ROLE OF THE NMDA RECEPTOR SUBUNIT, NR3A, IN GLUTAMATERGIC SYNAPSE DEVELOPMENT

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ABSTRACT

Maile Anne Henson: Role of the NMDA receptor subunit, NR3A, in glutamatergic synapse development
(Under the direction of Benjamin D. Philpot, Ph.D.)

Proper functioning of neuronal networks relies on the refinement of immature synaptic contacts, although many aspects of this process are poorly understood. NMDA-type glutamate receptors (NMDARs) are crucial mediators of brain development and function, especially excitatory synapse development. Accumulating evidence suggests that the NR3A subtype of NMDARs has an unappreciated and particularly important role during early postnatal development. These developmentally regulated receptor subunits serve a prominent role in CNS development soon after birth, and their role is likely less important prenatally and in adulthood. Inclusion of the nonconventional NR3A subunit in NMDARs may serve to alter cortical microcircuitry in the brain anatomically, through reduced numbers of dendritic spines, and functionally, through weakened synaptic connections due to decreased glutamatergic neurotransmission and calcium influx through NMDARs. In this dissertation, using molecular and biochemical techniques in human and mouse model systems, I explored (1) how developmental changes in the complement of glutamate receptors, specifically NR3A subunits, enable synapses to mature or to be eliminated in the mouse forebrain, and (2) whether an increase in NR3A subunit expression could provide an endogenous basis for the NMDAR hypofunction observed in schizophrenia. Because the profound effects that NR3A is likely to have on

synapse development have not been fully considered, my research addresses this gap in our knowledge. Dissecting the contributions of specific NMDAR subunits to synapse development will increase our understanding of the maturation of forebrain circuitry and its effects on learning and memory processes, as well as guide therapeutic strategies for treating pathologies associated with NMDAR dysfunction, such as schizophrenia.

DEDICATION

This dissertation is dedicated to my husband, Steve, and my children, Leiana and Deryck, without whose unwavering support and loving encouragement I would never have completed this epic journey. To my parents, James and Regina Silver, thank you for instilling in me the love of learning and for believing I could accomplish this goal. To my parents-in-law, Harold and Marjorie Henson, thank you for your loving support all these years.

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This dissertation would not have been possible if my advisor, Ben, had listened to his faculty advisors who urged him not to take on yet another student – me. Ben warned me up front that it was a huge risk for both of us and I would have to work very hard to prove them wrong. I think I have. He taught me to have cautious optimism in my findings, only after they were meticulously scrutinized, and to ask the big picture questions. His enthusiasm for teaching and grooming scientists is contagious and I hope I can impart these qualities to others that I will mentor in the future. I thank him for taking a chance on me and I appreciate his support and confidence in me, especially in pursuing projects outside of his expertise (and lab!).

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Ben strongly encourages collaborations and pro-actively seeking out the expertise of others to gain a more complete, well-defined picture. To this end, I took the initiative to find complementary resources and guidance for various aspects of my graduate training by forming alliances with other labs throughout UNC and in Spain, including those of Dr. Isabel Pérez-Otaño, Dr. John Gilmore, Dr. Fred Jarskog, Dr. George Breese,

Dr. James Anderson, Dr. Bill Snider, and Dr. Rick Meeker. These scientists graciously opened their doors to let me learn techniques, troubleshoot experimental problems, analyze and interpret data. The postdocs, graduate students, and staff members within these groups generously gave of their time and resources to help me with my work. In particular I thank Dr. Bonnie Blake, Dr. Darin Knapp, Rani Vadlamudi, Dr. Kayvon Salimi, Dr. Leisa Glantz, Beth Cox, Dr. Alan Fanning, Dr. Rebeca Martinez-Turrillas, and Dr. Elena Marcello.

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PREFACE

In my graduate training, I sought to explore the molecular mechanisms of synaptic development and function in the healthy and diseased brain. My aim was to receive a broad range of training in cellular and molecular neuroscience, scientific writing, and critical evaluation of experimental design and analysis. In the Philpot Lab, I gravitated towards the molecular side of neurobiology, studying the role of NMDA-type glutamate receptors (NMDARs) in synaptic development. Because glutamate receptors are critical for synaptic plasticity and neuronal cell death, understanding their function has relevance for neurological conditions, such as schizophrenia, stroke, and neurodegenerative diseases. My work explored the contributions of NMDAR subunits to the strengthening and weakening of connections during excitatory synapse development, and may shed light on the mechanisms for glutamate receptor dysfunction seen in schizophrenia. These studies have opened up questions about glutamate receptor trafficking in synapse assembly and elimination, especially as they relate to neurodevelopmental disorders such as schizophrenia, and have helped me to gain a deeper understanding of the relationship between synaptic formation and abnormal development of neuronal microcircuits.

My initial project in the lab involved using RNA interference (RNAi)-mediated gene silencing to provide temporal and regional control for modifying NMDAR subunits in the neocortex. By selectively knocking down expression of NMDAR subunit, NR2A,

in cortical neurons both *in vitro* and *in vivo*, I was able to produce encouraging preliminary data demonstrating the powerful potential of cell-penetrating peptides for RNAi delivery. This technology may be useful in future projects requiring a method to selectively downregulate NMDAR subunits, and thus parse NMDAR subtype-specific roles in cortical development.

I then turned to examining the role of the novel NMDAR subunit, NR3A, in the neurodevelopmental disorder, schizophrenia. Behavioral problems occurring in schizophrenics are thought to arise from aberrant development, stabilization, and plasticity of synapses in the prefrontal cortex. Several lines of evidence suggest that hypofunction of the NMDAR may play a particularly important role in the underlying pathophysiology, although the molecular basis for this is still unknown. Because the NR3A subunit acts in a dominant-negative fashion to reduce NMDAR function, I hypothesized that while transient expression of NR3A is important for brain development, the persistent expression of NR3A might contribute to disease states. Indeed, increased NR3A expression in schizophrenia could be an endogenous mechanism and account for the observed NMDAR hypofunction and improper formation of cortical circuits in this disease. My data resulted in a first-author manuscript in *Cerebral Cortex*, detailing the novel findings of NR3A expression during human development and in schizophrenic postmortem human tissue.

