these identified genes in the GluN1Cre mice. In terms of rescue, the full spectrum of variability was seen; from no rescue to complete rescue, including intermediate forms. As seen in Figure 24A, the largest proportion of perturbed
gene expression saw only partial rescue following the excision of the insertion mutation. Interestingly, 68 genes showed ‘unique’ expression in the GluN1Cre mice, potentially highlighting targets for mechanisms mediating the ‘re-wiring’ of dysfunctional circuits, or plastic mechanisms needed for the rescue of behavioural dysfunctions. This requires further investigation and validation. Nonetheless, even within the cortex, an established ‘highly-plastic’ structure, molecular rescue remained variable, despite full rescue in cortex-mediated behaviours, and high levels of NMDA receptor rescue. The deciding factor for whether a target undergoes full, partial or no rescue has yet to be determined. However, if discovered, it could open numerous possibilities for the targeting of plasticity mechanisms.

To better understand, and visualize, the level of change that occurs at the molecular level as circuits develop with decreased NMDA receptor levels, genes with the greatest change in expression were highlighted. Gene targets with the greatest change in expression (either increase or decrease) due to the knockdown of the GluN1 subunit (WT vs GluN1) were quantified and expressed as log2 fold change relative to WT (Figure 24B). Although the levels of change are variable, it is important to note that regardless of degree, or direction, of change, genes with the greatest fold change showed a more normalized expression in the GluN1Cre mice following rescue, bringing them closer to WT levels. This is visualized as a lightening of colour in the heat map (Figure 24B); darker blocks in the GluN1 column vs. lighter blocks of colour in the GluN1Cre column. This highlights that genes with the greatest perturbations in the GluN1 mice, molecularly, tend to shift expression levels closer to those seen in WT once expression of the GluN1 subunit is rescued in the GluN1Cre mice.

Finally, to better understand how changes in molecular expression, due to GluN1 knockdown, impacted biological processes, pathways analyses were performed. The gene list used for these analyses can be found in Table III. For this study, focus was placed on pathways that were specific to the nervous system; pathways enriched for full, partial and no rescue are highlighted in Table IV. It is important to note that within the pathway analysis, common enriched pathways were identified in all levels of rescue; the same pathways that contain genes which are fully rescued, also contain genes that are partially rescued and even not rescued at all. Therefore, within the same pathway, variable levels of rescue in gene expression are observed. This is demonstrated schematically in Figure 24C. Within the ‘neuron differentiation’ pathway, all three levels of rescue are observed; full, partial, and no rescue. This further corroborates that there may
exist some underlying mechanism that determines level of rescue. Within the cortex, there are variable levels of molecular rescue, that manifest into a relatively high level of biochemical rescue, and a full behavioural rescue. The underlying mediator of this variability remains to be identified, and requires further investigation.

### 30.4 Interpretation of Results

It is not unreasonable that our study highlighted variable rescue across different behaviours, involving different brain regions. Previous studies have shown that adult rescue of neurodevelopmentally-disrupted behaviours is possible (Guy *et al.*, 2007; Silverman *et al.*, 2012; Yin *et al.*, 2013; Sztainberg *et al.*, 2015; Mei *et al.*, 2016). These findings corroborate the behavioural results observed in this study; rescue of sociability and sensorimotor gating deficits, and partial rescue of hyperlocomotion, increased stereotypic movements, decreased anxiety, and impaired executive function. Furthermore, this study determined whether similar levels of plasticity can be found in the young vs. adult brain, or if there is more profound rescue in the younger brain. This study highlights that plasticity of the behaviours tested may not be mediated by developmental age, but in fact, by underlying factors that are regionally and spatially determined (ie. brain region and circuit specific). Mei *et al.* (2016) substantiate these findings and the variability in rescue, since their study showed similar findings; deficits in sociability were fully rescued, whereas locomotor and anxiety dysfunction were not rescued. Where Mei *et al.* (2016) saw no amelioration in locomotor or anxiety behaviours, this study demonstrated partial rescue. Whether a partial rescue of these behaviours is functionally relevant (partial rescue can be sufficient for functional gain), is a question that is beyond the scope of this study, however, clinically quite relevant and pressing for treatment strategies. It is important to note that final behavioural outputs of rescue in this study may not be solely due to the restoration of GluN1 subunit levels, but rather can be due to other neurotransmitter systems compensating for the marked decrease in GluN1 subunit levels, and ultimate NMDA receptor hypofunction.

It has been well-established that the cortex and hippocampus, as functional structures, are both quite plastic in nature, and therefore, our findings are not surprising. Most studies that have been done on LTP and LTD have focused on excitatory synapses onto excitatory neurons in both the hippocampus and the neocortex (Bliss and Collingridge, 1993; Froemke, 2015). The cortex is a region that is particularly relevant in terms of plasticity as it regulates and performs sensory,
motor, and cognitive tasks with a strong underlying learning component. Studies, and past literature, have proposed a number of mechanisms underlying plasticity in the cortex, including physiological mechanisms (functional modification of existing synapses and neurons), and structural mechanisms (physical rewiring of cortical circuits via formation, elimination and morphological change at the synapse level) (Feldman, 2009). The hippocampus is a heterogeneous structure (neuron-wise) and can be mapped via morphological and connectivity characteristics of neurons. It has been long established that the synapses and dendrites of even mature neurons in the hippocampus continuously undergo rearrangement, with entirely new neurons being formed throughout life. These structural changes that are normally found to take place during development, continue to occur postnatally, well into young adulthood and even into middle age (Leuner and Gould, 2010). Therefore, knowing that the cortex and hippocampus are classically well-established ‘highly plastic’ brain structures, it is not surprising that this study found the highest levels of rescue of the GluN1 protein and NMDA receptor in the cortex and hippocampus. With this level of biochemical rescue, full rescue of cortically-relevant social behaviours ((Hamilton and Brigman, 2015); also rescued pharmacologically, Appendix B)) and hippocampus-associated sensorimotor gating is also predictable (Bast and Feldon, 2003; Osumi et al., 2015).

Although synaptic plasticity is often studied in the cortex and hippocampus, the striatum also exhibits LTP/LTD that is associated with behavioural plasticity. Traditionally, studies involving the basal ganglia, including the striatum, have focused on the control of movement. Only later did it include research about control of cognition and decision making. But, it is important to note that the striatum can also participate in a number of circuits and loops connected to areas of the cortex, further being implicated in cognitive or sensory outputs (Middleton and Strick, 2000). The striatum is primarily involved in two pathways: the direct and indirect. Both of these pathways are influenced by fast excitatory and inhibitory synaptic outputs, with a slower modulation occurring via dopamine and other signaling molecules. The net effect of the activation of the indirect pathway is the reduction of premotor drive and inhibition of movement, while the opposite is true of direct pathway activation (Kreitzer and Malenka, 2008). The most significant inputs into the striatum include cortical glutamatergic and nigral dopaminergic afferents onto medium spiny neurons. A combination of both glutamate and dopamine receptor activation is required to produce long-lasting modifications to synaptic excitability in the
striatum, whereas individual activation of either leads to short-term effects. A large body of work has demonstrated that LTD is the predominant form of plasticity in the cortico-striatal pathway, mediated by calcium influx via L-type channels, not NMDA receptors (Centonze et al., 2001; Wang et al., 2006). Therefore, literature shows that the striatum, as a brain structure, mediates a number of circuits that control anywhere from motor function to cognition, but does not use the typical synaptic mechanisms for relaying information. Perhaps the striatum does not rely heavily on NMDA receptors for synaptic transmission.

Following the concept that the striatum may not rely on NMDA receptors for synaptic plasticity, this would corroborate the results seen in this study. Since the striatum does not express NMDA receptors as much as the cortex and hippocampus do, the striatum exhibits lower levels of NMDA receptor ‘knockdown’, and subsequently a more modest ‘rescue’. This reasoning would help explain the partial rescue seen in hyperlocomotion and increased stereotypic behaviours since, underlying neural circuits of OCD (the human-equivalent endophenotype to increased stereotypy in mice) have been shown to involve the cortex as well as the caudate nucleus and thalamus (Aouizerate et al., 2004), and movement is known to be controlled via the striatum (Middleton and Strick, 2000). Therefore, with a lower reliance on NMDA receptors, the striatum may have the innate mechanisms to employ plasticity to adapt to NMDA receptor levels.

