Molecular and Synaptic Alterations in the Striatum of GluN1-Deficient Mice

by

Yuxiao Chen

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

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Abstract

N-methyl-D-aspartate receptors (NMDARs) are essential for proper neurodevelopment and cognitive function. NMDAR dysfunction contributes to many neurological disorders, with striatal changes linked to psychosis and cognitive impairment. GluN1 knockdown (GluN1KD) mice have low NMDAR expression, striatal synaptic deficits, and cognitive behavioural abnormalities. How these phenotypes functionally relate to one another is unclear. We studied molecular and synaptic changes in the GluN1KD striatum to understand the molecular underpinnings of GluN1KD behaviours. From a screen of Rho GTPase pathway proteins, which regulate synapses and cognition, Ras-related C3 botulinum toxin substrate 1 (Rac1) signaling proteins were specifically identified. GluN1KD mice at 6 and 12 weeks of age consistently had less Rac1 and its downstream effector Wiskott-Aldrich syndrome protein-family verprolin homologous protein 1 (WAVE-1). These deficits were developmentally aligned with medium spiny neuron (MSN) spine density deficits and previously published behavioural abnormalities. To assess the biological and behavioural significance of these findings, we restored WAVE-1 levels by intercross-breeding GluN1KD mice and WAVE-1 overexpressing (WAVE-Tg) mice. GluN1KD-WAVE-Tg F1 hybrids had improved WAVE-1 levels and spine density at the striatum, as well as better Y-maze and 8-arm radial maze performance. These phenotypic recoveries were not mirrored by WAVE-1 levels in the hippocampus nor by neuronal dendritic
spines in cornu ammonis 1. From a broader RNAseq analysis of the GluN1KD striatal transcriptome, both neurodegeneration-related and cell-growth signaling pathways were distinguished as affected by NMDAR knockdown. These transcript changes were greatly sex-dependent, potentially mediated by aberrations of estrogen receptor α signaling. Our data suggest that different molecular changes in male and female GluN1KD mice contribute to similar behavioural abnormalities. Restoring WAVE-1 at the striatum improved some of these phenotypes, pointing to a nuanced relationship between its function, striatal synaptic plasticity, and cognition. Specifically, striatal WAVE-1 and MSN spine density may be important for goal-directed maze exploration in mice.
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Appendix 1: Supplemental Figures and Tables

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List of Abbreviations

2-DG : 2-deoxyglucose
AD : Alzheimer's disease
AKT : protein kinase B
AMPA : α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid
AMPA receptor
ARC : activity-regulated cytoskeleton-associated protein
Arp2/3 : actin related protein 2/3
ASD : autism spectrum disorder
ATD : amino-terminal domain
BH : Benjamini-Hochberg
CA1 : cornu ammonis 1
Cacnb2 : Ca\(^{2+}\) voltage-gated channel auxiliary subunit β2
CaMK : Ca\(^{2+}\)/calmodulin-dependent protein kinase
cAMP : cyclic adenosine monophosphate
CBP : CREB-binding protein
Cdc42 : cell division control protein 42 homolog
CDK5 : cyclin-dependent kinase 5
CNS : central nervous system
CREB : cAMP response element-binding protein
CTD : carboxy-terminal domain
CTX : cortex
CytC : cytochrome c
d\(\text{D}1\)R : DA receptor D1
d\(\text{D}2\)R : DA receptor D2
DA : dopamine
DAT : DA transporter
Ddx3y : DEAD-Box Helicase 3, Y-linked
DISC1 : disrupted in schizophrenia 1
Drd2 : DA receptor D2 (gene)
Drp1 : dynamin related protein 1
EEG : electroencephalography
Eif2s3y : eukaryotic translation initiation factor 2 subunit 3, Y-linked
EPSP : excitatory postsynaptic potential
ERK : extracellular signal–regulated kinases
ERP : event-related potentials
Erα : estrogen receptor α
Esr1 : estrogen receptor α gene
FoxO : forkhead box, class O
GAP : GTPase-activating protein
GEF : guanine nucleotide exchange factor
GluN : glutamate NMDAR subunit
GluN1KD : GluN1 knockdown
Grin/GRIN : glutamate receptor ionotropic, NMDA
GTP : guanosine triphosphate
HD : Huntington’s disease
HPC : hippocampus
YLD : years lived with disability
Chapter 1
Introduction

1.1 The N-methyl-D-aspartate Receptor

1.1.1 N-methyl-D-aspartate Receptor Characterization and Organization

The N-methyl-D-aspartate (NMDA) receptor (NMDAR) is an ionotropic glutamate receptor and a nonspecific cation channel (Pachernegg et al., 2012; Paoletti et al., 2013; Scheefhals and MacGillavry, 2018). It is named after an early-identified potent agonist that was also a mimic of glutamate (Curtis and Watkins, 1961; Sucher et al., 1996). NMDARs are expressed in organisms throughout the animal kingdom (Sakarya et al., 2007; Alsaloum et al., 2016), and occupy a special place in neuroscience due to their essential roles in learning and memory (Newcomer et al., 2000; Whitlock et al., 2006; Baez et al., 2018).

NMDARs are heterotetrameric receptors. NMDAR subunit proteins are called glutamate NMDAR subunits (GluNs), which are encoded by genes named glutamate receptor ionotropic, NMDA (GRIN in humans; Grin in mice, which will be the major focus in this thesis). NMDARs are typically composed of two GluN1 subunits and some combination of GluN2 or GluN3 subunits (Köhr, 2006; Paoletti et al., 2013). There are four isoforms of GluN2 (A-D) and two isoforms of GluN3 (A-B), each encoded by their own gene (Grin2a-d and Grin3a-b, respectively) (Andersson et al., 2001; Teng et al., 2010). In contrast, GluN1 subunit variation is predominantly through alternative splicing of Grin1, resulting in eight different isoforms named GluN1-1a to GluN1-4a and GluN1-1b to GluN1-4b (Vrajová et al., 2010; Paoletti et al., 2013). For a complete NMDAR, subunits are likely assembled first into GluN1-GluN2/3 heterodimers before the two dimers are subsequently combined (Schüler et al., 2008). Variation in subunit combinations is a common mechanism by which NMDARs differ in signaling or function between different brain regions or cell types (Paoletti et al., 2013; Swanger et al., 2015; Alsaad et al., 2018), different locations in neurons (Köhr, 2006; Bouvier et al., 2015), and between biological samples of different ages (Dumas, 2005; Xing et al., 2006; Henson et al., 2008) and sex (Dong et al., 2007; Ridge et al., 2009; Gray et al., 2015).
Recently, crystal structures of NMDARs containing GluN1-GluN2B subunits have been solved (Karakas and Furukawa, 2014; Lee et al., 2014), though they required stabilizing mutations. One of these studies described the NMDAR as mushroom-shaped and being approximately 150 Å in height and 125 x 115 Å in width (Lee et al., 2014). In general, NMDARs have four main domains on top of one another: an amino-terminal domain (ATD) furthest away from the cell membrane; an extracellular ligand-binding domain (LBD) containing orthosteric binding sites right “below” the ATD; a transmembrane domain (TMD) that anchors the receptor and regulates cation permeability via Mg$^{2+}$ blockade; and an intracellular carboxy-terminal domain (CTD) for downstream signaling interactions (Burnashev et al., 1992b; Zhu and Gouaux, 2017; Gu and Wang, 2018). A representative schematic of NMDARs is shown in Figure 1.1. Beyond individual receptors, NMDARs have been reported to be partitioned into ~0.8MDa complexes or ~1.5 MDa supercomplexes at mammalian forebrain synapses, a synapse being the primary signaling connection between neurons (Jessell and Kandel, 1993; Rozental et al., 2000; Frank et al., 2016).
Figure 1.1. Representative schematic of NMDARs. NMDARs are heterotetrameric non-selective cation channels whose subunits determine much of their characteristics. In general, NMDA receptors are composed of two GluN1 and two GluN2 subunits (left). Incorporation of GluN3 subunits generally decreases Ca\(^{2+}\) influx and strength of Mg\(^{2+}\) block (right). Different roles and functions of NMDARs in different tissues, cell positions, and other contexts can largely be facilitated by varying subunit compositions. The CTDs of GluN subunits can bind to various scaffolding and downstream signaling proteins, not detailed in specifics in this image. Binding sites in LBDs are orthosteric sites for endogenous glutamate, glycine, and D-serine while various drugs can bind to NMDARs at either the ATD or LBD. Abbreviations: ATD - amino-terminal domain, LBD - ligand-binding domain, TMD - transmembrane domain, CTD - carboxy-terminal domain. Figure was constructed with information from current NMDAR literature (Cull-Candy and Leszkiewicz, 2004; Paoletti et al., 2013; Sanz-Clemente et al., 2013; Pérez-Otaño et al., 2016; Alam et al., 2017; Zhu and Gouaux, 2017).
NMDARs are not limited to synapses on neurons, nor to only neurons (Murugan et al., 2013; Bouvier et al., 2015; Hogan-Cann and Anderson, 2016). Indeed, current knowledge of NMDARs points to a high level of complexity and nuance – such as subunit composition in crystallography and brain region specificity in protein-interaction studies exemplified above. In addition to the complexity presented by different subunits (Paoletti et al., 2013; Sanz-Clemente et al., 2013) and brain region differences (Newcomer et al., 2000; Frank and Grant, 2017), NMDARs have multifaceted activation mechanisms (Traynelis et al., 2010; Cummings and Popescu, 2015), location-dependent functions and signaling in neurons (Bouvier et al., 2015; Scheefhals and MacGillavry, 2018) and non-neuronal cells of the brain and periphery (Du et al., 2016; Hogan-Cann and Anderson, 2016). The research presented in this thesis investigates the molecular, cellular, and functional consequences of a whole-body NMDAR deficit in mice as well as how such consequences may be attenuated. Thus, while some NMDAR complexities are not specifically addressed in this experimental body of work, it is still important to appreciate how NMDARs can vary in structure, organization, and function in order to establish context.

While a vast number of investigations relate NMDARs of various subunit compositions to different experimental outcomes in specific biological contexts, some general trends have emerged. The LBD of GluN2 subunits accept glutamate at its orthosteric binding site while GluN1 and GluN3 bind to glycine (Yao et al., 2013). GluN1 isoforms are expressed throughout the central nervous system (CNS) and primarily arise from differential splicing of exons 5, 21, and 22 (Okabe et al., 1999; Hansen et al., 2017; Yi et al., 2018). GluN1 isoforms that differ in exon 5 are particularly important, as those without exon 5-coded motifs in their ATDs (GluN1-4a) are more sensitive to pH changes and have slower deactivation speed (Regan et al., 2018). GluN2 subunits have been much more heavily studied. In general, higher conductance, higher Ca$^{2+}$ permeability and faster deactivation is seen with GluN2A- and GluN2B-containing NMDARs than GluN2C or GluN2D NMDARs (Wyllie et al., 2013). GluN3 subunits are reported to negatively regulate NMDARs by decreasing Ca$^{2+}$ permeability and may play nuanced roles in complex biological processes like synaptogenesis, the formation of new synapses (Matsuda et al., 2002; Henson et al., 2010; Pachernegg et al., 2012; Nelson et al., 2015). These are the general characteristic differences between GluN subunits and their different isoforms. There are also spatial and temporal variations in GluN expression, which when combined with their distinctive characteristics, may imply much about specific NMDAR functions.
There are established general patterns of where and when NMDARs with certain subunits are found, as summarized in Table I-I. As the obligatory subunit, GluN1 is found throughout the brain, though the significance of specific isoform distributions, both spatial and developmental, is not currently clear (Laurie and Seeburg, 1994; Sheng et al., 1994; Yi et al., 2018). GluN2A and GluN2B are expressed throughout the brain, especially at the cortex (CTX) and hippocampus (HPC) (Naassila and Pierrefiche, 2018; Sun et al., 2018) while GluN2C and GluN2D are more limited to the midbrain and cerebellum or brainstem, respectively (Paoletti et al., 2013; Tovar and Westbrook, 2017). There appears to be no unifying broad theme for the peripheral distribution of NMDAR subunits, as GluN2 (as well as GluN3) subunits have been reported in various organs and cell types (Chen et al., 2005; Sproul et al., 2011; Hogan-Cann and Anderson, 2016). In terms of GluN2-NMDAR expression differences by age, GluN2B-GluN2A switching is a well-established phenomenon for developing brains, where primarily GluN2B-containing NMDARs that are abundant in early development are replaced with primarily GluN2A-containing NMDARs (Sanz-Clemente et al., 2013; Baez et al., 2018). GluN2D and GluN2C are similar to GluN2B and GluN2A, respectively, as GluN2D is expressed much more in embryonic brains while GluN2C expression starts after birth (Watanabe et al., 1992; Monyer et al., 1994; Wenzel et al., 1997). Like GluN2A and GluN2B, GluN3 subunits have been investigated and found primarily in the CTX and HPC, though reports of expression in the hind- and midbrain exist as well (Matsuda et al., 2002; Wong et al., 2002; Wee et al., 2008). GluN3A is generally considered to be highly expressed in early development while GluN3B is more highly expressed in later developmental stages (Wong et al., 2002; Prithviraj and Inglis, 2008; Pachernegg et al., 2012). Sex differences in the context of NMDAR research have been investigated often (Dong et al., 2007; McRoberts et al., 2007; Ikeda et al., 2010), though relatively few consider the role of different NMDAR subunits. Two studies have specifically investigated NMDAR expression differences between males and females to some degree, studying postmortem human samples in the context of alcoholism (Ridge et al., 2009) or major depression (Gray et al., 2015). Neither reported significant differences between male and female control samples. Instead, they reported a unique trend of increase for Grin2b in the primary motor CTX of male alcoholics and that only female patients with depression had increased Grin1 and Grin2a-d expression (Ridge et al., 2009; Gray et al., 2015). Taken together, the different NMDAR subunits and their combinations offer a robust mechanism of variation and regulation in vivo. This variation can greatly influence what signals an active NMDAR sends.
Table I-I. Summary of NMDAR subunit distribution patterns in the brain and their typical characteristics. There are some reports of the 8 GluN1 isoforms varying in brain region expression, though the biological significance is unknown. There does not currently appear to be any unifying broad theme or pattern for subunit distributions between sexes or amongst peripheral organs and cell types.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Orthosteric site ligand</th>
<th>Brain region expression</th>
<th>Developmental expression</th>
<th>Other general characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GluN1</td>
<td>glycine or D-serine</td>
<td>Throughout</td>
<td>Both early and late in development</td>
<td>Obligatory subunit, 8 isoforms by alternative splicing</td>
</tr>
<tr>
<td>GluN2A</td>
<td>glutamate</td>
<td>Throughout; particularly at cortex and hippocampus</td>
<td>Late in development</td>
<td>High conductance, fast deactivation (relative to other GluN2 subunits)</td>
</tr>
<tr>
<td>GluN2B</td>
<td>glutamate</td>
<td>Throughout; particularly at cortex and hippocampus</td>
<td>Early in development</td>
<td>High conductance, fast deactivation (relative to other GluN2 subunits)</td>
</tr>
<tr>
<td>GluN2C</td>
<td>glutamate</td>
<td>Midbrain and cerebellum</td>
<td>Late in development</td>
<td>Low conductance, slow deactivation (relative to other GluN2 subunits)</td>
</tr>
<tr>
<td>GluN2D</td>
<td>glutamate</td>
<td>Midbrain and brainstem</td>
<td>Early in development</td>
<td>Low conductance, slow deactivation (relative to other GluN2 subunits)</td>
</tr>
<tr>
<td>GluN3A</td>
<td>glycine or D-serine</td>
<td>Throughout</td>
<td>Early in development</td>
<td>Incorporation in NMDARs generally decreases Ca$^{2+}$ conductance</td>
</tr>
<tr>
<td>GluN3B</td>
<td>glycine or D-serine</td>
<td>Throughout</td>
<td>Late in development</td>
<td>Incorporation in NMDARs generally decreases Ca$^{2+}$ conductance</td>
</tr>
</tbody>
</table>

1.1.2 NMDAR Activation and Subsequent Downstream Signaling

The activation of NMDARs and subsequent downstream effects are also complex, with general trends reported in the literature. In terms of ligands, as mentioned in the discussion of subunit compositions above, NMDARs have binding pockets for both glutamate and glycine (Johnson and Ascher, 1990; Yao et al., 2013). From their initial discovery and characterization in the 1980s, NMDARs have been considered glutamate receptors with unique characteristics, such as the Mg$^{2+}$ block (McLennan et al., 1981; Coyle, 2012). There have since been many studies of the interplay between glutamate and NMDARs in neurons, astrocytes, and microglia at the brain (Murugan et al., 2013; Pál, 2018) as well as in peripheral tissues, particularly in the context of disease and injury (Seidlitz et al., 2009; Du et al., 2016; Mahieu et al., 2016). However, it was
also discovered early on that glycine binding is necessary for NMDAR activation (Kleckner and Dingledine, 1988; Hirai et al., 1996; Cummings and Popescu, 2015). In fact, GluN1-GluN3 heterotetramers, formed when *Grin1* and *Grin3a/b* are co-expressed in *Xenopus* oocytes or human embryonic kidney cells, are functional excitatory glycine receptors that are unresponsive to glutamate (Chatterton et al., 2002; Smothers and Woodward, 2007; Madry et al., 2010). This is particularly intriguing as canonical glycine receptors are chloride channels whose activation would lead to hyperpolarization of cells (Lynch et al., 2017). The specific biological significance of pure GluN1-GluN3 NMDARs is unclear and may be minimal, but such an atypical combination has been found *in vivo* in rodent optic nerve myelin (Pina-Crespo et al., 2010). The glycine binding site is also activated endogenously by D-serine and both transmitters may be released by glia or neurons, though glycine may be more likely released by glia onto extrasynaptic NMDARs and D-serine by neurons onto synaptic NMDARs (Papouin et al., 2012; Li et al., 2013; Balu and Coyle, 2015). Evidence points to glycine and D-serine both being important for NMDAR signaling with each potentially coding for different information depending on the biological context (Li et al., 2009, 2013; Acton and Miles, 2017). Finally, it is important to mention that glutamate, glycine, and D-serine are considered the major endogenous NMDAR ligands, but many other endogenous molecules may bind to and regulate NMDARs as well, such as Zn$^{2+}$ (Rachline, 2005; Madry et al., 2008), cholesterol (Paul et al., 2013; Korinek et al., 2015), and glutathione (Sucher and Lipton, 1991; Varga et al., 1997). A representative list of reported endogenous ligands is shown in Table I-II.
**Table I-II. Representative list of reported endogenous NMDAR ligands.** The list is a representative highlight of commonly studied and biologically relevant ligands. Specific isomers are not listed, though both can function as ligands in most cases. Related metabolites of listed ligands, such as those processed by redox mechanisms, may also bind to and affect NMDAR activity. Some of the listed ligands are structurally and/or metabolically related while other isoforms and metabolites are not listed for clarity and brevity. Designations of ligands as agonists vs positive allosteric modulators (or antagonists vs negative allosteric modulators) are primarily based on how they are treated in the literature, as competition binding studies have not been completed for all. Similarly, whether ligands increase or decrease NMDAR activity (designation as agonist vs antagonist) is based on consensus in the literature – context-specific, conflicting reports may exist. Abbreviations: PAM - positive allosteric modulator, NAM - negative allosteric modulator.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Function</th>
<th>Sample Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Agonist</td>
<td>(Mayer et al., 1989; Chatterton et al., 2002; Mitani et al., 2006)</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Agonist</td>
<td>(Schell et al., 1997; Morland et al., 2012, 2013)</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Agonist</td>
<td>(McLennan et al., 1981; Traynelis et al., 2010; Coyle, 2012)</td>
</tr>
<tr>
<td>Glycine</td>
<td>Agonist</td>
<td>(Kleckner and Dingledine, 1988; Li et al., 2009; Cummings and Popescu, 2015)</td>
</tr>
<tr>
<td>Homocysteate</td>
<td>Agonist</td>
<td>(Olney et al., 1987; Yuzaki and Connor, 1999; Lewerenz and Maher, 2015)</td>
</tr>
<tr>
<td>Quinolinate</td>
<td>Agonist</td>
<td>(Monaghan and Beaton, 1991; Guidetti et al., 2004; Barton et al., 2015)</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>Agonist</td>
<td>(Zhang et al., 2009a; Lee et al., 2017)</td>
</tr>
<tr>
<td>Serine</td>
<td>Agonist</td>
<td>(Papouin et al., 2012; Li et al., 2013; Balu and Coyle, 2015)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Mixed</td>
<td>(Levy et al., 1991; Sucher and Lipton, 1991; Varga et al., 1997; Ogita et al., 2002)</td>
</tr>
<tr>
<td>Kynurenate</td>
<td>Antagonist</td>
<td>(Birch et al., 1988; Barton et al., 2015)</td>
</tr>
<tr>
<td>Agmatine</td>
<td>Antagonist/ NAM</td>
<td>(Yang and Reis, 1999; Gibson et al., 2002; Wang et al., 2006)</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>PAM</td>
<td>(Paul et al., 2013; Korinek et al., 2015)</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>PAM</td>
<td>(Compagnone and Mellon, 2000; Nicolas et al., 2001; Mellon and Griffin, 2002)</td>
</tr>
<tr>
<td>Pregnenolone sulfate</td>
<td>PAM</td>
<td>(Wu et al., 1991; Bowby, 1993; Emnett et al., 2015)</td>
</tr>
<tr>
<td>Spermidine</td>
<td>PAM</td>
<td>(Mony et al., 2011; Carvalho et al., 2012; Zhu and Paoletti, 2015)</td>
</tr>
<tr>
<td>H⁺</td>
<td>NAM</td>
<td>(Traynelis and Cull-Candy, 1990; Low et al., 2000; Chizh et al., 2001; Mony et al., 2011)</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>NAM</td>
<td>(Hatton and Paoletti, 2005; Rachline, 2005; Takeda and Tamano, 2009; McAllister and Dyck, 2017)</td>
</tr>
</tbody>
</table>

Beyond ligands, NMDARs are often termed “coincidence detectors” (Seeburg et al., 1995; Banerjee et al., 2016; Baez et al., 2018) because they also require concurrent local depolarization
for pore opening (Clarke et al., 2013; Sweatt, 2016). This concurrent depolarization may be facilitated by nearby α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs) at the synapse, which are also glutamate-gated cation channels (Burnashev et al., 1992a; Rao and Finkbeiner, 2007; Rogawski, 2011). The requirement for concurrent depolarization is due to the presence of a Mg$^{2+}$ block in NMDARs (Mayer et al., 1984; Nowak et al., 1984; Johnson and Ascher, 1990). NMDAR pores have binding sites for Mg$^{2+}$ and the strength of Mg$^{2+}$ blockade is greatly affected by GluN2 subtype (Clarke et al., 2013; Lee et al., 2014). A key exchange between leucine and serine at an amino acid position between 632 and 657 (specific locations dependent on the specific GluN2 subunit), termed the “GluN2 S/L site”, greatly influences blockade strength. GluN2A- and GluN2B-containing NMDARs, which have serine at the site, demonstrate stronger Mg$^{2+}$ block while the opposite is true in GluN2C- and GluN2D-containing NMDARs (Retchless et al., 2012; Clarke et al., 2013). An NMDAR bound with activating ligands without concurrent depolarization is not necessarily inert, however. Such a phenomenon is generally studied in the context of biologically relevant “silent synapses”, which express NMDARs without AMPARs (Isaac et al., 1995; Malinow et al., 2000; Yoon and Choi, 2017). Stimulation of silent synapses generate different types of action potentials (Huang et al., 2015; Li et al., 2017) and trigger their own downstream signaling cascades (Kerchner and Nicoll, 2008; Lee and Dong, 2011). Therefore, the presence or absence of concurrent depolarization is a key factor in eliciting activity from NMDARs.

NMDAR activity and subsequent downstream signaling cascades manifest in two main ways: cation, primarily Ca$^{2+}$, influx (Xia et al., 1996; Tao et al., 1998; Hardingham and Bading, 2003) and interaction with signaling proteins, particularly through their CTDs (Niethammer et al., 1996; Gardoni et al., 1999; Krapivinsky et al., 2003). These two mechanisms of activity are not necessarily exclusive to one-another, as CTD binding of signaling proteins could help facilitate Ca$^{2+}$-regulated effects (Strack and Colbran, 1998; Gardoni et al., 1999). However, metabotropic effects of NMDARs independent of channel opening have also been reported and may serve important biological functions (Dore et al., 2016; Gray et al., 2016; Weilinger et al., 2016). Many signaling networks have been associated with NMDAR activation and specific pathways may vary greatly depending on the physiological context (Hardingham and Bading, 2003; Luo et al., 2011). One striking example is the linking of NMDAR activation to potassium channels in neurons and in pancreatic islet cells to produce meaningful inhibitory K+ efflux – opposite to the
canonical excitatory Ca\textsuperscript{2+} influx of NMDARs (Isaac et al., 1995; Yu et al., 1999; Marquard et al., 2015). It is important to understand downstream signaling mechanisms as they may have a drastic impact on the ultimate physiological consequences of NMDAR activation.

Various molecular consequences have been reported downstream of NMDAR stimulation. Multiple studies have reported an important role for the extracellular signal–regulated kinase (ERK) pathway in NMDAR-mediated synapse development and maintenance (Thomas and Huganir, 2004; Tian et al., 2004; Schwechter et al., 2013). As part of this process, Rho GTPases are downstream effectors of NMDAR-regulated actin dynamics in dendritic spines – the physical location of most excitatory synapses on neurons (Nakayama et al., 2000; Dillon and Goda, 2005; Saneyoshi and Hayashi, 2012). Another broad type of signaling network that operates downstream of NMDARs is caspase signaling (Girling et al., 2018; Szychowski et al., 2018), which is part of regulated cell death (Allen et al., 1999; McIlwain et al., 2013). For both cell growth- and death-related physiological contexts, NMDAR activation also regulates phosphoinositide 3-kinase (PI3K) – protein kinase B (AKT) – mammalian target of rapamycin (mTOR) signaling (Gong et al., 2006; Li et al., 2010a; Heras-Sandoval et al., 2014; Wang Yansong et al., 2014). In line with these downstream signals, NMDAR signaling is also interconnected with the signaling of neurotrophins (Ortega et al., 2010; Fukuchi et al., 2015) and cytokines (Jander et al., 2000; Wang et al., 2009). Furthermore, downstream NMDAR signaling can involve Ca\textsuperscript{2+}/calmodulin-dependent protein kinases (CaMKs) and cAMP response element-binding protein (CREB) that regulate gene transcription (Penzes et al., 2008; Hardingham and Bading, 2010; Bustos et al., 2017). It may also involve proteins that couple NMDARs with traditional metabotropic glutamate receptor signaling (Naisbitt et al., 1999; Tu et al., 1999; Kaizuka and Takumi, 2018), nitric oxide (NO) destined for nearby cells’ cyclic-guanosine-monophosphate signaling pathways (Belsham et al., 1996; Araújo et al., 2018), and more (Ko et al., 2002; Banko et al., 2004; Rodríguez-Durán and Escobar, 2014). Finally, NMDAR signaling can also regulate, and be regulated by, sex hormones (Hsu et al., 2000; Bi et al., 2001; Hojo et al., 2008; van den Buuse et al., 2017) and the activity of other receptors (Mao, 1999; Pei et al., 2004; Kruse et al., 2009; Chen et al., 2018). A representative schematic of commonly studied NMDAR signaling pathways is shown in Figure 1.2. Overall, NMDAR signaling spans a multitude of molecular mechanisms dependent on cellular context and physiological function.
Figure 1.2. Representative schematic of downstream molecular signaling after NMDAR activation. NMDAR activation plays an integral role in generating, developing, maintaining, and eliminating cells and synapses. In general, synaptic NMDAR signaling represents pro-survival, pro-growth, and synaptic strengthening signals while extrasynaptic NMDAR signals largely serve opposing biological processes. The former is primarily mediated by GluN2A-containing NMDARs while the latter uses GluN2B-NMDARs. Chronic or excessive NMDAR stimulation often overlaps with extrasynaptic NMDAR signaling mechanisms. These pathways mainly represent neuronal NMDAR signaling; nonneuronal signaling may involve different molecules or molecular interactions and have been omitted for clarity. Similarly, other NMDAR signaling
contexts have been omitted for clarity, such as molecular mechanisms involved with LTD, reactive oxygen species, and crosstalk pathways with other receptors. Green pointed arrows indicate a positive relationship of some form, such as enhanced activation or release from a sequestering factor. Different shades of green only serve to clarify overlapping arrows and do not represent any meaningful difference. Red blunted lines indicate a negative relationship, causing some form of inhibition, decreased activity, or sequestering of the target. Black arrows indicate a more complex regulatory relationship from mixed or limited experimental results. Blue proteins indicate those that are affected by both synaptic and extrasynaptic NMDAR signaling.

Abbreviations are the same as those used in text, in addition to the following: RSK2 – ribosomal S6 kinase 2; PHLPP1β – pleckstrin homology domain and leucine rich repeat protein phosphatase 1β; STEP – striatal-enriched protein tyrosine phosphatase; PKC – protein kinase C; RAF – rapidly accelerated fibrosarcoma; ARC – activity-regulated cytoskeleton-associated protein; RHEB – Ras homolog enriched in brain; CBP – CREB-binding protein; FoxO – forkhead box, class O; RhoA – Ras homolog gene family, member A; DISC1 – disrupted in schizophrenia 1; KAL7 – kalirin-7; Rac1 – Ras-related C3 botulinum toxin substrate 1; WAVE-1 – Wiskott-Aldrich syndrome protein-family verprolin homologous protein 1; Arp2/3 – actin related protein 2/3; Pak1 – p21-activated kinase 1; LIMK1 – LIM domain kinase 1; CytC – cytochrome c; nNOS – neuronal nitric oxide synthase; CDK5 – cyclin-dependent kinase 5; Drp1 – dynamin related protein 1; LTP – long-term potentiation. This figure was constructed with literature cited within text as well as additional articles (Vallano, 1998; Snyder and Ferris, 2000; Korhonen et al., 2004; Jocoy et al., 2011; Uribe and Wix, 2011; Baudry and Bi, 2016; Haiying et al., 2017; Mackay et al., 2018).

To sum, the NMDAR is a heterotetrameric cation channel that is canonically activated with three concurrent events: glutamate binding to pockets involving GluN2 subunits, glycine binding to GluN1/GluN3 subunits, and local membrane depolarization to relieve a Mg$^{2+}$ block (Kleckner and Dingledine, 1988; Clarke et al., 2013; Paoletti et al., 2013). NMDARs are composed from 14 different subunits that have different spatial and developmental distributions and determine the unique characteristics of each receptor (Paoletti et al., 2013; Wyllie et al., 2013; Hogan-Cann and Anderson, 2016). Upon activation, NMDARs canonically allow Ca$^{2+}$ entry into the cell and trigger downstream signaling cascades that regulate gene expression and cell survival (Xia et al.,
1996; Hardingham and Bading, 2003; Luo et al., 2011). These fundamental characteristics lend themselves to the various physiological roles of NMDARs.

1.1.3 Physiological Roles of NMDARs

NMDARs play numerous roles depending on the context of their signaling (Shouval et al., 2002; Luo et al., 2011; Hogan-Cann and Anderson, 2016), as shown in Figure 1.2. As mentioned above, NMDAR signaling is involved with the maintenance of synapses (Tian et al., 2004; Schwechter et al., 2013) and with regulated cell death (Allen et al., 1999; Szychowski et al., 2018). NMDARs, particularly at the synapses of cortical and hippocampal neurons, are important for learning and memory behaviour (Newcomer et al., 2000; Whitlock et al., 2006; Baez et al., 2018; Roelfsema and Holtmaat, 2018). As such, they also play an important role in molecular and cellular correlates of learning and memory, such as the commonly-studied long-term potentiation (LTP) and synaptic plasticity (Morris et al., 1986; Calabresi et al., 1992; Shouval et al., 2002; Bouvier et al., 2015). The role of NMDARs in signaling cell death has also been well established, particularly in the context of excitotoxicity where overactivation of neurons leads to neuronal death (Hardingham and Bading, 2003; Weilinger et al., 2016; Girling et al., 2018). These two major functions of NMDARs may seem discordant and perhaps unrelated to one another. However, a simple reframing of NMDARs’ role in learning and memory as a role in selecting which neurons and synaptic connections should survive and thrive may help connect it to cell death signaling. Put another way, the core underlying principle of NMDARs’ physiological roles is determining when a cell (or a part of a cell, like a synapse) should grow and when it should die. This paradigm is further supported by the fact that NMDARs also have roles beyond the synapse that affect learning and memory, such as regulation of neurogenesis – the proliferation and development of new neurons (Gould et al., 1997; Nacher and McEwen, 2006; Lai et al., 2016). Commonly proposed physiological roles for NMDARs outside the brain also often involve cell growth or death, though research on peripheral NMDARs is relatively limited (Du et al., 2016; Hogan-Cann and Anderson, 2016). It is therefore not surprising that many researchers discuss NMDAR signaling and function as a contrasting combination of cell survival and cell death (Hardingham and Bading, 2003; Zhang et al., 2007; Luo et al., 2011).
The gating and activation properties of NMDARs particularly lend themselves to determining the fates of cells and synapses. Whether a cell or synapse would survive and grow or weaken and die may be determined by the context of NMDAR activation (Hardingham and Bading, 2003; Zhang et al., 2007). As mentioned above, NMDARs are coincidence detectors that are opened with concurrent local membrane depolarization but can also signal without allowing a Ca$$^{2+}$$ influx (Seeburg et al., 1995; Dore et al., 2016; Weilinger et al., 2016; Baez et al., 2018). This ability for NMDARs to detect and respond differentially to distinct levels of stimulation may be a key mechanism by which they signal synaptic strengthening or cell death. LTP, the enhancement of synaptic transmission efficiency as determined by changes in excitatory postsynaptic potentials (EPSPs), is induced at synapses with high frequency stimulation. LTD (long-term depression, the opposite weakening of synaptic connections) is induced with low or moderate frequency stimulation (Herron et al., 1986; Bliss and Collingridge, 1993; O’Dell and Kandel, 1994; Shouval et al., 2002). Coupled with cell death signaling being triggered by NMDARs when there is overstimulation (Shan et al., 1997; Carvajal et al., 2016; Girling et al., 2018), there is ample evidence of NMDARs facilitating synaptic strengthening or cell death as a result of the magnitude of stimulation. However, the gating properties of NMDARs are not the only mechanisms by which cell growth or cell death signaling can be chosen. The location of NMDARs may activate specific signaling pathways: synaptic NMDARs promote cell survival while extrasynaptic NMDARs, potentially responding to glutamate spillage from overactive synapses, promote cell death (Hardingham et al., 2002; Papadia et al., 2008; Okamoto et al., 2009; Xu et al., 2009). The selectivity of these signaling pathways may be facilitated by predominantly GluN2A-NMDARs at the synapse and predominantly GluN2B-NMDARs outside of the synapse, though this has not been established definitively (Hardingham and Bading, 2010; Luo et al., 2011). Finally, the fact that NMDARs are non-selective cation channels may also allow them to signal cell death, as apoptosis has been reported with K$$^+$$ efflux mediated by NMDARs (Yu et al., 1999). There is an overall abundance of experimental evidence that links cell survival or cell death signaling to different, specific contexts of NMDAR activation.