Because the developmental profiles of NR3A showed strong parallels between humans, non-human primates, and rodents, this gave me confidence in the relevance of NR3A expression across mammalian species. My focus then shifted to using mice to model the consequences of NR3A loss and overexpression in synapse development in a

controlled environment, without confounding variables found in humans such as medication history. The goal was to carefully characterize the synaptic presence of NR3A. Two pressing issues were (1) to define the subcellular localization of NR3A-containing receptors at peak expression levels, and (2) to determine whether the influence of NR3A is to serve as a 'synaptic brake' to limit synapse/spine formation or to act as a 'synaptic marker' to promote those processes. These two hypotheses are not mutually exclusive. The data from these experiments are still being analyzed, but they suggest that NR3A acts as a molecular brake to negatively regulate expression of several key synaptic proteins in the maturation of excitatory synapses.

Portions of this work have been previously published or are in preparation for publication:

CHAPTERS 1 and 4

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CHAPTER 2

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CHAPTER 3

Roberts, A.C., Diez-Garcia, J., Rodriguiz, R.M., Lopez, I.P., Lujan, R., Martinez-Turrillas, R., Pico, E., **Henson, M.A.**, Bernardo, D.R., Jarrett, T.M., Clendeninn, D.J., Lopez-Mascaraque, L., Feng, G., Lo, D.C., Wesseling, J.F., Wetsel, W.C., Philpot, B.D., Perez-Otano, I. (2009). Downregulation of NR3A-containing NMDARs is required for synapse maturation and memory consolidation. *Neuron* 63(3):342-56.

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APPENDIX

Larsen, R.S.*, Corlew, R.J.*, **Henson, M.A.**, Roberts, A.C., Perez-Otano, I., Nakanishi, N., Lipton, S.A., Weinberg, R.J., Philpot, B.D. NR3A-containing

NMDA receptors promote tonic neurotransmitter release and spike timing-dependent plasticity. *Nature Neuroscience, in revision.*

* These authors contributed equally.

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NMDAR, N-methyl-D-aspartate receptor

NR1, N-methyl-D-aspartate receptor subunit 1

NR2A, N-methyl-D-aspartate receptor subunit 2A

NR2B, N-methyl-D-aspartate receptor subunit 2B

NR2C, N-methyl-D-aspartate receptor subunit 2C

NR2D, N-methyl-D-aspartate receptor subunit 2D

NR3A, N-methyl-D-aspartate receptor subunit 3A

NR3B, N-methyl-D-aspartate receptor subunit 3B

AMPAR, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor

GluR1, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor subunit 1

PKA, protein kinase A

PKC, protein kinase C

PP2A, protein phosphatase 2A

PTK, protein tyrosine kinase

CaMKII, calcium/calmodulin-dependent kinase II

mRNA, messenger ribonucleic acid

cDNA, complementary deoxynucleic acid

RT-PCR, reverse transcriptase polymerase chain reaction

TM, transmembrane region

P, postnatal day

ER, endoplasmic reticulum

LTP, long-term potentiation

LTD, long-term depression

mEPSC, miniature excitatory postsynaptic current

DLPFC, dorsolateral prefrontal cortex

SP, signal peptide

glyc, glycosylation

S1-S2, extracellular ligand-binding domain/glycine binding pocket of NMDARs

mo, months

yr, year

EC, entorhinal cortex

VC, visual cortex

FC, frontal cortex

Hpc, hippocampus

Str/GP, striatum / globus pallidus

Olf. Bulb, olfactory bulb

PNS, postnuclear supernatant

LM, light membranes fraction

CYT, cytosolic fraction

P2, second pellet fraction, crude synaptosomes

P3, third pellet fraction, lysed synaptosomal membranes

TSF, triton-soluble fraction

SPM, purified synaptic plasma membranes

PSD, postsynaptic density

CNS, central nervous system

MAGUK, membrane-associated guanylate kinase

PSD-95, postsynaptic density protein, 95kD

SAP-102, synapse-associated protein, 102kD

Ca^{2+}, calcium

Mg^{2+}, magnesium

pre, presynaptic bouton

post, postsynaptic spine

KO, knockout

OE, overexpressor

CHAPTER 1

Introduction and Background

Synapses form the basis for neuronal communication

The fundamental unit for information processing in the brain is the synapse. The establishment of a functional synapse that provides reliable glutamate neurotransmission requires the participation of pre- and postsynaptic elements that are stabilized through coordinated activity (Katz and Shatz, 1996). In early brain development, synaptogenesis results in a massive overproduction of synapses and the subsequent culling of weak, uncoordinated processes (Hua and Smith, 2004). The remaining synaptic junctions undergo structural and molecular maturation of the postsynaptic spines.

Developing synapses undergo alterations in spine/synapse size, receptor complement, synaptic activity, and synaptic plasticity

Nascent excitatory synapses undergo remarkable transformations in spine morphology, the composition and numbers of glutamate receptors, and the ability to undergo activity-driven synaptic plasticity mechanisms that lead to strengthening or weakening of connections. Immature dendritic spines are small, thin structures which grow in size into full, mushroom-shaped heads that are capable of supporting increasing demands of the mature synapse (Matsuzaki et al., 2001). Other changes include the stabilizing and clustering of membrane receptors by scaffolding/anchoring proteins of the postsynaptic density (PSD), and enabling lifelong potentiation of the synapse

(Matsuzaki et al., 2004). However, when threshold activity levels are not reached and synapses are not strengthened and stabilized, weakened synapses no longer can support neurotransmission and may become depressed and eliminated (Zhou et al., 2004; Bastrikova et al., 2008; Becker et al., 2008).

NMDAR activation is required for synapse maturation and, because its subunits impart different properties to the receptor (Cull-Candy and Leszkiewicz, 2004; Paoletti and Neyton, 2007), the transition between 'immature' and 'mature' forms of NMDARs is thought to regulate the stabilization of NMDARs in the PSD and the subsequent decline in plasticity of the synapse (Burgard and Hablitz, 1994). Immature synapses are characterized by the presence of predominantly NR2B-containing NMDARs, which are replaced with the more stable subtype, NR2A (Philpot et al., 2001a; Barria and Malinow, 2002; Bellone and Nicoll, 2007). Importantly, the upregulation of NR2A is accompanied by the synaptic incorporation of AMPARs that are required to respond to increased neuronal activity from the presynaptic terminal. This is crucial because most immature synapses do not contain AMPARs, only NMDARs (Isaac et al., 1995; Liao et al., 1995; Rumpel et al., 1998; Shi et al., 1999). Because AMPARs are responsible for the majority of excitatory neurotransmission, these electrically 'silent' synapses have functional consequences in early brain development that have only begun to be addressed.