Lastly, in our study, large-scale fluctuations of NMDA receptors levels were not seen in the cerebellum; a brain region with low levels of NMDA receptors endogenously (Magnusson, 1995). GluN1 mice showed the lowest levels of knockdown in the cerebellum, and the lowest level of rescue in the GluN1Cre mice. NMDA receptors contribute a substantial proportion of total synaptic current in the cerebellum, even at negative voltages, due to the expression of GluN2D NMDA receptor subunits (large decay constant, weakly blocked by Mg\(^{2+}\)) (Audinat et al., 1992; Anchisi et al., 2001; Zheng and Raman, 2010). Therefore, due to the fact that the cerebellum does not have a large concentration of NMDA receptors to begin with, and the ones it does express contain GluN2D subunits that are easily activated and do not decay rapidly, perhaps the effect of GluN1 knockdown has a muted effect in the cerebellum. It is important to note that the cerebellum is not only involved in motor coordination behaviours, but also has been implicated in emotional behaviour and memory (Strata et al., 2011). Therefore, perhaps any rescue at all (intermediate) seen in some emotional behaviours (anxiety, Figures 19 and 20) and memory (puzzle box, Figure 18) could potentially be driven by the low-scale, yet nominally
affected, NMDA receptor-mediated signaling that takes place in the cerebellum (Strata et al., 2011).

Anxiety is a complex behaviour, involving a number of brain regions that form connections to mediate this emotionally driven behaviour. Anxiety encompasses both cognitive processes and emotional responses. Brain structures known to be involved in anxiety (as well as fear-related responses) include the central nucleus of the amygdala, the periaqueductal gray, and the paraventricular nucleus of the hypothalamus (Arnold et al., 2001; Viveros et al., 2007). Anxiety is a behaviour that manifests due to the presentation of a conflict between potential response options. In laboratory animals, this conflict corresponds to the choice between approach and avoidance with respect to exploration (ex. the open arms of an elevated plus maze). Fear and anxiety are two behaviours that are closely linked, but functionally distinct. Whereas fear is the response to a threat that is present, anxiety is the response to a potential threat (Gray and McNaughton, 2003; Barkus et al., 2010). This is an important distinction, as different responses are mediated differentially by the amygdala and the ventral hippocampus (present danger, amygdala; potential danger, hippocampus). The hippocampus allows for the detection of a ‘conflict’, and then the resolution of the conflict via increased levels of attention and arousal and inhibition of locomotion. The combination of these anxiety-like responses allows the animal to gather more information before deciding how to proceed.

The partial rescue of anxiety-like behaviour is complicated to explain, but not unreasonable. As already mentioned, the hippocampus is an NMDA-rich brain structure, and it is reasonable to expect that a decrease in NMDA receptors in the hippocampus can lead to an anxiolytic phenotype, as seen in the elevated plus maze with the GluN1 mice (Figure 19). The GluN1Cre mice exhibited intermediate rescue of their anxiety-like behaviour. Although we saw high levels of NMDA receptor rescue in the hippocampus, perhaps the partial rescue in behaviour is driven by the restoration of NMDA receptors in this brain region, but limited by a lack of rescue outside of the hippocampus. In other words, partial rescue of anxiety-like behaviours may be due to an increase in NMDA receptors in one part of the circuit necessary for the behaviour, but not another. Spending equal amounts of time in the open and closed arms (as seen in the GluN1Cre mice) could be interpreted as a conflict between taking time to evaluate the situation (while in the closed arms, rescued NMDA receptors in the hippocampus) and a lack of fear (exploration in
the open arms and therefore, little to no rescue of NMDA receptors in structures like the amygdala).

To test this hypothesis of conflict between exploration and fear, it would be important to determine the level of rescue of NMDA receptors in the amygdala, both via saturation binding and specific behavioural tests. It would also be beneficial to better distinguish between ventral (anxiety) and dorsal (spatial memory) hippocampal NMDA receptor rescue. To better determine these variables, spatial memory tasks like the Morris Water Maze could be tested to elucidate dorsal hippocampal rescue. Fear-conditioning could be tested for rescue in the amygdala. A study done by another student in the lab has shown that the GluN1Cre mice have intermediate rescue of cue- and context-mediated fear conditioning (data not shown, study by R. Islam). A second test of anxiety-like behaviour should also be undertaken. Further investigation into specific sub-regions within discrete structures is warranted for this complex behaviour. As a note on experimental design, although center-time showed intermediate rescue, similar to elevated plus maze, the arena used was too small. An open field to measure center time should be 61x61cm for a mouse, with light levels at ~600lux, whereas our dimensions were 20x20cm with dim light at ~16lux (Crawley, 1999; 2004). Therefore, although the parameters were adequate for the measurement of locomotor activity, and we were able to measure center time with the apparatus, there are confounding factors that do not allow us to accurately represent center time. Therefore, a larger open field is necessary for center time measurement in relation to anxiety-like behaviour.

31 Epigenetic Control of the Grin1 Gene

An unexpected result of this study was the variable, and highly restrictive, levels of excision of the insertion mutation following treatment with tamoxifen (Figure 14). Limited excision lead to partial rescue of the GluN1 subunit at both the molecular (Table III) and biochemical (Figure 15, Table II) level. This result could be due to insufficient doses of administered tamoxifen, bioavailability of tamoxifen in the brain, or regional modifications of Cre-recombinase expression. To address these concerns, a number of thorough steps were taken to ensure that the variable excision rates observed in this study were not due to experimental design or technical components involved in the study.
First, the amount of tamoxifen used in this study to induce Cre-recombinase activation was much higher than previous studies done involving inducible Cre-recombinase in the brain (Weber et al., 2011; Shimada et al., 2015). Usual dosages of tamoxifen used for induced Cre-recombinase expression in the brain range from 5mg-12.5mg, a much lower dose than the 20mg given to mice in this study. Furthermore, the dose and route of administration used in this study were chosen following an extensive period of optimization of administration routes and drug preparations (see Appendix A). Lastly, to ensure that tamoxifen induction of Cre-recombinase was not the variable confound leading to non-uniform levels of excision of the insertion mutation, a Cre reporter line was used (\textit{ROSA26\textsuperscript{tdTomato}}). As seen in Figure 13, there is robust and uniform expression of the tdTomato protein (visualized as red fluorescence) in all tested brain regions following administration of tamoxifen. Therefore, it is with confidence that we can exclude confounding factors of tamoxifen dose, route of administration, or Cre-recombinase induction as the cause for the variable excision rates observed in this study.

The reason behind the variable and relatively low excision rates of the insertion mutation remains unknown, however is hypothesized to be due to epigenetic regulation of the \textit{Grin1} locus. It seems that the restrictive mechanism is not based on brain region, tissue, Cre-recombinase induction or tamoxifen dose, therefore, it is rational to assume that the confound must be at the DNA level. A hypothesis involving epigenetic regulation of the \textit{Grin1} locus is a highly reasonable mechanism to further explore. Perhaps Cre-recombinase has limited accessibility to the insertion mutation (and its flanked loxP sites), due to epigenetic regulation of the chromatin structure. This would in theory force the \textit{Grin1} locus into a closed formation, and therefore not allow access for the activated Cre-recombinase to excise the mutation.

An important way to regulate gene expression is through the remodeling of chromatin structure, a complex made up of DNA, and histone proteins that DNA wraps around. The functional unit of chromatin, the nucleosome, is made up of 147 base pairs that are wrapped around core histone octamers that consist of 2 copies of each of the following histone proteins; H2A, H2B, H3 and H4 (Walker et al., 2015). Gene expression is controlled via the wrapping density of the DNA around the histones; tight wrapping around the histone lends itself to a closed conformation, and therefore a decrease in gene transcription, the same being true for the opposite. Histone modifications, which control the wrapping density of DNA, are numerous and include the following: acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, ribosylation.
and citullintion, and ADP-ribosylation (Volmar and Wahlestedt, 2015). Histone modifications largely occur at H3 and H4, and more specifically along the histone tails, targeting amino acids lysine and arginines for methylation, and lysine only for acetylation (Grayson and Guidotti, 2013).