Moving away from the general concepts of cell survival and death, as well as synaptic strengthening and weakening, towards specific physiological contexts will reveal how NMDAR signaling affects living, multi-cellular organisms. As mentioned before, proper NMDAR signaling in the brain is important for cognition, particularly learning and memory (Malhotra et
al., 1996; Newcomer et al., 2000; Barria and Malinow, 2005; Gilmour et al., 2012). Many studies reporting a significant role for NMDARs in cognition operate with outcomes related to synaptic plasticity (Gilbert and Mack, 1990; Barria and Malinow, 2005), brain development and maintenance (Papadia et al., 2008; Komuro et al., 2015; Lai et al., 2016), and in vivo cognitive behaviour (McHugh et al., 2007; Morris et al., 2013).

Synaptic plasticity is a general term for the ability of synapses to change their transmissive strength and efficiency in response to local activity (Shouval et al., 2002; De Pittà et al., 2016). LTP is a form of synaptic plasticity and is commonly studied as a cellular correlate of learning and memory (Abbott and Nelson, 2000; De Pittà et al., 2016). As mentioned above, one method of LTP induction is through high frequency stimulation. This stimulation increases, and often depends on, NMDAR activity to induce post- and pre-synaptic changes that facilitate the more efficient synaptic transmission that is characteristic of LTP (Bliss and Collingridge, 1993; Shouval et al., 2002; Lisman and Raghavachari, 2006). These NMDA-mediated changes involve, but are not limited to, colocalized CaMK-II activity (Barria and Malinow, 2005), presynaptic actin and scaffolding protein rearrangement (Antonova, 2001; Colicos et al., 2001), trafficking of AMPARs to the synapses to enhance transmission (Malinow and Malenka, 2002; Bredt and Nicoll, 2003; Kim et al., 2005), activation of the Rho GTPases RhoA and Cdc42 to expand hippocampal dendritic spines (Murakoshi et al., 2011), and CaMKII- and protein-synthesis-dependent stabilization of dendritic spines (De Roo et al., 2008; Hill and Zito, 2013). Pharmacologically antagonizing NMDARs, fittingly, has been reported to block the LTP between rat perforant path and dentate gyrus (Gilbert and Mack, 1990), in mature and newborn mouse dentate granule cells (Ge et al., 2007), and in rat hippocampal neurons (Barria and Malinow, 2005). As to the propriety of studying LTP and synaptic plasticity as cellular correlates of learning and memory, recent experiments with optogenetics provide strong support for this paradigm. Association of a neutral optogenetics-based conditioned stimulus with a foot shock in rats was NMDAR-dependent, extinguishable by an LTD protocol, and restorable with an LTP protocol (Nabavi et al., 2014). Furthermore, LTP induction in dentate gyrus cells attenuated spine density deficits and memory impairments in the mutant β-amyloid precursor/presenilin-1 double-transgenic mouse model of Alzheimer's disease (AD) (Roy et al., 2016). NMDARs play an important role in facilitating and regulating LTP and synaptic plasticity, and thus learning and memory.
There are numerous studies that link NMDARs to cognition and brain homeostasis beyond LTP and the synapse. NMDAR activation can suppress apoptotic neurodegeneration in perinatal developing mouse brains (Ikonomidou et al., 1999; Heck et al., 2008), promote neurogenesis in neonatal rats after hypoxic-ischemic injury (Lai et al., 2016), act as a signal for mouse cerebellar granule cell migration during brain development (Komuro and Rakic, 1993; Komuro et al., 2015), and protect rat cortical neurons from oxidative insult by increasing thioredoxin activity (Papadia et al., 2008). NMDAR activation may also regulate sleep-wake cycles (Manfridi et al., 1999; Kawai et al., 2015) as well as facilitate proper sleep architecture (Armstrong-James and Fox, 1988) and sleep-dependent memory consolidation (Feld et al., 2013).

In terms of direct assessments of learning and memory, there is an abundance of behavioural evidence that points to an important role for NMDARs. Many of these studies involve investigating the consequences of perturbing NMDAR signaling. Acute NMDAR antagonism in mice resulted in diminished in vivo perforant path – to – dentate gyrus LTP, impaired spatial memory formation, and impaired avoidance learning (Walker and Gold, 1991). Chronic NMDAR blockade in rats resulted in both suppressed in vivo LTP at the dentate gyrus and impaired spatial memory formation in multiple configurations of the Morris water maze (Morris et al., 1986, 2013). Distinct from a pharmacological approach, knocking out GluN1 subunits in the granule cells of the mouse dentate gyrus produces mice with impaired contextual pattern learning and discrimination (McHugh et al., 2007). Taken together, numerous studies operating with a wide range of experimental approaches and outcomes indicate that NMDARs are important for synaptic strengthening, neuronal maintenance, and learning and memory.

Another major role of NMDARs is to propagate signals related to cell death and synaptic weakening (Shouval et al., 2002; Hardingham and Bading, 2003; Luo et al., 2011; Girling et al., 2018). NMDAR-mediated cell death signaling cascades are often studied and reported in the context of injury and disease, such as excitotoxic processes during ischemia (Seubert et al., 1989; Gao et al., 2005) and other neuropathological states (Weilinger et al., 2016; Wong et al., 2016). While regulated cell death is certainly an important part of normal brain development (Oppenheim, 1991; Vaux and Korsmeyer, 1999; Kuan et al., 2000), the role of NMDARs in this developmental context is primarily to promote neuronal survival after activation (Fiske and Brunjes, 2001; Martins et al., 2005; Heck et al., 2008). In the context of adult neurogenesis, NMDAR activation may be considered as indirectly supporting cell-death signaling by
suppressing growth-related mechanisms (Cameron et al., 1995; Gould and Cameron, 1996). Generally, nonpathological NMDAR-mediated cell death requires further characterization and study as current evidence is limited.

Synaptic weakening by synapse pruning or LTD are normal physiological processes mediated by NMDARs. Synaptic pruning is an important part of brain development whereby redundant connections between neurons, overabundant in youth, are reduced to establish efficient, mature neuronal networks (Uesaka et al., 2014; Lieberman et al., 2018; Millán et al., 2018). Many experiments have shown that NMDAR blockade disrupts synaptic pruning and leaves more aberrant synaptic connections intact (Cline and Constantine-Paton, 1989; Rabacchi et al., 1992; Hickmott and Constantine-Paton, 1997; Lüthi et al., 2001). It is difficult to explicitly and specifically state what purpose synaptic pruning serves on a macroscopic level, but disruptions in synaptic pruning have been linked to autism spectrum disorders (ASDs) (Tang et al., 2014; Kim et al., 2017), schizophrenia (Sekar et al., 2016; Johnson and Stevens, 2018), and other neurological disorders (Johnston et al., 2001; Stertz et al., 2013). NMDAR-mediated synapse elimination can involve molecular signals related to apoptosis without fully prompting cell death, such as caspase-3 activation, and shares similar molecular mechanisms with NMDAR-mediated LTD (Li et al., 2010b p.3; Jiao and Li, 2011; Erturk et al., 2014; Piochon et al., 2016).

LTD of synapses can be induced with low frequency stimulation of presynaptic neurons, different than LTP that needs high frequency stimulation and both pre- and post-synaptic neuronal activity (Herron et al., 1986; Shouval et al., 2002; Luscher and Malenka, 2012). As mentioned before, optogenetically-induced LTD can extinguish associative memory in rats (Nabavi et al., 2014). Furthermore, specific blockade of GluN2B-NMDARs prevents LTD induction and conditioned fear memory extinction in rats (Dalton et al., 2012). LTD is not only important for erasing a memory, however, but also memory formation de novo. Hippocampal LTD, for example, may work together with LTP changes to encode memories of objects in new environments (Kemp and Manahan-Vaughan, 2004) or social stress (Artola et al., 2006) in rats. Combined with NMDARs’ robust involvement with LTP and neuronal health, NMDAR-mediated synaptic pruning and LTD greatly affect cognition in vivo. It is therefore not surprising that there is a rich scientific literature regarding NMDARs in physiological responses to diseases and insults as well as NMDAR dysfunction in neurological disorders.
1.1.4 NMDARs in Health and Disease; Links to Neurological Disorders

Disruptions of NMDARs can lead to various neurological disorders and associated symptoms (Luby et al., 1959; Irani et al., 2010; Burnashev and Szepetowski, 2015). A summative diagram of how NMDAR disruptions relate to pathological processes and states is shown in Figure 1.3. Some of the earliest evidence supporting a significant role of NMDARs in mental illness come from pharmacological studies. Pharmacological blockade of NMDARs with ketamine or phencyclidine (PCP) produces positive, negative, and cognitive symptoms similar to those seen in schizophrenia (Luby et al., 1959; Allen and Young, 1978; Krystal et al., 1994; Malhotra et al., 1996). NMDAR agonism can mediate the effects of glutamatergic excitotoxins (Giulian et al., 1990; Berman and Murray, 1997) and can induce seizures and convulsions (Schoepp et al., 1991; Toscano et al., 2008). More chronic and permanent NMDAR disruptions, such as by genetic mutations of NMDAR subunit genes, can also serve as a causal factor in neurological disorders. Many studies have linked human GRIN mutations to epilepsy, schizophrenia, ASDs, attention deficit hyperactivity disorder, and other forms of cognitive impairment (Soto et al., 2014; Burnashev and Szepetowski, 2015; Yuan et al., 2015). Anti-NMDAR autoimmune encephalitis, where patients produce antibodies targeting their own NMDARs, also produce neuropsychiatric symptoms, cognitive impairments, and seizures (Dalmau et al., 2007; Florance-Ryan and Dalmau, 2010; Irani et al., 2010). Overall, abundant evidence suggests that NMDAR disruption plays a very important role in many neurological disorders. Both too much and too little NMDAR signaling can be detrimental and may even lead to similar outcomes (Hardingham and Bading, 2003; Parsons et al., 2007; Schmeisser et al., 2012; Won et al., 2012), but each direction of dysfunction is more closely associated with certain disorders than others. It is therefore useful to categorize the health-related contexts and consequences of NMDAR hyperfunction and hypofunction.
Figure 1.3. Summary of NMDAR-mediated biological processes and their dysregulation in key representative neurological disorders. Mentioned thus far, proper NMDAR signaling is important for the genesis, development, maintenance, and elimination of cells and synapses. Altered synaptic plasticity can have multiple manifestations, such as an altered excitation-inhibition balance that is epileptogenic or an impairment of synaptic strengthening that weakens learning and memory. Cell growth and survival mechanisms are especially important for neurodevelopment while cell death contributes to late-onset neurodegenerative disorder. Green and red arrows denote positive and negative relationships, as denoted in Figure 1.2. Black, purple, and grey arrows only serve to establish a relationship between generalized NMDAR signaling pathways, the biological processes they serve, and the disorders both contribute to
NMDAR hyperactivity and subsequent excitotoxicity is a major component of the body’s responses to physiological insults. NMDAR-mediated excitotoxicity is considered part of physiological responses to ischemia (Seubert et al., 1989; Gao et al., 2005), stroke (Weilinger et al., 2016; Wong et al., 2016), infections (Haughey et al., 2001; Costa et al., 2017), and trauma (Lynch and Dawson, 1994; Aiba and Shuttleworth, 2012; Estrada-Rojo et al., 2018). It has been demonstrated in rat lung tissue (Said et al., 1996) and postulated as a potential pathological mechanism in other peripheral tissues (Gill et al., 2000; Ortiz et al., 2006). Excitotoxicity may very well be the most prominent NMDAR-related pathological process in peripheral tissues, but this may be because peripheral NMDAR studies are relatively scarce (Du et al., 2016; Hogan-Cann and Anderson, 2016). The main commonality between different physiological insults, occurring anywhere in the body, that can cause NMDARs to respond similarly is very likely the excessive glutamate release from tissue damage or stress (Seubert et al., 1989; Weilinger et al., 2016; Costa et al., 2017; Estrada-Rojo et al., 2018). Indeed, many clinical trials were conducted to evaluate NMDAR antagonists for preventing cell death, particularly after ischemia, though these trials generally produced negative results (Morris et al., 1999; Davis et al., 2000; Albers et al., 2001; Sacco et al., 2001; Yurkewicz et al., 2005). It is thought that these NMDAR antagonists failed clinical trials because they also disrupted important nonpathological NMDAR functions, including neuroprotection (Ikonomidou and Turski, 2002; Muir, 2006; Shohami and Biegon, 2014). This is consistent with previously mentioned reports of NMDAR activity promoting neurogenesis after hypoxic ischemia (Lai et al., 2016) and enhancing antioxidant mechanisms (Papadia et al., 2008), as well as other reports of NMDAR-dependent mechanisms for adapting to acute ischemia (Grabb and Choi, 1999; Gonzalez-Zulueta et al., 2000). Fittingly, the only NMDAR antagonist used to treat AD, memantine, is largely thought to be effective because it has a rapid off-rate and preferentially blocks excessive NMDAR activity (Tariot et al., 2004; Lipton, 2006; Parsons et al., 2007). NMDARs therefore clearly play a prominent role in pathological, preservation, and recovery processes (Petrovic et al., 2005; Bading, 2017; Olloquequi et al., 2018). However, it is also clear that simply blocking NMDARs during or after injury may not be effective in improving health outcomes (Ikonomidou and Turski, 2002; Lipton, 2006; Shohami and Biegon, 2014). Manipulating NMDAR activity to prevent
excitotoxicity and promote survival may be the ideal therapy, but more research is required to understand when, where, and with what drug NMDARs need to be regulated.

Excessive and/or inappropriate NMDAR activation has also been linked to neuropathic pain (Alles and Smith, 2018; Inquimbert et al., 2018) and neurodegenerative diseases such as AD (Danysz and Parsons, 2003; Okamoto et al., 2009; Tönnies and Trushina, 2017). In the case of neuropathic pain, the involvement of NMDAR signaling in its etiology is well supported (Zhuo, 2008; Alles and Smith, 2018). Rather than excitotoxicity, NMDAR-mediated synaptic changes such as LTP are key facilitators of enhancing pain signaling (Svendsen et al., 1998; Ren and Dubner, 2007; Alles and Smith, 2018). Treatment of neuropathic pain using the NMDAR antagonist ketamine is generally effective, but clinically limited due to undesirable side effects such as hallucinations and cognitive impairment (Hewitt, 2000; Aiyer et al., 2018). Inappropriate NMDAR hyperactivity may contribute to AD via excitotoxic mechanisms and neurodegeneration (Hynd et al., 2004; Zhang et al., 2016; Tönnies and Trushina, 2017). Amyloid-β protein, the aggregates of which are a hallmark of AD, can directly activate NMDARs (Texidó et al., 2011), increase glutamate release from microglia (Noda et al., 1999), disrupt Ca\(^{2+}\) homeostasis to enhance NMDAR-mediated excitotoxicity (Mattson et al., 1992; Bezprozvanny and Mattson, 2008), and decrease the ratio of synaptic-to-extrasynaptic NMDAR activity (Snyder et al., 2005; Li et al., 2011; Talantova et al., 2013). A relative increase in extrasynaptic NMDAR activity is particularly meaningful for signaling excitotoxicity and cell death (Hardingham et al., 2002; Parsons and Raymond, 2014) and is consistent with reports of synaptic NMDAR inhibition by amyloid-β (Snyder et al., 2005; Shankar et al., 2007; Zhang et al., 2009b). As mentioned before, memantine may be useful for treating AD by suppressing excessive NMDAR activity (Tariot et al., 2004; Parsons et al., 2007). Memantine may also preferentially block active extrasynaptic NMDARs, making it particularly suitable for preventing NMDAR-mediated neurodegeneration (Lipton, 2006; Xia et al., 2010). The differential status of synaptic and extrasynaptic NMDARs in AD reinforces the idea of multi-faceted NMDAR changes in neurological disorders, as both increased and decreased NMDAR activity is reported. Much of the evidence supports a strong role of enhanced NMDAR activation leading to excitotoxic neurodegeneration (Hynd et al., 2004; Parsons and Raymond, 2014; Tönnies and Trushina, 2017). However, decreased synaptic NMDAR signaling may also simultaneously contribute to LTD and memory loss in AD (Snyder et al., 2005; Shankar et al., 2007; Zhang et al., 2009b). NMDAR hyperactivity, particularly
through excitotoxic mechanisms, can overall cause great damage to surrounding tissue in many contexts, but other forms of NMDAR disruption can also contribute to disease symptoms.

Epilepsy and ASDs are two illustrative examples of neurological disorders with mixed-direction NMDAR disruptions. Despite epilepsy being a neurological disorder of hyperexcitability in the brain (Devinsky et al., 2013), most of the functionally-assessed Grin mutations related to epilepsy resulted in unchanged or decreased NMDAR currents (Xu and Luo, 2018). Similarly, most animal models of epilepsy had unchanged or decreased levels of Grin mRNA when assessed (Ghasemi and Schachter, 2011). It is not completely clear how loss-of-function NMDAR mutations contribute to a hyperexcitable brain (Oyrer et al., 2018), but NMDAR hypofunction can lead to unstrengthened inhibitory synaptic connections that allow for excessive non-NMDAR glutamatergic signaling (Moscato et al., 2014; Chiu et al., 2018). That is not to say that only NMDAR deficits have been associated with epilepsy, however. Firstly, NMDAR-mediated excitotoxicity and cell death is, expectedly, a concerning mechanism in epilepsy (Quincozes-Santos et al., 2014; Salazar and Tapia, 2015). NMDAR activation has also been reported to be necessary for developing epilepsy in rats (Rice and DeLorenzo, 1998).

Endogenous NMDAR activity is increased in resected tissue from mesial temporal lobe epilepsy patients (Banerjee et al., 2016), NMDA-mediated EPSPs were prolonged in epileptic hippocampal tissue from humans (Isokawa and Levesque, 1991), and NMDAR density was increased in epileptic human cortical tissue when assessed by radioligand binding (Steffens et al., 2005). Furthermore, use of memantine to block excess NMDAR has been suggested as a personalized therapy option for some patients (Pierson et al., 2014) while NMDAR enhancers may have a very narrow therapeutic window (Oyrer et al., 2018). However, D-cycloserine, a glycine-site partial agonist of NMDARs, has been reported to have anticonvulsant effects in preclinical models (Peterson and Schwade, 1993; De Sarro et al., 2000).

Both memantine (Chez et al., 2007; Erickson et al., 2007; Ghaleiha et al., 2013) and D-cycloserine have also been shown to be beneficial for patients with autism in clinical trials (Posey et al., 2004; Urbano et al., 2014, 2015; Minshawi et al., 2016). Increased and decreased NMDAR function has been linked to animal models of ASDs. Mice with a SH3 and multiple ankyrin repeat domains 2 (Shank2) mutation that models ASDs showed impaired NMDAR-mediated LTP and LTD in the HPC along with a decreased NMDAR/AMPAR signal ratio (Won et al., 2012). However, Shank2 knockout mice had increased GluN subunits at the HPC and
striatum (STR) as well as increased hippocampal NMDAR/AMPAR signaling ratio (Schmeisser et al., 2012). Knockout of *Grin1* in parvalbumin interneurons of mice resulted in autism-relevant social behaviour deficits (Saunders et al., 2013) while prenatal valproic acid exposure in rats, a model of teratogenic autism, resulted in increased cortical protein levels of GluN2A and GluN2B as well as improved LTP (Rinaldi et al., 2007). In the case of ASDs, some of the variation in NMDAR disruption may be due to age, as early brain development aberrations can differ from consequent mature brain abnormalities (Contestabile, 2000; Rinaldi et al., 2007; Spooren et al., 2012). Increased and decreased NMDAR activity may therefore contribute to the same neurological disorder via altering different physiological functions at different sites and timepoints.

Schizophrenia is the prime example of a neurological disorder associated with NMDAR hypofunction. To start, the glutamatergic theory of schizophrenia, first proposed by Arvid Carlsson (Carlsson and Carlsson, 1990; Carlsson et al., 1999), is centered on a hypothesis of NMDAR hypofunction. NMDAR hypofunction is a current, major paradigm in schizophrenia research (Gilmour et al., 2012; Moghaddam and Javitt, 2012; Nakazawa et al., 2017). The core findings underlying the NMDAR hypofunction hypothesis are the reports of schizophrenia-like positive, negative, and cognitive symptoms seen in humans after pharmacological NMDAR blockade (Luby et al., 1959; Krystal et al., 1994; Gilmour et al., 2012; Moghaddam and Javitt, 2012). Furthermore, when given to patients with schizophrenia, ketamine exacerbated their symptoms (Lahti et al., 1995, 2001; Malhotra et al., 1997). The role of genetic NMDAR dysfunction in schizophrenia is likely more complex and could include many disrupted physiological functions. Reported *GRIN* mutations of schizophrenia patients are fairly evenly distributed amongst non-obligatory GluN subunits, though *GRIN2A* and *GRIN2B* mutations may be good general representations of NMDAR dysfunction in schizophrenia (Paoletti et al., 2013; Yuan et al., 2015). *GRIN2A* mutations are particularly common in epilepsy and, consistent with the NMDAR hypofunction hypothesis, are often loss-of-function mutations (Xu and Luo, 2018). Thus, underactive NMDARs may also facilitate a hyperglutamatergic state in schizophrenia, which is supported by evidence like the attenuation of ketamine-induced psychosis via inhibition of glutamate release (Anand et al., 2000). An increased glutamatergic tone may act as noise in the brain to disrupt neuronal networks, manifesting as psychosis (Corlett et al., 2011; Moghaddam and Javitt, 2012). *GRIN2B* mutations have been considered particularly common to
neurodevelopmental disorders and cognitive impairment, potentially pointing to disrupted brain development and synaptic pruning in schizophrenia (Ohno et al., 2010; Soto et al., 2014; Yuan et al., 2015). This would be consistent with perinatal NMDAR blockade in animals producing long-lasting, schizophrenia-relevant phenotypes (du Bois and Huang, 2007) and with the disrupted synaptic connectivity found in post-mortem brains of schizophrenia patients (Garey et al., 1998; Glantz and Lewis, 2000; Black et al., 2004; Weickert et al., 2013). Clinical studies of D-cycloserine treatment for schizophrenia, often as adjunct therapy, reported efficacy in some cases (Evins et al., 2002; Heresco-Levy et al., 2002; Goff et al., 2008), no effect in others (Heresco-Levy et al., 1998; Duncan et al., 2004a; Goff et al., 2005), and even symptom worsening in still other cases (Cascella et al., 1994; Berckel et al., 1999; Goff et al., 1999). While these studies varied greatly in design, the complex etiology of schizophrenia may ultimately mean that D-cycloserine is best suited as a personalized therapy for some patients.

Overall, many studies strongly link NMDAR function to proper brain health and its disruption to neurological disorders (Newcomer et al., 2000; Paoletti et al., 2013; Burnashev and Szepetowski, 2015). The illnesses listed above are just some of the more well-studied, illustrative examples of how NMDAR dysfunction can contribute to neuropsychiatric pathology and symptoms. Briefly, other examples of NMDAR involvement in mental illnesses include the rapid antidepressant effects of NMDAR blockade in major depression (Li et al., 2010a), investigations of NMDAR-mediated synaptic plasticity to understand and treat post-traumatic stress disorder (Yamamoto et al., 2008; Mikics et al., 2017), and evidence of dysregulated glutamate transmission in obsessive-compulsive disorder (Alonso et al., 2012). Disrupted NMDAR function can have many consequences and manifest as various symptoms in humans. Current evidence suggests that moderating NMDAR activity, rather than net activation or inhibition, with drugs like memantine and D-cycloserine, may improve mental health outcomes. However, the reported benefits of these drugs are often only moderate for some patients, if at all (Herrmann et al., 2011; Schade and Paulus, 2016). Further research is therefore warranted to understand the molecular and cellular mechanisms underlying neurological disorders related to NMDAR dysfunction; this in turn will help the development of future treatments that are more specific and efficacious. My research uses a genetic mouse model of NMDAR deficiency to study such mechanisms.
1.2 Mouse Model of NMDAR Deficiency by GluN1 Knockdown

1.2.1 Background and Overview of GluN1 Knockdown Mice

The GluN1 knockdown (GluN1KD) mouse model was generated by Dr. Amy J. Ramsey in the lab of Dr. Beverly H. Koller and was first published in 1999 (Mohn et al., 1999). As the name implies, GluN1KD mice have decreased levels of the obligatory GluN1 subunit of NMDARs. This is a global decrease that has been confirmed by assessments of RNA (Mohn et al., 1999; Bodarky et al., 2009), protein (Mohn et al., 1999; Bodarky et al., 2009; Ramsey et al., 2011), and radioligand binding (Mohn et al., 1999; Duncan et al., 2002, 2004b). GluN1KD heterozygotes are less characterized, but have demonstrated WT-like levels of NMDARs in all brain regions assessed by radioligand binding (Duncan et al., 2002). Functionally, NMDAR deficits have been less characterized in GluN1KD mice. When measured as evoked EPSPs in striatal medium spiny neurons (MSNs), NMDAR-specific currents were reduced (Jocoy et al., 2011). Injection of the direct and potent NMDAR agonist (tetrazol-5-yl)glycine (TZG) in GluN1KD mice produced muted Fos protein expression, a marker of increased intracellular Ca\(^{2+}\), in many brain regions (Inada et al., 2007; Duncan et al., 2008). NMDAR function was thus demonstrated to be generally deficient in GluN1KD brains. However, Fos induction by TZG and other methods in the GluN1KD STR was often similar to wild-type (WT) littermates (Miyamoto et al., 2004; Inada et al., 2007; Duncan et al., 2008). Such an absence of NMDAR hypofunction contrasts with decreased NMDAR currents in GluN1KD MSNs (Jocoy et al., 2011), which represent more than 90% of neurons in the STR (Rymar et al., 2004). These conflicting results may point to limitations of using Fos to assess NMDAR activity, the remaining NMDARs being sufficient for striatal Fos induction, or effective compensatory mechanisms. Further studies are needed to more directly assess NMDAR activity and functionality in GluN1KD mice. As a whole, however, the current evidence confirms a global reduction of NMDAR quantity and function in the GluN1KD brain.

The GluN1KD hypomorphic mutation is an insertion of a neomycin resistance cassette into intron 19 of the \textit{Grin1} gene. GluN1KD mice were initially of mixed background, containing alleles from the 129/Ola, C57BL/6, and DBA/2 mouse lines. This background heterogeneity was controlled by tracking 11 microsatellite markers on 10 chromosomes (Mohn et al., 1999). In contrast, later studies used a defined genetic background to compare GluN1KD mice and WT
littermates (Fradley et al., 2005; Duncan et al., 2006a; Milenkovic et al., 2014). The defined genetic backgrounds of experimental animals have been F1 hybrids of C57BL/6J and either 129/Ola (Moy et al., 2008), 129/SvEv (Duncan et al., 2006a; Wesseling et al., 2014), or 129X1/SvJ (Mielnik et al., 2014; Milenkovic et al., 2014). Despite the differences in genetic background between some studies, findings have been largely consistent, particularly for commonly-studied GluN1KD behavioural deficits (Gandal et al., 2012; Milenkovic et al., 2014; Islam et al., 2017). To date, more than 30 studies have been published using GluN1KD mice; these include characterization studies (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012), drug discovery studies (Gregory et al., 2013; Grannan et al., 2016), and studies addressing other basic science questions (Moy et al., 2008; Jocoy et al., 2011). Our lab has worked on characterizing GluN1KD mice and using them to answer basic science questions related to NMDAR hypofunction (Mielnik et al., 2014; Milenkovic et al., 2014; Islam et al., 2017).

On a gross, macroscopic level, GluN1KD mice do not present with very overt physical or neurological abnormalities (Mohn et al., 1999; Miyamoto et al., 2004). They survive to adulthood (Mohn et al., 1999; Miyamoto et al., 2004; Ramsey et al., 2008) and have WT-like body temperature (Fradley et al., 2005), but are not considered fertile – GluN1KD males have seemingly normal testicular histology but severe deficits in reproduction-related behaviour (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012). The hypomorphic mutation can take a toll on the survivability and health of mice, however, as generation of GluN1KD mice using only one parental strain can result in less homozygous knockdown mice, stunted growth, and higher mortality (Duncan et al., 2006a; Ramsey et al., 2011; Wesseling et al., 2014). Studies using GluN1KD mice on a predominantly C57BL/6 background have reported reduced bodyweights, particularly in males, compared to WT littermates (Fradley et al., 2005; Moy et al., 2012) while others have noted reduced body size or weight even when using F1 GluN1KD hybrids (Mohn et al., 1999; Islam et al., 2017). Other signs of poorer health in GluN1KD mice include reports of self-injury by excessive grooming and scratching (Moy et al., 2008; Gandal et al., 2012), increased incidence of seizures and seizure-like electrical activity (Dzirasa et al., 2009; Halene et al., 2009a), poor pre-weaning survival (Fradley et al., 2005), and increased mortality in males when fed an ω-3 fatty acid deficient diet (Islam et al., 2017). Overall, however, the GluN1KD mice would generally appear healthy if small, unless the experimenter comes upon one of the minority that is coincidentally seizing or damaged from excessive
grooming. They have no major anatomical or neuro-anatomical abnormalities (Mohn et al., 1999; Miyamoto et al., 2004).

Several assessments of the sensory and motor systems of GluN1KD mice have been conducted, generally pointing to functional but abnormal systems (Bickel et al., 2007; Moy et al., 2008; Halene et al., 2009a). The main direct piece of evidence pointing to a functional visual system in GluN1KD mice comes from electrophysiological experiments. Investigators have detected and reported timely event-related potentials (ERP) after visual stimulation from a flash box. However, these ERPs had higher amplitudes compared to WT littermates, suggesting abnormal sensory processing (Halene et al., 2009a). Secondary evidence of a functioning visual system in GluN1KD mice comes from their ability to identify visual components of behavioural tests such as an open door in puzzle box tests (Milenkovic et al., 2014) and a visible platform in swim tests (Fradley et al., 2005). Auditory ERPs are much more commonly studied than visual ERPs in GluN1KD mice (Bickel et al., 2007, 2008; Bodarky et al., 2009; Halene et al., 2009a, 2009b). They are again present and timely but also generally greater in amplitude or longer lasting and less modulated by endogenous mechanisms compared to WT littermates (Bickel et al., 2007; Bodarky et al., 2009; Halene et al., 2009a; Gandal et al., 2012). In one of these experiments, GluN1KD test mice can even produce appropriate reductions in auditory ERP amplitude when presented with follow-up secondary auditory stimuli (Halene et al., 2009a). Behavioural secondary evidence also points to a functional auditory system in GluN1KD mice, particularly via the presence of timely, and even enhanced, acoustic startle (Fradley et al., 2005; Duncan et al., 2006b; Islam et al., 2017). Olfaction has been less directly assessed in GluN1KD mice, with the most direct experiment reporting appropriate auditory ERP modulation in the presence of fox urine, a predator odorant (Halene et al., 2009b). Acoustic startle is altered in GluN1KD mice exposed to soiled rat bedding, another predator odorant (Duncan et al., 2004b), while a study using an exploratory hole-board reported an abnormal preference for the scent of novel cage bedding in a particular GluN1KD male (Moy et al., 2008). Multiple studies have also reported normal social sniffing behaviour frequency in GluN1KD mice (Duncan et al., 2004b, 2009), but this does not hold for time spent on social investigation or sniffing (Mohn et al., 1999; Halene et al., 2009a; Moy et al., 2012). In general, these behavioural studies convey a working olfactory system whose signals are not always handled appropriately (Duncan et al., 2004b; Moy et al., 2008; Halene et al., 2009b). The senses of taste and touch, to our knowledge, have not
been directly assessed in GluN1KD mice. GluN1KD mice may show some preference for chocolate chips in the exploratory board task, but the data were not statistically significant and odor is a confounding factor (Moy et al., 2008). GluN1KD mice were quick to move away from a hot plate and displayed altered ERPs and motor responses during fear conditioning with foot shocks (Bickel et al., 2007), but pain is also not the same as general tactile sensation. Finally, balance and motor-coordination in GluN1KD mice have been reported to be WT-like when assessed by swimming and rotarod tests (Duncan et al., 2004b; Fradley et al., 2005). GluN1KD are therefore generally healthy, functional, and, as already implied by the above examples, suitable for behavioural testing.

1.2.2 GluN1KD Behavioural Abnormalities and Their Pharmacological Treatments

GluN1KD mice present with behavioural abnormalities that span multiple domains of cognition and are considered relevant to neuropsychiatric disorders (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012; Milenkovic et al., 2014). A summary of these abnormalities is listed in Table I-III. Our lab has previously reported deficits in locomotor activity, habituation, sensorimotor gating, stereotypy, social behaviour, and higher cognitive functions like learning and memory (Mielenik et al., 2014; Milenkovic et al., 2014; Islam et al., 2017). Others have reported the same or comparable deficits using similar behavioural testing methodology (Dzirasa et al., 2009; Halene et al., 2009a; Gandal et al., 2012; Grannan et al., 2016) as well as other abnormalities in behavioural correlates of anxiety and motivation (Duncan et al., 2004b; Moy et al., 2008; Halene et al., 2009a). The only behavioural study that tested GluN1KD-heterozygotes generally pointed to the Grin1 gene being haplosufficient (Moy et al., 2012). The GluN1KD mouse model and its reported behavioural deficits in homozygous knockdown mice have been considered relevant in particular to schizophrenia and ASDs (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012). Locomotor activity and sensorimotor gating, for example, may be considered behavioural correlates for the positive symptoms of schizophrenia (Fradley et al., 2005; Hazlett et al., 2007). Furthermore, the relatively rarer studies of sex and age differences in GluN1KD behavioural phenotypes also support a connection to schizophrenia and autism (Moy et al., 2012; Milenkovic et al., 2014). GluN1KD behavioural deficits can be treated by currently approved antipsychotics (Mohn et al., 1999; Duncan et al., 2006a) as well as experimental therapies for schizophrenia that target related molecular signaling mechanisms (Gregory et al.,
Overall, GluN1KD mice present a model of NMDAR hypofunction that is relevant and useful in a variety of behavioural and neuropsychiatric contexts.