Synapse stabilization is driven by sensory experience. NMDAR-dependent synaptic plasticity is a remarkable phenomenon whereby synapses can rapidly alter the efficiency with which they convey information from one neuron to another, resulting in their strengthening or weakening. Strong or weak electrical activity between neurons results in the induction of long-term potentiation (LTP) and long-term depression (LTD),

respectively (Malenka and Bear, 2004). Both processes depend upon coincident activation of NMDARs by glutamate binding and membrane depolarization, which permits low and high levels of calcium influx, respectively, and the rapid insertion or removal of AMPARs.

The N-methyl-D-aspartate type glutamate receptor (NMDAR)

NMDARs are activated by glutamate, the most common excitatory neurotransmitter in the central nervous system, and are essential for the proper development of cortical circuitry and synaptic function (McBain and Mayer, 1994; Cull-Candy et al., 2001; Perez-Otano and Ehlers, 2004; Lau and Zukin, 2007). Dysfunction of NMDARs has been implicated in a variety of pathological conditions including schizophrenia (Mueller and Meador-Woodruff, 2004), neurodegenerative disease (Bonuccelli and Del Dotto, 2006; Wenk, 2006; Fan and Raymond, 2007), stroke (Martin et al., 1998), white-matter injury (Karadottir et al., 2005; Salter and Fern, 2005; Micu et al., 2006), and neuropathic pain (Dubner and Ruda, 1992).

Great diversity exists in NMDAR subtypes. The NMDAR is thought to be a tetrameric transmembrane channel (Laube et al., 1998; Rosenmund et al., 1998; Ulbrich and Isacoff, 2007) composed of combinations of the obligatory NR1 subunit with NR2 and/or NR3 subunits (Schorge and Colquhoun, 2003; Furukawa et al., 2005). The NR1 subunit exists as eight functional splice variants (Durand et al., 1992; Sugihara et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Mori and Mishina, 1995), while separate genes produce four types of NR2 (NR2A-D) and two types of NR3 (NR3A-B) subunits (Moriyoshi et al., 1991; Meguro et al., 1992; Monyer et al., 1992; Mishina et al., 1993; Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001). Each receptor

subtype exhibits temporal and regional specificity (Monyer et al., 1994; Sheng et al., 1994) and unique functional properties (Monyer et al., 1994; Paoletti and Neyton, 2007) which are determined by the specific combination of subunits assembled to form the heteromer. For instance, subunit composition determines receptor properties such as glutamate affinity, receptor desensitization, and pharmacological sensitivity (Cull-Candy and Leszkiewicz, 2004; Paoletti and Neyton, 2007). A major goal in neuroscience is to characterize the contributions of individual subunit types to NMDAR function. Such knowledge will increase our understanding of how NMDARs contribute to normal brain development, as well as guide therapeutic strategies for treating pathologies associated with NMDAR dysfunction.

While attention has focused on the role of NR2 subunits in neural function, much less is known about how the more recently identified NR3 subunits, NR3A and NR3B, modify NMDAR functions. This is surprising, given that NR3 subunits act in a novel, dominant-negative manner to suppress NMDAR activity (Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Matsuda et al., 2002). Interest in NR3A has grown with the observations that the subunit influences dendritic spine density (Das et al., 1998; Roberts et al., 2009), synapse maturation (Roberts et al., 2009), memory consolidation (Roberts et al., 2009), cell survival (Nakanishi et al., 2009), and may be involved in certain neuropathologies (Zhang et al., 2002; Mueller and Meador-Woodruff, 2004; Karadottir et al., 2005; Salter and Fern, 2005; Micu et al., 2006).

Although NR3A was identified over a decade ago, there have been few efforts to consolidate knowledge of its functions and possible disease roles (although see (Eriksson et al., 2007b; Cavara and Hollmann, 2008). Given the large number of disorders

associated with NMDAR dysfunction, and the paucity of studies that have examined the putative contribution of NR3 subtypes, we anticipate that its unique structure, expression patterns, and function will make it an important therapeutic target.

NR3A amino acid sequence and structure

NR3A was identified through sequence and structure homology to other *GRIN (Glutamate Receptor Ionotropic N-methyl-D-aspartate)* genes, and originally termed 'χ-1' or 'NMDAR-like' (NMDAR-L) (Ciabarra et al., 1995; Sucher et al., 1995). The *GRIN3A* gene localizes to human chromosome 9q34.1, and consists of 9 exons spanning 1115 amino acids (Andersson et al., 2001) (**Figure 1.1**). The high level of sequence homology between human and rodent NR3A (93%) indicates that its function is likely similar between mammalian species (Andersson et al., 2001; Eriksson et al., 2002). Most consensus sites are conserved between rodents and humans, although the lack of gene equivalents in *D. melanogaster* and *C. elegans* suggests that evolutionary changes may have necessitated the emergence of NR3 subtypes in vertebrates (Matsuda et al., 2002). By sequence alignment, NR3A shares the greatest homology with NR3B (*GRIN3B;* 57%), having low identity with NR1 (27%) and NR2 subunits (24-29%) as well as non-NMDA glutamate receptors (23%) (Andersson et al., 2001).

NR3A shares structural features with other ionotropic glutamate receptor subunits (**Figure 1.2**), including a 4-pass transmembrane topology which forms the channel pore, a large extracellular N-terminal ligand-binding domain with a putative signal peptide and multiple glycosylation consensus sites, and a cytoplasmic tail. Like other NMDAR subunits, the intracellular C-terminus of NR3A contains sites potentially phosphorylated by protein kinases A and C (PKA, PKC), protein tyrosine kinase (PTK), and

calcium/calmodulin-dependent kinase II (CaMKII) (Ciabarra et al., 1995; Sucher et al., 1995; Andersson et al., 2001; Nishi et al., 2001; Eriksson et al., 2002), suggesting that phosphorylation could play critical roles in regulating NR3A trafficking, signaling, and channel properties (Chen and Roche, 2007). NR3A also contains a motif with two vicinal cysteine residues, CC(Y/K)G(Y/F)CID(I/L)L, required for redox modulation (Sullivan et al., 1994; Sucher et al., 1995; Andersson et al., 2001), as well as an intracellular RXR motif which may serve as an endoplasmic reticulum (ER) retention signal (Perez-Otano et al., 2001).