We hypothesize that, in a state of NMDA receptor hypofunction, the *Grin1* locus undergoes epigenetic regulation via methylation or acetylation, maintaining it in a closed conformation, not allowing the Cre-recombinase to have access to the floxed insertion mutation. Although it seems counter-intuitive that the brain would want to further restrict expression of the GluN1 subunit when the system is already in a hypofunctioning state, it is also not unreasonable. As the primary excitatory neurotransmitter, glutamate levels are tightly regulated. The proteins involved in the regulation of excess glutamate levels include sodium-dependent glutamate transporters, found on surrounding astrocytes at the synapse. If allowed to reach elevated extracellular concentrations, glutamate becomes neurotoxic in nature, triggering excitotoxicity and cell death in surrounding postsynaptic neurons (Choi, 1994; Miladinovic et al., 2015). Therefore, due to the brain developing in a state of NMDA receptor hypofunction, perhaps a number of compensatory mechanisms occur throughout development, making the overall system more sensitive to glutamate. By being more sensitive, this places the system in a more vulnerable state to fluctuations in glutamate signaling.

Conceivably, by having the *Grin1* locus in a more closed conformation, via either hypermethylation or hypoacetylation, neurons prevent ‘excessive’ rescue that the compensating system might be incapable of regulating. This incapability to regulate a sudden increase in NMDA receptor expression could be due to already established compensatory mechanisms that developed due the state of hypofunction. If the system develops with less glutamate signaling, and compensatory mechanisms emerge over time, perhaps the newly established equilibrium is incapable of monitoring and regulating higher, physiological levels of glutamate. Evidence shows that the *Grin1* locus does undergo epigenetic control, specifically methylation (Oh et al., 2013; Zhang et al., 2013). Alternatively, studies have shown that histone deacetylase (HDAC) inhibitors are also being applied to a number of disorders, including neurodevelopmental and psychiatric ones. Reduced histone acetylation could be a final common endpoint for a number of clinical disorders (Qiu et al., 2017). Furthermore, epigenetic regulation of glutamate receptors occurs during development, and is also region specific (Stadler et al., 2005). Therefore, it is
realistic to hypothesize that the Grin1 locus is a possible target for epigenetic regulation in this study, and prevents the full excision of the insertion mutation via tamoxifen-inducible Cre-recombinase.

32 Limitations of the Study

32.1 Tamoxifen Off-Target Effects

Inducible activation of Cre/loxP-recombination in a temporal fashion via tamoxifen is a commonly used strategy to study circuits and plasticity in the adult brain (Morozov et al., 2003; Schönig and Bujard, 2003; Vogt et al., 2008). This study took advantage of the Cre-recombinase protein fused to a mutated ligand binding domain of the human estrogen receptor (CreERT2). Cre-recombinase, under the control of a specific promotor, is sequestered in the cytoplasm until tamoxifen (estrogen receptor ligand) binds and Cre-recombinase translocates into the nucleus, excising any loxP flanked DNA sequences (Erdmann et al., 2007; Vogt et al., 2008). The sequestering of Cre-recombinase in the cytoplasm allows for temporal control of “rescue” in this study. The tamoxifen-inducible Cre-recombinase system has allowed for the successful induction, or reversal, of mutations in neurons, astrocytes, and oligodendrocytes (Leone et al., 2003; Ganat et al., 2006; Hirrlinger et al., 2006; Mori et al., 2006; Erdmann et al., 2007; Vogt et al., 2008).

Although temporal- and spatial-control of the CreERT2 system is beneficial, the use of tamoxifen as the induction ligand is problematic due its off-target effects. Disruption of estrogen signaling can lead to large scale effects in numerous brain regions, resulting in changes in mood, cognition, and behaviour (Gogos et al., 2015). This presents the possibility for confounding drug effects when using tamoxifen, compromising the ability to test rescue of behaviour, plasticity, and rewiring in the brain. Specifically, in behaviours strongly linked to hormonal influence, such as social behaviour, administration of an exogenous estrogen ligand could have profound effects on sociability. In male mice particularly, a large dose of an estrogen ligand could greatly disrupt physiological hormonal levels and signaling, leading to baseline changes in testosterone-facilitated behaviours. Studies have shown conflicting results regarding changes in aggressive and social behaviours following tamoxifen treatment, indicating that caution needs to be taken when using tamoxifen in behaviour experiments (Hasan et al., 1988; Simon and Perry, 1988). However, as all mice in this study were exposed to tamoxifen, any changes in behaviour would
have been seen across all genotypes, still allowing for the comparison across genotypes and an interpretation of behavioural rescue.

Genotype specific effects in response to tamoxifen may confound behavioural results in this study. As previously mentioned, effects of drug on behaviour and biochemistry were mitigated for by treating all experimental animals with tamoxifen, and controlling for Cre-recombinase transgene effects by including WT mice both with and without the transgene. This allowed for the comparison of GluN1 and GluN1Cre mice to WT, looking at levels of behavioural rescue without confounding tamoxifen effects. However, we do have to be careful because we cannot rule out the possibility of genotype-drug interactions; that one genotype may be more sensitive to tamoxifen than others. It is important to note that we did observe the same phenotypic differences between WT and GluN1 mice that had been previously reported (Mohn et al., 1999; Duncan et al., 2004; Halene et al., 2009; Ramsey et al., 2011; Gandal, Anderson, et al., 2012; Mielnik et al., 2014; Milenkovic et al., 2014). Therefore, we do not believe that tamoxifen masked the phenotype of the GluN1 mice, or caused an exacerbation of their phenotype.

Nonetheless, it would have been valuable to have a vehicle (corn oil) control group for this study to allow for the assessment of tamoxifen effects. A vehicle control was not included in this study due to limitations in study size; 4 genotypes, 2 sexes, 3 time points and 2 behaviour groups per time point were included in this study, totaling 480 mice (528 mice were included in the end). Therefore, in adding the vehicle control group across 2 genotypes (WT vehicle and GluN1 vehicle), and 2 sexes for each time point (3 time points in total) with 2 behaviour groups (A and B), this would have added 240 mice, totaling 720 mice. This number of animals was not feasible for this study, therefore, a vehicle (corn oil) control group was not included. However, comparisons across genotype and levels of rescue were still possible due to the fact that every mouse tested was treated with tamoxifen.

32.1.1 Tamoxifen and Cognitive Effects

The cognitive effects of tamoxifen could present a serious confound in the results involving this behavioural function. In terms of brain development and function, estrogens are implicated in a number of brain functions, including migration of neurons during development, protection against brain injury and even improvement in memory, learning and the growth of synapses (Sharma and Thakur, 2006). Estrogens have also been shown to have neuroprotective properties,
that continue to be exerted over a lifetime (Gogos et al., 2015). Tamoxifen is both an agonist and antagonist of the estrogen receptor (tissue dependent), and has been shown to increase glutamate reuptake, helping regulate the availability of glutamate in the synaptic cleft (Phillis et al., 1998; Gogos et al., 2015). Therefore, by administering tamoxifen to test animals, there is a disruption of estrogen signaling for at least the length of time the drug is on board, but potentially longer. This could manifest in drastically confounding behavioural and cognitive effects.

A study completed by Vogt et al. (2008) addressed these concerns regarding tamoxifen and its effects on behavioural testing. This study showed that following a 4-week wash-out period after final treatment with tamoxifen (treatment lasting one-week), tamoxifen treated animals showed no difference in the following behavioural tests: Open Field (locomotion), Dark-Light box and Elevated O-maze (anxiety-like behaviours), T-maze (spatial short-term working memory), Fear Conditioning (emotional learning), Morris Water Maze (spatial reference memory), and Tail Suspension and Learned Helplessness (depression-like behaviours). Mice did show significant alteration in the forced swim test, that lasted after the 4-week latency period. It is also important to note that due to the lack of locomotor or exploratory behavioural alterations, there is confidence that these will not represent confounding factors in more complex behavioural tasks. It is important to note that the described study was done in male mice only. Therefore, this does not preclude the possibility of confounding factors, due to tamoxifen treatment, affecting female mice. However, we remain confident that tamoxifen effects continued to be minimal in this study.