**Table I-III. Major behavioural abnormalities found in previous GluN1KD studies.**

GluN1KD mice present with behavioural abnormalities across various domains of behaviour and cognition. Such a diversity of irregular behaviours resemble the multiple types of schizophrenia symptoms (positive, negative, cognitive).

<table>
<thead>
<tr>
<th>Behaviour domain or type</th>
<th>Behavioural test paradigm</th>
<th>Test measurement</th>
<th>GluN1KD abnormality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxiety-like behaviour</td>
<td>Elevated plus maze; open field locomotion</td>
<td>Time in open spaces</td>
<td>Increased</td>
</tr>
<tr>
<td>Memory; cue/context learning</td>
<td>Fear conditioning</td>
<td>Conditioned freezing</td>
<td>Decreased</td>
</tr>
<tr>
<td>Memory; exploratory behaviour</td>
<td>8-arm radial, Y-, and T-mazes</td>
<td>Maze-arm exploration pattern</td>
<td>Decreased</td>
</tr>
<tr>
<td>Memory; exploratory behaviour</td>
<td>8-arm radial maze</td>
<td>8-arm entries-to-repeat</td>
<td>Decreased</td>
</tr>
<tr>
<td>Memory; exploratory behaviour</td>
<td>Novel object recognition</td>
<td>Investigation time</td>
<td>Decreased</td>
</tr>
<tr>
<td>Memory; puzzle solving</td>
<td>Puzzle box</td>
<td>Puzzle box solution time</td>
<td>Decreased</td>
</tr>
<tr>
<td>Motivation-based conditioning</td>
<td>Conditioned place preference</td>
<td>Place-preference</td>
<td>Mixed abnormalities</td>
</tr>
<tr>
<td>Motor activity; habituation</td>
<td>Open field locomotion</td>
<td>Horizontal locomotor activity</td>
<td>Increased activity; Decreased habituation</td>
</tr>
<tr>
<td>Motor activity; stereotypy</td>
<td>Open field locomotion</td>
<td>Stereotypic fine movements</td>
<td>Increased</td>
</tr>
<tr>
<td>Sensorimotor gating</td>
<td>Prepulse inhibition</td>
<td>Prepulse inhibition</td>
<td>Decreased</td>
</tr>
<tr>
<td>Sensorimotor response</td>
<td>Prepulse inhibition</td>
<td>Acoustic startle</td>
<td>Increased</td>
</tr>
<tr>
<td>Social cognition</td>
<td>Social approach; resident-intruder</td>
<td>Social investigation time</td>
<td>Mixed abnormalities</td>
</tr>
<tr>
<td>Social cognition</td>
<td>Resident-intruder</td>
<td>Aggressive behaviour count</td>
<td>Decreased</td>
</tr>
</tbody>
</table>

Elevated locomotor activity and deficits in sensorimotor gating are considered particularly relevant to the positive symptoms of schizophrenia (Fradley et al., 2005; Hazlett et al., 2007). Locomotor activity and its decrease over time as a result of habituation are assessed by open field tests where horizontal mouse movements are tracked by motion sensors or video recording (Duncan et al., 2006a; Halene et al., 2009a; Milenkovic et al., 2014). The most consistent finding regarding GluN1KD locomotor abnormalities is the delayed habituation of GluN1KD mice in novel environments. In other words, GluN1KD mice move around exploring new cages or arenas longer than WT-littermates (Mohn et al., 1999; Duncan et al., 2006a, 2010; Gregory et al., 2013). This often means a net increase in overall travelled distance over the course of the experiment (Mohn et al., 1999; Duncan et al., 2006a; Milenkovic et al., 2014). To note, closer examination
of the tracked time-courses do not always show GluN1KD mice moving more at the beginning of open field tests, reinforcing the idea of a specific GluN1KD deficit of habituation (Duncan et al., 2002, 2006a, 2012; Miyamoto et al., 2004; Moy et al., 2012). The GluN1KD mice do habituate to WT levels of movement after 4 hours (Mohn et al., 1999), but they have also been reported to travel more in home cages during light cycle hours (Gandal et al., 2012). Finally, there are still other reports of WT-like movement by GluN1KD mice in home cages and arenas of other behavioural tests, which the authors believe may be due to the size of experimental spaces (Halene et al., 2009a; Gandal et al., 2012). In general, GluN1KD mice display hyperlocomotion and decreased habituation on a consistent basis.

Impaired sensorimotor gating is another behavioural change that is consistently observed in GluN1KD mice across multiple labs. Sensorimotor gating is assessed in the prepulse inhibition (PPI) paradigm wherein mice are placed in chambers that measure the auditory startle response (how much a mouse jumps when exposed to loud auditory stimuli). The startle-inducing stimuli may vary in intensity and are sometimes randomly preceded by a less intense auditory stimulus (the prepulse). How much each test mouse jumps after the main auditory stimuli is recorded as acoustic startle responses while how much this startle is decreased in the presence of a prepulse is recorded as PPI % (Moy et al., 2006; Bickel et al., 2008; Islam et al., 2017). To date, GluN1KD mice have consistently presented with increased acoustic startle and decreased PPI (Duncan et al., 2004b, 2010; Fradley et al., 2005; Bickel et al., 2008; Gandal et al., 2012; Islam et al., 2017). This finding has been replicated electrophysiologically as the same GluN1KD mice with PPI impairments also demonstrated impaired attenuation of second-tone ERPs when pairs of auditory stimuli were presented. However, the link between sensory gating of ERPs and PPI is not robust in the literature (Bickel et al., 2008). This specific electro-physiological finding has also not been specifically replicated, though other such experiments in GluN1KD mice still reported auditory ERP abnormalities. The authors of these studies attributed the discrepancies in findings to differences in the interval between auditory stimuli (Halene et al., 2009a; Gandal et al., 2012). Interestingly, habituation was also impaired in GluN1KD mice when PPI experiments were repeated with the same test mice (Bickel et al., 2008). GluN1KD deficits in PPI, locomotor activity, and the habituation of both thus paint an overall picture of neuronal dysregulation and information misprocessing (Halene et al., 2009a; Gandal et al., 2012; Grannan et al., 2016). Fittingly, neuronal disinhibition and signal processing deficits are thought to be neurological
underpinnings of the positive symptoms of schizophrenia (Gilani et al., 2014; Litman et al., 2016; Stevenson et al., 2017). This is again consistent with both PPI deficits and hyperlocomotion being behavioural correlates of positive schizophrenia symptoms (Fradley et al., 2005; Hazlett et al., 2007).

Studies on how antipsychotics affect GluN1KD mouse behaviour has focused on the open field and PPI paradigms. Enhanced startle response in GluN1KD mice has been shown to be attenuated by risperidone, olanzapine, and quetiapine while impaired PPI was improved by haloperidol, risperidone, olanzapine and quetiapine (Duncan et al., 2006a, 2006b). Acoustic startle and PPI abnormalities were also improved with administration of 3 mg/kg clozapine to GluN1KD mice that were C57BL/6 x 129/SvEv hybrids (Duncan et al., 2006b) but not with 1 mg/kg clozapine to mice on a mostly C57BL/6 genetic background (Fradley et al., 2005). GluN1KD hyperlocomotion was also improved with administration of haloperidol, olanzapine, and clozapine (Mohn et al., 1999; Duncan et al., 2006a). It is important to note, however, that these antipsychotics sometimes also decrease locomotor activity, decrease startle response, and increase PPI in WT littermates (Duncan et al., 2006a, 2006b). Thus, while GluN1KD locomotor and PPI abnormalities, related electrophysiology, and their improvement with antipsychotics present a consistent picture for modeling positive symptoms, more research is needed to investigate how these correlates specifically relate to GluN1KD mice. For example, blocking the kainate subtype of glutamate receptors in GluN1KD mice can also improve their locomotor, acoustic startle, and PPI behaviours (Duncan et al., 2010, 2012). However, only PPI is increased in WT littermates when LY382884 was used for kainate receptor antagonism (Duncan et al., 2010). It would therefore be interesting to further investigate glutamate or kainate receptor signaling in GluN1KD mice, along with other instances where responses differ between GluN1KD mice and WT littermates.

GluN1KD mice also consistently show increased stereotypic movements and impaired social behaviour (Mohn et al., 1999; Moy et al., 2008; Gandal et al., 2012; Milenkovic et al., 2014). Both behavioural abnormalities have been considered relevant to the core symptoms of autism (Moy et al., 2008; Gandal et al., 2012) and schizophrenia, with stereotypic behaviours being a positive symptom (Fradley et al., 2005) and reduced sociability being a negative symptom (Cox et al., 2016). Increased stereotypy in GluN1KD mice has been demonstrated in three experimental methodologies: concurrent tracking of fine repetitive movements during the open
field test (Mohn et al., 1999; Miyamoto et al., 2004; Milenkovic et al., 2014), video recording of mouse behaviour while suspended by their tails (Gandal et al., 2012), and manual tracking of grooming behaviour across experiments (Moy et al., 2008). When reported, GluN1KD mice consistently show increased total repetitive fine movements in open field tests (Mohn et al., 1999; Milenkovic et al., 2014; Islam et al., 2017). One study also found this specifically with rearing movements (Duncan et al., 2010). Further observation of time-courses again reveals decreased habituation of stereotypic movements in GluN1KD mice (Mohn et al., 1999; Miyamoto et al., 2004; Milenkovic et al., 2014; Islam et al., 2017). An instance of WT-like stereotypic movement levels in GluN1KD mice at the beginning of open field tests has been shown (Miyamoto et al., 2004), but such instances are rarer than what is seen for horizontal locomotor activity. This may be due to stereotypic movement time-courses being less reported. Also unlike horizontal locomotor activity, increased repetitive behaviour has been more consistently replicated in other behavioural tests. More GluN1KD mice demonstrated handclasping behaviour, where mice repeatedly clasped and shook their forepaws in front of their faces, during tail suspension tests than WT littermates (Gandal et al., 2012). Furthermore, self-injurious repetitive grooming has been documented in GluN1KD mice with an incidence of 25-30%, abnormally high compared to WT littermates (Moy et al., 2008; Gandal et al., 2012). While open field stereotypic behaviour has been shown to be treatable with haloperidol and clozapine (Mohn et al., 1999), consistent with increased stereotypy being a correlate of positive schizophrenia symptoms, GluN1KD handclasping and excessive grooming have not been assessed pharmacologically to date. Non-antipsychotic medications for autism, such as methylphenidate or selective serotonin reuptake inhibitors (LeClerc and Easley, 2015), have not been tested on GluN1KD mice. The kainate receptor antagonist LY382884, which improves GluN1KD horizontal locomotor activity, also attenuates GluN1KD stereotypic movements.

Multiple forms of social behaviour abnormalities have been demonstrated in GluN1KD mice (Mohn et al., 1999; Duncan et al., 2009; Mielnik et al., 2014). From the beginning, the first studied and reported GluN1KD mice were noted as having increased social withdrawal and impaired mating (Mohn et al., 1999). Mating behaviour, as a subset of social behaviour, has been primarily demonstrated in GluN1KD males via decreased mating frequency (Mohn et al., 1999) and ultrasonic vocalizations in the presence of a receptive female (Gandal et al., 2012). However, the majority of investigations into GluN1KD social behaviour did not focus on mating behaviour
but instead focused on social approach tests and resident-intruder assays (Mohn et al., 1999; Duncan et al., 2009; Mielnik et al., 2014; Milenkovic et al., 2014). The social approach test measures how long mice spend investigating novel mice, which are often matched for age and sex or are gonadectomized, relative to a control setup like an empty wire cage (Halene et al., 2009a; Milenkovic et al., 2014). When assessed by this method, GluN1KD mice consistently spent less time investigating the presented social stimulus mouse compared to WT littermates (Duncan et al., 2004b; Halene et al., 2009a; Mielnik et al., 2014). Less time spent on social investigation has also been demonstrated for male GluN1KD mice in the resident-intruder paradigm, where mouse behaviour is monitored after a genotype- and sex-matched intruder mouse is introduced to the home cage of a resident mouse (Mohn et al., 1999). Another way to perform the resident-intruder assay is to more specifically score mouse social interactions. When assessed this way, investigators reported that male and female GluN1KD mice perform less acts of aggression and dominance compared to WT littermates; male mice fight less while female mice mount less, but neither show decreased frequency of investigative social sniffing (Duncan et al., 2004b, 2009). Concurrent Fos induction studies have associated social approach deficits with decreased Fos at the cingulate CTX and lateral septal nuclei (LSN) (Mielnik et al., 2014) and resident-intruder deficits with decreased Fos at the same regions along with others like the hypothalamus and medial preoptic area (Duncan et al., 2009). Both the cingulate CTX and LSN have fittingly been associated with social cognitive function in animals and humans (Apps et al., 2016; Lockwood and Wittmann, 2018; Rilling et al., 2018; Weger et al., 2018), supporting the use of GluN1KD mice to model neuropsychiatric social impairments. Social behaviour pharmacology has been mixed in GluN1KD mice: 0.5 mg/kg clozapine improved resident-intruder behaviour and mating frequency in initially-generated mixed-background GluN1KD mice (Mohn et al., 1999) but 1 mg/kg clozapine did not improve social approach behaviour in C57BL/6J x 129X1Sv/J GluN1KD mice (Mielnik et al., 2014). While this is an inconsistency, it also reflects clinical trials of clozapine in patients with schizophrenia; clozapine had little or no benefits for treating the negative symptoms of schizophrenia (Breier et al., 1994; Buchanan et al., 1998). Non-antipsychotic medications for ASDs have not been tested on GluN1KD social behaviours. Overall, the evidence of social behaviour deficits in GluN1KD mice is robust and may be particularly useful as a model of the negative symptoms of schizophrenia.
There is also convincing evidence of cognitive impairments in GluN1KD mice, but the experiments involved are more varied compared to previously discussed behavioural abnormalities. Through these various assessment methods, GluN1KD mice consistently demonstrated impairments in learning and memory, puzzle solving, and attention (Dzirasa et al., 2009; Milenkovic et al., 2014; Grannan et al., 2016). In the Y-maze and T-maze tests, where mice must remember most-recently visited maze arms to alternately visit each of three arms consecutively (Deacon and Rawlins, 2006; Mandillo et al., 2008), GluN1KD mice consistently had impaired alternation performance (Gandal et al., 2012; Gregory et al., 2013; Milenkovic et al., 2014). In the similar but more complicated 8-arm radial maze, where mice learn to efficiently find food rewards at the end of each arm by strategically and quickly visiting each arm once, GluN1KD mice failed to learn over weeks of repeated trials. Instead, they readily and persistently explore already-visited maze arms (Dzirasa et al., 2009). GluN1KD mice also demonstrated impaired learning of obstacle solutions in the puzzle box test, as well as an impaired ability to bypass obstacles in the first place (Milenkovic et al., 2014; Islam et al., 2017). Exposure to the puzzle box test as a juvenile 3-week old can improve GluN1KD performance 4 weeks later when compared to naïve 6-week old GluN1KD mice, but this was not a complete behavioural rescue (Milenkovic et al., 2014). Finally, in the fear conditioning paradigm, where mice are expected to decrease their movements when exposed to a context or cue they have learned to associate with a foot shock, GluN1KD mice showed a lack of freezing behaviour (Grannan et al., 2016). As mentioned before, altered electrophysiological responses to fear conditioning cues have been reported, but these responses were abnormal and considered an impairment of attention (Bickel et al., 2007). Abnormal modulation of ERPs in GluN1KD brains when exposed to various types of stimuli (Bodarky et al., 2009; Halene et al., 2009b) can similarly be considered forms of attentional deficits, as can previously mentioned sensorimotor gating (Moy et al., 2006), social approach (Moy et al., 2012), and habituation (Bickel et al., 2008). The common concept between these different behavioural and electrophysiological measures is the inability of a nervous system to distinguish between important or novel stimuli from background environmental noise (Bickel et al., 2007, 2008; Bodarky et al., 2009; Halene et al., 2009b; Moy et al., 2012). To date, the main report of pharmacological rescue of GluN1KD cognitive deficits, aside from PPI and habituation improvements with antipsychotics, is the improvement of cue-conditioned freezing with VU6004256. VU6004256 is a potent positive allosteric modulator of the M1 muscarinic acetylcholine receptor and a drug being evaluated for
treating positive and cognitive symptoms of schizophrenia (Grannan et al., 2016). Cholinesterase inhibitors, the foremost class of drugs for treating cognitive impairments and dementia (Gauthier et al., 2006; Deardorff et al., 2015), have not been tested in GluN1KD mice. Memantine, the main approved non-cholinesterase-inhibitor drug for AD (Tariot et al., 2004; Deardorff et al., 2015), may not be suitable to try in GluN1KD mice due to its function as a NMDAR antagonist (Parsons et al., 2007). The NMDAR partial agonist D-cycloserine may be interesting to test in GluN1KD mice, however, as it has been reported, though rarely, to improve cognitive outcomes in human patients (Tsai et al., 1999; Goff et al., 2008). The data overall point to a rescuable cognitive deficit in GluN1KD mice, but more experiments are needed to study it in detail.

The behavioural deficits mentioned above are the major groups of findings from behavioural studies of GluN1KD mice, but there are a few other odd reports as well. GluN1KD mice have been reported to spend more time in the open arms of an elevated zero maze and the center of open field arenas, indicative of decreased anxiety-related inhibition (Halene et al., 2009a). In behavioural test paradigms involving olfactory appetitive stimuli, mainly chocolate chips, GluN1KD mice can detect and obtain them but do not demonstrate a strong preference for them when compared to WT littermates (Duncan et al., 2004b; Moy et al., 2008). For one of these studies, the authors offered an interpretation of possible motivational deficits in GluN1KD mice, at least in the context of desire for a chocolate chip (Duncan et al., 2004b). In contrast, GluN1KD mice can perform like WT littermates in the conditioned place preference behavioural paradigm; in response to repeated injections of amphetamine, GluN1KD mice demonstrate a preference for being in chambers were the injections took place when given the choice. When more closely examined with cocaine conditioning, however, GluN1KD mice developed the place preference more slowly compared to WT mice. This may be another indication of cognitive or motivational deficits, but cocaine and amphetamine also elicit multiple different responses in GluN1KD mice (Ramsey et al., 2008). Amphetamine generally produces WT-like behavioural responses in GluN1KD mice at lower doses and enhanced or altered responses at higher doses while cocaine is less often studied (Miyamoto et al., 2004; Moy et al., 2006; Ramsey et al., 2008). How these findings correlate to motivation is unclear, but it will be useful to keep them in mind when considering dopamine (DA) system changes in GluN1KD mice later. Finally, normal rotarod performance in GluN1KD mice was mentioned previously, indicative of a functional motor nervous system (Duncan et al., 2004b; Fradley et al., 2005). One of the studies employing
the rotarod test conducted multiple trials and demonstrated that GluN1KD mice, like their WT littermates, were able to stay on rotarods longer with repeat trials. The study did not report a statistically significant effect of trial repetition on rotarod performance for WT or GluN1KD mice, but it also did not explicitly state whether such an effect was significantly detected and the experiment design was susceptible to ceiling effects. If both WT and GluN1KD mice significantly improved their rotarod performance with repeated trials, such a result may indicate that GluN1KD mice are not impaired in learning primarily motor tasks (Fradley et al., 2005). No pharmacological interventions have been tested or reported for these behavioural paradigms in GluN1KD mice. Even more so than the established behavioural abnormalities mentioned earlier, GluN1KD deficits related to anxiety, motivation, and motor skill learning need to be replicated and investigated further before any robust interpretations or conclusions can be made.

Gene dose-, sex-, and age-based differences in GluN1KD behavioural abnormalities also need further investigation, as studies that account for these factors are relatively rare. The studies that have been completed report Grin1 haplosufficiency, worse abnormalities in males, and nuanced results on the effects of age (Moy et al., 2006, 2012; Milenkovic et al., 2014). To date, only one published study includes behavioural assessments of GluN1KD-heterozygotes. These heterozygotes largely did not behave differently compared to WT littermates in all the behavioural tests assessed: social approach, PPI, and open field. The only reported difference was that heterozygous males spent more time sniffing a novel mouse than an empty cage at 34-38 days of age while the time-spent difference in WT males was in the same direction but not statistically significant (Moy et al., 2012). This is consistent with the main molecular characterization of GluN1KD-heterozygotes: NMDAR binding was unchanged in their brains relative to WT littermates (Duncan et al., 2002). In all, there is no evidence of a gene dose effect for the neomycin insertion mutation into Grin1.

A few more GluN1KD behavioural studies have considered sex as a factor in their study design or at least their statistical analyses. The results from these studies often pointed to greater abnormalities in males (Moy et al., 2006, 2012; Milenkovic et al., 2014). Such a pattern may not be surprising, as GluN1KD males may be more likely to have smaller body sizes (Moy et al., 2012) and die on an ω-3 deficient diet (Islam et al., 2017). For example, deprivation of ω-3 fatty acids in GluN1KD mice also produces a male-specific exacerbation of puzzle box deficits (Islam et al., 2017). In the PPI test paradigm study, GluN1KD males have been shown to have more
enhanced acoustic startle responses than females (Moy et al., 2006). They also have different acoustic startle and PPI changes in response to exposure to predatory odor compared to GluN1KD females and WT mice of both sexes (Duncan et al., 2004b). Only male GluN1KD mice displayed significant hyperlocomotion at 12 days of age, increased open-field center time at 18-20 days of age, and impaired PPI at 13 and 17 days of age in one wide-ranging study of how GluN1KD behaviours change with sex and age (Moy et al., 2012). In a similarly thorough study from our lab, we found that GluN1KD males had greater total horizontal locomotor activity and stereotypy measures as well as lower social-approach investigation time and Y-maze alternation percentage than GluN1KD females at 6 weeks of age. Furthermore, we found that 12-week old GluN1KD males had a significantly worse Y-maze alternation score than WT males while the deficit in same-aged GluN1KD females was not significant (Milenkovic et al., 2014). One piece of potentially conflicting evidence is the finding that GluN1KD males preferentially investigated novel social stimulus mice at 34-38 days of age when GluN1KD females and WT mice of both sexes did not (Moy et al., 2012). However, this can be interpreted as a GluN1KD abnormality only manifesting in males rather than the GluN1KD males demonstrating an advantageous behaviour compared to GluN1KD females. Overall, most if not all reports of sex-GluN1KD interaction effects point to the males presenting with a more severe phenotype. This increased severity is highly age-dependent, as most sex differences presented above disappear by 12 weeks of age (Moy et al., 2012; Milenkovic et al., 2014).

The overall effect of sex and age on GluN1KD behavioural phenotypes can be summarized as abnormality manifestation or exacerbation with age along with earlier and more severe phenotypes in GluN1KD males (Moy et al., 2012; Milenkovic et al., 2014). Particularly in the study from our lab, the results seem to reflect earlier symptom presentation and worse outcomes in male schizophrenia patients (Eranti et al., 2013; Milenkovic et al., 2014; Thara and Kamath, 2015). However, there are some nuances presented by the two comprehensive studies discussed. In particular, while Y-maze and social approach data from our lab reflect schizophrenia quite well, other behaviours do not (Moy et al., 2012; Milenkovic et al., 2014). Multiple GluN1KD behavioural abnormalities are already robustly present in the youngest tested GluN1KD males and females: acoustic startle in 13-day old mice (Moy et al., 2012) and stereotypic movement count and puzzle box performance in 3-week old mice (Milenkovic et al., 2014). However, PPI was only impaired in 13-day old GluN1KD males with certain prepulse decibels and the amount
of time spent on stereotypic movements was not significantly different in 3-week old GluN1KD mice compared to WT littermates (Moy et al., 2012; Milenkovic et al., 2014). Such nuanced abnormalities need further investigations for meaningful interpretations, but the enhanced startle and stereotypy counts could still be indicative of abnormal behaviours manifesting in the early infancy of GluN1KD mice. Indeed, infant GluN1KD mice of at least one sex also demonstrate significantly increased horizontal locomotor activity (Moy et al., 2012; Milenkovic et al., 2014), open field center time, and social approach behaviour (Moy et al., 2012). This is inconsistent with the early-adulthood or later onset of schizophrenia in humans (Sham et al., 1994; Thorup et al., 2007; Eranti et al., 2013), but the behavioural abnormalities may be more representative of the childhood onset seen in autism (Baxter et al., 2015). This is also not a complete resolution, however, as the age-of-onset and age trajectories in autism patients are similar between the sexes. There are also reports of greater symptom severity in male autism patients, but this is not firmly established due to a potential underdiagnosis of female patients and their subsequent underrepresentation in research studies (Baxter et al., 2015; Halladay et al., 2015). There is a higher prevalence of autism in males, however (Baxter et al., 2015). The results paint a complex picture overall, but the early-onset and late-onset groups of GluN1KD behavioural phenotypes may support the idea of using GluN1KD mice to model autism and schizophrenia, respectively.

Similarly complex are the specific effects of age on GluN1KD social approach time and horizontal locomotor activity. Recall for social approach behaviour that 3-week old GluN1KD mice behaved like WT littermates, 5-week old males were more social, 6-week old males were less social, and GluN1KD females were only significantly different (less social) after adulthood. For horizontal locomotor activity, 2-week old GluN1KD males were hyperactive, 3-week old mice of both sexes were hyperactive, and this hyperactivity was then persistent but decreased significantly by 6- and 12-weeks of age (Moy et al., 2012; Milenkovic et al., 2014). The decreased horizontal locomotor activity with age may be due to the mice spending more time engaged in stereotypic behaviour, but more research is needed to investigate this explicitly (Milenkovic et al., 2014). Overall, such patterns need to be replicated in GluN1KD mice of one genetic background using one consistent methodology. And if replicated, future research into the relationship between NMDAR deficiency and behaviour may benefit from targeting the neurodevelopmental windows presented by these results. Male GluN1KD brains between 5 and 6 weeks of age, for example, may be undergoing neurodevelopmental changes very important for
social behaviour. Unfortunately, no other study to date has assessed the effects of age on GluN1KD behaviour. This also means no studies to date have investigated how drug effects might vary in GluN1KD mice of different ages. The current results of age studies using GluN1KD mice point to most behavioural abnormalities being present by adulthood and persisting or worsening with age. Some of these phenotypes start manifesting during adolescence (5-6 weeks of age) while other are already present before weaning (3 weeks or younger). The results also currently point to a strong interaction between sex and age effects on GluN1KD behaviours.

There is a robust body of evidence demonstrating a variety of behavioural abnormalities in GluN1KD mice. These abnormalities have been considered relevant to positive, negative, and cognitive symptoms of schizophrenia as well as symptoms seen in ASDs (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012; Moy et al., 2012; Milenkovic et al., 2014). The relatively rarer GluN1KD studies that consider the effects of age and sex on behavioural phenotypes offer some trends that may reflect both schizophrenia (Eranti et al., 2013; Milenkovic et al., 2014; Thara and Kamath, 2015) and autism (Moy et al., 2012; Baxter et al., 2015). Many of the GluN1KD behavioural phenotypes have been improved with antipsychotic treatment (Mohn et al., 1999; Duncan et al., 2006a, 2006b) while studies with other approved medications for neurological disorders are few (Duncan et al., 2006b; Mielnik et al., 2014). Studies with drugs that act on molecular systems known to interact with NMDAR functions have also reported improvements of GluN1KD phenotypes (Duncan et al., 2010; Gregory et al., 2013; Grannan et al., 2016). However, there is still a lack of molecular experiments confirming these drugs’ mechanisms of action in GluN1KD mice (Duncan et al., 2010; Gregory et al., 2013). Investigating the effects of other classes of drugs on GluN1KD mice will be important in understanding the relationship between GluN1KD phenotypes and the human symptoms they likely model, as well as how the test drug’s mechanism of action is involved. To facilitate an improved understanding of this last relationship, studies characterizing GluN1KD cellular and molecular phenotypes are important. Such studies will also help inform the decision and prioritization of what drugs to test next in GluN1KD mice.
1.2.3 Cellular and Molecular Changes in GluN1KD Mice

Investigations into the cellular and molecular alterations of GluN1KD mice mainly focus on the brain, as illustrated in Figure 1.4. They are fewer than behavioural studies, though there are overlaps; two such overlaps were mentioned with GluN1KD behavioural abnormalities. Impaired secondary auditory ERPs were detected in GluN1KD mice PPI deficits (Bickel et al., 2008) and decreased Fos induction at distinct brain regions was detected during social behaviour test paradigms (Duncan et al., 2009; Mielnik et al., 2014). Other studies focus purely on cellular and molecular changes; they are primarily characterization studies (Duncan et al., 2002; Wesseling et al., 2014; Cox et al., 2016). Studies that account for sex, and age differences in GluN1KD mice are again few but very useful, especially when considered in the context of results from behavioural studies (Duncan et al., 2009; Ramsey et al., 2011; Islam et al., 2017). Other than WT-like NMDAR binding in the brains of GluN1KD-heterozygotes (Duncan et al., 2002), no other cellular or molecular study published to date has investigated gene dose effects. Overall, the results from these non-behaviour-focused GluN1KD studies are disconnected and focused on their individual research contexts, but a few general trends can be discerned. While there is evidence of hypofunctional GluN1KD brains, particularly and expectedly with NMDAR-specific measures (Jocoy et al., 2011), there are also striking reports of neuronal hyperexcitability (Jocoy et al., 2011; Ferris et al., 2014). These two conflicting types of findings are likely related via inappropriate disinhibition and altered neuronal circuits in GluN1KD mice (Ferris et al., 2014; Grannan et al., 2016). Fittingly, of all the biological processes regulated by NMDARs, synaptic connectivity and plasticity may be particularly and importantly disrupted in GluN1KD mice (Ramsey et al., 2011; Wesseling et al., 2014). These trends may be unsurprising due to previously discussed behavioural abnormalities and may indeed also benefit from being considered with behavioural test results in mind (Halene et al., 2009b; Ramsey et al., 2011).
Figure 1.4. Major cellular and molecular changes reported in previous GluN1KD studies.

There is evidence for both increased and decreased neuronal activity in the GluN1KD brain. Such conflicting or complex alterations may be due to more general changes of impaired synaptic plasticity. The most studied brain region in GluN1KD mice to date is the STR, followed by the CTX and HPC.

Several pieces of evidence point to hypofunctionality in GluN1KD brains (Duncan et al., 2002, 2008; Jocoy et al., 2011). NMDAR hypofunction, as mentioned before, has been demonstrated directly in striatal MSNs (Jocoy et al., 2011). Uptake of 2-deoxyglucose (2-DG) and induction of Fos expression, both commonly used markers of brain activity (Duncan et al., 1993; Lundgaard et al., 2015; Renier et al., 2016), are generally muted in GluN1KD mice (Duncan et al., 2002, 2008; Inada et al., 2007; Mielnik et al., 2014). Basally, multiple layers and nuclei in the GluN1KD CTX, STR, and other brain regions show reduced 2-DG uptake compared to WT littermates. No assessed region displayed increased 2-DG uptake in GluN1KD mice (Duncan et al., 2002). Compared to WT littermates, GluN1KD mice have demonstrated decreased Fos induction in various brain regions after pharmacological NMDAR agonism with TZG (Inada et al., 2007; Duncan et al., 2008), amphetamine administration (Miyamoto et al., 2004), cocaine administration (Ramsey et al., 2008), and social behaviour testing (Duncan et al., 2009; Mielnik et al., 2014). There were also no assessed brain regions in any of these studies that showed increased Fos induction in GluN1KD mice compared to WT littermates. As discussed
before, these differences in Fos induction might be of vague biological significance, such as when WT-like levels of Fos at the GluN1KD STR is considered with impaired 2-DG uptake and MSN EPSPs (Duncan et al., 2002, 2009; Miyamoto et al., 2004; Jocoy et al., 2011). This may be in part due to a brain region resolution issue, as there is one report of differential GluN1KD Fos induction responses between dorsal and ventral STR (Ramsey et al., 2008). But Fos induction in GluN1KD LSN may serve as a robust counter-example of when the indirect neuronal activity marker works as intended: two independent studies confirmed GluN1KD Fos induction deficits after two different social behaviour test paradigms (Duncan et al., 2009; Mielnik et al., 2014) and an increased Fos induction coincided with improved social behaviour in GluN1KD mice (Mielnik et al., 2014). Overall, these studies present many indicators of decreased activity in the GluN1KD brain. However, one study did report increased Fos induction in GluN1KD mice after kainic acid administration. The authors also reported GluN1KD behavioural improvements in the open field and PPI tests with systemic administration of a kainate receptor antagonist (Duncan et al., 2010). This is one example of evidence for a hyperglutamatergic state in GluN1KD mice.