Despite similarities to other NMDAR subunits, NR3A exhibits a number of distinctive features. As with other NMDAR subunits, the NR3A amino acid sequence encodes an extracellular bi-lobed domain (split into S1 and S2 segments by membrane domains TM1, TM2, and TM3) that determines ligand binding specificity. But unlike NR2 subunits, which bind glutamate, the S1 region of the extracellular N-terminus and the S2 region of the extracellular loop between TM3 and TM4 form a glycine binding pocket, a clamshell-like structure (Paas, 1998; Yao and Mayer, 2006; Yao et al., 2008). Additionally, the second membrane pore loop (TM2) and the third membrane pore loop (TM3) in both NR3A and NR3B are substantially different from other NMDAR subunits. In NR1/NR2 NMDARs, an ion selectivity filter at TM2 is formed by an interaction between asparagine (N) residues of NR1 (N-site) and NR2 subunits (N+1 site) (Kuner et al., 1996). In NR3A subunits, however, the N-site is replaced by a glycine (G) (Sucher et al., 1995; Nishi et al., 2001; Matsuda et al., 2002), and this residue apparently has little influence upon ion selectivity (Wada et al., 2006). While NR3A-containing NMDARs apparently lack the N and N+1 site selectivity filter, they appear to have a novel

construction of the outer vestibule that can influence ionic currents. Unlike NR1/NR2 NMDARs, the TM3 domain between NR1 and NR3A subunits has a symmetrical configuration that forms a ring of threonines. This ring constricts the external vestibule and may account for the observed reductions in Ca^{2+} permeability, ionic currents, and magnesium (Mg^{2+}) sensitivity of NR3A-containing NMDARs (Wada et al., 2006). Another distinct characteristic is that the C-termini of NR3A and NR3B lack consensus sequences for PDZ domain protein-binding (Matsuda et al., 2002; Eriksson et al., 2007b), prominent features of other glutamate receptor subunits that permit stable anchoring to the postsynaptic density. Finally, with a calculated molecular weight of ~125kD, NR3A is the most heavily glycosylated glutamate receptor subunit (Ciabarra and Sevarino, 1997). The functional significance of this robust glycosylation is unclear, but it may alter channel gating kinetics (Covarrubias et al., 1989; Chazot et al., 1995).

Alternative mRNA splicing is another means to modify NR3A functions. A 60-base pair insert encoding an additional 20 amino acid sequence at the intracellular C-terminus has been described in rodents, resulting in short (NR3A-s or NR3A-1) and long (NR3A-l or NR3A-2) forms of NR3A (Sun et al., 1998; Eriksson et al., 2002; Sasaki et al., 2002)(**Figures 1.1** and **1.2**). The long splice variant NR3A-l, which contains putative PKA, PKC, and CaMKII phosphorylation sites, is apparently lacking in human NR3A (Andersson et al., 2001; Eriksson et al., 2002).

Developmental and regional expression

NR3A levels change dramatically over development and exhibit a unique spatiotemporal expression pattern when compared to other NMDAR subunits. While

NR3A expression is grossly similar across mammalian species, there are some notable differences. Understanding these distribution patterns will help predict how prospective NR3A agonists and antagonists are likely to affect neural function. Because the existing data on NR3A expression patterns are sometimes contradictory, the information is summarized here in tabular format to present an overview of the consensus findings from brain tissue (**Table 1.1**).

Ontogenetic profiles

Overall, NR3A expression is low embryonically, peaks during early postnatal life, and diminishes to much lower levels in adulthood. This expression profile is observed in many regions of the brain and in different species (**Table 1.1**), suggesting that it may play a specific role during postnatal neural development amongst mammals (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Sun et al., 1998; Goebel and Poosch, 1999; Sun et al., 2000; Al-Hallaq et al., 2002; Eriksson et al., 2002; Naassila and Daoust, 2002; Sasaki et al., 2002; Wong et al., 2002; Mueller and Meador-Woodruff, 2003, 2004, 2005; Perez-Otano et al., 2006; Eriksson et al., 2007a; Nilsson et al., 2007b; Henson et al., 2008; Bendova et al., 2009; Roberts et al., 2009). Despite having a roughly similar expression profile across species, future studies with a finer level of analysis may reveal important differences. Further heterogeneity of NR3A expression across species and brain regions could also arise from differential expression of its two splice variants (discussed above).

In contrast to NR3A, NR3B levels are low around postnatal day (P) 10 and gradually increase into adulthood within the neocortex, hippocampus, striatum,

cerebellum, brainstem, and spinal cord (Andersson et al., 2001; Nishi et al., 2001; Eriksson et al., 2002; Matsuda et al., 2002; Bendel et al., 2005; Wee et al., 2008) (**Figure 1.3a**). Given the functional similarities and high sequence homology between NR3A and NR3B (57%) (Andersson et al., 2001), it is surprising that these dominant-negative subunits have distinct temporal expression patterns in many of the same brain regions.

Each NMDAR subunit (NR1, NR2A-D, and NR3A-B) exhibits a unique ontogenetic profile in the rodent (Watanabe et al., 1992; Monyer et al., 1994; Sheng et al., 1994; Ciabarra et al., 1995; Dunah et al., 1996; Laurie et al., 1997; Sun et al., 1998; Matsuda et al., 2002; Fukaya et al., 2005) (**Figure 1.3a**), consistent with its functional "signature" (Goebel and Poosch, 1999; Al-Hallaq et al., 2002). The NR1 subunit is obligatory for NMDAR function and is present throughout life. NR3A is developmentally expressed following a time course most similar to NR2D; both are prominent in the first two postnatal weeks in overlapping brain regions (Watanabe et al., 1992; Monyer et al., 1994; Ciabarra et al., 1995; Dunah et al., 1996; Laurie et al., 1997; Sun et al., 1998). The developmental decreases of NR3A and NR2D expression are in contrast to that of NR3B, NR2A, and NR2C, whose expression levels increase developmentally and peak during the third postnatal week (Monyer et al., 1994; Sheng et al., 1994; Matsuda et al., 2002; Fukaya et al., 2005) (**Figure 1.3a**). While both NR3A and NR2B are strongly expressed early in development, NR3A (but not NR2B) levels exhibit a pronounced reduction during postnatal maturation.