32.1.2 Tamoxifen and Sex Effects

Being a ligand of the estrogen receptor, tamoxifen is likely to have an effect on estrogen-mediated signaling pathways and processes, particularly sex-dependent effects that could interfere with physiological sexual maturation processes. Studies have found that in males, disruption of estrogen receptor signaling leads to infertility, a decrease in aggressive behaviours and male-typical offensive attacks, however motivation to mount females was not altered (Ogawa et al., 1997). In females, there is a reduction in sexual behaviour observed, that can last for several months (Csaba et al., 2001). These behaviours, although altered, do not confound the behaviours tested in this study.
32.1.3 Tamoxifen and Peripheral Effects

Tamoxifen administration has been shown to affect bodyweight of experimental animals. Dependent on route of administration, drug preparation and age of administration, tamoxifen treatment can lead to both weight gain, and weight loss in mice. Animals treated in adulthood have been shown to gain more bodyweight following tamoxifen treatment (Vogt et al., 2008). On the other hand, if given in a food preparation, or given at a young age, tamoxifen administration can lead to body weight loss (Mei et al., 2016). Tamoxifen is bitter in taste when prepared in food, so sucrose is added to the pellet preparation, which can also aid in preventing bodyweight loss due to decreased consumption. No exaggerated changes in bodyweight were observed in this study.

32.2 RNAseq Analysis Constraints

Due to a large data set, and limited software resources, we encountered analysis constraints when analyzing our RNAseq data. More specifically, we were unable to compare all three genotypes simultaneously as the data files were too large and the Galaxy server would time-out when analysis was attempted with all three genotypes. To overcome this, we were forced to perform three pairwise comparisons between genotypes, instead of comparing across all three genotypes simultaneously. We placed gene transcripts into one of five categories that described their rescue status, and the categorization was based on these three pairwise analyses (WT vs. GluN1, GluN1 vs. GluN1Cre, and WT vs. GluN1Cre). For example, genes that were considered to be “fully rescued” were those whose transcript levels were significantly different between WT and GluN1 mice, and also significantly different between GluN1 and GluN1Cre mice, but were not significantly different between WT and GluN1Cre mice. By completing three pairwise analyses, this did not allow for quantitative comparison between genotypes, but only directional comparison. The study was able to identify which genes changed and if they were rescued based on the three pairwise comparisons, but was unable to provide direct quantitative comparisons of the three groups. In future studies, comparison between all three genotypes is necessary.
33 Strengths of the Study

33.1 Sex Differences

In designing this study, it was ensured that both sexes were included and that a sufficient number of animals were tested to assess differences due to sex. Sexual dimorphism in mammals, stemming from both genetic and hormonal events, begins at an early age and continues throughout life. Sex differences, physiologically, account for a number of differences when it comes to disease; incidence, susceptibility, time of onset, manifestation, prognosis, treatment and drug response are all affected by sex (Becker et al., 2005). Within the brain, there exist differences in structure and function based on sex, a result of complex response cascades that are initiated by genes and prolonged by the effects of gonadal hormones (MacLusky et al., 1997; Sharma and Thakur, 2006).

The results of our study indicate that all trends observed in behavioural rescue (as described above) remained the same between sexes. However, some differences were seen. Most notably, there was an effect of sex, and an interaction of sex and genotype, observed in executive function, which was tested in the puzzle box assay (Figure 18). GluN1Cre mice showed an intermediate phenotype in both adolescent- and adult-rescue. Overall, however, females did perform better in this problem-solving and learning task. Furthermore, it seems that GluN1Cre females show better amelioration of executive function impairment when rescued in adulthood. Although the rescue remains intermediate in nature, the level of rescue is greater in GluN1Cre females when compared to GluN1Cre males (interaction between sex and genotype). Assessment of potential differences in plasticity, mediated by sex factors, requires much further investigation in future studies. However, thoughtful planning of this study was undertaken, with sufficient group sizes to ensure that any potential sex differences in behaviour or biochemistry could be elucidated. When delving further into sex effects, it is important to consider a female’s reproductive status and ovarian cycle when studying disease and/or pharmacological effects of drugs (Becker et al., 2005). This was not taken into account in this study, and should be considered for future work.
33.2 Tight Temporal Regulation of Cre Induction

An important component for the accuracy of this study is the tight regulation and control of Cre-recombinase induction following tamoxifen administration. As previously mentioned, tight regulation of Cre-recombinase activation was confirmed via the use of a Cre reporter line \textit{(ROSA26^{tdTomato})}. As seen in Figure 13, there is robust and uniform expression of the red fluorescent tdTomato protein following treatment with tamoxifen, and no expression following treatment with corn oil (vehicle). Therefore, we are confident that the Cre-recombinase protein is tightly regulated via tamoxifen and does not become endogenously activated in its absence. Furthermore, the presence and expression of the Cre-recombinase protein itself does not have any effect on behaviour or biochemical expression of the NMDA receptor. We confirmed this by having all experiments completed with a WTCre control group. This study showed that WT and WTCre were both behaviourally and biochemically equivalent. Testing demonstrated that there was no effect of the presence of Cre-recombinase alone on behaviour or expression of the NMDA receptor.

34 Future Directions and Experiments

34.1 Untested Behavioural Paradigms in GluN1-IR Study

This study covered a wide breadth of behavioural tests, to ensure a thorough snapshot of behavioural rescue following intervention at two key developmental time points. However, there still remain a number of behavioural paradigms that need to be tested to gain a fuller understanding of the rescue of specific circuits and brain regions. As previously mentioned in section 30.4, it would be highly beneficial to understand rescue of spatial memory (Morris water maze, 8-arm radial maze), anxiety behaviours (Light-Dark box, larger arena for Center Time), as well as behaviours not previously mentioned; rescue of reward circuitry (conditioned place preference, Pavlovian conditioning), and depression-like behaviours (Porsolt forced swim test or tail suspension test). A larger battery of behavioural testing would give a better superficial understanding of the state of rescue in specific circuits and brain regions.

34.2 Epigenetic Control of the \textit{Grin1} Locus

As mentioned in section 31, we hypothesize that the limited levels of excision seen in regards to the insertion mutation could potentially be due to epigenetic modification of the \textit{Grin1} locus. To
test this, a study needs to be undertaken that investigates the levels of tamoxifen-induced Cre-recombinase-mediated excision following pre-treatment with either a methyl-transferase inhibitor or an HDAC inhibitor. Due to the fact that epigenetic modifying pharmacological agents result in behavioural modifications themselves (Qiu et al., 2017), it would not be practical to repeat all the behavioural paradigms listed above in the presence of either HDAC inhibitors or methyl transferase inhibitors. Any behavioural rescue observed could not be distinguished from NMDA receptor rescue or epigenetic modification. However, it would be reasonable to treat animals concomitantly with either a methyl transferase inhibitor or an HDAC inhibitor, and then the established tamoxifen treatment. Following a two-week washout period (as that seen in the adult+4 week intervention group), the excision levels of the insertion mutation could be measured via the previously described qPCR strategy (section 24). If excision levels increase in the presence of epigenetic modifying pharmacological agents, then it would be reasonable to conclude that limited excision is due to secondary chromatin structure; epigenetic modifications that block access to the loxP sites flanking the insertion mutation.