There are several pieces of evidence for a hyperexcitable or hyperglutamatergic brain in GluN1KD mice. One of the most overt pieces is the noticeable incidence of seizures and seizure-like brain activity in GluN1KD mice (Dzirasa et al., 2009; Halene et al., 2009a). Furthermore, 20 mg/kg kainic acid was reported to reliably induce lethal seizures in GluN1KD mice but not WT littermates (Duncan et al., 2010). But still consistent with the idea of NMDAR hypofunction in GluN1KD mice, they were not more susceptible than WT mice to seizures induced by 1.25-1.5 mg/kg TZG (Duncan et al., 2008). Another blatant sign of hyperexcitability in GluN1KD brains is the enhanced AMPAR-mediated EPSPs in MSNs – the same type of neurons that demonstrated inhibited NMDAR-mediated responses (Jocoy et al., 2011). To note, no changes of AMPA or kainate receptors were detected in any brain region of GluN1KD mice by quantitative autoradiography (Duncan et al., 2002). AMPAR subunit protein levels were also unchanged when assessed by western blotting while GluN2A and GluN2B were decreased in the GluN1KD STR, HPC, and prefrontal CTX (Ramsey et al., 2008). This would imply that glutamatergic activity or excitability increases in GluN1KD mice may well be mediated by alterations in function or trafficking of AMPA or kainate receptors – the two main non-NMDA ionotropic glutamate receptors (Duncan et al., 2002; Fradley et al., 2005). There are also the ERP studies that have been mentioned and discussed multiple times now. GluN1KD ERPs to both visual and
auditory stimuli are enhanced and less inhibited (Bickel et al., 2008; Halene et al., 2009b, 2009a; Gandal et al., 2012), consistent with increased excitability and disinhibition at the brain and somewhat reinforced by the reports of GluN1KD PPI deficits (Bickel et al., 2008). Also of behavioural relevance, GluN1KD mice have an absence of muscarinic-receptor induced LTD and an increase of prefrontal CTX pyramidal neuron firing. Normalization of these measures was related to GluN1KD cognitive improvements after administration of VU6004256 (Grannan et al., 2016). Overall, studies using electroencephalography (EEG) and electrophysiology consistently demonstrate increased brain activity in GluN1KD mice, which is also reflected by seizures and behavioural changes.

Aside from general or glutamatergic electrical activity in the brain, the DA system in GluN1KD mice may serve as another example of hyperactivity. Like AMPA and kainate receptors in GluN1KD mice, many measures of DA system homeostasis were reported as unchanged compared to WT littermates (Mohn et al., 1999; Duncan et al., 2002; Ramsey et al., 2008). From their initial characterization, GluN1KD mice had WT-like levels of DA and its metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid at the STR (Mohn et al., 1999). This was true for both tissue content and extracellular levels and was later reconfirmed in a stimulant challenge study where DA release was measured in vivo by microdialysis (Mohn et al., 1999; Ramsey et al., 2008). GluN1KD mice also had WT-like numbers of MSNs along with unperturbed protein levels for DA transporters (DAT), D₁ receptors (D₁Rs), and D₂ receptors (D₂Rs). The DA receptors were also determined to be functional in vivo when direct agonism resulted in horizontal locomotor activity changes similar to what is seen in WT mice (Ramsey et al., 2008). The first report of a dopaminergic disruption in GluN1KD mice was the finding that DA enhancement of NMDAR currents in MSNs via D₁Rs was weaker, but inhibition via D₂Rs was not (Jocoy et al., 2011). Furthermore, when electrically-evoked DA release was measured in brain slices by fast-scan cyclic voltammetry, GluN1KD mice demonstrated deficits at both the nucleus accumbens (NAc) and caudate. Such a finding was reported alongside decreased synaptic tyrosine hydroxylase (TH) protein levels and function, meaning less DA synthesis, as well as increased synaptic DAT protein and function, meaning more clearance of extracellular DA (Ferris et al., 2014). These results point to decreased DA signaling, but further experiments also revealed impaired D₂R-mediated autoinhibition of DA release and higher tonic firing rate of ventral tegmental area (VTA) neurons, which release DA (Taber et al., 2002; Ferris et al., 2014).
VTA neurons in GluN1KD mice were also more excitable but less likely to produce burst-firing patterns, similar to previous results of pharmacological NMDAR blockade on in vivo rat cortical neurons (Jackson et al., 2004; Ferris et al., 2014). The authors of this study ultimately interpreted these findings as GluN1 knockdown leading to chronically hyperactive VTA neurons, desensitized D2 autoreceptors, and an ultimately altered DA system with decreased transmission (Ferris et al., 2014). Overall, studies of the GluN1KD DA system found subtle changes pointing to a slightly hypodopaminergic state resulting from chronic hyperactivity.

Behavioural changes in GluN1KD mice after administration of DA-system-targeting and related drugs can further help understand their dopaminergic state. When treated with amphetamine, which blocks DAT and increases DA release, GluN1KD open field and PPI responses were WT-like at low doses but enhanced or altered at higher doses (Miyamoto et al., 2004; Moy et al., 2006; Ramsey et al., 2008). Cocaine, which affects the DA system primarily by blocking DAT, produced more muted locomotor activity changes and delayed place-preference conditioning when compared to amphetamine (Ramsey et al., 2008). Finally, all instances of differential benefits between haloperidol, which potently and selectively blocks D2Rs, and atypical antipsychotics, which affect multiple neurotransmitter systems, point to the typical antipsychotic being less effective (Duncan et al., 2006b, 2006a; Wesseling et al., 2014). These results help interpret the findings from DA system studies of GluN1KD mice. The dopaminergic deficits in GluN1KD mice do not seem to be enough to diminish amphetamine responses up to the highest dose tested of 10 mg/kg (Moy et al., 2006) but enough to affect cocaine (Ramsey et al., 2008). This is consistent with the findings of increased DAT in GluN1KD mice and suggests that the decreased TH was not enough to affect amphetamine-mediated DA release (Ferris et al., 2014). The combined results from DA-related GluN1KD studies also confirm an important role of DA in open field and PPI behavioural abnormalities, as haloperidol produces robust improvements, but DA is also not the only player as atypical antipsychotics seem to generally do better (Mohn et al., 1999; Duncan et al., 2006b, 2006a). This may be a reflection of human schizophrenia patients, but the advantage of atypical antipsychotics over haloperidol in humans may be more due to less side effects and/or better efficacy on non-positive symptoms (Leucht et al., 2009; Nandra and Agius, 2012). More specific conclusions will need further research. Currently, DA may be the best characterized neurotransmitter system in GluN1KD mice due to many studies investigating the mice for modelling schizophrenia (Mohn et al., 1999; Moy et al.,
Other reports of cellular or molecular changes in GluN1KD mice are more disparate in research context.

The altered DA transmission mentioned above can also serve as an example of disrupted synaptic and neuronal connectivity in GluN1KD mice, which is a common theme in several reports of GluN1KD brain changes (Duncan et al., 2002; Dzirasa et al., 2009; Gandal et al., 2012). The differential 2-DG uptake and Fos-induction deficits between brain regions in GluN1KD mice can be interpreted as a disruption of neuronal networks as well (Duncan et al., 2002, 2008; Inada et al., 2007). For example, this was suggested for 2-DG uptake deficits in the GluN1KD CTX: uptake is impaired in layer 6 output neurons but not in layers 3 and 4 that receive thalamic input (Duncan et al., 2002). On a network-activity level, auditory-evoked synchronization of γ brain waves was deficient in GluN1KD mice, likely reflecting excitation-inhibition imbalances (Gandal et al., 2012). Brain wave synchronization patterns were also abnormal in GluN1KD mice during exploration of an 8-arm radial maze. Specifically, intra-structure synchrony at the HPC and prefrontal CTX was disrupted along with an abnormally increased synchronization between hippocampal θ waves and cortical γ waves (Dzirasa et al., 2009). Coincidental impairments of maze performance and neuronal synchrony are consistent with previous reports connecting synchronous brain activity to memory formation and rodent spatial navigation (Fell et al., 2001; Jones and Wilson, 2005; Herrmann et al., 2010). Disrupted neuronal synchrony in human patients has been reported in both ASDs and schizophrenia (Dzirasa et al., 2009; Gandal et al., 2012). Finally, direct measurements of MSN dendritic spine density revealed a deficit in GluN1KD mice at 6 weeks of age but not 2 weeks (Ramsey et al., 2011). Dendritic spines, to reiterate, are the physical locations of most excitatory synapses – they are therefore also representations of specific neuronal connections with behavioural relevance (Moser et al., 1994; Sanders et al., 2012; Saneyoshi and Hayashi, 2012). The reported deficit was primarily in mature, mushroom-shaped spines (Ramsey et al., 2011) and was coincidental with the onset of some GluN1KD behavioural abnormalities: stereotypy time, social approach time, and Y-maze alternation (Milenkovic et al., 2014). It was also found with concurrent striatal decreases of the proteins 14-3-3ε and disrupted in schizophrenia 1 (DISC1), which are related to Rho GTPase regulation, neurite growth, cognitive function, and risk for schizophrenia (Hayashi-Takagi et al., 2010; Brandon and Sawa, 2011; Ramsey et al., 2011).
This investigation into striatal DISC1 and MSN spine density of 2- and 6-week old mice is also the primary molecular study considering the effects of age in GluN1KD mice. Along with spine, 14-3-3ε and DISC1 deficits, 6-week old mice also had increased synaptic levels of the lissencephaly-1 (LIS1) protein (Ramsey et al., 2011). However, total STR level of LIS1 was decreased, pointing to dysregulation and disruption of its work with 14-3-3ε and DISC1 for neurodevelopmental processes like axon elongation and neuronal migration (Toyo-oka et al., 2003; Taya et al., 2007; Ramsey et al., 2011). The juvenile 2-week old GluN1KD STR had more modest decreases in DISC1 – only significant for one isoform at the synapse – with no other deficits of DISC1 interactor proteins (Ramsey et al., 2011). Total tissue and synaptic 14-3-3ε decreases were also confirmed in the STR of adult GluN1KD mice later, though the study focused on 14-3-3ε’s ability to increase TH activity and affect DA synthesis (Ferris et al., 2014). These molecular changes, like the spine deficits, generally mirror GluN1KD behaviour abnormality onset and progression – signs of disruption appear at 2 weeks of age and worsen by 6 weeks (Ramsey et al., 2011; Moy et al., 2012; Milenkovic et al., 2014). Overall, several independent studies using various distinct methodologies have demonstrated disrupted synaptic connectivity in some form in GluN1KD mice.

The last main group of molecular studies in GluN1KD mice are proteomic studies (Wesseling et al., 2014; Cox et al., 2016). The first is a characterization study of GluN1KD mice investigating protein level differences in blood serum, frontal CTX, and HPC (Wesseling et al., 2014). The second included GluN1KD mice in a proteomic comparison of multiple animal models of schizophrenia with the profiles of human patients, focusing on the anterior prefrontal CTX (Cox et al., 2016). The identified protein differences from both studies are compiled in Supplemental Table SI, using raw data from their supplementary materials. In the characterization study, dozens of proteins were differentially expressed between GluN1KD mice and WT littermates. The authors emphasized that various neurotrophic factors are increased in the serum of GluN1KD mice and that computational pathways analyses pointed to proteomic changes in both brain regions being related to synaptic LTP, myelination, and ERK-signaling (Wesseling et al., 2014). Combined with the fact that ERK signaling is often related to NMDAR-mediated development and maintenance of synapses, the proteomic characterization study offers consistent and robust evidence for synaptic disruption in GluN1KD mice (Thomas and Huganir, 2004; Tian et al., 2004; Wesseling et al., 2014). The second proteomic study did not investigate
GluN1KD mice quite as specifically or deeply. Instead, the authors considered changes in schizophrenia patients and animal models together to find consensus pathways and functional domains that they then used to evaluate the similarity between each animal model and human patients. The five consensus functional domains of protein changes were generally vague, but two bared some resemblance to the characterization study results: “development and differentiation” and “nucleic acid metabolism and ATP/GTPase activity”. Closer examination of specific pathways altered in GluN1KD mice reinforced this echoing of results: “cell projection organization”, “regulation of cell projection organization”, and “small GTPase mediated signal transduction” (Cox et al., 2016). Note that small GTPases include Rho GTPases as a subfamily, which were previously mentioned as part of the mechanism relating DISC1 to synaptic plasticity and cognition (Luo, 2000; Hayashi-Takagi et al., 2010; Brandon and Sawa, 2011). Taken together, the patterns of proteomic changes reported in GluN1KD mice offer some consistent support for the idea of synaptic disruptions in the GluN1KD brain.

Cellular and molecular studies of GluN1KD mice that assess sex differences are limited, but a few sex differences have been published (Duncan et al., 2009; Islam et al., 2017). Basal activation of Fos was only decreased in the male cingulate CTX and basolateral nucleus while female GluN1KD mice were not different from female WT littermates. This difference was found in control mice of the investigation into differential Fos induction after resident-intruder mice. It is important to point out then that these differences may not be completely reflective of a basal state; they may instead be differential responses to a week of social isolation that is part of the protocol for control mice (Duncan et al., 2009). Such an interpretation is possible as both brain regions have been reported to be altered with social isolation or rejection (Somerville et al., 2006; Wang et al., 2012). To note, no sex differences in Fos induction after resident-intruder testing was reported (Duncan et al., 2009). The other reported molecular sex difference in GluN1KD mice relates to the increased mortality of males on an ω-3 deficient diet. Screening of more than a dozen fatty acids in the brain and serum of GluN1KD mice in this study revealed significant increases of α-linolenic acid and linoleic acid in the brain as well as decreased gondoic acid in serum. Supplementation or deficiency of ω-3 fatty acids resulted in many changes to the GluN1KD lipid profile, but supplementation did not affect behaviours tested. GluN1KD mice on an ω-3 deficient diet, to reiterate, had worse puzzle box performance along with their increased mortality. Changes in GluN1KD fatty acids after an ω-3 deficient diet were
mostly the same between males and females however: increases of various ω-6 fatty acids in the brain and serum, decreases of α-linolenic and docosahexaenoic acids (both ω-3) in the brain and serum, and decrease of oleic acid (ω-9) in the serum (Islam et al., 2017). While these changes may have various specific meanings, increased ω-6 fatty acids or ω-6/ω-3 ratios are generally associated with poorer health measures (Simopoulos, 2002). Complementing this idea in GluN1KD mice, ω-3 supplementation has been reported to improve mental health outcomes in patients with depression or schizophrenia (Peet and Stokes, 2005). Despite all these changes however, the main sex-dependent change was an increase of serum eicosadienoic acid in GluN1KD males after ω-3 deficiency. A specific role for eicosadienoic acid may be to serve as negative feedback in prolonged inflammation (Huang et al., 2011), potentially pointing to a worse inflammatory state in ω-3 deficient GluN1KD males. Further research is needed to understand the presented molecular sex differences in GluN1KD mice, as well as answer other questions like why ω-3 supplementation did not improve GluN1KD phenotypes.

Cellular and molecular studies of GluN1KD mice present evidence of both hypofunction and hyperactivity in their brains (Duncan et al., 2002; Jocoy et al., 2011; Ferris et al., 2014; Grannan et al., 2016). Evidence also suggests that the net effect of developmental and chronic NMDAR deficiency is a disinhibited brain with disrupted neuronal signaling connections (Dzirasa et al., 2009; Ramsey et al., 2011; Wesseling et al., 2014). It is important to note here that these general trends are based on current, limited evidence, and may be affected by factors such as research interest bias. As an example, other molecular pathways and consensus functional domains were represented by GluN1KD proteomic changes aside from those discussed above (Wesseling et al., 2014; Cox et al., 2016). These pathways or domains can be vague and broad, such as the “biosynthetic processes and energy metabolism” consensus domain (Cox et al., 2016). It is possible that this domain is related to 2-DG uptake deficiencies in the CTX of GluN1KD mice, but more research is needed than one uptake and two proteomic studies (Duncan et al., 2002; Wesseling et al., 2014; Cox et al., 2016). There is also a report of the miR-219 being decreased in the prefrontal CTX and HPC of GluN1KD mice, which the authors related to NMDAR-antagonist-induced hyperlocomotion, but the whole field of microRNAs has been largely unexplored so far in the GluN1KD model (Kocerha et al., 2009). It is reassuring, however, that the currently available evidence points to consistent themes that can direct future research.
1.3 Study Overview

1.3.1 GluN1KD Model Validity, Utility and Research Impact

Results of many studies support the validity of using GluN1KD mice to model neurological disorders, particularly schizophrenia and ASDs (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012). There is strong support for the construct validity of GluN1KD mice for these disorders, as NMDAR dysfunction has been often related to both (Moghaddam and Javitt, 2012; Won et al., 2012; Burnashev and Szepetowski, 2015). Molecular evidence also supports the validity of the GluN1KD model, such as when proteins or biological processes altered in GluN1KD mice were also reported as changed in human patients (Ramsey et al., 2011; Wesseling et al., 2014; Cox et al., 2016). GluN1KD PPI deficits and hyperactivity, social behaviour deficits, and cognitive impairments may represent the positive, negative, and cognitive symptoms of schizophrenia, respectively (Mohn et al., 1999; Halene et al., 2009a; Milenkovic et al., 2014; Grannan et al., 2016). Social and cognitive impairments in GluN1KD mice can also be relevant to ASDs along with multiple forms of stereotypic behaviour, a hallmark of autism (Moy et al., 2008; Gandal et al., 2012; Milenkovic et al., 2014). To the extent that mouse behaviour can seem like human behaviour, the behavioural phenotypes seen in GluN1KD mice presents a compelling degree of face validity. Fittingly, antipsychotics improve multiple GluN1KD behavioural abnormalities (Mohn et al., 1999; Duncan et al., 2006b, 2006a) and are used to treat both schizophrenia and ASDs (Anderson et al., 1984; Miyamoto et al., 2005; Posey et al., 2008). The GluN1KD model thus also presents a degree of pharmacological predictive validity. Taken together, there are many pieces of evidence supporting the use of GluN1KD mice to model schizophrenia and ASDs.

However, the utility of GluN1KD mice is not necessarily limited to studying schizophrenia and autism. The reports of seizures and seizure-like activity in GluN1KD mice (Dzirasa et al., 2009; Halene et al., 2009a), which might reflect a higher susceptibility to seizures in autism patients (Volkmar and Nelson, 1990; Qadir et al., 2017), may also indicate a relevance to epilepsy. GluN1KD mice may be especially useful to study how NMDAR hypofunction can lead to a hyperactive or hyperexcitable brain. This would be important for understanding the many epileptogenic GRIN mutations that result in a loss of function (Xu and Luo, 2018). If GluN1KD brains are indeed in a generally hyperglutamatergic or hyperexcitable state, which certainly
needs more experimental confirmation, then GluN1KD mice may also be used to study the excitotoxic processes related to various pathologies (Parsons and Raymond, 2014; Weilinger et al., 2016; Tönnes and Trushina, 2017; Estrada-Rojo et al., 2018). While GluN1KD brains may generally have decreased NMDARs, about 10% still remain and can respond (Mohn et al., 1999; Duncan et al., 2008; Jocoy et al., 2011). If the distribution of remaining NMDARs in GluN1KD mice is WT-like, which is another interesting hypothesis to test, a concurrent hyperglutamatergic state may translate to a considerable level of excitotoxic signaling via relatively increased extrasynaptic NMDAR signaling (Hardingham et al., 2002; Parsons and Raymond, 2014). Furthermore, GluN1KD cognitive impairments may be useful to model similar impairments in AD patients, as NMDAR hypofunction can contribute to cognitive decline (Snyder et al., 2005; Shankar et al., 2007; Zhang et al., 2009b). Overall, the utility of GluN1KD mice likely extends beyond schizophrenia and ASDs. The various GluN1KD behavioural, cellular, and molecular phenotypes may, individually and in combinations, be useful as animal correlates of many human disorders.

Many people suffer from the neurological disorders that GluN1KD mice can model, even if just considering schizophrenia and ASDs (Kessler et al., 2005b; Melville et al., 2008; Simeone et al., 2015; McKenzie et al., 2016). Estimates of lifetime prevalence for schizophrenia and schizophreniform disorder range between 0.3 to 1.6% (Kessler et al., 2005a), with 1% being generally accepted as a good approximation (Vanasse et al., 2012). More recent global lifetime prevalence estimates for schizophrenia range from 0.48 to 0.72% of the population, though there is much variation between epidemiological studies from all over the world (McGrath et al., 2008; Simeone et al., 2015). In Canada, a lifetime prevalence of up to 1.46% was estimated in a 2012 study based on a sample size of 6 million people from Quebec (Vanasse et al., 2012). Prevalence estimates of ASDs have been in a similar range, from about 0.58 to 1.16% (Melville et al., 2008; Elsabbagh et al., 2012). Moreover, the prevalence of autism has been increasing in recent decades (Chien et al., 2011; Idring et al., 2015). Such prevalence increases have been reported in Canada and the USA (Newschaffer, 2005; Ouellette-Kuntz et al., 2014), and may not be completely explained by increased diagnoses or reporting (Ouellette-Kuntz et al., 2014; Hansen et al., 2015). From the 2010 Global Burden of Disease Study, schizophrenia was estimated to result in 13 million years lived with disability (YLDs) and 0.6 million years of life lost to premature mortality for its patients, the most severe after depressive and anxiety disorders.
Pervasive developmental disorders, which includes ASDs and Asperger's syndrome, were estimated to have a burden of 7.7 million YLDs (Whiteford et al., 2013). Beyond the patients, caregivers of patients with neurological disorders also bear significant emotional, social, and economical burdens (Kessler et al., 2008; Zahid and Ohaeri, 2010; Mak and Cheung, 2012). On a societal level, annual costs of 94 million to 102 billion US$, or 0.02% to 1.65% of gross domestic product, have been estimated for schizophrenia depending on country and study (Chong et al., 2016). Studies publishing such large-scale estimates are less common for ASDs, but they similarly report cost estimates on the scale of billions of US$ (Järbrink and Knapp, 2001; Ganz, 2007; Knapp et al., 2009; Horlin et al., 2014). Given the scale of the problem, treatment options are relatively limited for schizophrenia and autism patients, particularly for cognitive impairments (Green, 2016; Kishi et al., 2017; Masi et al., 2017). Impaired cognition greatly contributes to patients’ decreased quality of life and worse long term prognoses (Carpenter and Strauss, 1991; Howlin et al., 2004; Mitchell et al., 2010; Tolman and Kurtz, 2012). Thus, there is a great need for further research into the etiology, pathophysiology, and treatments of these disorders. Again, this is not to mention other disorders that GluN1KD mice can model or the more recently reported cases of autoimmune NMDAR encephalitis (Dalmau et al., 2007; Florance-Ryan and Dalmau, 2010; Irani et al., 2010). Using GluN1KD mice to study schizophrenia, ASDs, and other disease contexts related to NMDAR hypofunction can help address the current need for further mental health research.

1.3.2 Rationale and Purpose of the Study

A major goal for research with GluN1KD mice is to facilitate new treatment development. As part of this process, new research should build on and complement what is known and published previously. The most studied drug class in GluN1KD mice are approved antipsychotics, which improves some behavioural abnormalities (Mohn et al., 1999; Duncan et al., 2006b, 2006a). Given that these drugs are already used in patients with limited effects on non-positive schizophrenia symptoms (van Os and Kapur, 2009; Green, 2016; Masi et al., 2017), other classes of drugs and mechanisms of action should be explored. Other drugs tested on GluN1KD mice to date have targeted kainate receptors (Duncan et al., 2010, 2012), metabotropic glutamate receptors (Gregory et al., 2013), muscarinic receptors (Grannan et al., 2016), and γ-aminobutyric acid receptors (Mielenk et al., 2014). These drugs only resulted in limited improvement for a few behaviours (Duncan et al., 2012; Gregory et al., 2013) or were only tested in a few behavioural
paradigms (Mielnik et al., 2014; Grannan et al., 2016) and most had limited cellular or molecular GluN1KD data informing their drug use or explaining drugs’ mechanisms of action. To increase the chances of finding effective treatments for GluN1KD phenotypes and the interpretability of experimental findings regardless of efficacy, more studies of cellular and molecular changes in GluN1KD mice are needed. Preferably, these studies would also consider the behavioural relevance of molecular changes. The is part of my first research aim.

As discussed before, current cellular and molecular GluN1KD data point to a few general themes such as hyperexcitability, disinhibition, dysregulated DA signaling, and synaptic dysconnectivity (Dzirasa et al., 2009; Jocoy et al., 2011; Ramsey et al., 2011; Ferris et al., 2014). All of these can be, at least partially, subsumed under the last theme of synaptic dysconnectivity since excitatory, inhibitory, and DA signaling disruptions may well contribute to and manifest from dysfunctional synapses. The most in-depth knowledge about GluN1KD neuronal changes and synaptic disruptions relate to the STR: 2-DG uptake is inhibited (Duncan et al., 2002); Fos-induction is WT-like in almost all experimental paradigms (Miyamoto et al., 2004; Inada et al., 2007; Duncan et al., 2008; Ramsey et al., 2008); DA system abnormalities were found in the STR, where it also receives input from overactive VTA neurons (Voorn et al., 2004; Ferris et al., 2014); NMDAR currents are decreased and less enhanced by DA but AMPAR currents are increased in MSNs (Jocoy et al., 2011); and age-dependent DISC1 and spine density deficits were also found in the STR and its MSNs, respectively (Ramsey et al., 2011). Furthermore, striatal Fos-induction was associated with open field behaviour changes (Miyamoto et al., 2004; Ramsey et al., 2008) while MSN spine deficits were developmentally consistent with the onset of multiple GluN1KD behavioural abnormalities (Ramsey et al., 2011; Milenkovic et al., 2014). Thus, we chose synaptic plasticity as a major concept of focus for the research in this thesis and also focused our studies on the STR to build on previous knowledge.

To build on and complement previous knowledge of GluN1KD molecular, cellular, and behavioural phenotypes, my research includes a hypothesis-driven research aim and an unbiased bioinformatics-based aim. These two types of approaches reflect those of previous GluN1KD studies: those that focused on more specific research questions (Duncan et al., 2008; Mielnik et al., 2014) and those that were broader characterizations of GluN1KD phenotypes (Duncan et al., 2002; Wesseling et al., 2014). Firstly, we aimed to further investigate the relationship between DISC1, synaptic connectivity, and behaviour in GluN1KD mice. As noted previously, DISC1
can regulate Rho GTPases that develop and shape dendritic spines; all steps and components of this process are important for proper cognition (Dillon and Goda, 2005; Hayashi-Takagi et al., 2010; Brandon and Sawa, 2011). Our lab therefore asked whether levels of Rho GTPase signaling proteins were altered in the STR of GluN1KD mice concurrently with MSN spine deficits. This work was largely completed by Marija Milenkovic, a previous student of the Ramsey lab, who found general decreases of the proteins Ras-related C3 botulinum toxin substrate 1 (Rac1) and Wiskott-Aldrich syndrome protein-family verprolin homologous protein 1 (WAVE-1; data shown in results section 3.1). This was expected given the close functional relationship between DISC1 and Rac1 – DISC1 regulates guanine nucleotide exchange factors (GEFs) that activate Rac1 (Hayashi-Takagi et al., 2010; Brandon and Sawa, 2011; Chen et al., 2011). WAVE-1 changes were also consistent with DISC1 and Rac1 changes since WAVE-1 acts downstream of Rac1 (Eden et al., 2002; Soderling et al., 2003). WAVE-1 acting downstream of Rac1 is also a major reason for why we further focused our attentions on studying WAVE-1.

WAVE-1 is a scaffolding protein with a molecular weight of approximately 85 kDA (Kim et al., 2006a; Soderling et al., 2007). Some of its known regulators and interactions with NMDARs and Rac1 are outlined in Figure 1.5. It is a regulator of actin dynamics in mammalian cells, and is more enriched in the CNS and neurons compared to the isoforms WAVE-2 and WAVE-3 (Kim et al., 2006b, 2006a). WAVE proteins contain three main domains: an amino-terminal Scar homology domain, a central polyproline domain, and a carboxy-terminal Verprolin–Cofilin–Acidic (VCA) domain (Kim et al., 2006a; Soderling et al., 2007). The ATD and polyproline regions of WAVE-1 bind to phospholipids for localization to the cell membrane as well as other proteins for actin and GTPase regulation (Soderling et al., 2007; Pollitt and Insall, 2009). The VCA domain is considered the main executor of WAVE-1 functions; it binds to and activates the actin-related protein (Arp) 2/3 complex, serving together as a scaffold for actin polymerization (Kim et al., 2006a; Soderling et al., 2007). Rac1 activation leads to the dissociation of cytoplasmic FMRP-interacting protein 1 from WAVE-1 complexes and a subsequent disinhibition of WAVE-1 (Pathania et al., 2014). Besides Rac1, there are also other regulators of WAVE-1 function, such as CDK5, WAVE-associated Rac GTPase-activating protein (WRP), and more (Kim et al., 2006b; Sung et al., 2008; Ceglia et al., 2010; Huang et al., 2017). Many of these regulators act by changing the phosphorylation state of WAVE-1, with phosphorylated
WAVE-1 generally considered as inhibited (Kim et al., 2006b; Sung et al., 2008). WAVE-1 and Arp2/3 activation induces actin polymerization and ultimately plays a role in dendritic spine growth, trafficking of mitochondria into spines, and other neurodevelopmental processes (Kim et al., 2006a, 2006b; Sung et al., 2008; Ito et al., 2018). It is therefore unsurprising for WAVE-1 dysregulation to contribute to neurological disorders.

Figure 1.5. Representative schematic of WAVE-1 molecular mechanisms in relation to NMDARs. Multiple reports have related WAVE-1 function to NMDAR signaling and synaptic plasticity. Proteins disrupted in GluN1KD mice are denoted in red text. Green pointed arrows indicate a positive relationship of some form, such as enhanced activation or release from a
sequestering factor. Red blunted lines indicate a negative relationship, causing some form of inhibition, decreased activity, or sequestering of the target. Black double-ended arrows indicate limited evidence that suggest some sort of relationship – such as bioinformatics-based associations. Abbreviations are the same as those used in text. This figure was constructed with literature cited within text as well as additional articles (Soderling et al., 2002; Camargo et al., 2007).

While WAVE-1 alterations do not cause Wiskott-Aldrich syndrome, an x-linked disease with cytoskeletal abnormalities and immunodeficiency (Soderling et al., 2003; Takenawa and Suetsugu, 2007), they do result in clinically relevant pathophysiological changes. In vitro experiments with cultured neurons have demonstrated that decreased WAVE-1 function, via phosphorylation or RNA interference, results in less mature dendritic spines (Kim et al., 2006b; Sung et al., 2008; Sanchez et al., 2009). WAVE-1 knockout mice, similar to GluN1 knockout mice, have been reported to die postnatally (Forrest et al., 1994; Li et al., 1994; Dahl et al., 2003) while other in vivo experiments have associated disruption of WAVE-1 function with decreased dendritic spines (Kim et al., 2006b; Soderling et al., 2007). Targeted WAVE-1 disruption can also result in mice with sensorimotor abnormalities and cognitive deficits (Soderling et al., 2003). Overall, the reports of spine density deficits and mouse behavioural abnormalities after WAVE-1 alterations echo previous studies of GluN1KD mice (Dzirasa et al., 2009; Halene et al., 2009a; Ramsey et al., 2011; Milenkovic et al., 2014). In humans, genetic or transcriptional disruption of the WAVE-1 encoding gene, WASF1, has been associated with schizophrenia and autism-like symptoms (Maycox et al., 2009; Ito et al., 2018), also similar to NMDAR dysfunction and GluN1KD mice (Mohn et al., 1999; Halene et al., 2009a; Gandal et al., 2012). We therefore asked whether restoring WAVE-1 improves GluN1KD spine density and behavioural abnormalities. In other words, were the discovered Rho GTPase signaling alterations in GluN1KD mice biologically significant and/or feasible targets for behavioural improvement?

We also wanted to more broadly investigate the molecular changes of the GluN1KD STR using an unbiased, bioinformatics approach. More specifically, we aimed to characterize the GluN1KD transcriptome using RNAseq, which is more sensitive compared to proteomic approaches and traditional microarrays (Hegde et al., 2003; Seyednasrollah et al., 2015). Studying the GluN1KD STR this way fills an important knowledge gap because bioinformatics-based assessments so far only investigated non-striatal brain regions (Wesseling et al., 2014; Cox
et al., 2016) despite many other studies including or focusing on the STR (Miyamoto et al., 2004; Ramsey et al., 2011; Ferris et al., 2014). For example, previous proteomic studies offered evidence that protein changes in GluN1KD mice likely represented changes related to synaptic plasticity at the frontal CTX and HPC (Wesseling et al., 2014; Cox et al., 2016). It would be interesting to see if similar outcomes appear for the STR when RNAseq results are analyzed with gene list analyses (Chen et al., 2013; Krämer et al., 2014; Kuleshov et al., 2016). We wanted to determine the molecular changes of the GluN1KD STR, the biological processes and pathways those changes most likely represent, and how the overall results align with what we currently know about GluN1KD mice.

Sex was included as a factor for statistical analyses in both studies when sufficiently powered because both schizophrenia and ASDs show elements of sexual dimorphism (Eranti et al., 2013; Baxter et al., 2015; Halladay et al., 2015). Again, schizophrenia has an earlier onset in males, who may also have more severe symptoms and worse outcomes (Häfner, 2003; Thorup et al., 2007; Eranti et al., 2013). ASDs are more prevalent in males (Baxter et al., 2015). Considering sex differences is also important because previous GluN1KD studies infrequently accounted for sex differences, forming another gap of knowledge. Bioinformatics-scale data are notably absent for female GluN1KD mice as previous proteomic studies only assessed males (Wesseling et al., 2014; Cox et al., 2016).

To sum, this thesis aims to investigate the relationship between Rho GTPase signaling proteins, synaptic connectivity, and behaviour in GluN1KD mice as well as to characterize the GluN1KD striatal transcriptome.

1.3.3 Specific Research Objectives

This section is meant to reiterate explicitly and specifically the research goals of the work in this thesis. There are two major research aims, both are directed at understanding the cellular and molecular changes in the GluN1KD STR. The first aim also has a specific focus on changes related to synaptic plasticity while the second involves a more unbiased bioinformatics approach.

Aim 1: Investigation into the effect of restoring of WAVE-1 on MSN spine density and behaviour in GluN1KD mice. We hypothesize that decreased striatal WAVE-1 in GluN1KD mice is biologically significant and that restoring WAVE-1 protein will improve spine density
and GluN1KD behaviours: Y-maze, 8-arm radial maze, puzzle box, open field, and social approach tests will be employed.