Cellular, laminar, and subcellular localization

NR3A and NR3B are expressed by multiple neuronal cell types, including

interneurons, pyramidal cells, motor neurons, trigeminal neurons, retinal ganglion and amacrine cells (Ishihama *et al.*, 2005; Ishihama and Turman, 2006; Matsuda *et al.*, 2002; Mueller and Meador-Woodruff, 2005; Nishi *et al.*, 2001; Paarmann *et al.*, 2005; Sucher *et al.*, 2003). Although NR3A is present in oligodendrocytes, it does not seem to be expressed in astrocytes (Ishihama *et al.*, 2005; Karadottir *et al.*, 2005; Matsuda *et al.*, 2002; Paarmann *et al.*, 2005; Perez-Otano *et al.*, 2006; Salter and Fern, 2005). NR3B localization has been observed in most of the cells located in adult cerebral cortex, hippocampus, striatum, cerebellum, and lumbar spinal cord. This ubiquitous expression of NR3B parallels that of NR1 and suggests a role for NR3B in adult NMDAR function (Wee et al., 2008). Data from NR3B knockout mice will be required to determine the extent to which NR3B influences NMDAR-mediated transmission in the adult central nervous system.

Because of the inside-out pattern in which cortical lamination occurs, the high levels of NR3A observed in layer 5 have raised the possibility that NR3A may play a role in establishing early cortical circuits, perhaps by modulating Ca^{2+} influx and cell vulnerability to excitotoxicity (Mueller and Meador-Woodruff, 2005; Sucher *et al.*, 1995). NR3B also shows predominant expression in layer 5, but the implications of this laminar specificity in the adult have not yet been investigated.

Ultrastructurally, NR3A colocalizes with NR1 and NR2 subunits at postsynaptic membranes of asymmetric (excitatory) synapses (Perez-Otano *et al.*, 2006; Wong *et al.*, 2002), although it appears to be more abundant at perisynaptic and extrasynaptic sites than at the postsynaptic density (PSD) in both juvenile and adult animals (Perez-Otano *et al.*, 2006). The localization of NR3A contrasts sharply with the PSD-centric localization

of NR2A, but is similar to the more lateral and extrasynaptic positioning of NR2B-containing receptors (Groc *et al.*, 2006; Racca *et al.*, 2000). Biochemical fractionation studies confirm the ultrastructural data, and show that NR3A is associated with membranous fractions, such as ER, Golgi, endosomes, and synaptic membranes, but is not as enriched in PSDs as NR1 or NR2 subunits (Perez-Otano *et al.*, 2006). Future experiments are needed to determine (1) whether NR3A-containing receptors shift from synaptic to extrasynaptic sites during development, as has been suggested for other NMDAR subtypes (Kohr, 2006), or (2) whether their peri/extrasynaptic localization indicates a graded and reciprocal organization of different NMDAR subtypes within the PSD that could be fitted for sensing the varied patterns of glutamate release.

NR3 subunits exert a dominant-negative effect upon NMDAR function

The properties of typical NMDARs containing NR2A or NR2B subunits include channel block by Mg^{2+} at hyperpolarized potentials, high permeability to Ca^{2+}, and a subunit-specific complement of protein binding partners (Kennedy *et al.*, 2005; Matute *et al.*, 1997). These properties are critical to many forms of plasticity (Gustafsson and Wigstrom, 1988; Madison *et al.*, 1991) and profoundly influence the wiring of the nervous system and memory processes (Constantine-Paton et al., 1990; Bliss and Collingridge, 1993). Receptors containing NR3A or NR3B subunits have unique properties that differ from conventional NR1/NR2 heteromers. In heterologous expression systems, NR3 subunits assemble into two functional receptor combinations: (1) a heterodimer of NR1 and NR3 subunits that unexpectedly forms an excitatory glycine receptor, and (2) a heterotrimeric complex of NR1, NR2, and NR3 subunits that forms an NMDAR with novel properties and attenuated currents compared to NR1/NR2

NMDARs (Chatterton *et al.*, 2002; Ciabarra *et al.*, 1995; Das *et al.*, 1998; Sucher *et al.*, 1995).

Triheteromeric NR3A-containing glutamate receptors

NR3A-containing NMDARs only sense glutamate when glutamate-binding NR2 subunits are included (Ciabarra *et al.*, 1995; Perez-Otano *et al.*, 2001; Sucher *et al.*, 1995) **(Figure 1.4)**. When NR1, NR2A, and NR3 subunits are co-expressed in heterologous expression systems, single-channel recordings reveal two populations of NMDA-evoked currents: one characterized by a large conductance resembling the prototypical channel response of NR1/NR2A heteromers, and a second population of smaller conductance, presumably due to channels containing NR3 (Das *et al.*, 1998; Perez-Otano *et al.*, 2001; Sasaki *et al.*, 2002; Ulbrich and Isacoff, 2008) **(Figure 1.4B, C)**. The putative NR3-containing receptors exhibit dramatically reduced single-channel open probabilities and longer mean open times (Perez-Otano *et al.*, 2001; Sasaki *et al.*, 2002, but see (Das et al., 1998).

Along with generally decreasing ionic conductance through NMDARs, inclusion of NR3 subunits alters two of the most prominent properties of traditional NMDARs (NR1/NR2 heteromers). First, it causes a five- to ten-fold decrease in Ca^{2+} permeability, with the variation of the estimates likely reflecting differences between the recombinant systems employed (Das *et al.*, 1998; Matsuda *et al.*, 2002; Perez-Otano *et al.*, 2001; Sasaki *et al.*, 2002; Tong *et al.*, 2008) (**Figure 1.4A**). Second, NR3A-containing NMDARs are also almost completely insensitive to Mg^{2+} block at hyperpolarized potentials (Sasaki *et al.*, 2002, but see Nishi *et al.*, 2001). Both the reduced Mg^{2+} sensitivity and Ca^{2+} permeability of NR3A-NMDARs have recently been confirmed in

neuronal cultures or hippocampal slices from transgenic mice overexpressing NR3A and knockout mice lacking NR3A (Roberts *et al.,* 2009; Tong *et al.,* 2008) (**Table 1.2**). As previously discussed in Section 2, the observed physiological characteristics of NR3A-containing NMDARs most likely result from the formation of a narrow constriction of the outer vestibule along the TM3 pore-forming region of the NR3 subunits.