34.3 Other Cre-recombinase Mouse Lines

In future studies it would be very interesting to study the consequences of cell-selective rescue of NMDA receptors using other Cre-recombinase mouse lines. For example, we can study the contribution of interneurons to the phenotypes of GluN1 knockdown mice. Compared to the abundant glutamatergic projection neurons found in the cortex, GABAergic interneurons only make up about 20% of the population of cortical neurons. However, GABAergic interneurons have been shown to play a crucial role in regulating the balance, flexibility, and functional architecture of cortical circuits (Taniguchi et al., 2011). As previously mentioned in section 5.1, glutamate dysfunction at the NMDA receptor leads to GABAergic dysfunction, resulting in a state of ‘disinhibition’. A large body of research suggests that this GABAergic dysfunction leads to perturbed inhibitory outputs and network oscillations, which manifest as behavioural symptoms that contribute to social and cognitive abnormalities (Gandal, Sisti, et al., 2012; Gandal, Anderson, et al., 2012). Therefore, by taking advantage of Cre-recombinase expression under the regulation of a GABA interneuron specific promotor, one could study the role that GABAergic interneurons play in mediating these behavioural dysfunctions. More succinctly, one could address whether rescue of disinhibition caused by NMDA receptor hypofunction could lead to rescue of cognitive and social deficits seen in neurodevelopmental disorders. Plasticity of
these inhibitory circuits could be further elucidated by using a tamoxifen-inducible Cre-recombinase under a GABAergic specific promotor, allowing for the investigation of how plastic adult inhibitory circuits are.

To specifically express Cre-recombinase in GABAergic interneurons only, a GABAergic promotor is necessary. GABA is synthesized by two isoforms of glutamic acid decarboxylases in mammals, GAD67 and GAD65. These decarboxylases are encoded by Gad1 (GAD67) and Gad2 (GAD65), and are co-expressed in most brain regions (Soghomonian and Martin, 1998). Taniguchi et al. (2011), have generated both a Gad2-ires-Cre and Gad2-CreER driver mouse lines. The Gad2-ires-Cre mouse line has Cre-recombinase expression throughout development in GABAergic neurons, as well as certain non-neuronal cells. The Gad2-CreER mouse line has robust expression of Cre-recombinase in GABAergic neurons throughout the brain following tamoxifen administration. Recombination efficiency can be adjusted based on tamoxifen dosage. Together, these two Cre-recombinase mouse lines, under GABAergic promotor control, provide robust and flexible genetic tools to manipulate GABAergic neurons in the mouse CNS.

35 Clinical relevance of study

Although previous studies have sought to investigate adult plasticity and rescue (Guy et al., 2007; Silverman et al., 2012; Yin et al., 2013; Mei et al., 2016), to the best of our knowledge, this is the first study to investigate a global rescue of NMDA receptor deficiency, specifically in a temporal fashion. We sought to determine how plastic the brain is and if there is a difference in plasticity in adolescence versus adulthood. We found that while plasticity is not dependent on age, it is dependent on brain region. This study highlights the potential for accessing intrinsic plasticity mechanisms present in specific circuits, in specific brain regions that underlie specific behaviours. From a therapeutic stand point, this information is instrumental in helping to focus strategies in the amelioration of symptoms that arise from miswiring during development in neurodevelopmental disorders. Some symptoms may inherently be more amenable to change, and therefore respond better to treatment, while others may be more resistant. It is reasonable to infer that treating disrupted molecular and cellular mechanisms in adulthood can result in dramatic improvements in some behavioural phenotypes and function. While other mechanisms may, at best, have intermediate improvement. This would translate into not quite physiological levels, but a level of improvement that may still be clinically relevant. This study helps to
identify these specific symptoms that may be more plastic in nature, and therefore capable of rescue. In terms of neurodevelopmental disorders, it is important to acknowledge that despite developmental miswiring of circuits, at least in terms of the glutamate system, adult rescue results in some degree of plasticity and the correction of specific developmental pathologies.

As mentioned above, it is not unreasonable that this study did not find a straight-forward answer to the question of whether or not developmental impairments can be overcome in adulthood. The answer is far more complicated. A number of studies have shown that both pharmacological and genetic rescue strategies in adulthood can lead to the correction of phenotypic dysfunctions (Ehninger et al., 2008). However, a majority of these studies have reported diverse results in terms of rescue; some report full, partial, and even no change in deficits, similar to what was seen in this study. When attempting to correct neurodevelopmental impairments in the brain, it is important to consider that the brain exhibits multiple critical periods, spread across different brain regions, supported by different neurotransmitter systems, with synapse-specific impairments (Meredith, 2015). Even more specifically, schizophrenia is a very variable disorder, manifesting differently between patients. No one single molecular target has high reproducibility for the cause or treatment of schizophrenia, and it remains etiologically diverse (Horváth and Mirnics, 2014). Therefore, when looking to target specific symptoms, it is important to consider the unique nature of each symptom, its reinstatement parameters, and the intrinsic plasticity mechanisms that may be specific to that symptom.
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## Appendix A. Optimization of Tamoxifen Administration

<table>
<thead>
<tr>
<th>ROUTE OF ADMINISTRATION</th>
<th>Intraperitoneal (i.p. Injection)</th>
<th>Oral Gavage</th>
<th>Subcutaneous (s.c.) Injection</th>
<th>Tamoxifen Chow</th>
<th>Tamoxifen Chow &amp; Oral Gavage</th>
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<tr>
<td></td>
<td>Alternating day</td>
<td>Every day</td>
<td>Student</td>
<td>DOM</td>
<td>Every day for 14 consecutive days</td>
</tr>
<tr>
<td>Cohort Name</td>
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<td>COHORT B</td>
<td>COHORT C</td>
<td>COHORT D</td>
<td>COHORT E</td>
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<td></td>
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<td></td>
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<td>Days 1, 3, 6</td>
<td>Days 1, 3, 5</td>
<td>Days 1 - 5</td>
<td>Days 1 and 2</td>
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</tr>
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<td>Difficult, single injection needed daily</td>
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  - No rescue
  - CTX - 5% increase SSTR - 2% increase NR1 - 5% increase
  - CTX - 9% increase SSTR - 2% increase NR1 - 5% increase
  - No rescue
  - CTX - 9% increase SSTR - 2% increase NR1 - 5% increase
- **RESCUE OF BEHAVIOUR**
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  - No rescue
  - No rescue
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  - No rescue
- **PPI**
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  - Not tested
  - Not tested
  - Not tested
  - Not tested
  - Not tested

### Available as a regimen
- No
- Maybe
- Maybe
- No
- No
- Yes
Appendix B.

Diazepam improves aspects of social behaviour and neuron activation in NMDA receptor-deficient mice

C. A. Mielnik, W. Horsfall and A. J. Ramsey

Department of Pharmacology and Toxicology, University of Toronto, Toronto, ON, Canada

*Corresponding author: A. Ramsey, Department of Pharmacology and Toxicology, University of Toronto, J. King’s Circle, Rm 4302, Medical Sciences Building, Toronto, ON, Canada M5S 1A8. E-mail: a.ramsey@utoronto.ca

NRI1 knockout (NRI1KD) mice are genetically modified to express low levels of the NR1 subunit of N-methyl-D-aspartate (NMDA) receptors, and show deficits in affiliative social behaviour. In this study, we determined which brain regions were selectively activated in response to social stimulation and asked whether differences in neuronal activation could be observed in mice with reduced sociability. Furthermore, we aimed to determine whether brain activation patterns correlated with the amelioration of social deficits through pharmacological intervention. The cingulate cortex, lateral septal nuclei, hypothalamus, thalamus and amygdala showed an increase in c-Fos immunoreactivity that was selective for exposure to social stimuli. NRI1KD mice displayed a reduction in social behaviour and a reduction in c-Fos immunoreactivity in the cingulate cortex and septal nuclei. Acute clonazepam did not significantly alter sociability; however, diazepam treatment did increase sociability and neuronal activation in the lateral septal region. This study has identified the lateral septal region as a neural substrate of social behaviour and the GABA system as a potential therapeutic target for social dysfunction.

Keywords: Antipsychotic, benzodiazepine, GABA, glutamate, neural substrate, NMDA, social behaviour

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Sociality is defined as the tendency to seek out social interactions (Caldwell 2012). In terms of the neural substrates that regulate sociability, there are striking similarities across the vertebrate species (Goodson 2005, Newman 1999). A number of studies have outlined the ‘social behaviour network’, which is comprised of the medial amygdala, bed nucleus of stria terminalis (BNST), lateral septum, medial preoptic area, the anterior hypothalamus, the ventromedial hypothalamus and the paraventricular grey (Goodson 2005, Newman 1999). Each of these nodes of the ‘social behaviour network’ have all been implicated in the mediation of a number of social behaviours including aggression, sexual behaviour, various forms of communication, social recognition, affiliation, bonding, parental behaviour and responses to stressors (Goodson 2005).