Aim 2: Characterization of the striatal transcriptome in GluN1KD mice. As this was an unbiased RNAseq characterization study, no specific hypothesis was established \textit{a priori}. Pathways analyses will be run on the resulting transcriptomic differences between GluN1KD mice and WT littermates. In general, we expect both the initial RNAseq results and the subsequent pathways analyses results to be consistent with current knowledge about GluN1KD mice.
2.1 Animals Used in the Study

2.1.1 GluN1KD Mice

GluN1KD mice and WT littermates aged 3, 6, or 12-14 weeks were used in this study. Male and female mice were used for all experiments comparing only WT and GluN1KD mice. GluN1KD mice generation was previously described (Mohn et al., 1999). Briefly, a neomycin cassette was inserted into intron 19 of \textit{Grin1}, which encodes GluN1 subunits, resulting in a reduction of full-length \textit{Grin1} mRNA by more than 90%. The resulting mouse line was backcrossed for more than 20 generations onto two genetic backgrounds, C57B1/6J and 129X1/SvJ. Specifically, mice used for comparisons between GluN1KD and WT littermates in this study were F1 progeny from the intercross breeding of C57Bl/6J \textit{Grin1}+/- and 129X1/Sv \textit{Grin1}+/- heterozygotes. This breeding strategy was chosen to minimize the potential confound of homozygous mutations in parent strains, in accordance with Banbury Conference recommendations (Silva et al., 1997). Experiment mice were genotyped by polymerase chain reaction analysis of tail sample DNA: the primers used to amplify GluN1KD \textit{Grin1} were 5′ AAG CGA TTA GAC AAC TAA GGG T 3′ and 5′ GCT TCC TCG TGC TTT ACG GTA T 3′ while those used for WT \textit{Grin1} were 5′ TGA GGG GAA GCT CTT CCT GT 3′ and 5′ AAG CGA TTA GAC AAC TAA GGG T 3′.

2.1.2 WAVE-Tg Mice

WAVE-Tg mice were generated by the Soderling lab from Duke University. They were constructed by the pronuclear injection of BAC DNA (id: RP11-1072O1) containing the human \textit{WASF1} locus, with tens of kbp upstream and downstream, into fertilized eggs of C57B1/6J mice. Mice used to compare between the WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE genotypes were F1 progeny generated by intercross breeding between C57B1/6J \textit{Grin1}+/- WAVE-Tg mice and 129X1/Sv \textit{Grin1}+/- non-WAVE-Tg mice. Male and female mice were used for all experiments involving all four genotypes except for RNAscope and dendritic spine experiments, which used only males. For genotyping, the \textit{Wasf1}-targeting primers 5′ CAA CTC
ATT GCA AGA ACG TGT GGA C 3’ and 5’ AAT AAA AAA ATT AGC CAG GCG TGG TG 3’ were used along with the *Grin1* targeting primers mentioned above.

### 2.1.3 Animal Handling and Ethics

All animal housing conditions and experimentation protocols were in line with institutional (University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee, approved protocol #20011988) and federal (Canadian Council on Animal Care) guidelines. Behavioural tests were performed during the light cycle (between 9am and 6pm local time) using mice that were previously naïve to all behavioral tests. To minimize animal suffering, all mouse brains were either collected after quick and precise cervical dislocation for biochemical measures or after terminal anesthesia with 250 mg/kg tribromoethanol for dendritic spine measures.

### 2.2 Dendritic Spine Analyses

#### 2.2.1 Dendritic Spine Density Analysis of WT and GluN1KD Mice at Three, Six, and Twelve Weeks of Age

Dendritic spine density was assessed using DiOLISTIC labeling and confocal microscopy described previously (Seabold et al., 2010; Ruddy et al., 2015). After deep tribromoethanol anesthesia, mice were perfused transcardially with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in PBS over 10 minutes. Brains were then collected and kept in 4% PFA for an hour on ice before being stored in PBS at 4°C. Brains were then sliced coronally at a thickness of 100 μm within two days. Slices focusing on the STR (lateral 1.2 mm) were collected and their neurons were randomly labeled with DiI (1-1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate). Specifically, slices were impregnated with DiI coated gold beads using a Helios gene gun (Bio-Rad, Hercules, CA, USA). Z-stack images with steps of 0.5 μm at 60X magnification were collected using an IX81 confocal microscope and Fluoview FV 1000 (Olympus, Tokyo, Japan). Dendritic spine counting was performed on these images using NIS-Elements Basic Research (Version 3.10, Nikon, Tokyo, Japan). Striatal MSN dendrite segments were chosen mostly randomly for analysis, only systematically avoiding branching points. Three to seven z-stack image compositions were used for each mouse assessed. Spine density analysis was blinded.
2.2.2 Dendritic Spine Density and Morphology Analyses of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE Mice

Dendritic spine density and morphology for WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice were also assessed via DiOLISTIC labeling and confocal microscopy (Seabold et al., 2010; Ruddy et al., 2015). Anesthetized mice were perfused transcardially with PBS followed by 4% PFA over 10 minutes before brain collection. Brains were submerged in 4% PFA for one hour before storage in PBS at 4°C and sectioned coronally at a thickness of 150 µm within two days. Slices centering on the STR and cornu ammonis 1 (CA1) region of the HPC were collected and their neurons were randomly labeled via impregnation with DiI coated tungsten beads delivered by Helios gene gun (Bio-Rad). Z-stack images with steps of 0.9 µm at 60X magnification were collected via an IX81 confocal microscope and Fluoview FV 1000 (Olympus). Z-stack composite images were analyzed with ImageJ [Version 1.50d; (Schneider et al., 2012) from the National Institutes of Health (NIH, Bethesda, MD, USA)] using the NeuronJ (Version 1.4.3) plug-in (Meijering et al., 2004). Striatal spine density and morphology assessments comparing WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice were focused on 20 µm dendrite segments starting approximately 20 µm away from the soma. For hippocampal slices, 20 µm segments of secondary or tertiary dendrites from the basolateral and apical sides of CA1 pyramidal neurons were analyzed. Basolateral dendrites were assessed using segments starting at 30-45 µm away from the soma while apical dendrites were assessed near the initial branching points of secondary or tertiary dendrites closest to the soma – which varied in distance from soma. All dendritic segments selected for analysis avoided including actual branching points as much as possible. Three to six z-stack image compositions were used for each mouse assessed. Both spine density and spine morphology measurements were blinded.

2.3 Western Blotting

Western blot analysis was performed to assess the levels of proteins of interest. Dissected mouse STR and HPC samples were homogenized in 400 µl of PHEM buffer (0.5% TritonX 100, 60 mM PIPES, 25 mM HEPES, 10 mM EGTA, and 2 mM MgCl2) with added protease and phosphatase inhibitors (1.5 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 0.1 mg/ml benzamidine, 0.25 mM PMSF, 5 mM Na orthovanadate, 10 mM NaF, 2.5 mM Na
pyrophosphate, 1 mM β-glycerophosphate). Loading samples were then prepared from these protein homogenates using standard SDS protein sample buffer with 5% β-mercaptoethanol, followed by heating at 100°C for 5 minutes. Depending on the specific protein being measured, 15-25 μg of loading sample protein were electrophoresed in 7.5-12% bis-acrylamide gels at 80-100 V for 1-3 hours and then transferred onto polyvinylidene difluoride membranes at 100 V for 1 hour. The resulting protein-bearing membranes were blocked with 5% milk or bovine serum albumin in tris-buffered saline and Tween 20, according to primary antibody supplier instructions, before overnight incubation with appropriate primary antibodies at 4°C. Membranes were incubated with fluorescent secondary antibodies the following day and imaged with the Odessey Infrared Imaging System (LI-COR, Lincoln, NE, USA). Densitometry was performed using Odessey (Version 3, LI-COR), Image Studio (Version 5, LI-COR), or ImageJ (Version 1.45, NIH). Primary antibodies used were as follows: α-WAVE-1 (1:1000-2000, catalog number 75-048, NeuroMab, Davis, CA, USA), α-pS397-WAVE-1 (1:2000, catalog number W2768, Sigma, St. Louis, MO, USA), α-Rac1 (1:500, catalog number ARC03, Cytoskeleton, Denver, CO, USA), and α-GAPDH (1:4000, catalog number G8795, Sigma, St. Louis, MO, USA). The secondary antibodies used were as follows: donkey α-mouse IgG-800 (1:5000, catalog number 610-731-002, Rockland, Pottstown, PA, USA), goat α-rabbit IgG-680 (1:5000, catalog number A21076, Invitrogen, Carlsbad, CA, USA), and goat α-rabbit IgG-800 (1:15000, catalog number 926-32211, LI-COR, Lincoln, NE, USA). Blots were normalized to control loading protein (GAPDH) bands, provided in the results figures, or to REVERT total protein stain (LI-COR, Lincoln, NE, USA) before analysis.

2.4 Fluorescent in situ hybridization via RNAscope

Mouse and human WASF1 expression in WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice was visualized by a chromogenic in situ hybridization technique called RNAscope. The experiment largely followed the RNAscope Multiplex Fluorescent Reagent Kit v2 protocol from ACD Bio (Newark, CA, USA). Fresh mouse brains were frozen by submersion in cold 2-methylbutane and sectioned sagittally (lateral 1.2 mm Bregma) at a thickness of 20 µm. Selected sections were mounted onto charged slides, fixed by submersion in 4% PFA (in PBS), and dehydrated via serial submersion in 50%, 70%, and 100% ethanol. Afterwards, the slices were
treated with hydrogen peroxide, followed by RNAscope Protease IV. The multiplex fluorescent assay was then performed: sections were hybridized to both human- (product reference ID 533691, ACD Bio) and mouse- (ref ID 533701) specific Wasf1 probes or to one of two control probe mixtures (positive control probes ref ID 320881, negative control probes ref ID 320871); amplification reagents 1-3 were then used on the slides to boost signal strength; finally, fluorescent signals were developed using TSA Plus cyanine 3 and 5 (PerkinElmer, Llantrisant, UK) and channel-specific horseradish peroxidases for channels 1 and 2, respectively. Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) was then applied to the slices before glass coverslip placement. Slides were dried overnight and then imaged with an Axio Scan.Z1 slide scanner (Zeiss, Oberkochen, DE) at 20X magnification.

2.5 Behavioural Tests of Cognition in Mice

2.5.1 Y-Maze Spontaneous Alternation Test

The Y-maze spontaneous alternation test was used to assess the cognitive function of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Its procedure and equipment have been described in previous studies (Mandillo et al., 2008; Milenkovic et al., 2014). As shown in Figure 2.1, the Y-maze consisted of three identical arms measuring 38 × 7.6 × 12.7 cm that were separated by 120° between each pair (San Diego Instruments, San Diego, CA, USA). Test mice were placed at the end of one arm and tracked with Bioobserve Viewer (Version 2; St. Augustin, DE) for 8 minutes as they explored the maze. Digital zones were defined for each arm in Viewer 2 starting at approximately 5 cm away from the center of the maze. Spontaneous 3-arm alternations were defined as instances where the mouse enters each of the three zones consecutively – that is, without a repeat entry into any one zone. After the first two zone entries were excluded, the percentage of spontaneous 3-arm alternation zone entries relative to all zone entries was taken as the Y-maze performance score.
Figure 2.1. Schematic of Y-maze test (a) and potential exploration pattern results (b-d).
Test mice explored the maze for 8 minutes. b-d) Red paths follow black paths. b) When the mouse enters each arm consecutively without a repeat, it is an instance of a spontaneous 3-arm alternation. c-d) When a mouse re-enters a recently-explored arm, it does not count as a 3-arm alternation, potentially indicating an error of working memory or exploration strategy.

2.5.2 Eight-Arm Radial Maze Test

The cognitive function of test mice was also assessed using the 8-arm radial maze, particularly to complement and contrast the Y-maze test. Previously, GluN1KD mice have also been reported to perform poorly in the 8-arm radial maze test (Dzirasa et al., 2009). The test maze consisted of
8 identical arms measuring 22.86 × 7.62 × 15.24 cm that met together at the center of the maze as equal sides of an octagon (San Diego Instruments). The experiment lasted 6 weeks for each test mouse cohort, which contained at least 2 mice of each genotype. Each Monday, mice were started on a restricted diet of 2-3g of food per mouse per day and no preparation sessions or test trials were performed. On subsequent Tuesdays to Fridays, food was given only after the day’s habituation session or test trial. Mice were allowed to feed ad libitum on weekends and monitored to ensure no mouse dropped below 90% of their free-feeding bodyweight through the week. The first week consisted of habituating test mice to the maze center with or without a cut-up piece of cereal for 5-10 minutes. Weeks 2-6 were 5 blocks of 4 daily maze trials. Each trial consisted of 1 minute of habituation to the maze center followed by the maze arms opening up and free maze exploration for 5 minutes or until all 8 cereal piece-baited arms were visited. A schematic of the set-up is shown in Figure 2.2. Measures of cognition were termed working memory error (WME, the number of mouse entries into already-visited arms) and entries-to-repeat (ETR, the number of maze arms visited before the test mouse makes a repeat entry into any arm) and were recorded by Biobserve Viewer (Version 3).
Figure 2.2. Schematic of 8-arm radial maze. A piece of cereal was placed at the end of each arm, offering a goal for diet-restricted test mice. Mice explored the maze for up to 5 minutes. Every entry into an already-visited (within the trial) maze arm counts as a working memory error. The number of maze arm entries before a repeat entry is taken as a score termed “entries-to-repeat”.

2.5.3 Puzzle Box Test

The puzzle box test was used to assess problem solving, short-term memory, and long-term memory in mice, as described previously (Ben Abdallah et al., 2011; Milenkovic et al., 2014). As shown in Figure 2.3, the puzzle box was an arena with two adjacent compartments measuring
58 × 28 × 27.5 cm and 14 × 28 × 27.5 cm. The two compartments could be separated by a divider with an opening that allowed free passage for mice or by one that only leaves a mouse-sized underpass connection between the compartments. For each trial, test mice were placed in the larger compartment under bright-light conditions facing away from the divider. As the smaller compartment has a roof providing dim-light conditions, mice are motivated to enter it. Test performance was defined by the amount of time it took each mouse to enter the dim-light compartment, which was manually recorded. There were 9 trials spread to 3 per day with new obstacles introduced between the bright- and dim-light compartments during the second trial of each day. The open-door divider was replaced with the door-less divider for trial 2 and all subsequent trials. The underpass was blocked with cage bedding in trials 5-7 or with a cardboard plug in trials 8-9. A summary of the obstacle arrangements is shown in Table II-I. A maximum of 5 minutes was given for each trial and 2-minute breaks were given between trials on the same day. Mice that did not reach the dim-light compartment during the first trial with an open-door divider were excluded from the experiment.

Figure 2.3. Schematic of puzzle box test. Test mice are set in the brightly-lit and larger arena compartment (depicted on the right), face away from the dimmer goal compartment (left). One of two dividers separate the compartments – with or without a door. An underpass also connects both arena compartments, below the middle of the divider. Divider and underpass states for each puzzle box trial are listed in Table II-I. The amount of time it takes for test mice to enter the goal compartment (up to 5 min) is taken as test performance scores.
Table II-I. Puzzle box configuration over all trials. Increasingly difficult challenges are presented to test mice: dividers are switched from open-door to doorless from trial 2 onwards, the underpass is filled with cage bedding for trials 5-7, and the underpass is blocked with a cardboard plug for trials 8-9. The introduction of new challenges tests the puzzle-solving ability of mice while the repeat-trials are assessments of short- and long-term memory.

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<td>Empty</td>
<td>Filled with cage bedding</td>
<td>Filled with cage bedding</td>
<td>Filled with cardboard plug</td>
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2.5.4 Open Field Locomotion and Stereotypy Test

The open field test was used to assess horizontal motor activity, habituation, and stereotypy of mice, as described previously (Choleris et al., 2001; Milenkovic et al., 2014). Test mice were placed in clear Plexiglas chambers with the dimensions 20 × 20 × 45 cm, a novel environment to them, for 2 hours. Mouse locomotor activity and measures of stereotypy were tracked and recorded by the infrared beam sensors of digital activity monitors from Omnitech Electronics (Columbus, OH, USA). The 2-hour time-course of horizontal distance travelled and stereotypy event count were analyzed to assess the overall activity of test mice.

2.5.5 Social Approach Behaviour Test

WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice were also assessed for their social cognition via the social approach behaviour test. Specifically, a modified social approach behaviour test that was previously described (Moy et al., 2004; Mielnik et al., 2014; Milenkovic et al., 2014) was used. The test uses a white Plexiglas arena (62 × 40.5 × 23 cm) containing two inverted wire cups that were placed symmetrically. One cup was empty while the other contained novel age- and sex- matched mice, which served as social stimuli. The set-up is diagrammed in Figure 2.4. Test mice were placed in the arena at an equal distance away from both cups and their subsequent interactions with the wire cups were recorded with a video camera for 10 minutes. Biobserve Viewer 2 was used to define circular zones (5 cm radius) centered on each cup, to track each test mouse’s center of body mass, and to quantify the amount of time spent by
test mice in each zone. The amount of time spent in the social stimulus and nonsocial zones were taken as measures of social cognition and object-novelty control, respectively.

Figure 2.4. Schematic of modified social approach behaviour test. Mice were placed equidistant and facing away from both wire cups. Afterwards, test mice explored the arena for 10 minutes. The position of social stimulus mice alternated between the two cups for different test mice. The amount of time test mice spent investigating each cup is recorded as a measure of social cognition (mouse-containing cup) or as a control for object novelty (empty cup).

2.6 RNAseq Analyses

2.6.1 Sample Preparation and Differential Gene Expression Analysis

Total RNA was isolated from dissected whole-striatal tissue from 6 WT and 6 GluN1KD mice, 3 of each sex for each genotype and at 12 weeks of age. RNA isolation was performed with Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) and sample RNA purity was verified with OD260/OD280 ratios taken with an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT, USA). For each sample, 2-6 µg of RNA was sent to The Centre for
Applied Genomics (TCAG; Toronto, Canada) for poly-A isolation, library generation, amplification, and sequencing. TCAG generated raw sequence data using the Illumina HiSeq 2500 protocol (Illumina, San Diego, CA, USA).

Resulting FASTQ files from TCAG were analyzed using the tuxedo protocol (Trapnell et al., 2012) and the public Galaxy platform (http://usegalaxy.org) (Afgan et al., 2018). After uploading, FASTQ files were prepared for use on Galaxy with FASTQ Groomer (Version 1.0.4) and each file’s raw sequence reads were aligned to the GRCm38/mm10 mouse genome assembly with TopHat (Version 2.1.0). Aligned reads were then used to generate and predict transcripts for each sample via Cufflinks (Version 2.2.1.0) while referencing a RefSeq annotation from the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA). Resulting cufflinks assemblies for each sample were then combined together with Cuffmerge (Version 2.2.1.0). Gene expression quantifications and comparisons were performed with Cuffdiff (Version 2.2.1.3) using the aligned sequence reads for every sample, combined transcript index from Cuffmerge, and GRCm38/mm10 genome assembly to correct for organism expression bias. Two types of comparisons were performed: WT vs GluN1KD and WT male vs WT female vs GluN1KD male vs GluN1KD female. P and q (false discovery rate-adjusted p) value cut-offs were set to 0.05 and 0.1 respectively.

Genes that were expressed differentially between WT and GluN1KD mice for at least one sex were identified and annotated for their specific significant differences between the four genotype X sex groups. Unrecognized entries that were significantly different between WT and GluN1KD mice were manually double-checked for an identifier via cross-referencing their genomic positions with data from the University of California, Santa Cruz Table Browser (https://genome.ucsc.edu/cgi-bin/hgTables) (Karolchik et al., 2004), Ensembl BioMart (accessed through http://www.ensembl.org/index.html) (Kinsella et al., 2011), and NCBI gene database (https://www.ncbi.nlm.nih.gov/gene) (O’Leary et al., 2016).

2.6.2 Gene List Analyses

All identified genes based on sex-matched genotype comparisons were further analyzed as gene lists with Ingenuity Pathway Analysis (IPA; QIAGEN Bioinformatics, Aarhus, Denmark) and Enrichr (http://amp.pharm.mssm.edu/Enrichr/) (Chen et al., 2013; Kuleshov et al., 2016). IPA was run to predict key molecules and pathways connected to and represented by the
transcriptomic differences between mice of different GluN1KD genotype and sex. Four gene lists served as inputs for IPA – for each sex-matched genotype comparison and each genotype-matched sex comparison, a list of genes with significant differences was included. Each list included gene names, fold changes and q-values. IPA was run including all information from the Ingenuity Knowledge Base (including all node types, mutation studies, and data sources) that was experimentally observed and considering endogenous chemical effects and relationships. Analyses were however constrained to knowledge specific to mice, nervous-system-related tissues and cell lines, and other cell types that could be in the original sample of dissected mouse STR (such as endothelial cells). Gene lists were also analyzed with Enrichr to identify transcriptome signatures of curated databases or specific study subjects that are the most similar. Four gene lists, consisting simply of gene names, were entered to Enrichr: genes that had increased expression in GluN1KD males compared to male WT littermates, those that were decreased in GluN1KD males, those that were increased in GluN1KD females relative to female WT littermates, and those that were decreased in GluN1KD females. Done this way, the input gene lists could be matched with Enrichr entries that specify the direction of transcriptomic changes.

2.7 Statistical Analysis

Prism (Version 6.01, GraphPad Software, La Jolla, CA, USA) was used to graph and analyze most experimental data. Exceptions include repeated measures data that were analyzed by two-way ANOVAs with repeated measures using SPSS (Version 20.0.0, IBM, Armonk, NY, USA) and RNAseq data, which were analyzed with derivatives of the Jensen-Shannon divergence (Trapnell et al., 2010) or the Fisher exact test (Chen et al., 2013; Krämer et al., 2014; Kuleshov et al., 2016) followed by Benjamini-Hochberg (BH) procedural corrections for multiple testing. Assumptions of equal variance and sphericity were tested where appropriate. WT and GluN1KD comparisons were mostly assessed with independent, two-tailed t-tests. Comparisons between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice were mostly assessed with one-way ANOVAs, Kruskal-Wallis tests when variance was not equal, or two-way ANOVAs with repeated measures. Comparisons of repeated-measures data included locomotor activity, stereotypy, and 8-arm radial maze performance (WME and ETR). Bonferroni corrections were
used after ANOVAs for post hoc comparisons between all groups. Dunn’s post hoc multiple comparisons tests were used after Kruskal-Wallis tests. Kolmogorov–Smirnov (KS) Tests were also used to assess spine morphology differences as distribution comparisons. As KS tests could only be performed for pairs of groups, an a priori testing strategy was decided to decrease the chance of false positives: WT vs GluN1KD mice would be compared first; if significantly different, GluN1KD vs GluN1KD-WAVE and WT vs GluN1KD-WAVE would be compared to assess rescue. A cut-off $p$-value of 0.05 was chosen for statistical significance while a cut-off $q$-value of 0.1 was chosen for RNAseq results. Where appropriate, sample sizes are indicated within results figures. Data are expressed as mean ± standard error of the mean. Post hoc power analyses were performed with G*Power [Version 3.1.9.2; (Faul et al., 2007, 2009) from the Heinrich Heine University Düsseldorf].
Chapter 3
Results

3.1 GluN1KD Mice Show MSN Spine Density Deficits and Rac1 Signaling Protein Aberrations that are Age-Dependent

Note: Data presented in all of section 3.1.1 and most of 3.1.2 were produced by and part of the thesis of Marija Milenkovic, a previous student of the Ramsey Lab.

3.1.1 MSN Dendritic Spine Density is Decreased in Six- and Twelve-Week-Old GluN1KD Mice

We first compared the MSN dendritic spine densities of 3-, 6-, and 12-week-old WT and GluN1KD mice to establish a time course of synaptic deficits. As shown in Figure 3.1, GluN1KD mice show spine density deficits by 6 weeks of age, which persists when they are 12 weeks old. Specifically, 6-week-old WT mice had $138.2 \pm 1.5$ spines/100 μm while GluN1KD littermates had a spine density of $123.8 \pm 2.1$ spines/100 μm, a 11% reduction (independent, two-tailed $t(4) = 5.55, p < 0.01$). At 12 weeks, spine densities were $135.5 \pm 1.7$ spines/100 μm and $114.3 \pm 1.4$ spines/100 μm for WT and GluN1KD mice, respectively (16% reduction in GluN1KD mice, $t(4) = 9.74, p < 0.01$). Our results point to MSN spine loss manifesting between 3 and 6 weeks of age for GluN1KD mice and persisting at 12 weeks of age.
Figure 3.1. Representative images (left) and graphs (right) showing MSN dendritic spine density of WT and GluN1KD mice aged 3, 6, and 12 weeks. GluN1KD mice have significant spine density deficits at 6 and 12 weeks of age compared to WT littermates (11% and 16% decreases, respectively). 3-7 dendrite images were quantified for each mouse. Mouse sample sizes are indicated in each bar. The scale bar for representative images represents 20 µm. Data in this figure were analyzed by two-tailed, independent t-tests. **p < 0.01.
3.1.2 Rac1 and WAVE-1 Protein Levels are Decreased in Six- and Twelve-Week Old Mice but Differentially Altered in Three Week Olds

Striatal Rho GTPase pathway proteins in 3-, 6-, and 12-week-old WT and GluN1KD mice were measured by western blotting. Rac1, RhoA, WAVE-1, LIMK1, actin, cortactin, coflin, pS3-cofilin, cell division control protein 42 homolog (Cdc42), and neural Wiskott-Aldrich syndrome protein (N-WASP) were investigated. Of these proteins of interest, most were not changed in GluN1KD mice compared to WT littermates. However, significant differences were found consistently for Rac1 and WAVE-1, as shown in Figure 3.2. Rac1 was expressed at 125.8 ± 2.8 % (independent, two-tailed t(6) = 6.00, p < 0.01), 69.7 ± 10.1 % (t(6) = 2.45, p = 0.05), and 76.3 ± 1.5 % (t(6) = 5.07, p < 0.01) relative to WT levels at 3, 6, and 12 weeks of age, respectively. WAVE-1 was expressed at 74.5 ± 4.9 % (independent, two-tailed t(6) = 3.04, p = 0.02), 63.3 ± 9.0 % (t(6) = 2.44, p = 0.05), and 79.5 ± 2.4 % (t(6) = 2.94, p = 0.03) of WT levels at 3, 6, and 12 weeks of age, respectively. From these results, we saw that Rac1 signaling was particularly altered relative to all the Rho GTPase signaling proteins of interest, as WAVE-1 is a downstream effector of Rac1 (Eden et al., 2002; Bompard and Caron, 2004).
Figure 3.2. Representative western blots (top) and their quantifications (bottom) measuring Rac1 and WAVE-1 at the STR of 3-, 6-, and 12-week-old WT and GluN1KD mice. Rac1 was significantly higher in 3-week-old GluN1KD mice before decreasing to levels significantly lower than WT by 6 and 12 weeks of age. WAVE-1, a downstream effector of Rac1, was consistently lower in GluN1KD mice at all ages tested. Rac1 and WAVE-1 bands were first normalized to GAPDH loading control bands. As indicated in the graph legends, a sample size of 4 mice was used for every group. Data in this figure were analyzed by two-tailed, independent t-tests. * $p \leq 0.05$, ** $p < 0.01$.

3.2 WAVE-Tg and GluN1KD-WAVE Mice Express Wasf1 and WAVE-1 Reliably, Predictably, and Abundantly

3.2.1 WASF1 Transgene Expression is Abundant and Similar to Endogenous Wasf1 at the STR and HPC of WAVE-Tg and GluN1KD-WAVE Mice

Previous studies have reported a causal relationship between Rac1 and WAVE-1 reductions and subsequent dendritic spine loss (Soderling et al., 2007; Haditsch et al., 2009), both observed
in older GluN1KD mice. Chronic Rac1 activity increase, which may be present in GluN1KD mice between 3 and 6 weeks of age, can however cause different spine abnormalities, including increased or unchanged spine densities but deteriorated spine morphology (Luo et al., 1996; Tashiro et al., 2000; Hayashi-Takagi et al., 2010). We therefore considered that Rac1 and WAVE-1 decreases could contribute to GluN1KD spine loss or be compensatory for spine loss prevention. To assess these possibilities, we aimed to restore WAVE-1 in GluN1KD mice by intercross breeding with WAVE-1-overexpressing, WASF1 transgenic mice.

WAVE-1-overexpressing, WASF1 transgenic mice (WAVE-Tg) were generated by pronuclear injection of the entire the human WASF1 genomic sequence (including 86.5 kb of upstream and 70.3 kb of downstream sequence) via a bacterial artificial chromosome. Progeny of the GluN1KD/WAVE-Tg intercross breeding were viable and survived to adulthood. As we observed GluN1KD spine density deficits in older mice, similar to previous reports of GluN1KD behavioural abnormalities (Ramsey et al., 2011; Milenkovic et al., 2014), we focused on adult GluN1KD/WAVE-Tg intercross progeny for further experiments. WASF1 transgenic mice (both WAVE-Tg and GluN1KD-WAVE-Tg hybrids, hereafter termed GluN1KD-WAVE) have increased WASF1 mRNA compared to non-transgenic (WT and GluN1KD) littermates at the STR (Figure 3.3) and HPC (Figure 3.4). The WASF1 mRNA increases of transgenic mice are specific to the human transgene (green signal) and similar to endogenous mouse Wasf1 expression (red signal) in pattern.
Figure 3.3. RNAscope visualization of Wasf1 expression at the STR of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Endogenous mouse Wasf1 mRNA is visualized in red.
(left) while transgenic human \textit{WASF1} is in green (middle). Transgenic \textit{WASF1} was expressed abundantly and specifically in WAVE-Tg and GluN1KD-WAVE mice with an expression pattern similar to endogenous \textit{Wasf1}.

\textbf{Figure 3.4.} RNAscope visualization of \textit{Wasf1} expression at the HPC of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Endogenous mouse \textit{Wasf1} mRNA is visualized in red (left) while transgenic human \textit{WASF1} is in green (middle). Transgenic \textit{WASF1} was expressed abundantly and specifically in WAVE-Tg and GluN1KD-WAVE mice with an expression pattern similar to endogenous \textit{Wasf1}. 
3.2.2 WAVE-1 Protein Levels are Increased in WAVE-Tg Mice and Improved in GluN1KD-WAVE Littermates at the STR

We also measured striatal Rac1 and WAVE-1 protein levels in WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice by western blots. To get a clearer sense of WAVE-1 signaling in test mice, we also measured pS397-WAVE-1, which is considered an inactive form of WAVE-1 (Kim et al., 2006b). The results are shown in Figure 3.5. One-way ANOVA revealed a significant effect of genotype on levels of WAVE-1 and pS397-WAVE-1 ($F_{3,20} = 33.59, p < 0.01; F_{3,20} = 10.37, p < 0.01$; respectively) but not Rac1 ($F_{3,20} = 1.51, p = 0.24, \beta = 0.66$). Rac1 was expressed less in GluN1KD genotypes ($85.6 \pm 4.5 \%$ and $92.0 \pm 6.4 \%$ for GluN1KD and GluN1KD-WAVE mice, respectively) compared to non-GluN1KD genotypes ($100 \pm 6.0 \%$ and $99.6 \pm 5.4 \%$ for WT and WAVE-Tg mice, respectively) but, again, these differences were not statistically significant. Intercrossing the GluN1KD and WAVE-Tg mouse lines produced transgenic progeny with increased expression of WAVE-1. Compared to WT littermates, WAVE-1 was expressed at $119.7 \pm 4.6 \%$ in WAVE-Tg mice (Bonferroni adjusted $p < 0.01$), $68.6 \pm 2.3 \%$ in GluN1KD mice ($p < 0.01$), and $87.1 \pm 3.5 \%$ in GluN1KD-WAVE hybrids ($p = 0.14$). pS397-WAVE-1 was expressed at $116.4 \pm 6.1 \%$ of WT levels in WAVE-Tg mice (Bonferroni adjusted $p = 0.32$ vs WT), $143.3 \pm 8.1 \%$ in GluN1KD mice ($p < 0.01$ vs WT), and $125.9 \pm 2.2 \%$ in GluN1KD-WAVE hybrids ($p = 0.02$ vs WT) after normalization to total WAVE-1. pS397-WAVE-1 was not significantly different between GluN1KD and GluN1KD-WAVE mice ($p = 0.24$). These results point to significantly reduced WAVE-1 function in GluN1KD mice as total WAVE-1 is reduced and what is left is highly phosphorylated. However, our results also indicate that the Wasf1 transgene improved total WAVE-1 levels in GluN1KD-WAVE hybrids.
Figure 3.5. Representative western blots (top) and their quantifications (bottom) measuring Rac1, WAVE-1, and pS397-WAVE-1 in the STR of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. No significant effect of genotype was detected statistically for Rac1. Total WAVE-1 was confirmed as decreased in GluN1KD mice, increased in WAVE-Tg mice, and improved towards WT levels in GluN1KD-WAVE hybrids. The percentage of pS397-WAVE-1, normalized to total WAVE-1, was higher in GluN1KD and GluN1KD-WAVE mice. All blots were normalized to REVERT total protein stain as loading control. Indicated in bars, a mouse sample size of 6 was used for every genotype. All data in the figure were analyzed by one-way ANOVAs followed by Bonferroni post hoc tests for all pairings. * $p < 0.05$, ** $p < 0.01$. 
3.3 Spine Density Deficits and Maze Exploration Abnormalities are Improved in GluN1KD-WAVE Mice

3.3.1 Striatal MSN Dendritic Spine Density is Improved in GluN1KD-WAVE Hybrids

As total WAVE-1 protein levels were improved in GluN1KD-WAVE hybrids, we also asked whether their spine density was improved. Furthermore, we assessed MSN spine morphology, which could also reflect alterations in Rho GTPases and behavioural abnormalities (Nakayama et al., 2000; Schubert et al., 2006; Arellano et al., 2007). Dendritic spine density results are shown in Figure 3.6a. One-way ANOVA yielded a genotype effect on spine density trending towards significance \(F_{3,32} = 2.79, p = 0.06\). GluN1KD mice had a spine density of 144.9 ± 8.7 spines/100 μm, lower than WT levels of 184.8 ± 11.0 spines/100 μm (Bonferroni adjusted \(p = 0.05\)). GluN1KD-WAVE hybrids, in contrast, had a spine density of 172.3 ± 6.3 spines/100 μm \(p > 0.99\) vs WT. No morphological differences were detected between MSN spines of WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice (Supplemental Figures S1 and S2). Thus, the improved WAVE-1 levels of GluN1KD-WAVE mice may be contributing to MSN spine density recovery. These results further led us to ask whether GluN1KD-WAVE mice perform better than GluN1KD littermates in cognitive behaviour tests – a more definitive assessment of the biological significance of increasing WAVE-1 in NMDAR-deficient mice.