Diheteromeric NR3A-containing excitatory glycine receptors

The physiological significance of NR1/NR3 excitatory glycine receptors remains an open question because of conflicting reports *in vitro,* and this subunit combination has yet to be documented *in vivo*. Despite evidence that NR1/NR3 complexes are targeted to the plasma membrane in heterologous systems (Madry *et al.*, 2007; Perez-Otano *et al.*, 2001), early reports failed to record NMDAR-mediated responses to glutamate or NMDA application in the absence of NR2 subunits (Ciabarra *et al.*, 1995; Das *et al.*, 1998; Perez-Otano *et al.*, 2001; Sucher *et al.*, 1995). Instead of exhibiting responsiveness to glutamate, NR1/NR3 complexes were found to be sensitive to glycine application (Chatterton *et al.*, 2002). This finding is consistent with the observation that the NR3A ligand binding domain has very high affinity for glycine, but is relatively insensitive to glutamate (Nilsson *et al.*, 2007a; Yao and Mayer, 2006). Glycine application to NR1/NR3 diheteromers evokes a large inward current, indicating that these receptors form an excitatory glycine receptor. The ion permeability of NR1/NR3 diheteromers was similar to that of NR3-containing triheteromers, in that the currents were relatively impermeable to Ca^{2+} and insensitive to Mg^{2+} block (Chatterton *et al.*, 2002; Das *et al.*, 1998; Nishi *et al.*, 2001; Perez-Otano *et al.*, 2001; Sasaki *et al.*, 2002). Additionally, while both NR1/NR3A and NR1/NR3B receptors form in *Xenopus* oocytes (Chatterton *et*

al., 2002), NR3 subunits fail to assemble with NR1 subunits in HEK cells unless both NR3A and NR3B are present (Smothers and Woodward, 2007).

Chatterton and colleagues (2002) reported that bath application of glycine to cerebrocortical cultures elicited a bursting response, which they interpreted as evidence that excitatory glycine receptors exist in the brain. Furthermore, single-channel recordings from outside-out patches from these cultured neurons exhibited channel properties similar to NR1/NR3 receptors in heterologous systems. However, this bursting response to glycine can be found in cultures in which NR1 subunits, and hence all NMDARs, are lacking (Matsuda et al., 2003). Given that the presence of NR1 is required for NR3 subunits to exit the ER and form stable complexes in the plasma membrane (Perez-Otano et al., 2001), these data indicate that the glycine-induced bursting may be independent of NR3 subunits. The existence of glycinergic NR1/NR3 receptors was also questioned by studies finding no evidence for excitatory glycine currents in cultured neurons from mice genetically engineered to overexpress NR3A (Tong *et al.*, 2008) (**Table 1.2**).

The high affinity of NR1/NR3 receptors for glycine suggests that, if found *in vivo*, this receptor combination might be saturated by physiological concentrations of glycine (Yao and Mayer, 2006). Thus, these receptors would serve to keep neurons in a more depolarized state and, depending upon their subcellular localization, could have a profound influence upon synaptic transmission and action potential firing.

NR3A pharmacology

Agonists

The study of NR3A pharmacology is still in its infancy. However, it is clear that

NR3A shares more commonalities with NR1 than it does with NR2 subunits. For instance, NR1 binds glycine (Hirai *et al.*, 1996; Yao and Mayer, 2006) whereas NR2 subunits bind glutamate (Laurie et al., 1997), and receptors formed by NR1 and NR3A bind glycine with much higher affinity than do NR1/NR2 receptors (Chatterton *et al.*, 2002). Studies of the rodent ligand binding domain and human full-length protein confirm that glycine binds NR3A with much higher affinity than it binds NR1 [rodent NR3A, *K*d = 40 nM, 650 times less than that for NR1 (Yao and Mayer, 2006), and human NR3A, *K*d = 535 nM (Nilsson et al., 2007a)]. The lower affinity for glycine of human NR3A compared to rodent NR3A could be due to species differences or differences between the full-length protein and the truncated soluble ligand binding domain. In both human and rodent, NR3A is likely saturated by its preferred endogenous ligand, glycine, at physiological concentrations (Yao and Mayer, 2006). The NR3A ligand binding domain has a low affinity for glutamate (*K*d = 9.6 mM) (Yao and Mayer, 2006), and the presumptive activation of NR1/NR2/NR3A triheteromeric receptors by glutamate or NMDA is almost certainly due to the binding of these agonists to NR2 subunits.

D-serine is a potent glycine site agonist of NMDARs (Kleckner and Dingledine, 1988), and accordingly, binding assays demonstrate that D-serine also binds human and rat NR3A with high affinity (Nilsson *et al.*, 2007a; Yao and Mayer, 2006). One electrophysiological study indicated that D-serine behaves as a functional *antagonist* of NR1/NR3A receptors (Chatterton *et al.*, 2002). This differs from other reports, and may be due to the fact that D-serine produces a rapid desensitization resembling an antagonist block (Yao and Mayer, 2006). Another possibility is that assembly of NR3A with other subunits (e.g. NR1) produces allosteric interactions that alter ligand binding properties

(Laurie and Seeburg, 1994). The ligand binding properties of NR3A, and if/how they differ when NR3A co-assembles with other NMDAR subunits have yet to be determined. Until these limitations are overcome, caution should be used when interpreting whether NR3A-containing NMDARs are present or absent based on the effects of pharmacological agonists/antagonists such as D-serine (Li and Han, 2006).

While NR3A shares more attributes with NR1 than with NR2 subunits, there are nonetheless striking pharmacological differences between NR1 and NR3A subunits (Awobuluyi *et al.*, 2007; Nilsson *et al.*, 2007a; Yao and Mayer, 2006). For example, NR1 binds the partial agonist ACPC (1-aminocyclopropanecarboxylic acid) with higher affinity than ACBC (1-aminocyclobutane-1-carboxylic acid), while the converse is true for NR3A (Yao and Mayer, 2006). Moreover, neither the partial agonist D-cycloserine nor the antagonist 7-chlorokynurenic acid displace glycine binding to human NR3A expressed in HEK cells (Nilsson et al., 2007a), in contrast to what is observed for the binding of glycine to NR1. The differences between NR1 and NR3A subunits raise the exciting possibility that NR3A-specific agonists or antagonists could be generated in the future to target diseases of NMDAR dysfunction.