Because humans are highly social animals, deficits in social interaction are often debilitating, and as such are diagnostic features of autism and schizophrenia. Social withdrawal in schizophrenia is one of the symptoms that does not respond well to pharmacological treatments and this precludes patients from participating in normal daily activities (Yogar 2012). To improve treatment for social withdrawal, it is important to delineate the areas of the brain that are responsible for different aspects of social interaction, and study how pharmacological interventions affect functioning of these areas.

In this study, we wanted to determine how the neural activity of the social brain is affected by impaired N-methyl-D-aspartate (NMDA) receptor signalling, which has been proposed to contribute to the social deficits observed in schizophrenia and autism (Gandal et al. 2012). NMDA receptor (NMDAR) antagonists, such as phencyclidine (PCP), are known to induce social withdrawal in humans and animal models (Corbett et al. 1999); however, acute antagonism is not likely to fully model chronic diseases such as schizophrenia. Therefore, we used a genetic mouse model of NMDAR deficiency, the NRI1 knockdow (NRI1KD) mouse, to understand how the social brain is impacted.

NRI1 knockdow mica (Kopp et al.) have only 10-15% of wildtype levels of NMDARs because of a hypomorphic mutation in the gene that encodes the essential NRI subunit of these receptors (Mohn et al. 1999). Studies from multiple laboratories have demonstrated robust alterations in social interaction in a number of behavioural paradigms, including resident-intruder assays and a modified three-chamber sociability paradigm (Dutson et al. 2004, 2009, Halene et al. 2009, Mohn et al. 1999, Ramsey et al. 2011). In this study, we compared the pattern of neural activation in NRI1KD mice with that in wildtype mice in response to a non-threatening, novel, social stimulus. This study focused on the lateral septum and cingulate cortex, two brain regions that have been previously identified to regulate social interaction (Goodson 2005). In addition, we studied the ability of two drugs, clozapine and diazepam, to normalize social interactions and patterns of neuronal activation within these brain regions.

Materials and methods

Animal subjects
Adult 0–15-weeks male mice (NRI1KD or wildtype littermates) were used. NRI1KD mice express low levels of the NRI subunit of the NMDAR, the hypomorphic mutation is achieved by the targeted insertion of a neomycin cassette into intron 17 of the NRI1 sequence.
Appendix B.

Figure 1: Identification of social regions in mouse brain. Neuron activation patterns were determined by o-Pos immunoreactivity 1 h post-exposure to a social stimulus (social) or an empty arena (non-social). The brain regions that were more activated by social stimulus, than non-social stimulus, include the amygdala, cingulate cortex, hypothalamus, lateral septum and thalamus. Illustrations of sagittal sections at 0.12 and 1.92 mm from the midline demonstrate the location of brain structures that were analysed. Coronal micrographs at 10X magnification depict representative images of o-Pos immunoreactivity.

Drug administration
Cloperil (Sigma Aldrich, St. Louis, MO, USA, Cat. No. C0205) was dissolved in saline acidified with glacial acetic acid (1%) to a final 0.1 mg/ml solution. Experimental animals were administered clozapine (1.0 mg/kg) via intraperitoneal ip injection 1 h prior to starting the social affilative behaviour paradigm. Diazepam (Toronto Research Chemicals, Toronto, ON, Canada, Cat. No. D16835) was dissolved in 35% ethanol to make a 5 mg/ml solution, subsequently prepared to a final concentration of 0.1 mg/ml in saline solution. Experimental animals were administered diazepam (2.0 mg/kg) via ip injection 30 min prior to starting the social affilative behaviour paradigm.

Social affiliative behaviour paradigm
Male wildtype and NR1KD mice aged 13–15 weeks were assessed in a social affiliative behaviour paradigm to measure sociability. All behavioural tests were completed between 0800 and 1200 h. Sociability was measured via video recording using gender and age-matched mice (C57BL/6J) as test subjects as social stimuli. The experimental mice (wildtype or NR1KD)
Appendix B.

![Image of a mouse with labeled regions]

Figure 2: Methods for the quantification of sociability in the social affiliation behaviour paradigm and the semi-automatic quantification of activated neurons. (a) Quantification of sociability via video recording using gender and age-matched mice (C57BL/6J). The mouse novel to the test subject is social stimulus. The experimental mouse (wild type or NR1 KD, drug-treated or vehicle) was allowed to explore the open arena (opaque white walls, 62×42×22 cm) for 10 min. The arena contained two inverted wire cups: one containing stimulus mouse (social) area and the other empty (non-social) area. (b) Semi-automatic quantification of activated c-Fos immunoreactive neurons in the cingulate cortex and the lateral septal region of c-Fos immunoreactive mice were quantified in a semi-automated manner using Nisham Elements software at a magnification of 20x. The two brain regions selected for quantification were cingulate cortex (0.13 mm²/field), 12 fields total (2 hemispheres), 1.6 mm² total area and lateral septal region (0.13 mm²/field), 4 fields total (2 hemispheres), 0.53 mm² total area, 40-μm coronal sections, 1.0–0.3 mm Bregma.

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drug-treated or vehicle) was allowed to explore the open arena (opaque white walls, 62×42×22 cm) for 10 min. The arena contained two inverted wire cups (as described by Crawley (2004)), one containing a stimulus mouse (social) area and the other empty (non-social) area. This was a modified version of the three-chamber social approach paradigm, as described by Nadler et al. (2004), where there were no dividing walls (forming three chambers) in the arena. Instead, the area is one chamber where the test mouse freely explores the open area and wire cups (social and non-social) (Fig. 2a). Over the 10-min period, the test mouse was video-recorded and its movements were tracked using Bioverview Viewer (version 2) software. Sociability was measured as follows: (1) time spent in social zone, which is the time spent in the 3 cm zone around the cup containing the social stimulus mouse, (2) social investigation, which is the difference between time spent in social investigation (social zone) and time spent in non-social area per visit, which is the time spent in the social zone per visit divided by the number of visits.

c-Fos immunohistochemistry
Neuronal activation was quantified using induction of c-Fos expression as a biochemical marker as described Bullitt (1991), protein product can be detected 20–90 min following neuronal activation. 60 minutes (median of time range) for the detection of protein product following completion of the social affiliation behaviour paradigm described above, mice were anesthetized with 250 mg/kg
Appendix B.

Diazepam improves social behaviour of NMDA receptor-deficient mice

![Graph showing the effect of diazepam on social behavior](image)

Statistical analysis

Data for sociability were analysed using a two-tailed Student's t-test comparing measures of sociability with respect to genotype (wild type or NR1KD) or drug treatment (NR1KD vehicle compared with NR1KD drug). All data for each brain region were analysed using a two-tailed Student's t-test, comparing factors of genotype (Fig. 4) or drug treatment (Figs. 6, 8). For all comparisons, data are represented as the mean ± SEM and significance set at P < 0.05. Statistically significant differences between behaviour and c-Fos immunohistochemistry were excluded from data using Grubb's test (Fig. 3c) – one excluded from NR1KD, Fig. 5a – one excluded from NR1KD, Fig. 5c – one excluded from NR1KD, Fig. 7b – one excluded from NR1KD, Fig. 8a – one excluded from NR1KD, and diazepam, Fig. 7b – one excluded from NR1KD diazepam. Statistical analyses were performed in GraphPad Prism 6.

Results

c-Fos immunoreactivity identifies brain regions that are selectively activated by interaction with a novel mouse
c-Fos immunoreactivity is routinely used as an indicator of neuronal activity, as nuclear c-Fos protein is produced 20–90 min following intense neuron activity (Buttimore 1990). In this study, wildtype mice were exposed to a novel mouse to measure c-Fos immunoreactivity and identify the brain regions that are activated following social stimulation. In this paradigm, the tested mouse was placed in an open arena with two inverted wire cups, one empty and one containing a novel male mouse, the social stimulus mouse. The genetic background of the social stimulus was C57BL/6J, which has been used in other social interaction paradigms because it displays highly sociable behaviour (Miyon et al. 2007). Test animals that spent at least 2 min engaged in social investigation were used for subsequent expression analysis (Fig. 1).