3.3.2 Y-Maze Spontaneous Alternation Performance is Normalized in GluN1KD-WAVE Hybrids

The Y-maze test was used to evaluate the cognitive function of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. One-way ANOVA showed a significant effect of genotype on Y-maze performance scores \(F_{3,88} = 5.70, p < 0.01\). As shown in Figure 3.6b, GluN1KD mice had a lower percentage of spontaneous 3-arm alternations (43.2 ± 2.4 %) relative to WT (51.3 ± 1.5 %, Bonferroni adjusted \(p = 0.04\)) and WAVE-Tg littermates (51.3 ± 1.8 %, \(p = 0.03\)). GluN1KD-WAVE hybrids performed better than GluN1KD mice (54.1 ± 2.0 %, \(p = 0.01\) vs GluN1KD) and were not significantly different from WT or WAVE-Tg mice \(p > 0.99\) when compared to either. No sex differences were observed in this behaviour test. Thus, in the context of Y-maze exploration, intercrossing GluN1KD and WAVE-Tg mice resulted in GluN1KD-WAVE hybrids that have improved cognitive function.
3.3.3 Eight-Arm Radial Maze WME is Improved in GluN1KD-WAVE Hybrids

To confirm and complement our Y-Maze results, we used the 8-arm radial maze to investigate the cognition of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. The results for both WME and ETR are shown in Figure 3.6c. Two-way repeated measures ANOVAs reported significant genotype effects on both WME and ETR ($F_{3,40} = 53.03, p < 0.01$; $F_{3,40} = 66.80, p < 0.01$; respectively). WME results indicated that, over the course of 5 trial blocks, GluN1KD mice entered already-explored arms more often than WT (Bonferroni adjusted $p < 0.01$), WAVE-Tg ($p < 0.01$), and GluN1KD-WAVE littermates ($p = 0.02$). In contrast to their Y-maze performance however, GluN1KD-WAVE hybrids also performed worse than WT ($p < 0.01$) and WAVE-Tg mice ($p < 0.01$). Furthermore, lower ETR scores indicated that GluN1KD and GluN1KD-WAVE mice were both quick to re-enter an arm that was already explored ($p < 0.01$ for each pairwise comparison between a non-GluN1KD and a GluN1KD genotype). GluN1KD and GluN1KD-WAVE mice did not have significantly different mean ETR scores. When tested by the 8-arm radial maze, GluN1KD-WAVE hybrids displayed a selective and partial improvement of WME scores.
Figure 3.6. GluN1KD-WAVE Hybrids have improved MSN spine density (a), Y-maze spontaneous 3-arm alternation (b), and 8-arm radial maze WME (c) compared to GluN1KD littermates. a) Representative MSN dendrite images (left) and spine density quantifications (right) show GluN1KD deficits, relative to WT littermates, that are improved in GluN1KD-WAVE hybrids. 3-6 dendrite images were analyzed per mouse. Mouse sample sizes for each genotype are indicated within the bars. Scale bar represents 20µm for dendrite images. MSN Spine density data was analyzed by one-way ANOVA with Bonferroni post hoc comparisons for all pairings. * p = 0.05. b) GluN1KD mice had significantly lower 3-arm alternation scores compared to WT, WAVE-Tg, and GluN1KD-WAVE littermates. GluN1KD-WAVE hybrids were not significantly different from WT and WAVE-Tg mice. Y-maze scores
were analyzed by one-way ANOVA with Bonferroni *post hoc* comparisons for all pairings. *p < 0.05, **p < 0.01. Group sample sizes indicated in each bar. No sex differences were found for Y-maze performance. c) 8-arm radial maze WME (top) and ETR (bottom) over time show different effects of overexpressing WAVE-1 in GluN1KD mice. WME and ETR were worse in both GluN1KD genotypes compared to either non-GluN1KD genotype. However, GluN1KD-WAVE hybrids also had less WMEs over the course of 5 trial blocks compared to GluN1KD littermates. Both WME and ETR were analyzed by two-way repeated measures ANOVAs. # Bonferroni adjusted p < 0.01 for each pairwise comparison between a non-GluN1KD and a GluN1KD genotype. *p = 0.02 for GluN1KD-WAVE vs GluN1KD WME. Sample sizes are denoted next to genotypes in the graph legend. No sex differences were found in 8-arm radial maze WME or ETR.

### 3.4 Other Behavioural Abnormalities Persist in GluN1KD-WAVE Hybrids

#### 3.4.1 Puzzle Box Solution and Memory Deficits Persist in GluN1KD-WAVE Mice

The puzzle box test was also used to assess the cognitive function of test mice. Different trials evaluated puzzle solving, short-term memory, and long-term memory to varying degrees. Therefore, the solution times for each trial were analyzed by Kruskal-Wallis tests, which consistently reported significant genotype effects ($\chi^2 = 25.87-69.29, N = 94, and p < 0.01$ for all puzzle box trials). Puzzle box completion times for all trials are shown in Figure 3.7a. GluN1KD and GluN1KD-WAVE mice were slower to complete all trials relative to WT and WAVE-Tg littermates. Furthermore, GluN1KD and GluN1KD-WAVE mice regularly failed to complete trials 5-9 within the 5-minute maximum exploration time. Dunn’s *post hoc* tests reported significantly different rankings for each pairwise comparison between a non-GluN1KD genotype and a GluN1KD genotype, for all trials ($p \leq 0.015$). GluN1KD-WAVE hybrids were not significantly different from GluN1KD littermates, indicating no improvement of cognition in the puzzle box context.
3.4.2 GluN1KD Horizontal Activity and Stereotypy Abnormalities Persist in GluN1KD-WAVE Mice

We performed the open field test to investigate the effects WAVE-1 rescue might have on other aspects of cognition – locomotor activity, habituation, and stereotypic behaviour. Two-way repeated measures ANOVAs revealed significant genotype effects on locomotor activity and stereotypic incidence number over time ($F_{3,83} = 53.59, p < 0.01; F_{3,83} = 55.42, p < 0.01$; respectively). Shown in Figure 3.7b, GluN1KD and GluN1KD-WAVE mice had more horizontal locomotor activity, decreased habituation (slower activity decrease over time), and more stereotypic behaviour. All post hoc pairwise comparisons between mice of a non-GluN1KD genotype and those of a GluN1KD genotype were significant (Bonferroni adjusted $p < 0.01$). GluN1KD and GluN1KD-WAVE mice were not significantly different, indicating no open field test improvement was associated with increased striatal WAVE-1.

3.4.3 GluN1KD Social Cognition Deficits Persist in GluN1KD-WAVE Mice

The social approach behavior test was used to investigate WAVE-1 rescue effects on social cognition, represented by how long test mice spend in zones containing social stimulus mice (Mielnik et al., 2014). One-way ANOVA revealed a significant effect of genotype on social approach behavior ($F_{3,92} = 5.69, p < 0.01$). GluN1KD mice (202.2 ± 12.4 s) and GluN1KD-WAVE hybrids (213.1 ± 15.9 s) spent significantly less time in social zones compared to WAVE-Tg littermates (269.9 ± 14.0 s, Bonferroni adjusted $p ≤ 0.01$ for both comparisons). WT mice also spent more time in social zones (258.7 ± 13.7 s) relative to GluN1KD mice ($p = 0.04$), though it was a trend compared to GluN1KD-WAVE mice ($p = 0.15$). Nonsocial zone time, the time spent by test mice in zones with empty wire cups, was also measured and reported as a control for general object novelty. Nonsocial zone time also had a significant genotype effect reported by one-way ANOVA ($F_{3,92} = 16.84, p < 0.01$). WT mice also spent more time investigating empty cups compared to GluN1KD (141.6 ± 9.3 s vs 63.2 ± 7.7 s respectively, $p < 0.01$) and GluN1KD-WAVE littermates (83.7 ± 7.7 s, $p < 0.01$). No sex differences were observed in either measures of the social approach test. Social cognition was not improved in GluN1KD-WAVE mice.
Figure 3.7. GluN1KD-WAVE hybrids did not show improvements in puzzle box (a), open field (b) or social approach behavior (c) test performance. a) GluN1KD and GluN1KD-
WAVE mice were significantly slower in completing puzzle box trials when compared to WT and WAVE-Tg littermates. Data for each trial, representing different cognitive aspects, was analyzed by Kruskal-Wallis tests with Dunn’s post hoc multiple comparisons. # indicates $p \leq 0.015$ for each pairwise comparison between a non-GluN1KD genotype and a GluN1KD genotype for each trial’s completion time. GluN1KD and GluN1KD-WAVE mice did not differ. Sample sizes are indicated in the graph legend next to their respective genotypes. No sex differences were found for any puzzle box trials. b) Distance traveled (top) and stereotypy number (bottom) were recorded over time in the open field test. GluN1KD and GluN1KD-WAVE mice travelled further and had more stereotypic incidents, not showing as much habituation over time, when compared to non-GluN1KD littermates. Data were analyzed by two-way repeated measures ANOVAs. # Bonferroni post hoc $p < 0.01$ for each pairwise comparison between a non-GluN1KD and a GluN1KD genotype. GluN1KD and GluN1KD-WAVE mice did not differ. Sample sizes are indicated in the graph legend next to their respective genotypes. No sex differences were found for the open field test. c) GluN1KD and GluN1KD-WAVE mice spent less time in social zones compared to non-GluN1KD littermates. GluN1KD-WAVE social zone time was significantly lower than WAVE-Tg social zone time and trended towards significance when compared to WT results ($\dagger p = 0.15$). WT nonsocial zone time was also higher compared to both GluN1KD and GluN1KD-WAVE mice. GluN1KD and GluN1KD-WAVE mice did not differ. Data were analyzed by one-way ANOVAs with Bonferroni post hoc comparisons for all pairings. * $p < 0.05$, ** $p < 0.01$. Sample sizes are indicated in the bars of their respective genotypes. No sex differences were observed in the social approach behaviour test.

3.5 Improvements in GluN1KD-WAVE Hybrids are not Reflected by WAVE-1 Levels at the HPC nor CA1 Spines

3.5.1 Hippocampal WAVE-1 Levels are not Different Between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE Mice

Finally, since Y-maze and 8-arm radial maze performance were improved in GluN1KD-WAVE mice, we further asked whether WAVE-1 and spine density improvements were seen at the HPC, a key brain region for learning and memory formation (McHugh et al., 2007; Neves et
al., 2008). Western blots measuring total WAVE-1 in the HPC of test mice are shown in Figure 3.8a. One-way ANOVA found no significant genotype effect ($F_{3,20} = 2.33, p = 0.11, \beta = 0.50$). WAVE-1 was expressed at 102.5 ± 13.2 % of WT levels in WAVE-Tg mice, 102.7 ± 11.4 % in GluN1KD mice, and 84.2 ± 19.9 % in GluN1KD-WAVE hybrids.

### 3.5.2 CA1 Pyramidal Neuron Spine Density is not Different Between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE Mice

One-way ANOVAs found no significant genotype effect on CA1 pyramidal neuron dendritic spines, whether they be apical or basolateral ($F_{3,28} = 0.93, p = 0.44, \beta = 0.77; F_{3,28} = 2.48, p = 0.08, \beta = 0.45$; respectively). Hippocampal spine density data are shown in Figure 3.8b. GluN1KD mice had an apical spine density of 130.0 ± 10.2 spines/100 μm while GluN1KD-WAVE hybrids had 131.3 ± 9.2 spines/100 μm. These measures were lower, but not significant, compared to WT and WAVE-Tg littermates, which had 150.4 ± 10.7 spines/100 μm and 141.9 ± 9.7 spines/100 μm, respectively. Basolateral spine density measurements were similar: GluN1KD mice had 106.0 ± 8.4 spines/100 μm and GluN1KD-WAVE hybrids had 105.0 ± 8.8 spines/100 μm, neither significantly lower than the 124.8 ± 5.3 spines/100 μm of WT mice or the 124.8 ± 4.6 spines/100 μm of WAVE-Tg mice. No major or consistent morphological differences were detected between test mice as well (Supplemental Figures S3-6).

Overall, we did not find GluN1KD deficits for hippocampal WAVE-1 or CA1 spine density. Intercrossing the GluN1KD and WAVE-Tg mouse lines also did not significantly affect either measure. However, our experiments investigating the HPC of test mice were found to be underpowered post hoc.
Figure 3.8. Assessments of Hippocampal WAVE-1 protein levels (a) and CA1 pyramidal neuron spine density (b) in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. 

a) Representative western blots of WAVE-1 (top) and their quantifications (bottom) show no significant difference between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Blots were normalized to GAPDH loading control. Indicated in bars, a mouse sample size of 6 was used for each genotype. Quantified WAVE-1 was analyzed by one-way ANOVA. 

b) Apical (top) and basolateral (bottom) dendritic spine analyses are shown with representative images (left) and
quantifications (right). Mice with a GluN1KD genotype had less apical and basolateral spines than non-GluN1KD littermates, but no significant genotype effect was found by one-way ANOVAs. Sample sizes are indicated within each bar. For each mouse, 3-6 dendrite images were analyzed. Scale bars represent 20µm for dendrite images.

3.6 RNAseq Analyses Find Sex-Dependent GluN1KD Gene Expression Changes, Likely Involving Sex Hormone Signaling

3.6.1 GluN1KD Gene Expression Changes from RNAseq Show Internal Validity and Depend on the Sex of Mice

To characterize the striatal transcriptome of GluN1KD mice, we used RNAseq analyses following a modified tuxedo protocol (Trapnell et al., 2012). When comparing WT and GluN1KD mice with sexes combined (sample size of 6 for each group), expression of 235 genes was significantly different. We also performed sex-factored RNAseq analyses to consider sex differences in GluN1KD gene expression changes, comparing WT males, WT females, GluN1KD males, and GluN1KD females (sample size of 3 for each group). Sex-factored RNAseq identified 784 genes with changed expression in GluN1KD mice for at least one sex. As an internal validation of these results, we checked the expression of *Grin1* and sex-linked genes in test mice. Shown in Table III-I, the expression of checked genes was consistent with expectations: *Grin1* was low in both male and female GluN1KD mice, the X-inactivation effector gene *Xist* was near-silent in male test mice, and Y-linked genes were near-silent in female test mice. Cross-referencing both sets of RNAseq results found an overlap of 195 genes, as illustrated in Figure 3.9. Thus, most differentially-expressed genes identified by non-sex-factored RNAseq were also identified via sex-factored RNAseq, another good marker of consistency for our results.
Table III-I. RNaseq-quantified expression of *Grin1*, *Xist*, and Y-linked genes demonstrate internal validity of results. *Grin1* is decreased in GluN1KD mice of both sexes, *Xist* is near-absent in male mice of both genotypes, and Y-linked genes (*Eif2s3y*, *Ddx3y*, *Kdm5d*, and *Uty*) are near-absent in female mice of both genotypes. Quantifications are in units of FPKM – number of Fragments Per Kilobase of transcript per Million mapped reads. Gene full names: *Xist* – X-inactive specific transcript; *Eif2s3y* – eukaryotic translation initiation factor 2 subunit 3, Y-linked; *Ddx3y* – DEAD-Box Helicase 3, Y-linked; *Kdm5d* – lysine demethylase 5D; *Uty* – ubiquitously transcribed tetratricopeptide repeat containing, Y-linked.

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Figure 3.9. Sex factored RNAseq identified more GluN1KD gene expression changes, including most non-sex factored RNAseq results. 195 differentially expressed genes were identified by both analyses, 589 were uniquely identified by sex factored analysis, and 40 were unique to non-sex factored analysis.

Our RNAseq results point to sex being a key factor for transcriptomic comparisons, at least in the context of GluN1KD gene expression changes. Firstly, also illustrated in Figure 3.9, sex factored RNAseq uniquely identified 589 differentially expressed genes along with most of the non-sex factored RNAseq results. Most of the genes identified from sex factored RNAseq also varied in how they were changed by GluN1 knock-down depending on the sex of the mice. As shown in Table III, only 35 of the 784 GluN1KD gene expression changes were in the same direction when comparing male and female mice. The rest were different between mice of the
opposite sex, with gene expression changes being present only in one sex or oppositely affected by GluN1 knock-down. GluN1KD transcriptomic changes at the STR were greatly sex-dependent.

**Table III-II. Breakdown of GluN1KD gene expression changes by sex and directionality of changes.** Totaled differentially-expressed genes in GluN1KD mice were relative to sex-matched WT littermates. 749 of 784 genes identified by sex-factored RNAseq showed sex-dependency. 35 GluN1KD gene expression changes were in the same direction for both sexes.

<table>
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<th>Not Changed in Male GluN1KD Mice</th>
<th>Decreased in Male GluN1KD Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased in Female GluN1KD Mice</td>
<td>2</td>
<td>161</td>
<td>48</td>
</tr>
<tr>
<td>Not Changed in Female GluN1KD Mice</td>
<td>192 (black)</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Decreased in Female GluN1KD Mice</td>
<td>28</td>
<td>201</td>
<td>33</td>
</tr>
</tbody>
</table>

To help us further understand sex-dependency of GluN1KD gene expression changes, we filtered our results to genes that were also significantly different between WT males and females by sex-factored RNAseq analysis. Out of 784 genes, expression of 293 were significantly different between male and female WT mice – 156 “Male Genes” were higher in WT males while 137 “Female Genes” were higher in females. As shown in Tables III-III and III-IV, GluN1KD transcription changes (relative to sex-matched WT littermates) of Male Genes, were only decreases in GluN1KD males, increases in GluN1KD females, or both. Similarly, expression of Female Genes was only decreased in GluN1KD females, increased in GluN1KD males, or both. In other words, when focusing on Male and Female Genes, male GluN1KD mice are demasculinized or feminized while female GluN1KD mice are masculinized or defeminized. The gene changes showing demasculinization or feminization patterns in GluN1KD males and those showing masculinization or defeminization patterns in GluN1KD females are listed in Supplemental Table SII. Over all, we have strong evidence of sex-dependency in GluN1KD gene expression changes. We therefore focused our post-RNAseq gene list and pathways analyses on sex-factored RNAseq results.
Table III-III. Breakdown of GluN1KD gene expression changes for “Male Genes” by sex and directionality of changes. Male Genes refer to those out of the 784 sex-factored RNAseq results that were also significantly higher in WT males than WT females. Totaled differentially-expressed genes in GluN1KD mice were relative to sex-matched WT littermates. Expression of Male Genes changed by GluN1 knockdown was limited to downregulation in male mice and upregulation in female mice.

Table III-IV. Breakdown of GluN1KD gene expression changes for “Female Genes” by sex and directionality of changes. Female Genes refer to those out of the 784 sex-factored RNAseq results that were also significantly higher in WT females than WT males. Totaled differentially-expressed genes in GluN1KD mice were relative to sex-matched WT littermates. Expression of Female Genes changed by GluN1 knockdown was limited to upregulation in male mice and downregulation in female mice.
3.6.2 Gene List Analyses Predict that Sex-Dependent GluN1KD Transcriptomic Changes are Related to Sex Hormone Signaling and Lead to Convergent Outcomes

To help us further understand and interpret the RNAseq-identified gene expression changes, we used IPA (Krämer et al., 2014) and Enrichr (Chen et al., 2013; Kuleshov et al., 2016). Pathways analysis with IPA reported multiple curated canonical pathways, upstream regulators, diseases, biological functions, and predicted networks that were significantly over-enriched in our results, some of which were associated with predicted activation scores. In the context of our investigation into sex-differences between male and female GluN1KD transcriptomic changes, many IPA results were different between males and females. Representative sex-dependent IPA results are shown with causal network analysis results in Tables III-V and III-VI and constructed networks in Figure 3.10.

Causal network analyses report upstream regulators most associated with input genes and predict the activation states of these regulators based on input gene expression changes. As shown in Tables III-V and III-VI, two of the top three causal network analysis results for male and female GluN1KD gene expression changes (with significant activation scores for at least one sex) are sex dependent. Neural cell adhesion molecule 2 (Ncam2) regulates 27 genes from our inputs and, based on the specific expression changes of these 27 genes relative to sex-matched WT mice, was predicted to be activated in GluN1KD males (activation z-score = 2.18) but inhibited in females (z = -2.50). Estrogen receptor α (ERα; Esr1 is its encoding gene, which IPA continues to use in its outputs since genes were initially entered) was also identified as a significant regulator of GluN1KD-changed gene expression (p = 5.94 x 10^{-12} for differentially-expressed genes in both male and female GluN1KD mice), particularly interesting as it is an effector of estrogen signaling. Based on the sex-dependent expression changes of 17 genes it regulates, Esr1 is predicted to be activated in GluN1KD males (z = 4.12) and neither activated or inhibited in females (z = -1.70). Low density lipoprotein receptor-related protein 1 (Lrp1) was predicted to be inhibited in both GluN1KD males and females, but with different activation scores (z = -2.58 and -2.32, respectively) based on different gene expression changes like Wnt9a and Wnt5a.

Figure 3.10 further illustrates the sex-dependency of GluN1KD transcriptomic changes via IPA-constructed networks that include Esr1 as a hub based on connectivity. In male GluN1KD
mice, genes connected to Esr1 have increased expression while a connected hub centered on histone H3 contains primarily downregulated genes on the right and primarily upregulated genes on the left. These patterns are largely flipped in female GluN1KD mice: most genes connected to Esr1 are downregulated, the right half of the H3 hub largely consists of upregulated genes, and the left half of the H3 hub mainly contains downregulated genes. Taken together, our IPA results also point to a strong effect of sex on GluN1KD gene expression changes. Esr1, in particular, may be a strong intermediate between mouse sex and GluN1 knockdown.

**Table III-V. Representative results from IPA causal network analysis of GluN1KD gene expression changes in male mice.** Listed are the top three upstream regulators that are related to male GluN1KD gene expression changes and that have significant activation z-scores.

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>Activation z-score</th>
<th>Gene list over-representation p-value</th>
<th>Predicted upstream regulator state</th>
<th>GluN1KD gene expression changes that predict activation</th>
<th>GluN1KD gene expression changes that predict inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ncam2</td>
<td>2.117</td>
<td>5.23E-12</td>
<td>Activated</td>
<td>↑ Tr; ↑ Tnem72; ↑ Sulf1; ↑ Sostdc1; ↑ Ptgs2; ↑ Malat1; ↑ Lbp; ↑ Kl; ↑ Igf2; ↑ Fosl1; ↑ Enpp2; ↑ Colla1; ↑ Clic6; ↑ Cldn2; ↑ Cldn1; ↑ Ace</td>
<td>Tr; ↑ Tnem72; ↑ Sulf1; ↑ Sostdc1; ↑ Ptgs2; ↑ Malat1; ↑ Lbp; ↑ Kl; ↑ Igf2; ↑ Fosl1; ↑ Enpp2; ↑ Colla1; ↑ Clic6; ↑ Cldn2; ↑ Cldn1; ↑ Ace</td>
</tr>
<tr>
<td>Esr1</td>
<td>4.123</td>
<td>5.94E-12</td>
<td>Activated</td>
<td>↑ Tr; ↑ Tnem72; ↑ Sulf1; ↑ Sostdc1; ↑ Slc13a4; ↑ Rhdx5; ↑ Obx2; ↑ Lbp; ↑ Kl; ↑ Igf2; ↑ Gal; ↑ Fosl1; ↑ Enpp2; ↑ Colla1; ↑ Clic6; ↑ Cldn2; ↑ Ace</td>
<td>None</td>
</tr>
<tr>
<td>Lrp1</td>
<td>-2.582</td>
<td>2.59E-11</td>
<td>Inhibited</td>
<td>↑ Wnt5a; ↑ Sfrp2; ↑ Serpinb1a; ↑ Ptgs2; ↑ Plin3; ↑ Pdp1; ↑ Mk67; ↑ Lsp1; ↑ Kif2; ↑ ker5; ↑ Hsp90b1; ↑ Hba-a2; ↑ Glap; ↑ E2f1; ↑ Dusp1; ↑ Dkk3; ↑ Colla2; ↑ Bok; ↑ Adcyap1; ↑ Ace</td>
<td>↑ Wnt5a; ↑ Tp73; ↑ Tgfb2; ↑ Sclc8a2; ↑ Serpin1; ↑ Ptnin; ↑ Per1; ↑ Pcolce; ↑ Nkra2; ↑ Nkra1; ↑ Neurof1b; ↑ Ndfp2; ↑ Nkra2; ↑ Myr4; ↑ Me2; ↑ Malat1; ↑ Mag; ↑ Jumb; ↑ Irl1; ↑ Igf2; ↑ Id1; ↑ Hsd11b1; ↑ Trn1; ↑ Egr4; ↑ Egr2; ↑ Egr1; ↑ Dusp14; ↓ Dlgap3; ↑ Col5a2; ↑ Clic4; ↑ Cck; ↑ Camk2n1; ↑ Cac3b8; ↑ Btg2; ↑ Bhlhe40; ↑ Arc; ↑ Aspg1; ↑ Apold1; ↑ Amot2; ↑ Adcy1</td>
</tr>
</tbody>
</table>

**Table III-VI. Representative results from IPA causal network analysis of GluN1KD gene expression changes in female mice.** Listed are the top three upstream regulators that are related to male GluN1KD gene expression changes and that have significant activation z-scores.

<table>
<thead>
<tr>
<th>Upstream regulator</th>
<th>Activation z-score</th>
<th>Overlap p-value</th>
<th>Predicted upstream regulator state</th>
<th>GluN1KD gene expression changes that predict activation</th>
<th>GluN1KD gene expression changes that predict inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ncam2</td>
<td>-2.502</td>
<td>5.23E-12</td>
<td>Inhibited</td>
<td>↑ Sulf1; ↑ Slc13a4; ↑ Rhdx5; ↑ Ptgs2; ↑ Malat1; ↑ Lbp; ↑ Kl</td>
<td>↑ Sulf1; ↑ Slc13a4; ↑ Rhdx5; ↑ Ptgs2; ↑ Malat1; ↑ Lbp; ↑ Kl</td>
</tr>
<tr>
<td>Esr1</td>
<td>-1.698</td>
<td>5.94E-12</td>
<td>Neither activated nor inhibited</td>
<td>↑ Sulf1; ↑ Slc13a4; ↑ Rhdx5; ↑ Lbp; ↑ Kl</td>
<td>↑ Sulf1; ↑ Slc13a4; ↑ Rhdx5; ↑ Lbp; ↑ Kl</td>
</tr>
<tr>
<td>Lrp1</td>
<td>-3.234</td>
<td>2.59E-11</td>
<td>Inhibited</td>
<td>↑ Wnt5a; ↑ Tp73; ↑ Tgfb2; ↑ Sfrp2; ↑ Serpinb1a; ↑ Ptgs2; ↑ Plin3; ↑ Pdp1; ↑ Mk67; ↑ Kif2; ↑ ker5; ↑ Hsp90b1; ↑ Hba-a2; ↑ Glap; ↑ E2f1; ↑ Dusp1; ↑ Clic4; ↑ Cck; ↓ Cab39b; ↑ Amot2; ↑ Adcy1</td>
<td>↑ Wnt5a; ↑ Tp73; ↑ Tgfb2; ↑ Sfrp2; ↑ Serpinb1a; ↑ Ptgs2; ↑ Plin3; ↑ Pdp1; ↑ Mk67; ↑ Kif2; ↑ ker5; ↑ Hsp90b1; ↑ Hba-a2; ↑ Glap; ↑ E2f1; ↑ Dusp1; ↑ Clic4; ↑ Cck; ↓ Cab39b; ↑ Amot2; ↑ Adcy1</td>
</tr>
</tbody>
</table>
Figure 3.10. IPA-constructed, Esr1 (implied ERα)-centric, networks of male (left) and female (right) GluN1KD gene expression changes have the same structures but are largely opposite in regulation. The legend for network components is shown on the bottom. Double-lined shapes represent complexes. Red shapes indicate upregulation while green shapes indicate downregulation. Shading reflects magnitude of change. Solid lines represent experimentally-determined direct relationships while dotted lines represent indirect relationships. Arrows denote direction of relationship – the component at the beginning of the arrow affects the component at the end.

Pathways analyses and gene list analyses are also useful for determining the biological function and relevance of sets of genes and gene changes. Gene list analyses performed with Enrichr are shown in Tables III-VII and III-VIII and show similar results despite four different input lists – differentially-expressed genes separated by sex and direction of change (increased or decreased, in GluN1KD males or females). In the context of diseases in Table III-VII, Huntington’s disease (HD) records show up frequently amongst the top 5 results for each list’s analysis. Table III-VIII contains Enrichr results based on chemical-induced gene expression changes, particularly focusing on what chemical-induced changes could reverse those in our input lists. Similar to Table III-VII, Table III-VIII also has top results that are common to
multiple gene lists’ analyses – records involving withaferin A. Furthermore, though the other records of Table III-VIII largely involve different drugs, many are used for similar purposes, particularly for cancer treatment. These gene list analysis results are an illustration of different gene expression change patterns converging to similar biological significance and outcomes, at least in the context of NMDAR deficits in mice.

**Table III-VII. Enrichr gene list analysis results associate similar disease-related records with multiple input lists.** Differentially-expressed genes from sex-factored RNAseq were separated into four input lists based on mouse sex and direction of change. Analyses referenced “Disease Perturbations from GEO up” and “Disease Perturbations from GEO down” datasets for upregulated and downregulated genes in GluN1KD mice, respectively. Gene-set records related to HD appeared often in the results of all four lists’ analyses. For each gene list analysis, the top 5 results based on (and ordered by) Enrichr combined scores are shown. Enrichr reported $p$-values were adjusted by BH method for multiple comparisons. Denominators of gene overlap ratios represent the number of genes in each matched Enrichr record.
<table>
<thead>
<tr>
<th>Input gene list</th>
<th>Matched record from Enrichr</th>
<th>Adjusted p-value</th>
<th>Gene overlap</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased in Males (222 genes)</td>
<td>Schizophrenia DOID-5419 human GSE25673 sample 892</td>
<td>1.5E-09</td>
<td>21/263</td>
</tr>
<tr>
<td></td>
<td>Huntington's disease DOID-12858 mouse GSE19780 sample 527</td>
<td>9.6E-08</td>
<td>20/306</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>Decreased in Females (262 genes)</td>
<td>Decreased in Females (262 genes)</td>
<td>Decreased in Females (262 genes)</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>Depression DOID-1595 mouse GSE66205 sample 463</td>
<td>4.6E-06</td>
<td>18/327</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>Spinal Muscular Atrophy C0026847 mouse GSE10599 sample 235</td>
<td>1.6E-06</td>
<td>20/368</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>pancreatic ductal adenocarcinoma DOID-3498 human GSE15471 sample 604</td>
<td>1.5E-05</td>
<td>21/487</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>Huntington's disease DOID-12858 mouse GSE3621 sample 703</td>
<td>1.5E-24</td>
<td>38/371</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>Huntington's disease DOID-12858 mouse GSE3621 sample 704</td>
<td>3.2E-23</td>
<td>36/356</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>Alzheimer's disease DOID-10652 human GSE69880 sample 519</td>
<td>2.6E-13</td>
<td>27/380</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>glaucoma associated with systemic syndromes DOID-1686 mouse GSE26299 sample 491</td>
<td>3E-09</td>
<td>21/341</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>Huntington's disease DOID-12858 mouse GSE9038 sample 717</td>
<td>1.3E-20</td>
<td>32/889</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>Huntington's disease DOID-12858 mouse GSE19780 sample 528</td>
<td>0.0018</td>
<td>16/328</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>multiple sclerosis DOID-2377 human GSE8010 sample 737</td>
<td>0.0003</td>
<td>15/310</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>multiple sclerosis DOID-2377 human GSE8010 sample 738</td>
<td>0.0011</td>
<td>14/309</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>endometriosis DOID-289 human GSE23339 sample 883</td>
<td>0.0011</td>
<td>14/315</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>Wolfram syndrome UMLS CUI-C0043207 mouse GSE15293 sample 695</td>
<td>4.2E-15</td>
<td>32/350</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>Niemann-Pick disease Type C DOID-14504 mouse GSE36621 sample 469</td>
<td>7.5E-15</td>
<td>29/290</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>Huntington's disease DOID-12858 mouse GSE3621 sample 703</td>
<td>7.7E-15</td>
<td>23/371</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>Huntington's disease DOID-12858 mouse GSE3621 sample 704</td>
<td>8.9E-13</td>
<td>29/356</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>Papillary Carcinoma of the Thyroid C0238463 human GSE3678 sample 306</td>
<td>2.5E-12</td>
<td>26/292</td>
</tr>
</tbody>
</table>
### Table III-VIII. Enrichr gene list analysis results associate similar chemical-perturbation records with multiple input lists.

Differentially-expressed genes from sex-factored RNAseq were separated into four input lists based on sex and change direction. Analyses referenced “LINCS L1000 Chem Pert down” and “LINCS L1000 Chem Pert up” datasets for upregulated and downregulated genes in GluN1KD mice, respectively. Opposite-directionality was chosen to identify drugs (in bold) that may reverse GluN1KD transcriptomic changes. Enrichr gene-set records related to withaferin A were identified in multiple gene list analyses. For each gene list analysis, the top 5 results based on (and ordered by) Enrichr combined scores are shown. Enrichr reported p-values were adjusted by BH method for multiple comparisons. Denominators of gene overlap ratios represent the number of genes in each matched Enrichr record.