Antagonists

Very little is known about how NR3A-containing receptors can be pharmacologically blocked. Studies to date have focused on the binding of NR1 antagonists to NR3A or have used electrophysiological measures to determine how NR1/NR3A or NR1/NR2/NR3A heteromers respond to typical NMDAR antagonists. When NR1/NR3A diheteromers are expressed in heterologous systems, they exhibit little electrophysiological block by the classic NMDAR antagonists APV (competitive), MK-

801 (non-competitive), or memantine (uncompetitive) (Chatterton *et al.*, 2002). Triheteromeric receptors containing NR1/NR2/NR3A are more difficult to isolate electrophysiologically because they must be distinguished from either NR1/NR2 or NR1/NR3A receptors. However, this has been possible using single-channel recordings, which showed that the small conductance NR1/NR2A/NR3A channel is blocked by D-APV (200 μM) (Sasaki *et al.*, 2002). Interestingly, NR1/NR2/NR3A triheteromeric receptors may also be blocked by antagonists targeting NR2 subunits, as receptors composed of NR1/NR2B/NR3A triheteromers and NR1/NR2B diheteromers are similarly blocked by high concentrations of the NR2B-antagonist ifenprodil (Smothers and Woodward, 2003). One might predict that, compared to NR1/NR2B diheteromers, NR1/NR2B/NR3A triheteromers might be less sensitive to lower concentrations of ifenprodil where NR2B-specificity is greater, as previous studies have shown that the magnitude of ifenprodil block depends on the number of NR2B subunits contained within the NMDAR complex (Hatton and Paoletti, 2005).

Studies of the ligand binding domain demonstrate that NR3A subunits interact with classical NMDAR antagonists. Six of eight NR1 subunit antagonists exhibit more than a 100-fold larger affinity for NR1 subunits compared to NR3A subunits (Yao and Mayer, 2006). For example, kynurenic acid binds the NR1 ligand binding domain with high affinity (Kd= ~53 μM) but only weakly binds the NR3A binding domain (*K*d = ~15 mM) (Yao and Mayer, 2006). These observations provide further evidence that NR1 and NR3A have distinct ligand binding properties and increase the likelihood that specific agonists/antagonists could be designed to target NR3A selectively. However, the extremely high affinity of NR3A for glycine indicates that the design of competitive

antagonists would require a particularly high affinity compound. Thus, it might be more feasible to design non-competitive antagonists that act via allosteric interactions with NR3A-containing NMDARs.

Intracellular binding partners

NMDAR-mediated effects can be initiated directly via ionic flow through the channel pore, and can also be shaped by intracellular partners tethered to the NMDAR complex. To date, few studies have examined interactions of NR3A with other proteins, but the existing reports have identified possible associations between NR3A and protein phosphatases, cytoskeletal proteins, and an adaptor protein implicated in receptor trafficking.

One specific C-terminal binding partner of NR3A is the catalytic subunit of protein phosphatase 2A (PP2A) (Chan and Sucher, 2001; Ma and Sucher, 2004) (**Figure 1.2**). PP2A plays important roles at postsynaptic sites, including dephosphorylating NR1 subunits on serine 897 (NR1-S897). Dephosphorylation of NR1-S897 by PP2A attenuates NMDAR single-channel currents and reduces permeability of the receptors to Ca^{2+}. Importantly, activation of NMDARs causes the physical uncoupling between NR3A and PP2A, decreasing PP2A activity at the synapse. By influencing NR1 phosphorylation through its interaction with PP2A, NR3A may provide a feedback mechanism to modulate NMDAR function based on the history of synaptic activity (Chan and Sucher, 2001). Intriguingly, brains from schizophrenic patients have increased dephosphorylation of NR1-S897 (Emamian et al., 2004) that might be related to overexpression of NR3A (Ma and Sucher, 2004), providing a potential mechanism that could contribute to the NMDAR hypofunction observed in schizophrenia.

In addition to modulating the activity of a phosphatase important to NMDARs, one intracellular NR3A binding partner appears to be crucial for the removal of nascent NMDARs during synapse maturation. NR3A binds PACSIN1/syndapin1, a neuron-specific intracellular adaptor (Perez-Otano *et al.*, 2006). PACSIN1 binds selectively to the C-terminus of NR3A (**Figure 1.2**) through tertiary structure interactions with its NPF (Asn/Pro/Phe) motifs, recruiting a larger complex with the endocytic proteins dynamin and clathrin and facilitating the rapid endocytosis of NR3A-containing NMDARs. Disruption of this interaction results in the synaptic accumulation of NR3A-containing NMDARs. The PACSIN1-mediated removal of NR3A-containing NMDARs is activity-dependent, providing a mechanism for regulated receptor replacement that could drive the functional maturation of synaptic NMDARs during postnatal development and the associated changes in the properties of synaptic plasticity.

Additional putative NR3A binding partners are beginning to be identified which could alter its localization, trafficking, and signaling (**Figure 1.2**), but these interactions were only shown *in vitro* and need to be extensively studied and verified. NR3A may interact with a cytoskeletal protein (MAP1S/C19ORF5), a scaffolding protein (plectin), a cell cycle and apoptosis regulatory protein (CARP-1), and a regulator of G-protein signaling (GPS2/AMF1) (Eriksson *et al.*, 2007a; Eriksson *et al.*, 2007b). Confirmation of these associations would position NR3A to play roles in intracellular processes such as trafficking and targeting of NR3A-containing receptors (MAP1S and plectin), PKC activation (plectin), and suppression of the MAPK pathway (GPS2/AMF1). Other NR3A binding partners will undoubtedly be identified, which may link NR3A to more signaling pathways. Notably, there is a conspicuous absence of NR3A interaction with the

postsynaptic scaffolding protein PSD-95 (Eriksson *et al.*, 2007a; Matsuda *et al.*, 2002), which may explain the labile synaptic expression of NR3A-containing NMDARs (Perez-Otano *et al.*, 2006). Alternatively, the clustering and stabilization of NR3A-containing receptors near synaptic sites may rely on NR2 anchoring with other membrane-associated guanylate kinase family scaffolding proteins, such as SAP102, which are present at high levels in developing synapses (Perez-Otano *et al.*, 2006; Sans *et al.*, 2000; Wong *et al.*, 2002).