Because the test was performed in a novel arena with other non-social elements, such as the wire cups, a control group of wildtype males was tested in the same arena with two wire cups, but without the presence of the social stimulus mouse. This was done to control for neuron activation surrounding...
Appendix B.

Figure 4: NR1KD mice show a decrease in neuronal activation in the cingulate cortex and lateral septal region following social exposure. Activation (cell counts) of c-Fos-labeled neurons in WT and NR1KD male adult (13–15 week old) mice (a). Representative images of c-Fos immunoreactivity in the cingulate cortex and the lateral septal region (b). (a) Quantification of neuronal activation following exposure to the social affiliative behaviour paradigm in the cingulate cortex and the septal region (b). Representative images of neuronal activation and its semi-automated quantification (40 μm coronal sections, 1.0 × 0.3 mm Bregma). All data are represented as mean ± SEM. Numbers for each group are as follows: WT = 8, NR1KD = 6. *P < 0.05, **P < 0.01.

NR1KD mice show a decrease in sociability and neuronal activation

As the cingulate cortex and lateral septum were engaged during social interaction, we next asked whether there would be a change in the level of neuron activation within these regions in a mutant mouse with decreased sociability, the NR1KD mouse (Duncan et al. 2004; Halgren et al. 2009; Mohlin et al. 1999; Ramsey 2009; Ramsey et al. 2011). Wildtype and NR1KD mice were tested in the social affiliative behaviour paradigm. NR1KD mice showed a marked decrease in social behaviour as quantified by a decrease in the amount of time spent in the social zone (Fig. 3a; WT 311.0 ± 10.24 seconds, NR1KD 229.73 ± 16.98 seconds, t\(_{14}\) = 3.792, P = 0.0016). NR1KD mice also showed a decrease in time spent per visit to the social zone (Fig. 3c; WT 4.26 ± 0.38 seconds vs. NR1KD 2.73 ± 0.38 seconds, t\(_{14}\) = 2.609, P = 0.0138). NR1KD mice were not significantly different from wildtype mice when social investigation was measured as the difference between time spent in social minus time spent in non-social investigation, although there was a trend towards a decrease in social investigation (Fig. 3b; WT 179.10 ± 16.01 seconds vs. NR1KD 130.50 ± 26.06 seconds, t\(_{14}\) = 1.548, P = 0.1390). Levels of neuronal activation in the cingulate cortex and the lateral septal region correlated with the levels of sociability, as there was a marked...
Appendix B.

Diazepam improves social behaviour of NMDA receptor-deficient mice

**Acute clozapine does not increase social investigation or neuron activation in NR1KD mice**

Our previous study indicated that the antipsychotic clozapine could improve some aspects of social interaction in NR1KD mice using a resident-intruder behavioural paradigm (Mohn et al. 1999). Therefore, we sought to normalize social investigation in NR1KD mice by acute clozapine treatment. Clozapine (0.1 mg/kg, i.p.) vehicle 30 min prior to testing in the social affilative behaviour paradigm. Acute clozapine treatment did not increase sociality in the three measures used in this study. There was no significant change in the total amount of time spent in the social zone (Fig. 5a, NR1KD vehicle 176.3 ± 19.12 seconds, NR1KD clozapine 191.18 ± 15.39 seconds, $t_{10} = 0.6097, P = 0.5618$), or in social investigation (Fig. 5b, NR1KD vehicle 101.4 ± 20.77 seconds, NR1KD clozapine 124.70 ± 27.58 seconds, $t_{10} = 0.6509, P = 0.5403$) or in time spent per visit to the social zone (Fig. 5c, NR1KD vehicle 2.43 ± 0.26 seconds, NR1KD clozapine 3.759 ± 1.283 seconds, $t_{10} = 0.9633, P = 0.3475$). Furthermore, neuronal activation in the cingulate cortex and the lateral septal region remained unchanged (Fig. 6a, cingulate cortex – NR1KD vehicle 452.44 ± 66.89/mm², NR1KD clozapine 449.11 ± 52.79/mm², $t_{10} = 0.04836, P = 0.9622$ lateral septal region – NR1KD vehicle 355.90 ± 41.49/mm² NR1KD clozapine 444.09 ± 57.98/mm², $t_{10} = 1.155, P = 0.2566$).

**Acute diazepam increases sociality and neuronal activation in the lateral septal region of NR1KD mice**

We next asked whether benzodiazepines, which reduce social anxiety in humans (Pollack 1999), could improve the social behaviour of NR1KD mice. NR1KD mice were treated acutely with the benzodiazepine diazepam (0.1 mg/kg, i.p.) vehicle 30 min prior to testing in the social affilative behaviour paradigm. Acute diazepam treatment markedly increased sociality in NR1KD mice as measured by an increase in the amount of time spent in the social zone (Fig. 7a, NR1KD vehicle 152.67 ± 19.28 seconds, NR1KD diazepam 253.75 ± 25.98 seconds, $t_{10} = 2.559, P = 0.0101$), the time spent in social investigation (Fig. 7b, NR1KD vehicle 161.64 ± 10.37 seconds, NR1KD diazepam 191.00 ± 26.88 seconds, $t_{10} = 3.064, P = 0.0007$) and the time spent per visit to the social zone (Fig. 7c, NR1KD vehicle 2.50 ± 0.25 seconds, NR1KD diazepam 5.07 ± 0.95 seconds, $t_{10} = 2.288, P = 0.0390$). Of the two brain regions studied, neuronal activation in the lateral septal region showed the stronger correlation with improved social behaviour. c-Fos immunoreactivity in the lateral septal region significantly increased with acute diazepam treatment prior to sociality testing (Fig. 8a, cingulate cortex – NR1KD vehicle 0.09 ± 24.76/mm², NR1KD diazepam 0.60 ± 24.12/mm², $t_{10} = 2.375, P = 0.0351$). This further supports a correlation between social behaviour (measured in terms of sociability) and neuronal activation patterns in two key neural substrates of social behaviour.

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[Image of Figure 5: Acute clozapine does not increase sociality in NR1KD mice.](https://example.com/figure5)

[Image of Figure 6: Acute clozapine does not increase neuron activation in NR1KD mice.](https://example.com/figure6)

[Image of Figure 7: Acute diazepam increases sociality and neuron activation in the lateral septal region of NR1KD mice.](https://example.com/figure7)
Appendix B.

Discussion
The NR1KD mouse line is a developmental model of NMDAR hypofunction, in which a genetic disruption of NMDAR signalling leads to exteroceptive behavioural abnormalities. Behavioural impairments include reduced social interactions and mating, locomotor hyperactivity, deficits in sensorimotor gating, self-injury and cognitive inflexibility (Duncan et al. 2004; Gandal et al. 2012a; Mohn et al. 1999). The profound reduction in social interactions within these mice is one of the most severe phenotypes observed (Gandal et al. 2012a). This decrease in sociability is not due to other confounding behaviours, such as hyperlocomotion, fear or increased anxiety; NR1KD mice exhibit a higher tendency to explore an open environment (elevated novel maze and open field tests) (Halene et al. 2009). They also show little preference for a novel social stimulus over a novel unmanipulated object based on the social investigation score in this study. Furthermore, these mutant mice exhibit reduced huddling in home cages with cagemates during sleep, reduced social investigation of a resident intruder and reduced mating behaviour (Halene et al. 2009; Mohn et al. 1999). Therefore, the NR1KD mouse model is a robust model of reduced sociability, and a useful tool to identify neural substrates implicated in social behaviour.