<table>
<thead>
<tr>
<th>Input gene list</th>
<th>Matched record from Enrichr</th>
<th>Adjusted p-value</th>
<th>Gene overlap</th>
<th>Overlapping genes (between input gene list and Enrichr record)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased in Males (222 genes)</td>
<td>LJP008_HT29_24H-KU-55933-10</td>
<td>0.00272</td>
<td>6/28</td>
<td>Postn, Gja1, Sparc, Peg10, Pdgfd, Wnt5A</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>LJP006_LNCApP_3H-withaferin-a-3.33</td>
<td>0.00272</td>
<td>7/39</td>
<td>Cpm, Postn, Sparc, Peg10, Igfbp2, Scg2, Follr1</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>LJP008_HEP32_24H-HOX2-10</td>
<td>0.0036</td>
<td>6/31</td>
<td>Tcf7l2, Igfbp2, Myol, Engpp2, Gabre, Esr1</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>LJP006_BT20_24H-KIN001-043-0.04</td>
<td>0.00272</td>
<td>8/64</td>
<td>Tppp3, Dab2, Sparc, Peg10, Pdgfd, Tsc22D3, Sgk1, Dlk1</td>
</tr>
<tr>
<td>Increased in Males (222 genes)</td>
<td>LJP005_MCF10A_24H-PHA-665752-10</td>
<td>0.01044</td>
<td>6/43</td>
<td>Cpm, Postn, Engpp2, Lamb1, Msx1, Sepp1</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>LJP006_SKBR3_24H-withaferin-a-3.33</td>
<td>8.6E-17</td>
<td>26/213</td>
<td>Btg2, Satb2, Fhl2, Ctgf, Dusp14, Rgs2, Junb, Ier5, Ier2, Kif10, Egr1, Egr3, Egr4, Dusp1, Fos, Cxcl10, Kif12, Fosl2, Ndr4A2, Ndr4A1, Per1, Arc, Ndr4A3, Bhlhe40, Fosl2, Junb, Ier5, Ier2</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>LJP006_HME1_3H-QL-XII-47-1.11</td>
<td>8.6E-17</td>
<td>22/135</td>
<td>Kif10, Egr1, Btg2, Egr2, Nm1, Egr3, Dusp1, Apold1, Kif12, Dusp6, Fosl2, Ndr4A2, Ndr4A1, Per1, Rgs2, Arc, Ndr4A3, Bhlhe40, Fosl2, Junb, Ier5, Ier</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>LJP006_HME1_3H-QL-XII-47-3.33</td>
<td>8.6E-17</td>
<td>22/135</td>
<td>Kif10, Egr1, Btg2, Egr2, Egr3, Dusp1, Fos, Kif12, Dusp6, Ctgf, Fosl2, Ndr4A2, Ndr4A1, Per1, Rgs2, Ndr4A3, Bhlhe40, Fosl, Junb, Ier5, Stx1A, Ier2</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>LJP005_HA1E_24H-withaferin-a-3.33</td>
<td>4.9E-16</td>
<td>21/130</td>
<td>Egr1, Ighm, Btg2, Me2C, Egr3, Dusp1, Cck, Fos, Dusp6, Ndr4A2, Ndr4A1, Rgs2, Arc, Ndr4A3, Frzb, Camk2N1, Fosl, Trib1, Junb, Ier5, Ier2</td>
</tr>
<tr>
<td>Decreased in Males (200 genes)</td>
<td>LJP005_MCF7_24H-withaferin-a-10</td>
<td>4.9E-16</td>
<td>22/149</td>
<td>Kif10, Egr1, Btg2, Egr2, Egr3, Egr4, Dusp1, Fos, Kif12, Dusp6, Ctgf, Fosl2, Ndr4A2, Ndr4A1, Rgs2, Arc, Ndr4A3, Bhlhe40, Fosl, Junb, Ier5, Stx1A, Ier2</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>LJP006_BT20_24H-dasatinib-0.04</td>
<td>3E-05</td>
<td>10/66</td>
<td>Col5A1, Coll1A1, Serpine1, Col5A2, Rbp1, Fhl2, Mical2, Sulf1, Ctgf, Dkk3</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>LJP008_MCF7_24H-(+)JQ1-0.04</td>
<td>0.02173</td>
<td>6/47</td>
<td>Ndr4A2, Coll5A1, Coll5A2, Coll6A1, Trbc1, Fhl2</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>LJP009_HA1E_24H-BI-2536-10</td>
<td>0.01063</td>
<td>8/75</td>
<td>Nm1, Lgals1, Coll5A1, Coll1A1, Serpine1, Mical2, Prss32, Ctgf</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>LJP007_HA1E_24H-HG-9-91-01-1.11</td>
<td>0.02173</td>
<td>5/27</td>
<td>Nm1, Serpine1, Mical2, Prss32, Ctgf</td>
</tr>
<tr>
<td>Increased in Females (211 genes)</td>
<td>LJP005_MCF10A_3H-PD-0325901-0.37</td>
<td>0.02173</td>
<td>7/68</td>
<td>Lgals1, Coll1A1, Fhl2, Mical2, Sox11, Stx1A, Mylk</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>LJP005_MCF10A_3H-NVP-AUY922-10</td>
<td>5.8E-06</td>
<td>11/59</td>
<td>Xbp1, Sdf2L1, Hspa5, Amig2a, Cdk2Ap2, Calr, Magt1, Crel1, Crel2, Pdia6, Pdia4, Hsp90B1</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>LJP005_MDAMB231_3H-NVP-AUY922-10</td>
<td>1.2E-05</td>
<td>11/67</td>
<td>Hspa8, Xbp1, Sdf2L1, Hspa5, Aldh1A1, Cdk2Ap2, Calr, Crel2, Coch, Pdia4, Hsp90B1</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>LJP005_MCF10A_3H-geldanamycin-3.33</td>
<td>2.1E-05</td>
<td>13/117</td>
<td>Hspa8, Xbp1, Sdf2L1, Hspa5, Magt1, Pdia6, Pdia4, Hsp90B1, Pdzd2, Tnnt1, Cdk2Ap2, Calr, Crel2</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>LJP005_HCC515_24H-NVP-AUY922-0.04</td>
<td>4.8E-05</td>
<td>11/89</td>
<td>Hsd11B1, Hspa8, Rgs2, Rcn1, Xbp1, Ddc, Mme, Hsp88, Cryab, Sepp1, Hsp90B1</td>
</tr>
<tr>
<td>Decreased in Females (262 genes)</td>
<td>LJP005_SKBR3_3H-withaferin-a-3.33</td>
<td>1.8E-05</td>
<td>14/133</td>
<td>Hspa8, Egr1, Egr2, Pkd2b, Egr3, Hspb8, Rnd3, Fosl2, Akap12, Ndr4A1, Arc, Fosl, Nefm, Cryab</td>
</tr>
</tbody>
</table>
4.1 Developmentally Correlated Synaptic and Molecular Deficits in GluN1KD Mice

As the overall aim of this study is to investigate the relationship between Rho GTPase signaling pathways, synaptic connectivity, and behaviour in GluN1KD mice, we first sought to more firmly establish the link between dendritic spine density and behaviour. Previously, GluN1KD studies have reported MSN spine density deficits at 6 weeks of age but not at 2 weeks (Ramsey et al., 2011). This deficit was developmentally aligned with the onset of some GluN1KD behavioural abnormalities at 6 weeks of age (Milenkovic et al., 2014). However, while spine density studies were conducted on two-week old mice, the behavioural studies were conducted on three-week old mice (Ramsey et al., 2011; Milenkovic et al., 2014). A one-week-age difference for the youngest mice in these studies may seem minimal, but this peri-weaning age gap may be developmentally important; female open field behaviour and male PPI have been shown to change between 2- and 3-weeks of age (Moy et al., 2012). We therefore sought to more definitively align MSN spine density and GluN1KD behavioural abnormalities by counting MSN spines at 3-, 6-, and 12-weeks of age.

As shown in Figure 3.1, GluN1KD mice started having significantly lower spine densities compared to WT littermates at 6-weeks of age, which persisted by 12-weeks of age. These results strongly mirror the developmental trajectories of GluN1KD behavioural abnormalities that manifest at 6 weeks of age (Milenkovic et al., 2014). Such a correlation is likely biologically relevant, as loss of synapses has also been associated with fear conditioning (Lai et al., 2012; Sanders et al., 2012) and cognitive impairment in AD (Tönnies and Trushina, 2017). More research will be needed to specifically understand how MSN spine changes contribute to GluN1KD behavioural phenotypes. The results presented in Figure 3.1 also narrow the possible developmental window for MSN spine loss in GluN1KD mice from the previous 2-6 weeks of age (Ramsey et al., 2011) to 3-6 weeks. Taken together, both age-related spine studies further suggest that synaptic deficits in GluN1KD mice are more likely due to problems with synaptic elimination than synaptogenesis. This could guide future research into the neurodevelopment of
GluN1KD brains. Overall, we have more fully characterized and established the course of age-dependent spine loss in GluN1KD MSNs.

We also report evidence for dysregulated Rho GTPase signaling in GluN1KD mice. Specifically, two proteins were consistently altered in GluN1KD mice at all three ages: Rac1 and WAVE-1. As mentioned before, changes in these two specific proteins are consistent with the previous reports of DISC1 deficits in GluN1KD mice (Ramsey et al., 2011). This is because DISC1 regulates the GEFs that activate Rac1 (Hayashi-Takagi et al., 2010; Chen et al., 2011), which in turn acts on WAVE-1 (Eden et al., 2002; Soderling et al., 2003; Kim et al., 2006b). Antagonism of NMDARs with PCP has also been reported to decrease cortical WAVE-1 in rats (Andrews et al., 2015) consistent with our findings and suggesting that WAVE-1 decreases in GluN1KD mice are indeed related to NMDAR hypofunction. The fact that primarily Rac1-related signaling is aberrant in GluN1KD mice is consistent with current, albeit limited, evidence for the relationship between DISC1 and Rho GTPases. RhoA, Cdc42, and Rac1 are the most characterized and emphasized Rho GTPases when it comes to cytoskeleton rearrangement during neuronal and synaptic development (Hedrick and Yasuda, 2017; Huang et al., 2017). Of the three, Rac1 has generally been considered the main interactor with DISC1 through Kalirin-7 (Dillon and Goda, 2005; Hayashi-Takagi et al., 2010; Saneyoshi and Hayashi, 2012), as illustrated with Figure 4.1. Proteomic screening of NMDAR-associated complexes in the mouse brain has also specifically reported the presence of Rac1 but not RhoA or Cdc42 (Husi et al., 2000). In general, Rac1 may be the most relevant or affected of the three Rho GTPases in a context of NMDAR and DISC1 deficiency. More specific experiments and focused studies also reinforce this idea.
Figure 4.1. Representative schematic of the Rho GTPase signaling pathways related to cytoskeletal rearrangement as part of synaptic plasticity. Green pointed arrows indicate a positive relationship of some form, such as enhanced activation or release from a sequestering factor. Red blunted lines indicate the opposite, causing some form of inhibition or decreased activity. Black arrows indicate a more complex regulatory relationship from mixed or limited experimental results. Abbreviations follow those used in text and previous figures along with newly mentioned proteins: GAP – GTPase-activating proteins; Tiam1 – T-lymphoma invasion and metastasis-inducing protein 1; ROCK – Rho-associated protein kinase; MLC – myosin light...
chain; MHCII – myosin heavy chain IIB. Figure was constructed from previously cited work (Nakayama et al., 2000; Dillon and Goda, 2005; Hayashi-Takagi et al., 2010; Saneyoshi and Hayashi, 2012).

There is evidence of connections between DISC1 and non-Rac1 Rho GTPases, though the evidence is more robust for DISC1 and Rac1. RhoA interacts with dynamin-related protein 1 (Drp1) and its protein level was decreased with knockdown of Drp1 in human glioma cells (Yin et al., 2016). DISC1 may therefore also be indirectly connected to RhoA since its knockdown in glioblastoma cells decreases Drp1, cell proliferation and self-renewal, and cell migration and invasion. The activity of DISC1 in this context was believed to be via mitochondrial mechanisms (Gao et al., 2016). DISC1 has also been shown to regulate the transport of NudE-like (NUDEL) and LIS1 in axons (Taya et al., 2007), which in turn can indirectly activate Cdc42 as part of cell migration mechanisms (Kholmanskikh et al., 2006; Shen et al., 2008). These connections between DISC1 and RhoA or Cdc42 are indirect, experimentally and molecularly, as well as more in the context of cell motility and proliferation than synaptic plasticity (Kholmanskikh et al., 2006; Shen et al., 2008; Gao et al., 2016). In contrast, the connection between DISC1 and Rac1 has been demonstrated more directly in single studies (Hayashi-Takagi et al., 2010; Chen et al., 2011). In both in vitro and in vivo experiments, DISC1 was shown to act at synapses by sequestering Kalirin-7 and inhibiting its activation of Rac1. Upon NMDAR activation, DISC1 and Kalirin-7 dissociate and Rac1 activity increases, resulting in more and larger spines as well as strengthened synapses (Hayashi-Takagi et al., 2010). DISC1 has also been demonstrated to increase Rac1 activity by releasing the GEF domain of triple functional domain protein (TRIO) in the axons of Caenorhabditis elegans and COS cells (Chen et al., 2011). These two studies thus demonstrated within one set of experiments a functional connection between DISC1 and Rac1 (Hayashi-Takagi et al., 2010; Chen et al., 2011). The second study in C. elegans and COS cells focused on the role of DISC1 and Rac1 in axon guidance and may therefore be less relevant to synaptic plasticity in a context of altered NMDAR signaling (Chen et al., 2011). In this sense, the DISC1/Kalirin-7/Rac1 pathway associated with NMDAR activity in dendritic spines may be particularly relevant to GluN1KD mice. It is also interesting to note that RhoA or Cdc42 was not activated by Kalirin-7 when Rac1 was (Hayashi-Takagi et al., 2010); this is also consistent with our findings. Our results could also be improved by looking more specifically at synaptic levels of Rho GTPase signaling proteins. This is because synaptic TH and DAT were previously
reported to be decreased in GluN1KD mice while striatal total-tissue levels of these proteins were not (Ferris et al., 2014). Even DISC1 deficits were more pronounced at the synapse (Ramsey et al., 2011). Furthermore, synaptic LIS1 was increased while tissue LIS1 was decreased in 6-week old GluN1KD mice (Ramsey et al., 2011), inviting the question of whether synaptic Cdc42 may be altered. As a whole, however, the current data point to Rac1 signaling deficits in the STR of GluN1KD mice that are consistent with the NMDAR/DISC1/Rac1/WAVE-1 literature (Soderling et al., 2003; Hayashi-Takagi et al., 2010; Ramsey et al., 2011).

Striatal Rac1 and WAVE-1 abnormalities in GluN1KD mice were also age-dependent, as shown in Figure 3.2. These proteins were both decreased in 6-week old adolescent mice and 12-week old adult mice but not in 3-week old juvenile mice. Specifically, Rac1 was increased while WAVE-1 was decreased in these younger GluN1KD mice. The fact that Rac1 signaling proteins were less overtly deficient in 3-week old mice may be consistent with how DISC1 was also more moderately decreased in 2-week old GluN1KD mice than 6-week olds (Ramsey et al., 2011). In other words, the mixed changes of Rac1 and WAVE-1 in 3-week old GluN1KD mice may be representative of a milder Rac1 signaling abnormality; the increase in Rac1 may be in some way compensatory for decreased DISC1 and WAVE-1. On the other hand, prolonged knockdown of DISC1 for 6 days resulted in sustained increases of Rac1 bound to guanosine triphosphate (GTP) in cultured rat cortical neurons, though total Rac1 level was not changed (Hayashi-Takagi et al., 2010). It would be interesting to study the time-course of Rac1 and WAVE-1 changes in response to chronic DISC1 deficiency in vivo. The transient increase of Rac1 at the GluN1KD STR also brings to mind the altered DA system in GluN1KD mice. Just as experiments with 12- to 15-week old brains found signs of previous hyperdopaminergia (Ferris et al., 2014), the current results present a period of increased Rac1. It is possible that the two findings are related. For example, as overactive VTA neurons project to the NAc in GluN1KD mice (Berton et al., 2006; Ferris et al., 2014), they may also increase the co-release of glutamate (Dal Bo et al., 2004; Stuber et al., 2010; Tecuapetla et al., 2010). This would produce an increased glutamatergic tone, activate the NMDAR/DISC1/Kalirin-7/Rac1 pathway discussed previously (Hayashi-Takagi et al., 2010) and potentially contribute to long-term protein-level changes for Rac1 and WAVE-1.

The mixed-direction changes of Rac1 and WAVE-1 in 3-week old GluN1KD mice, as well as milder DISC1 deficits in 2-week old GluN1KD mice, coincided with WT-like MSN spine density (Ramsey et al., 2011). Chronic Rac1 activation has been repeatedly demonstrated to
result in deficient dendritic spines (Luo et al., 1996; Tashiro et al., 2000; Hayashi-Takagi et al., 2010). As we did not see this in 3-week old GluN1KD mice, it is possible that the increase of Rac1, along with decreased WAVE-1, was starting to have a deleterious effect on GluN1KD MSN spines. WAVE-1 deficits are also consistent with dendritic spine disruptions, as it promotes actin polymerization at synapses (Bompard and Caron, 2004; Kim et al., 2006b; Soderling et al., 2007). Both in vitro neuronal studies and in vivo mouse studies have previously demonstrated decreased dendritic spines with WAVE-1 disruption (Kim et al., 2006b; Soderling et al., 2007). Mice with disrupted Arp2/3 complexes, which act with WAVE-1 (Bompard and Caron, 2004; Soderling et al., 2007), similarly demonstrated decreased dendritic spines (Kim et al., 2013). The changes of Rac1 and WAVE-1 are therefore generally consistent with a state of spine loss. However, it is also important to point out that the measures discussed so far are protein levels and spine numbers – not measures of functionality. It is possible that the effects of GluN1KD on the activity of Rac1 and WAVE-1 are not necessarily reflected in protein level changes. Indeed, both Rac1-GTP and phosphorylated WAVE-1, activity-dependent forms of their respective proteins, may represent only a portion of total Rac1 and WAVE-1 (Kim et al., 2006b; Hayashi-Takagi et al., 2010). Functional analyses of Rac1, WAVE-1, and MSN dendritic spines would improve the interpretability of the results in our study.

Together, the changes of Rac1, WAVE-1, and MSN spine density are generally consistent with GluN1KD behavioural abnormalities. The spine density deficits were absent in 3-week old GluN1KD mice, manifested by 6 weeks of age, and persisted into adulthood. Again, these spine density deficits mirrored the trajectories of some GluN1KD behavioral abnormalities – stereotypy time, social approach time, and Y-maze alternation performance (Milenkovic et al., 2014). If we interpret the mixed Rac1 and WAVE-1 changes in 3-week old GluN1KD mice as a moderate phenotype where increased Rac1 is compensatory for WAVE-1 deficits, then these molecular changes align with how MSN spines change with age. However, the increased Rac1 may instead represent a protracted increase in Rac1 activity that leads to atrophied spines (Luo et al., 1996; Tashiro et al., 2000; Hayashi-Takagi et al., 2010). If this is true, and the increased Rac1 compounds on the effects of a WAVE-1 decrease, then Rac1 signaling may explain some of the discrepancies between MSN spine density and GluN1KD behaviours. A worse or equally-disturbed Rac1 signaling status in 3-week old GluN1KD mice may be reflective of their demonstrated abnormalities in open field and puzzle box test performance (Milenkovic et al.,
The altered Rac1 signaling in juvenile mice in this case may be signs of synaptic anomalies more subtle than complete spine loss. Assessing the function of GluN1KD MSN spines would be especially helpful for determining whether this is true. If so, then there would be a more coherent picture: GluN1KD MSN synapses are abnormal at the molecular level at 3 weeks of age and these abnormalities are exacerbated to the point of spine loss in later ages.

The neurological disorder literature also offers some consistency with the Rac1, WAVE-1, and dendritic spine abnormalities we report here. Rac1 mRNA was reduced in the dorsolateral prefrontal CTX of schizophrenia patients, though the decrease was not significant after correction for multiple comparisons. As an interesting aside, Cdc42 decreases did remain significant and correlated with spine density deficits (Hill et al., 2006), again encouraging a second look at synaptic Cdc42 levels in GluN1KD mice. Results from an in silico bioinformatics study have also strongly pointed to the RAC1 gene as a key node associated with autism related biological processes, cellular components, and molecular functions (Zeidán-Chuliá et al., 2013). The relationship of these two disorders to WAVE-1 is more robust and direct: WASF1 was found to be commonly decreased in two large schizophrenia cohorts (Maycox et al., 2009) and de novo truncation mutations of WASF1 were found in humans with intellectual disability, autistic features, and seizures (Ito et al., 2018). Finally, just as GluN1KD behaviour onset was evocative of symptom onset in schizophrenia previously (Eranti et al., 2013; Milenkovic et al., 2014; Hardingham and Do, 2016), our observations of concurrently decreased Rac1 and WAVE-1 at later ages are also developmentally consistent. Overall, there is strong and consistent evidence of Rac1 signaling disruption leading to dendritic spine deficits and behavioural abnormalities in GluN1KD mice. These phenotypes are likely relevant to neurological disorders, thus supporting further investigations into treating them.

4.2 Effect of Restoring WAVE-1 on GluN1KD Phenotypes

Since WAVE-1 is generally acknowledged to act downstream of Rac1 (Eden et al., 2002; Soderling et al., 2003), we targeted its restoration for the improvement of GluN1KD molecular, synaptic, and behavioural phenotypes. Progeny from intercrossing the GluN1KD and WAVE-Tg mouse lines were viable and survived to adulthood. As shown in Figures 3.3 and 3.4, the expression of the transgenic human WASF1 was as expected in the STR and HPC of F1 mice:
transgene expression was abundant and similar to endogenous Wasf1 in WAVE-Tg and GluN1KD-WAVE mice and near absent in non-WAVE littermates. It would be useful in the future to further study these expression patterns via an additional co-labeling with Grin1 probes. Such labeling would allow us to more carefully discern interactions between knocking down Grin1 and WASF1/Wasf1 expression in our test mice. The protein levels of striatal WAVE-1 in F1 mice are shown in Figure 3.5 and were also consistent with expectations. WAVE-1 was confirmed as decreased in GluN1KD mice compared to WT littermates, higher than WT levels in WAVE-Tg mice, and recovered towards WT levels in GluN1KD-WAVE mice. However, the high proportion of pS397-WAVE-1 in GluN1KD-WAVE mice relative to WT littermates may represent a moderation of any effects from total WAVE-1 increases. This is because phosphorylated WAVE-1 is generally considered inactive, since neuronal stimulation via depolarization, D1R agonism, and cyclic adenosine monophosphate (cAMP) increases results in WAVE-1 dephosphorylation and spine growth (Kim et al., 2006b; Sung et al., 2008). Some of these experiments have also demonstrated WAVE-1 dephosphorylation after NMDAR stimulation, consistent with an idea of NMDAR hypofunction leading to more phosphorylated WAVE-1 (Sung et al., 2008; Ceglia et al., 2010). However, 17β-estradiol activation of ERα in rat cortical neurons was also associated with WAVE-1 phosphorylation, Arp2/3 colocalization to sites of spine formation, and increased dendritic spines (Sanchez et al., 2009). While our observations are likely more related to depolarization and D1R mechanisms, particularly since many of those experiments used mouse striatal MSNs (Kim et al., 2006b), we cannot rule out that established striatal ERα functions (Küppers and Beyer, 1999; Dubal et al., 2001; Schultz et al., 2009) are changed in GluN1KD mice. Further investigating how WAVE-1 in GluN1KD MSNs respond to different stimuli would help us understand the biological relevance of increased pS397-WAVE-1. GluN1KD striatal Rac1 was lower than WT levels, but the difference was unexpectedly not statistically significant. This could be due to insufficient statistical power (post hoc β = 0.66). Overall, the most important molecular characterization of our GluN1KD-WAVE mice was an intended and expected primary outcome: total striatal WAVE-1 was improved by intercrossing GluN1KD with WAVE-Tg mice.

The rescue of striatal WAVE-1 in GluN1KD-WAVE mice was also reflected by MSN spine density improvement, as shown in Figure 3.6a. GluN1KD-WAVE mice had a MSN dendritic spine density closer to WT mice than GluN1KD littermates, which again displayed a deficit. This
result is consistent with our hypothesis and supports the idea of targeting WAVE-1 specifically, and Rho GTPase or Rac1 signaling generally, to treat GluN1KD phenotypes. Achieving dendritic spine rescue is also consistent with previous experiments where cotransfection of knockdown-resistant Wasf1 improved the spine density of cultured neurons with endogenous Wasf1 knockdown (Kim et al., 2006b; Sung et al., 2008; Sanchez et al., 2009). Our results have now recapitulated these findings in vivo. The spine loss reported here is similar to the dendritic spine deficits reported in neuron-specific Rac1 knockout mice (Corbetta et al., 2009) and WAVE-1 knockout mice (Soderling et al., 2007). The spine density changes we observed are also similar in magnitude or proportion to those reported after fear conditioning and other behavioural tests (Roberts et al., 2009; Lai et al., 2012; Sanders et al., 2012). Thus it was possible that typical GluN1KD behavioral phenotypes were also altered in GluN1KD-WAVE mice.

GluN1KD behavioural abnormalities were retained in GluN1KD mice born from the intercross breeding of C57B1/6J Grin1+/− WAVE-Tg mice and 129X1/SvJ Grin1+/− non-WAVE-Tg mice. Our assessments of Y-maze, 8-arm radial maze, puzzle box, open field, and social approach test performance were consistent with previous GluN1KD studies (Mohn et al., 1999; Dzirasa et al., 2009; Milenkovic et al., 2014). These results, along with what happens with WAVE-1 rescue, are shown in Figures 3.6 and 3.7. GluN1KD mice displayed decreased Y-maze spontaneous alternations, 8-arm radial maze ETR, and social approach time compared to WT littermates. They also had increased 8-arm radial maze WME, puzzle box completion time, horizontal locomotor activity, and stereotypy incident number.

GluN1KD-WAVE mice demonstrated a selective improvement in maze exploration performance, consistent with the idea that striatal WAVE-1 and MSN spine improvements are behaviourally relevant. Specifically, GluN1KD-WAVE mice behaved like WT littermates in the Y-maze and had a significant improvement of 8-arm radial maze WME but not ETA compared to GluN1KD littermates. The GluN1KD-WAVE mice still had significantly more WMEs compared to WT littermates, indicating a selective and partial rescue in 8-arm radial maze performance. Such a combination of results is not unusual since the 8-arm maze is more complex than the 3-armed Y-maze. Furthermore, 8-arm WME is similar to 3-arm alternations as both measures are based on counts of incorrect or correct, respectively, arm entries (Mandillo et al., 2008; Dzirasa et al., 2009). ETR, on the other hand, is a measure of how quickly mice make their
first erroneous entry into an already-visited maze arm (Levin et al., 2009). An improvement in Y-maze alternations or 8-arm WME would therefore not necessarily predict better ETA scores. Our results are also limited due to potential confounds between hyperactivity and maze exploration performance – more errors may be due to more overall activity in GluN1KD mice, for example. However, these limitations are somewhat controlled in a few ways: Y-maze performance is a % of total arm entries rather than absolute counts of arm entries; 8-arm radial maze errors are counted until all maze arms are entered regardless of how quickly the entries were made. Furthermore, based on trends of hyperactivity in our open field test, we would expect GluN1KD-WAVE mice to perform as badly or worse than GluN1KD mice if our maze results were largely driven by horizontal movement differences. A limited behavioural rescue in GluN1KD-WAVE mice was also not unusual because more than WAVE-1 decreases could contribute to abnormal behaviours, such as the previously discussed GluN1KD-altered DA system (Ferris et al., 2014). The high proportion of pS397-WAVE-1, if truly inactive in our experimental context, would further predict a more modest behavioural improvement in GluN1KD-WAVE mice. The discovery of GluN1KD-WAVE behavioural improvements is consistent with previous reports of the STR being important for cognition and maze exploration (McDonald and White, 1993; Floresco et al., 1997; Simpson et al., 2010; Shu et al., 2015), though such evidence is fairly limited. Overall, it makes sense for striatal WAVE-1 and MSN spine increases in GluN1KD-WAVE mice to co-present with a modest improvement of maze exploration behaviour.

In contrast, the current literature does not elucidate why only maze exploration was improved in GluN1KD-WAVE mice. Striatal activity has also been linked to social (Báez-Mendoza and Schultz, 2013; Gunaydin et al., 2014) and locomotor behaviour (Burns et al., 1994; Patterson et al., 2015), though again the evidence is overall limited. The combination of GluN1KD-WAVE behaviours has not been replicated in any reports of GluN1KD mice, though the specific behavioural test battery we employed has also not been previously used in one GluN1KD study. There has been one prior instance of Y-maze behaviour being distinct in GluN1KD mice: it was the only behaviour that was not significantly worse in female adult GluN1KD mice (Milenkovic et al., 2014). This may mean that Y-maze deficits are later-onset than other GluN1KD behavioural abnormalities and, consequently, beneficial WAVE-1 increases further delayed or prevented its manifestation. However, we did not detect a significant effect of sex in our
behavioural tests. More Y-maze tests of male and female GluN1KD mice with greater age resolution around 12 weeks may help us understand its precise onset and trajectory. These and further experiments are needed to clarify why only maze exploration behaviours are improved in GluN1KD-WAVE mice. As part of this initiative, we further asked whether WAVE-1 and dendritic spine changes in the GluN1KD and GluN1KD-WAVE STR were mirrored at the HPC, which, along with the CTX, is very important for cognition and maze exploration (Choy et al., 2008; Morris et al., 2013).

4.3 Striatal and Hippocampal Differences in GluN1KD and GluN1KD-WAVE mice

We assessed total hippocampal tissue WAVE-1 and dendritic spines of CA1 pyramidal neurons, whose function has been previously associated with Y-maze and 8-arm radial maze performance (Markus et al., 1994; Li et al., 2005). Unchanged WAVE-1 expression in the HPC of our test mice was consistent with the previous report of WT-like DISC1 levels in the GluN1KD HPC (Ramsey et al., 2011). In the context of maze exploration improvement in GluN1KD-WAVE mice, however, a lack of WAVE-1 and spine density deficits in the HPC of GluN1KD mice (and its theoretical rescue with incorporation of a WASF1 transgene) is unexpected. This is because performance improvements in maze tests are cannonically considered improvements of learning and memory (Maurice et al., 1994; Dean et al., 2009; Kim et al., 2013), which is traditionally considered a main function of the HPC (McHugh et al., 2007; Neves et al., 2008; Buzsáki and Moser, 2013). To note, CA1 dendritic spine density experiments were found to be underpowered post hoc ($\beta = 0.45-77$). This does not resolve the inconsistency between our hippocampal and behavioural results, however: if CA1 spine density comparison results were false negatives, we would predict spine deficits in GluN1KD mice but not rescue in GluN1KD-WAVE littermates when considering our current data. GluN1KD-WAVE improvements of maze exploration thus remain incongruent with our WAVE-1 and spine data in the HPC.

Our synaptic and molecular data from the STR may still offer important clues about the relationship between WAVE-1, MSN spines, and mouse behaviour. In previous studies of striatal function and maze exploration, the dorsal STR was suggested to facilitate the reinforcement of
stimulus-response associations (McDonald and White, 1993), the NAc was associated with goal-directed exploration (Floresco et al., 1997), and the marginal division was associated with consolidation processes for long-term memory (Shu et al., 2015). As we did not employ any overt stimuli or cues in our maze tests, stimulus-response reinforcement may be a more minor factor in our study. In contrast, goal directed exploration may be especially applicable for food-restricted mice and long-term memory can form from learning by repetition. Both food restriction and repeat-testing were part of our 8-arm radial maze tests. Based on this information, the striatal improvements in GluN1KD-WAVE mice may be related to, for example, a recovered capacity for learning food-foraging strategies. The improved Y-maze alternation scores of GluN1KD-WAVE mice may similarly be due to the test’s similarity to nondelayed random foraging tasks, which depended on the NAc (Floresco et al., 1997). It is important to note, however, that neither the marginal division of the STR nor the NAc were previously linked to cognitive functioning independently of the HPC (Floresco et al., 1997; Shu et al., 2015). Therefore, what is happening in the HPC of GluN1KD-WAVE mice is still of great future research interest.

There are multiple leads that we can further investigate to elucidate the relationship between the HPC and behaviours in GluN1KD-WAVE mice. One simple, straightforward approach is to repeat our experiments with targeted WASF1 overexpression (location-specific virus injections or promoter-based specific transgene expression) in the STR or HPC of GluN1KD mice. As mentioned before for our Rac1, WAVE-1, and spine density results in the GluN1KD STR, our measures thus far are not measures of function. Another approach may thus be to consider hippocampal WAVE-1 activity along with CA1 neuron spine functionality in GluN1KD and GluN1KD-WAVE mice. Previous studies demonstrated that WAVE-1 activation by dephosphorylation occurs upon NMDAR stimulation while total WAVE-1 levels are unchanged (Sung et al., 2008; Ceglia et al., 2010). Though this was demonstrated in both the STR and HPC, it remains to be seen if these brain regions might differ with chronic NMDAR deficiency in vivo. In other words, one possible explanation for the observed differences between the two brain regions is that chronic NMDAR hypofunction may have more effects on the activity of WAVE-1 than its total protein level in the HPC. The clearest next step investigating this line of reasoning is to assess pS397-WAVE-1 in the HPC of WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. It may also be beneficial to assess other WAVE-1 phosphorylation sites such as serine
residues 310 and 441 in both the STR and HPC (Kim et al., 2006b). Similarly, it would be important to assess dendritic spine functionality in the STR and HPC of our test mice – did the previously reported NMDAR current deficit in MSNs (Jocoy et al., 2011) improve in GluN1KD-WAVE mice? Such information would provide insight along with an appraisal of CA1 dendrite functionality in all four genotypes of our test mice. CA1 pyramidal neurons with eliminated AMPAR and NMDAR signaling, but no physically altered dendritic spines, have been reported in mice before. The authors instead observed electrical and functional alterations (Lu et al., 2013). Finally, it is also possible that non-CA1 subdivisions of the HPC could be more affected by WAVE-1 rescue. Region 3 of the cornu ammonis or the dentate gyrus could be potential candidates as they are also important for cognition (Conrad et al., 1996; Manahan-Vaughan et al., 2008). Overall, many measures of WAVE-1 and synapse status in the HPC of our test mice can still be investigated.

Another way to reconcile the differences between our striatal and hippocampal data is to ask why WAVE-1 rescue worked in the STR rather than why it did not (by the measures we’ve assessed so far) in the HPC. Previous whole-brain assessments of functionality with Fos, though not 2-DG uptake (Duncan et al., 2002), pointed to the GluN1KD STR being a responsive, WT-like region (Miyamoto et al., 2004; Inada et al., 2007; Duncan et al., 2008). In contrast, Fos induction in hippocampal brain regions was consistently muted in GluN1KD mice along with 2-DG uptake abnormalities (Duncan et al., 2002, 2008; Inada et al., 2007). The STR may therefore be more functional and rescuable than the HPC in GluN1KD mice. On the other hand, DISC1, which was the changed protein that guided us to investigate Rho GTPases, was not altered in the HPC or CTX of 6-week old GluN1KD mice (Ramsey et al., 2011). This may simply point to Rac1 signaling being a somewhat STR-specific abnormality in GluN1KD mice. WAVE-1 has also recently been proposed to be part of a negative feedback mechanism for glutamatergic synapses in MSNs expressing D1Rs (Ceglia et al., 2017). Thus, WAVE-1 rescue in the STR may be attenuating a hyperglutamatergic state in GluN1KD mice, which was previously suggested by increased AMPAR currents in MSNs (Jocoy et al., 2011) and VTA neuron firing rate (Ferris et al., 2014). One way to address these questions, to various extents, is to attempt cell-specific WAVE-1 rescue in GluN1KD mice. For example, assessing GluN1KD phenotypes after WAVE-1 rescue only in D1R-expressing neurons may help us interpret our current results. This and other studies are needed to more fully understand GluN1KD phenotypes. In relation to our research
questions, more investigations into the GluN1KD HPC, STR, and other brain regions important for rodent cognition and behaviour would be especially helpful.

### 4.4 Sex-Dependency of Transcriptomic Changes in the GluN1KD STR

To further our understanding of the GluN1KD STR, we employed an unbiased bioinformatic approach – RNAseq analysis of mRNA changes. The number of genes that had significantly different expression between WT and GluN1KD mice was higher than previously reported proteomic differences (Wesseling et al., 2014; Cox et al., 2016), but within the range of expectations for RNAseq transcriptomic studies (Darlington et al., 2013; Hodes et al., 2015; Seyednasrollah et al., 2015). Such a high number of gene expression changes is consistent with our modest GluN1KD-WAVE behavioural rescue results; hundreds of gene expression changes in the GluN1KD STR likely means that WAVE-1 deficits are only part of the aberrations in GluN1KD mice. Table III-I provided initial support for us to trust our RNAseq results, as differential expressions between GluN1KD and WT mice and between male and female mice were as expected. However, the decrease of Grin1 by only about 50% in GluN1KD mice was unexpected quantitatively. Closer examination of our RNAseq data later explained this discrepancy as there was a substantial expression of incomplete Grin1 transcripts upstream of intron 19 in GluN1KD mice. This would be more wholly accounted for by RNAseq compared to specific primer-based polymerase-chain reaction experiments. Some of our detected genes were previously reported as mutated in schizophrenia patient genetic studies and are consistent with etiological evidence, such as Cacnb2 and Drd2 (Maycox et al., 2009; Ripke et al., 2014). Cacnb2 stands for Ca$^{2+}$ voltage-gated channel auxiliary subunit β2 and encodes a subunit of voltage gated Ca$^{2+}$ channels while Drd2 stands for DA receptor D2, encoding D2Rs. Mutations in both of these genes have been associated with schizophrenia previously (Berger and Bartsch, 2014; Vink et al., 2016). There were also general class-overlaps between our results and previous GluN1KD proteomic studies, such as alterations of growth factors and interleukins (Wesseling et al., 2014). This again offers a sense of consistency between results, but we would not necessarily expect greatly similar results because previous studies investigated protein changes in the HPC and prefrontal CTX of male mice (Wesseling et al., 2014; Cox et al., 2016) while we studied mRNA
changes in the STR of male and female mice. And as shown in Figure 3.9, sex was indeed an important factor in our transcriptomic assessments of the GluN1KD STR.

The great extent of sex-dependency observed in GluN1KD transcriptional changes at the STR is not surprising. Although less sexually dimorphic than other organs and tissues, hundreds of sex differences in gene expression have been reported for the brain (Rinn and Snyder, 2005; Yang et al., 2006; Hodes et al., 2015). Gonadal hormones can affect many changes through transcriptional regulation, alone or in conjunction with growth factors, while genes on sex chromosomes can likewise influence the CNS and behaviour (Reisert and Pilgrim, 1991; Ngun et al., 2011; Fester and Rune, 2015; McCarthy et al., 2015). NMDAR function, specifically, is also modulated by sex hormones (Bi et al., 2001; van den Buuse et al., 2017). For example, gonadectomy has been demonstrated to reduce NMDAR-antagonist-induced hyperactivity in male but not female mice (van den Buuse et al., 2017). The rat estrus cycle has also been demonstrated to regulate NMDAR phosphorylation and CA1 LTP (Bi et al., 2001). For an example with methodology similar to our work, a study of how stress affects transcription in the NAc of male and female mice reported hundreds of genes with sex-dependent expression changes (Hodes et al., 2015). This is akin to our reported results in Table III-II; apart from the expression of 35 genes that was changed in the same direction between male and female GluN1KD mice, hundreds of changes were sex-dependent. Finally, our results may be reflective of the sex differences seen in GluN1KD behavioural abnormalities, which have been reported multiple times (Duncan et al., 2004b; Moy et al., 2006, 2012; Milenkovic et al., 2014). The most robust behavioural sex differences demonstrated for GluN1KD mice were at younger ages (Moy et al., 2012; Milenkovic et al., 2014), but some differences remain in adulthood (Duncan et al., 2004b; Moy et al., 2006; Milenkovic et al., 2014). As we assessed the striatal transcriptome of adult GluN1KD mice, our findings may represent the molecular underpinnings of sex-dependent behavioural differences found in adulthood. It is also possible that our results include leftover signatures from neurodevelopmental processes that, at its peak, manifested as more severe behavioural differences between young GluN1KD males and females. Characterizing the striatal transcriptome of 3- and 6-week old, male and female, GluN1KD mice may help us distinguish these two interpretations. Over all, a strong effect of sex on the GluN1KD striatal transcriptome is consistent with previous evidence.
Our results displayed in Table III-III, Table III-VI and Figure 3.10 present further clues regarding the sex-dependency of GluN1KD gene expression changes in the STR. As shown in Tables III-III and III-IV, the directionality of transcriptomic changes becomes striking when considering “male” and “female” genes that are altered in GluN1KD mice – those genes that were more highly expressed in WT males or females, respectively, when compared to WT littermates of the opposite sex. To sum, all gene expression changes were opposite of what may be expected by biological sex: male genes were downregulated in male GluN1KD mice and/or upregulated in female GluN1KD mice while female genes were downregulated in female GluN1KD mice and/or upregulated in male GluN1KD mice. Put another way, male GluN1KD transcriptomes were more female-like and less male-like while female GluN1KD transcriptomes were more male-like and less female-like. Such a pattern may be reflective of multiple reports associating gender dysphoria and transsexualism with schizophrenia and ASDs (Gruzelier, 1994; Borras et al., 2007; de Vries et al., 2010; Saleem and Rizvi, 2017). However, clinical gender-factored studies are limited in number (Lewine et al., 2017; Saleem and Rizvi, 2017) and competing theories exist as well, such as hyper-masculinization in autistic brains (Baron-Cohen, 2005). It would be interesting to see how GluN1KD sex differences compare with findings from future studies that consider gender in a clinical setting. For now, this striking pattern of change represents to us a high likelihood that sex-dependent differences in GluN1KD striatal transcriptomes were due to sex-centered mechanisms.

Pathways analysis of our RNAseq results with IPA yielded further evidence of altered sex-related mechanisms in GluN1KD mice. IPA identified Esr1 as a key node that was connected to many other gene expression changes found in our GluN1KD STR samples, as shown in Tables III-V and III-VI. Specifically, it predicted ERα activity changes based on how genes it regulates were changed in our samples and based on how Esr1 expression itself was changed. Consistent with our results in Tables III-III and III-IV, GluN1KD Esr1 expression and ERα predicted activity changes were opposite of expectations based on sex: Esr1 was increased and ERα was predicted to be more active in GluN1KD males while Esr1 was decreased in GluN1KD females and their ERα activation z-score was negative, though statistically insignificant. This pattern was similar to algorithmically-determined Esr1 association networks shown in Figure 3.10. These networks, also generated by IPA, demonstrate visually a group of sex-dependent, Esr1/ERα-centered, gene expression changes in GluN1KD mice. Interestingly, a similar IPA network has
been previously published with one of the GluN1KD proteomic studies. Although the authors did not focus on \textit{Esr1}, they too reported an \textit{Esr1}-centered network that was largely downregulated or inhibited in the frontal CTX of male mice (Wesseling et al., 2014). Their network was associated with “developmental disorder, hematological disease, and molecular transport”, potentially reflecting the neurodevelopmental nature of schizophrenia and ASDs (Wesseling et al., 2014; Goldstein et al., 2018). The current striatal bioinformatics data have revealed ERα as a key target for future research to understand the molecular changes and sex differences in GluN1KD mice. Investigations into ERα signaling may also shed more light on our WAVE-1 rescue experiments due to its unique role in promoting both WAVE-1 phosphorylation and dendritic spine formation (Sanchez et al., 2009).

Lastly, we analyzed our RNAseq results with Enrichr to assess the diseases our transcriptomic changes most likely represented and the drugs most likely to reverse them. This was especially interesting to us because male and female GluN1KD mice presented with mostly the same behavioural phenotypes, if to different degrees (Moy et al., 2012; Milenkovic et al., 2014). The degree of sex differences in GluN1KD behaviours also often decreased by adulthood (Milenkovic et al., 2014). The diseases and drugs resulting from our analysis are shown in Tables III-VII and III-VIII, respectively. Generally, results in both tables present a fair degree of consistency despite being generated from 4 different gene list inputs. The consistency for Enrichr disease results was readily apparent as most came from mouse models of HD. On the other hand, most of the drug records looked to involve different drugs. However, closer examination of the drugs’ mechanisms of action pointed to some consistent molecular targets and clinical purposes: growth signaling and anti-cancer treatment, respectively. The results therefore suggest that various sex-dependent gene expression changes in GluN1KD mice generally contribute to similar larger-scale phenotypes.

Enrichr disease records associated with our RNAseq results are mostly consistent with the literature. The instances of schizophrenia and AD records are consistent with previously discussed associations between these disorders and GluN1KD mice or NMDARs (Mohn et al., 1999; Shankar et al., 2007; Halene et al., 2009a; Tönnies and Trushina, 2017). How NMDARs and GluN1KD mice relate to HD, which was our most common Enrichr result, needs further discussion. HD is a neurodegenerative disease caused by increased CAG repeats in the huntingtin (\textit{HTT}) gene that primarily targets striatal MSNs and cortical neurons (Shehadeh et al.,
An association between HD and GluN1KD mice is therefore feasible given the many alterations in MSNs we and previous GluN1KD studies have observed (Jocoy et al., 2011; Ramsey et al., 2011). Though GluN1KD mice that were 10- to 16-weeks old did not demonstrate reduced MSNs (Ramsey et al., 2008), we cannot rule out that they do with age or that surviving GluN1KD MSNs are simply less fit than WT MSNs. Patients with HD have been reported to have less NMDARs in the putamen subregion of the STR (Young et al., 1988). However, NMDAR hyperactivity and excitotoxicity are often considered associated with the neurodegeneration seen in HD (Cepeda et al., 2001; Fan and Raymond, 2007). This may seem incongruent with the decreased NMDARs of GluN1KD mice, but experiments with varying doses of memantine in cultured neurons demonstrated that increased extrasynaptic NMDAR activity resulted in greater vulnerability to mutant HTT protein. Furthermore, high-dose memantine that blocked both synaptic and extrasynaptic NMDARs worsened outcomes in a mouse model of HD (Okamoto et al., 2009). Our Enrichr disease results may therefore be considered consistent with the evidence from HD research, particularly with previous signs of a hyperexcitable brain in GluN1KD mice (Jocoy et al., 2011; Ferris et al., 2014). These results also demonstrate how different gene expression changes in male and female GluN1KD mice can still be related to one macroscopic phenotype.

The drug-related records identified from Enrichr databases also demonstrated how different molecular changes can affect similar processes to contribute to similar endpoints. In general, most of the drugs detected by Enrichr analysis were antineoplastic agents (Oh and Kwon, 2009; Ueno et al., 2012; Valianou et al., 2015; Bollard et al., 2017) that acted on signaling mechanisms related to cellular and vesicular growth (Mohan et al., 2004; Li and Yang, 2010; Valianou et al., 2015; Qiu et al., 2016). A general summary of these pathways and where our Enrichr-identified drugs act is illustrated in Figure 4.2. These drugs often act on ERK- (Lorusso et al., 2005; Fukuyo et al., 2010; Khalil et al., 2011) or AKT- (Li and Yang, 2010; Qiu et al., 2016; Suman et al., 2016) signaling pathways, which are commonly dysregulated in cancer (Steelman et al., 2011; De Luca et al., 2012). This is consistent with previous GluN1KD proteomic findings, as ERK aberrations have been reported for the GluN1KD HPC and CTX too (Wesseling et al., 2014). Furthermore, ERK and AKT pathways are regulated by NMDARs (Thomas and Huganir, 2004; Gong et al., 2006; Wang Yansong et al., 2014) and interact with estrogen signaling pathways (Hisamoto et al., 2001; Rocca et al., 2014; Filardo et al., 2018). A disruption of
NMDARs, which was strongly demonstrated in GluN1KD mice (Mohn et al., 1999; Duncan et al., 2002; Bodarky et al., 2009; Jocoy et al., 2011), or estrogen signaling, which was implied by our RNAseq data for the GluN1KD STR, could therefore result in dysregulated ERK and AKT pathways. These two pathways can interact with each other (Steelman et al., 2011; De Luca et al., 2012), potentially compensating for or exacerbating each other’s dysregulation. Both may also be affected by the same drug from our Enrichr results, such as KU-55933. KU-55933 administration has been shown to result in AKT phosphorylation and ERK dephosphorylation, ultimately presenting an anti-survival signal (Khalil et al., 2011). Beyond just AKT and ERK, drugs from our Enrichr results can have other targets and effects (Oh et al., 2008; Clark et al., 2012; Liang et al., 2017). For example, the frequently-reported Withaferin A has been demonstrated to have both antitumor and antiangiogenic effects through nitric oxide and other molecular mediators (Mohan et al., 2004; Oh and Kwon, 2009; Khan and Ghosh, 2010; Suman et al., 2016). However, the fact our identified drugs often have wide-ranging effects also means that more targeted studies on ERK and AKT pathways will be needed to confirm their specific dysregulation and biological significance in GluN1KD mice. Confirmatory experiments in the future could include quantitative polymerase-chain reaction experiments to study RNA and western blotting experiments to study protein changes.
Figure 4.2. Schematic of NMDAR signaling pathways commonly targeted by drugs from Enrichr analysis. ERK and AKT signaling, strongly associated with cell growth and survival, were distinctly common targets of Enrichr-result drugs. These signaling pathways were distinctly targeted out of the many that act downstream of NMDARs, such as those previously represented in Figure 1.2. Green and red arrows again indicate generally positive and negative relationships, respectively. Dashed lines with blunted ends for drugs represent indirect inhibition or currently unspecific mechanism of action. Solid lines with blunted ends (for drugs) denote more direct inhibition. Abbreviations follow those used in text and previous figures. This figure was constructed with literature cited in-text as well as other studies (Christensen et al., 2003; Ueno et al., 2012; Li et al., 2014; Hirabayashi and Cagan, 2015).
Our RNAseq and gene list analyses revealed a divergence of transcriptomic changes in male and female GluN1KD mice followed by a reconvergence of these changes into similar biological processes. Such results mirror GluN1KD behavioural phenotypes that are sex-dependent but largely very similar (Moy et al., 2012; Milenkovic et al., 2014). Estrogen, neurodegeneration and growth signaling pathways are some of the major themes that stand out from our assessment of the GluN1KD striatal transcriptome. Estrogen, due to its role as a sex hormone, may be a key mediator of GluN1KD sex differences. Neurodegenerative signaling may be related to imbalanced extrasynaptic-to-synaptic NMDAR function (Okamoto et al., 2009; Xu et al., 2009; Pál, 2018), which could be present in GluN1KD mice that showed signs of a hyperexcitable brain previously (Dzirasa et al., 2009; Jocoy et al., 2011; Ferris et al., 2014). Alterations in growth factor signaling is consistent with previous GluN1KD proteomic results (Wesseling et al., 2014), but need confirmation of specific pathway changes. GluN1KD growth factor signaling at the STR also need to be carefully considered and investigated in the context of potentially concurrent neurodegeneration mechanism changes. Overall, our unbiased bioinformatics investigation into the GluN1KD STR has provided several new clues regarding the molecular changes that occur with NMDAR deficiency and their biological significance.

4.5 Concluding Statements

4.5.1 Strengths of the Study

The present study investigates the effects of NMDAR hypofunction on the STR with multiple experimental approaches and perspectives. The initial assessment of MSN dendritic spines and Rho GTPase signaling proteins in GluN1KD mice covered three key ages: 3-week old juveniles, 6-week old adolescents, and 12-week old adults. Not only did this provide an age-trajectory of molecular and synaptic changes, it also allowed an assessment of developmental alignment with our previous behavioural study of similarly-aged mice (Milenkovic et al., 2014). This was an advantage because the comparisons allowed us a rough gauge of the biological and behavioural relevance of our findings. The age-trajectories of GluN1KD phenotypes also offered clues regarding the validity of using these mice to model early-onset neurological disorders like ASDs (Baxter et al., 2015; Halladay et al., 2015) or later-onset ones like schizophrenia (Sham et al., 1994; Thorup et al., 2007; Eranti et al., 2013). Our MSN spine density results seem to point to
the latter while our Rho GTPase results will require further study into the significance of mixed-direction changes for Rac1 and WAVE-1.

For our experiments with WAVE-1 rescue, a good range of neurological and behavioural measures were employed. In terms of different levels of biology, we surveyed molecular changes, spine density changes, and in vivo behavioural changes. Such a range of measures allowed us to associate phenotypes on all three levels and investigate a well-rounded and relatively holistic hypothesis – that restoring WAVE-1, a protein known to regulate dendritic spine formation (Kim et al., 2006b; Soderling et al., 2007; Ceglia et al., 2010), in GluN1KD mice would improve their MSN spine density and behavioural phenotypes. The range of behavioural tests employed was also a strength since it covered multiple domains of cognitive function. Such considerations are particularly important for disorders like schizophrenia where some symptoms are currently better treated than others (Green, 2016; Kishi et al., 2017). Finally, our WAVE-1 experiments looked at multiple brain regions, the STR and the HPC. Results from multiple brain regions are often useful for complementing each other and advancing the understanding of both regions’ function and status.

A bioinformatics approach often offers the advantage of yielding, analyzing, and interpreting large amounts of data. RNAseq experiments generally possess great sensitivity, often reporting hundreds if not thousands of significant changes that investigators can analyze by leveraging high computational power (Darlington et al., 2013; Hodes et al., 2015; Seyednasrollah et al., 2015). With our discovery of hundreds of gene expression changes, we surpassed the quantity of significant findings from previous GluN1KD proteomic studies (Wesseling et al., 2014; Cox et al., 2016). To be clear, this is not better per se. Having more potential targets to look at individually, for example, may instead be more time-consuming and distracting. However, it is often beneficial to have more data – more significant hits – to analyze computationally. In such cases, more data may result in better differentiation of results and more accurate identification of significant associations, clusters, or pathways. Results from analyses with large data-sets may better indicate the most biologically relevant changes occurring in tested samples.

4.5.2 Limitations of the Study

There are a few interpretive limitations on the results of our study, some of which were mentioned with previous discussion sections. As mentioned previously when discussing Rho
GTPases, WAVE-1, and dendritic spine changes in GluN1KD mice, our experimental measures did not explicitly indicate functional changes. There were more or less protein or spines, but such changes did not necessarily reflect how active or functional these CNS components were. As alterations in function have been reported independently of quantity differences before (Kim et al., 2006b; Lu et al., 2013), it stands to reason that functional changes in our mice could suggest different conclusions than our current results. While we did assess one form of WAVE-1 phosphorylation, more could be done. The functionality of dendritic spines also offers a different level of depth to explore experimentally: circuit and network-level phenomena that may represent more than the sum of individual neuronal activity. Network-level changes have been demonstrated in the past for neurological disorders and GluN1KD mice, specifically between the CTX and the HPC (Dzirasa et al., 2009). It would be interesting to investigate which synaptic connections or networks are different between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Furthermore, it may be beneficial to study EEG changes in our mice during behavioural test performance and contrast those findings with resting brain activity.

The interpretative limitations of our RNAseq analyses are similar to those in other bioinformatics studies (Wesseling et al., 2014; Cox et al., 2016). In general, the interpretability of our data was limited to what curated databases were present to serve as references in IPA and Enrichr. For example, without records of HD mouse models or withaferin A in Enrichr, our results would be drastically different. Our results and interpretations were largely limited to the set of possible outcomes defined in IPA and Enrichr. To go one step further, the databases and possible outcomes in IPA and Enrichr are limited to the current scientific literature. Connecting our list of transcriptomic changes to a record labeled “withaferin A” would be hard to interpret without previous studies into its mechanism of action and effects. Without previous studies (Oh and Kwon, 2009; Ueno et al., 2012; Valianou et al., 2015; Bollard et al., 2017), we would not have been able to connect the various drugs identified in our output to growth factor signaling and antineoplastic effects. This is the reliance of bioinformatics approaches on other types of experiments. The fact that we studied transcriptomic changes further compounds the limitations of our data’s interpretability. Despite the greater sensitivity we had with our approach, proteomic data are likely more indicative of functional changes in sample tissues (Cox et al., 2016). However, transcript levels have been reported to be more strongly correlated with clinically relevant mouse phenotypes than protein levels before (Ghazalpour et al., 2011). Our
transcriptomic data are therefore still very important for understanding GluN1KD mice. Overall, it is clear where we should take the results from our bioinformatics analyses: back to more hypothesis-based testing. To fully interpret our data, more experiments will be needed in the future to confirm and follow-up ERα-, neurodegeneration-, and growth factor-related signaling in the GluN1KD STR. This would include basic assessments like signaling component levels as well as more functional measures: how striatal proteins, dendritic spines, and neurons change after various estrogenic, damaging, or growth-promoting stimuli.

4.5.3 Future Directions

Many of the future experiments that could build upon our results have been mentioned previously. An assessment of functional outcomes will greatly improve our understanding of changes in the GluN1KD and GluN1KD-WAVE STR. Specifically, future investigators can execute something like the following in WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE: measure Rac1 and WAVE-1 function, such as via quantification of Rac1-GTP and all forms of phosphorylated WAVE-1 (Kim et al., 2006b; Hayashi-Takagi et al., 2010); culture MSNs and assess their ability to form new spines or larger spines after stimulation with glutamate or DA, as an assessment of ultimate Rac1 signaling outcomes; assess electrophysiological properties of these same cultured MSNs to see if synaptic signaling is directly improved. To help confirm that any observed differences are indeed due to WAVE-1 changes, cultured neuron experiments can be repeated with Wasf1 knockdown and WASF1 transfection, similar to previous studies (Kim et al., 2006b; Sung et al., 2008; Sanchez et al., 2009). Doing these same experiments in hippocampal neurons will also help clarify the state, function, and relevance of WAVE-1 in the GluN1KD HPC. If a similar pattern remains where striatal measures are improved but hippocampal ones are not, it may be helpful to change the direction of future experimental approach – look for signs of restored GluN1KD-WAVE brain function first. Like in previous GluN1KD studies, this can be done indirectly by using Fos-induction or 2-DG uptake as surrogates (Duncan et al., 2002, 2008; Miyamoto et al., 2004; Inada et al., 2007). A more direct method may be to investigate the coupling of local field potentials between different brain regions and the STR of test mice. This can be done in mice exploring a maze, like previously performed (Dzirasa et al., 2009), as well as at rest. Any brain region that has improved function in GluN1KD-WAVE mice compared to GluN1KD mice can then serve as a target for further assessing molecular and synaptic rescue in GluN1KD-WAVE mice. Any GluN1KD-WAVE
brain regions identified and confirmed this way will likely be working with the rescued STR to improve maze exploration behaviour. Overall, these experiments can provide many new clues about the relationship between WAVE-1, synaptic plasticity, NMDAR hypofunction, and mouse behaviour.

Future experiments related to our RNAseq, IPA, and Enrichr analyses have also been mentioned above, particularly at the end of the study limitations section. On top of what was suggested there, more RNAseq analyses of the GluN1KD striatal transcriptome in 6-, 3-, 5-, and 2-week old (in order of priority) male and female mice may have excellent value. Combining results from these experiments with our current results, we would have striatal transcriptomic profiles in alignment with the two main GluN1KD behavioural studies that included age and sex as factors (Moy et al., 2012; Milenkovic et al., 2014). Transcriptomic changes that align with striking age- and sex-dependent GluN1KD behavioural differences, such as increased sociability in 5-week old GluN1KD males (Moy et al., 2012) or generally worse phenotypes in males at 6-weeks of age (Milenkovic et al., 2014), may reveal signaling pathways particularly relevant for aligned behaviours. Completing a set of RNAseq experiments for WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice can also help us further understand the scope of molecular changes that can come from increasing total WAVE-1 levels. As part of this, however, it may be better to employ single-cell RNAseq methods (Tang et al., 2010; Hashimshony et al., 2012) to more specifically study MSNs. Results from these experiments may also shed more light on the altered DA system (Ferris et al., 2014) and MSN electrophysiology in GluN1KD mice (Jocoy et al., 2011). But like our current RNAseq results, however, more RNAseq analyses will likely result in more hypotheses that need non-bioinformatics approaches to test. In which case, more characterizations to confirm experimental targets would be needed. This would be followed by yet further studies, perhaps like our work with WAVE-1 rescue. And with every step, we would work towards a greater understanding of NMDAR deficiency and synaptic plasticity in the STR.

4.5.4 Conclusion

Our work has identified Rac1 signaling, particularly through WAVE-1, as a viable target for improving GluN1KD mouse phenotypes. This key finding is supportive of and consistent with the idea of targeting Rac1 signaling to improve schizophrenia-related outcomes, such as by inhibiting p21-activated kinases (Hayashi-Takagi et al., 2014). The behavioural improvements
we observed from WAVE-1 rescue were modest, specific to maze exploration, and potentially representative of better long-term memory or goal-directed exploration (Floresco et al., 1997; Shu et al., 2015). Concurrently, GluN1KD-WAVE mice showed an improvement of MSN spine density, which supported WAVE-1’s mechanism of action as regulating synaptic plasticity (Kim et al., 2006b; Soderling et al., 2007; Sung et al., 2008). In contrast, RNAseq, IPA, and Enrichr analyses emphasized growth factor- and neurodegeneration-related signaling alterations in the GluN1KD STR. These signaling alterations were based on drastically sex-dependent gene expression changes, which may be mediated by ERα signaling changes. More studies are needed to confirm and expand upon the results from our work, particularly the new leads coming from our unbiased bioinformatics approach. The primary strength of our study was the association of changes at three different levels of biology: molecular signaling, dendritic spines, and in vivo behaviour. This aided in the interpretation of our results, particularly regarding the behavioural significance of our findings at microscopic levels. Interpreting our behavioural data as a whole was a challenge, since maze exploration has been associated with spatial working memory, curiosity, and other aspects of cognition (Hughes, 2004). Our results of STR-specific improvements in WAVE-1 levels and spine density with better maze exploration provide more evidence for the importance of striatal function in proper cognition.
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Appendices

Appendix 1: Supplemental Figures and Tables

Supplemental Table SI. Protein expression changes in adult male GluN1KD mice relative to WT littermates from previous proteomic studies. These results are compiled from both previous studies (Wesseling et al., 2014; Cox et al., 2016). Any differences between cortical, hippocampal, and serum results regarding the number of identified proteins do not necessarily reflect biologically significant differences. Instead, they likely reflect the fact that each physiological location involved different studies and methods. Proteins in bold have been confirmed by both studies or by multiple experimental methods. Proteins in red represent findings that are conflicted between studies or experimental methods. Many of these protein changes have been related to synaptic plasticity.
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Supplemental Figure S1. Dendritic spine morphology measurements of striatal MSNs in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. Spine morphology measures (left) and associated spine-population cumulative-frequency distributions (right) were not significantly different between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Mouse sample sizes are indicated within each bar for bar graphs, which also apply to associated spine-population frequency data. 3-6 sample dendrite images were analyzed for each mouse. Mean morphology measures were analyzed by one-way ANOVAs. WT vs GluN1KD spine-population cumulative frequencies were compared using Kolmogorov–Smirnov tests. Since no significant WT vs GluN1KD differences were found, further pairwise frequency comparisons were not performed.
Supplemental Figure S2. Dendritic spine categorization of striatal MSNs in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. The number (left) and proportion (right) of mushroom, stubby, and thin spines were derived from dendritic spine density and morphology data. Spines were categorized as stubby if they were shorter than 0.8 µm. Of the longer ones,
mushroom spines had a head-to-neck ratio ≥ 1.1. The rest were categorized as thin spines. No significant differences were found between genotypes. Mouse sample sizes are indicated within each bar. 3-6 sample dendrite images were analyzed for each mouse. Data were analyzed by one-way ANOVAs.

Supplemental Figure S3. Apical dendritic spine morphology measurements of CA1 pyramidal neurons in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. Spine morphology measures are shown on the left while associated spine-population cumulative-frequency distributions are shown on the right. GluN1KD-WAVE hybrids had a significantly higher mean spine length compared to WT littermates while GluN1KD mice did not. By spine-
population distribution comparisons, both GluN1KD and GluN1KD-WAVE mice had more long spines than WT littermates. For both spine length analyses, GluN1KD and GluN1KD-WAVE mice were not different from each other. Thus, GluN1KD mice might have longer spines than WT mice, but any such abnormality would not be rescued in GluN1KD-WAVE hybrids. Spine head width and neck width were not significantly different between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. Mouse sample sizes are indicated within each bar for bar graphs, which also apply to associated spine-population frequency data. 3-6 sample dendrite images were analyzed for each mouse. Mean morphology measures were analyzed by one-way ANOVAs followed by Bonferroni post hoc comparisons for all pairings. * $p = 0.03$. Spine-population cumulative frequencies were compared using Kolmogorov–Smirnov tests, which were performed first for WT vs GluN1KD mice. GluN1KD vs GluN1KD-WAVE and WT vs GluN1KD-WAVE comparisons were performed only if a significant difference was found for WT vs GluN1KD, to decrease the chance for false positive results. # $p < 0.01$ vs WT distribution.
Supplemental Figure S4. Apical dendritic spine categorization of CA1 pyramidal neurons in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. The number (left) and proportion (right) of mushroom, stubby, and thin spines were derived from dendritic spine density and morphology data. Spines were categorized as stubby if they were shorter than 0.8 µm. Of the
longer ones, mushroom spines had a head-to-neck ratio $\geq 1.1$. The rest were categorized as thin spines. GluN1KD-WAVE hybrids had more mushroom spines by proportion and less stubby spines by number compared to WT mice. GluN1KD mice had less stubby spines by number compared to WT mice. No differences were significant between GluN1KD mice and GluN1KD-WAVE hybrids. Thus, GluN1KD mice might have fewer stubby spines than WT littermates, but any such deficit would not be rescued in GluN1KD-WAVE hybrids. No significant differences were found between genotypes for thin spines. Mouse sample sizes are indicated within each bar. 3-6 sample dendrite images were analyzed for each mouse. Data were analyzed by one-way ANOVAs followed by Bonferroni post hoc comparisons for all pairings. * $p \leq 0.05$, ** $p < 0.01$. 
Supplemental Figure S5. Basolateral dendritic spine morphology measurements of CA1 pyramidal neurons in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. Spine morphology measures are shown on the left while associated spine-population cumulative-frequency distributions are shown on the right. Mean spine morphology measurements were not significantly different between WT, WAVE-Tg, GluN1KD, and GluN1KD-WAVE mice. By spine-population distribution comparisons, both GluN1KD and GluN1KD-WAVE mice had more long spines than WT littermates. GluN1KD and GluN1KD-WAVE spine distributions were not different from each other. Thus, GluN1KD mice have more long spines than WT littermates, but this was not rescued in GluN1KD-WAVE hybrids. Mouse sample sizes are indicated within
each bar for bar graphs, which also apply to associated spine-population frequency data. 3-6 sample dendrite images were analyzed for each mouse. Mean morphology measures were analyzed by one-way ANOVAs. Spine-population cumulative frequencies were compared using Kolmogorov–Smirnov tests, which were performed first for WT vs GluN1KD mice. GluN1KD vs GluN1KD-WAVE and WT vs GluN1KD-WAVE comparisons were performed only if a significant difference was found for WT vs GluN1KD, to decrease the chance for false positive results. $\# p < 0.01$ vs WT distribution.
Supplemental Figure S6. Basolateral dendritic spine categorization of CA1 pyramidal neurons in WT, WAVE-Tg, GluN1KD and GluN1KD-WAVE mice. The number (left) and proportion (right) of mushroom, stubby, and thin spines were derived from dendritic spine density and morphology data. Spines were categorized as stubby if they were shorter than 0.8
μm. Of the longer ones, mushroom spines had a head-to-neck ratio ≥ 1.1. The rest were categorized as thin spines. No significant differences were found between genotypes. Mouse sample sizes are indicated within each bar. 3-6 sample dendrite images were analyzed for each mouse. Data were analyzed by one-way ANOVAs.

Supplemental Table SII. Full differentially-expressed gene lists showing demasculinization or feminization in GluN1KD males and masculinization or defeminization in GluN1KD females. These gene lists are the result of filtering the 784 sex-factored RNAseq results for those that were also different between male and female WT mice. Demasculinizing or feminizing gene expression changes did not show up at all in GluN1KD females. Similarly, masculinizing or defeminizing gene expression changes did not show up in GluN1KD males. All noted changes in GluN1KD mice are relative to sex-matched WT littermates unless specified otherwise.
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*Demasculinized (98)*: Changed in Male GluN1KD Mice

*Feminized (88)*: Changed in Female GluN1KD Mice

*Masculinized (103)*: Changed in Male GluN1KD Mice

*Defeminized (75)*: Changed in Female GluN1KD Mice

Demasculinized (98) Feminized (88) Masculinized (103) Defeminized (75)
Copyright Acknowledgements

No copyrighted works were used in this thesis as of September 26, 2018.