This study was set out to identify key neural substrates of social affiliative behaviour, and to determine whether there is a relationship between activation of these substrates and changes in social behaviour. In this study, we identified several neural substrates from regional in adult male wildtype mice that showed selective activation in response to a social stimulus, including the amygdala, cingulate cortex, hypothalamus, lateral septal region and the thalamus (Fig. 11). For this study, we focused on the cingulate cortex and the lateral septal region because of their known implications in social behaviour. NR1 knockdown adult male mice, a known model of decreased sociability (Duncan et al. 2004; Halene et al. 2000; Mohn et al. 1999, Ramsey 2009, Ramsey et al. 2011), showed a reduction in social affiliative behaviour (as measured in time spent in social zone, social investigation and time spent per visit) (Fig. 9) as well as a decrease in neuronal activation in both the cingulate cortex and the lateral septal region when compared with wildtype littermate controls (Fig. 4). Acute clozapine treatment did not ameliorate decreases in sociability in adult male NR1KD mice (Fig. 10).
Appendix B.

Diazepam improves social behaviour of NMDA receptor-deficient mice

Figure 7: Acute diazepam increases sociability in NR1KD mice. Behaviour in the social interaction behaviour paradigm during a 10 min trial in NR1KD male adult (13-15 weeks) mice treated with either acute diazepam (2.0 mg/kg) or vehicle (a, b, c). (a) Measure and comparison of total amount of time spent in social zone (black bars). (b) Measure and comparison of social interaction loop with social stimulus, social zone and time spent in non-social novel object investigation (empty cup, non-social zone) (investigation time in social zone—time in non-social zone). (c) Measure and comparison of time spent in social zone per visit made (total time spent in zone/number of visits; black bars). All data are shown as means ± SEM. Numbers for each group are as follows: (a) NR1KD vehicle = 12, NR1KD diazepam = 15; (b) NR1KD vehicle = 12, NR1KD diazepam = 14; (c) NR1KD vehicle = 12, NR1KD diazepam = 15. * P < 0.05, ** P < 0.01.

and caused no significant change in neuronal activation patterns in either the cingulate cortex or the lateral septal region when compared with vehicle controls (Fig. 6). Importantly, acute diazepam treatment did increase sociability in male NR1KD mice (Fig. 7), and also increased neuronal activation in the lateral septal region, but not in the cingulate cortex (Fig. 8).

To test sociability and quantify aspects of social behaviour, we used a modified version of the three-chamber social approach paradigm as previously described by Hadler et al. (2004). The social approach paradigm was chosen as a measure of sociability as there is no direct contact between mice (preventing confounding factors of aggression and fighting). Furthermore, this paradigm allows for automated scoring of sociability, rating out subjectivity and bias from the experimenter (Hanks et al. 2013). The modified version of the three-chamber social approach paradigm abolishes the ‘chamber’ aspect (two walls divide the arena and instead employs an open arena that contains two inverted vases cups, one representing the ‘social’ zone and the other the ‘non-social’ zone (Fig. 2a). This modified version of the social approach paradigm tracks ‘cylinder’ scores (time spent in a small area around the cylinder), rather than ‘chamber’ scores (time spent in respective chamber), to quantify sociability. These scores show a higher correlation with time spent in social interaction as well as a higher reliability and validity when compared with chamber scores (Ferreira et al. 2011). Moreover, chamber scores can be subject to confounds such as grooming in the ‘social’ chamber, without any actual social investigation of the stimulus mouse (Hanks et al. 2013).

The observed reduction in social behaviour in NR1KD mice, and reduction in neuronal activation in the identified neural substrates, was not ameliorated with acute diazepam. The failure of clozapine to improve social approach supports some previous studies in NR1KD mice with atypical antipsychotics, and is also similar to clinical findings in patients treated with clozapine (Hanks et al. 2013). Although clozapine was effective to reduce escape behaviours of NR1KD mice in the resident-intruder paradigm, it did not improve approach-oriented behaviours in that study (Mohn et al. 1999). Despite the fact that clozapine has been shown to be somewhat more effective than other antipsychotics in improving negative symptoms and cognitive deficits of schizophrenia (Ferreira et al. 2011), previous rodent studies have reported variable results in the ability of clozapine to ameliorate social deficits (Labone et al. 2000).

Unlike clozapine, acute diazepam increased sociability (time spent in social zone, social interaction and time spent per visit) (Fig. 7). Diazepam also increased neuronal activation in the lateral septal region (Fig. 8). Diazepam is a classic benzodiazepine, acting as a positive allosteric modulator at gamma-Aminobutyric acid (GABA_A) receptors. Interestingly, several recent studies have highlighted the potential for GABA modulation (both GABA_A and GABA_B receptors) as a treatment for symptoms of syndromic autism, specifically cognitive deficits and social withdrawal (Gandal et al. 2012b, Han et al. 2014, Sandhu et al. 2014). In fact, a recent study has shown that baclofen (GABA_B receptor agonist) improves social behaviour in NR1KD mice in a dose-dependent fashion (Gandal et al. 2012b).
Appendix B.

There is a long history of benzodiazepine use in patient populations with schizophrenia, either as a monotherapy or, more commonly, as an adjunctive therapy to antipsychotics (Bardhan et al. 2011; Cardet al. 2006; Dodd et al. 2002). It is evident that GABA modulation has some beneficial effects in ameliorating symptoms in this population; however, the underlying mechanism, whether as a monotherapy or in an adjunctive capacity, is not well known. In states of NMDAR hypofunction, low-dose GABA agonists may improve behaviors by increasing inhibitory transmission and correcting the imbalance between excitation and inhibition, as observed in NR1KD mice. This study provides additional support for the suggestion that non-sedating, non-anxiolytic doses of GABA-positive modulators could be effective for schizophrenia and autism symptoms (Gandal et al. 2012b; Levitt et al. 2004; Leves et al. 2005; Yizhar et al. 2011).

Diazepam also increased the activation of neurons in the lateral septum, a key component of the limbic system. The septum is involved in a number of functions, including learning and memory, emotions, fear and reward-seeking. More specifically, the lateral septal nucleus has strong projections to midbrain and hypothalamic regions, implicating strong associations in the modulation of social behaviour (Ophir et al. 2009; Vermeiren & Neumann 2008). Studies have shown that vasopressin-specific activation of the lateral septal region is critical for normal social recognition (Belsky et al. 2000). GABAergic projections from the lateral septal to the anterior hypothalamus are important for the regulation of anxiety-like behaviors, undoubtedly contributing to overall social behavior (Anthony et al. 2014). Therefore, by enhancing GABAergic signalling through diazepam treatment, it is reasonable to believe that a direct effect on the activation of neurons in the lateral septal region, as observed through neuronal activation patterns in this study (Fig. 8), reverses the decrease in sociability seen in the NR1KD mice.

Although we have identified the lateral septal region as a neural substrate of social behavior, and the GABA system as a potential therapeutic target for social dysfunction, there

Diazepam improves social behaviour of NMDA receptor-deficient mice

remain a number of other neural substrates that need to be investigated to gain a complete understanding of the circuitry and brain regions involved in social behaviour. Despite having solely investigated male–male non-aggressive social behaviour in this study, it is imperative to further examine the differing nuances of other social behaviours, such as maternal, aggressive behaviours, etc. Therefore, it will be beneficial to continue this study, investigating other neural substrates, as well as other social contexts, to gain a more rounded interpretation of possible targets in the treatment and amelioration of dysfunction in social behaviour.

References


Appendix B.
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Figure 1. Signaling pathways of metabotropic glutamate receptors (mGluRs). Taken from Pharmacological Research by Società italiana di farmacologia Reproduced with permission of ACADEMIC PRESS in the format Thesis/Dissertation via Copyright Clearance Center.

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Figure 4. Schematic outline and model of developmental brain dysfunction, including etiological factors and clinical symptomatic manifestations. Taken from Lancet Neurology by LANCET PUBLISHING GROUP. Reproduced with permission of LANCET PUBLISHING GROUP in the format Thesis/Dissertation via Copyright Clearance Center.
Figure 5. Disinhibition phenomenon in the glutamate hypothesis of schizophrenia.

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Confirmation Number: 11628198
List of Publications Completed During PhD

Peer Reviewed Publications
Journal Articles


Non-Peer Reviewed Publications
Book Chapters

(7) Mielnik CA and Ramsey AJ. (2015). Drugs that target the glutamate synapse: Implications for the glutamate hypothesis of schizophrenia. In Lipina TV and Roder JC (Eds.), Drug Discovery for Schizophrenia. Cambridge, United Kingdom: Royal Society of Chemistry.