Assembly, trafficking, and targeting of NR3-containing NMDARs

Developmental regulation of the membrane expression of different NMDAR subtypes shapes synaptic and behavioral plasticity (Isaac *et al.*, 1995; Liao *et al.*, 1995; Philpot *et al.*, 2001; Quinlan *et al.*, 1999; Roberts *et al.*, 2009; Rumpel *et al.*, 1998; Sawtell *et al.*, 2003; Tang *et al.*, 1999; Wu *et al.*, 1996). NMDARs can rapidly translocate in and out of synaptic regions by lateral diffusion in the plane of the membrane and by endo/exocytic trafficking. It is increasingly recognized that regulation of trafficking contributes to the synapse-specific and activity-dependent control of NMDAR expression at individual synapses, by favoring local receptor removal, insertion, or exchange (Lavezzari *et al.*, 2004; Montgomery *et al.*, 2005; Scott *et al.*, 2004; Washbourne *et al.*, 2004).

Although peak expression of rodent NR3A occurs in the second postnatal week, its expression levels and association with NR1 subunits may nonetheless be fairly limited compared to that of NR2 subunits. Immunoprecipitation experiments found that, despite the majority of NR3A associating with the obligatory NR1 subunit, NR3A subunits are likely to contribute only a small fraction of the total receptor complex composition (Al-

Hallaq et al., 2002). Specifically at age P10, approximately 80% of NR3A subunits are associated with NR1, but less than 10% of NR1, NR2A, and NR2B are bound to NR3A, and this association decreases further over development. Strikingly, NR3A genetic deletion studies demonstrate that even a low NR3A abundance is sufficient to have profound consequences on synapse formation and NMDAR-mediated transmission during early postnatal development (Das *et al.*, 1998; Perez-Otano *et al.*, 2001; Roberts *et al.*, 2009; Sasaki *et al.*, 2002; Tong *et al.*, 2008).

Protein degradation, fluorescence recovery after photobleaching, and fluorescence resonance energy transfer experiments suggest a central role for NR1 in the folding and assembly of NMDAR heteromers in the endoplasmic reticulum (ER) (Atlason *et al.*, 2007; Schuler *et al.*, 2008). Homomeric NR3 complexes fail to exit the ER, presumably because the C-terminus of NR3A/NR3B carries a putative ER retention signal (RXR) (**Figure 1.2**). For forward secretory trafficking and functional insertion into the plasma membrane to occur, the RXR motif is likely masked by heteromeric assembly with NR1 subunits (Matsuda *et al.*, 2003; Perez-Otano *et al.*, 2001; Schuler *et al.*, 2008; Scott *et al.*, 2001; Standley *et al.*, 2000)**.** However, NR3 subunits may be rapidly degraded, even in the presence of NR1, resulting in inefficient assembly mechanisms or extremely rapid turnover of NR3A-containing NMDARs lacking NR2 subunits (Atlason et al., 2007).

Once at the plasma membrane surface, the abundance and synaptic targeting of NR3A-containing NMDARs appears to be highly regulated by synaptic activation of NMDARs. In experiments using cultured hippocampal neurons transfected with GFP-tagged NR3A, intrinsic neuronal activity induces the movement of NR3A-containing NMDARs from membrane and synaptic locations to intracellular compartments (Perez-

Otano *et al.*, 2006).

Endocytic removal of NR3A can be facilitated by bath application of NMDA and prevented by disruption of synaptic activity (TTX) or antagonism of NMDARs with APV. Furthermore, removal relies upon a selective interaction with PACSIN1/syndapin1, a specialized accessory protein involved in clathrin-mediated endocytosis and enriched at synapses (discussed in more detail in Section 6). The synaptic expression of PACSIN1 is developmentally regulated and exhibits a reciprocal expression pattern to that of NR3A (Perez-Otano *et al.*, 2006), supporting a role for PACSIN1 in the developmental downregulation of NR3A subunits.

Another regulator of NR3A synaptic targeting is the protein phosphatase PP2A, which binds NR3A directly and influences the dephosphorylation state of NR1 subunits (Chan and Sucher, 2001; Ma and Sucher, 2004). PACSIN1 and PP2A binding domains on the C-terminus of NR3A overlap (**Figure 1.2**), making it unlikely that these proteins would bind concurrently (Chan and Sucher, 2001; Perez-Otano *et al.*, 2006). Interestingly, NMDAR activation disrupts the PP2A-NR3A protein interaction, and may unmask binding sites for PACSIN1, enabling NR3A endocytosis.

Finally, PDZ-domain binding motifs, which provide areas of attachment for prototypical PSD proteins and support the postsynaptic anchoring of other NMDAR subunits, are absent in the C-termini of NR3A and NR3B (Eriksson *et al.*, 2007a; Matsuda *et al.*, 2002). Perhaps as a consequence, these subunits are more loosely attached to the PSD than NR2 subunits (Perez-Otano et al., 2006; Roberts et al., 2009). Despite this emerging set of data, and in contrast to the large literature on NR2 subunits, little is yet known about how and where NR3 proteins traffic.

Rationale for Dissertation Research

Developmental and experience-dependent changes in NMDAR subtypes dictate the function of the receptor. Although the NR3 subfamily was discovered over fifteen years ago, most research has ignored the NR3A and NR3B subunits and has instead focused on the canonical subtypes predominant in the forebrain, NR2A and NR2B. However, recent studies have suggested that the NR3A subunit is also present and may have an important role in excitatory synapse development during early postnatal life.

Genetic manipulations of NR3A levels suggest this subunit influences synaptogenesis. NR3A knockouts exhibit a striking increase in the number of dendritic spines (Das et al., 1998), while NR3A-overexpressors have reduced synapse density (Roberts et al., 2009), suggesting that NR3A may play a key role in spine and synapse formation and elimination. Electron microscopy analysis shows not only reduced synapse numbers and postsynaptic density (PSD) length in the NR3A overexpressors, but also a sharp decrease in the number of NR3A particles with increasing synapse size (Roberts et al., 2009). This occurred while NR1 particles were roughly constant, suggesting that NR3A is normally absent from large synapses and is targeted to small synapses. Altogether, these data indicate a dominant role of NR3A in limiting synapse/spine growth.

The synaptic removal of NR3A-containing NMDARs is thought to be a trigger for synapses to gain a more mature complement of NMDAR subtypes and to enable robust forms of synaptic plasticity (Perez-Otano et al., 2006). NR3A has been shown to associate with NR1, NR2A, and NR2B at extra- and perisynaptic sites (Perez-Otano et al., 2006), although neither the subcellular localization nor the prevalence of NR3A-

containing receptors has been rigorously established. NR3A is in the right place at the right time to influence spine and synapse development, but it is unclear whether its influence is to serve as a 'synaptic brake' to limit synapse/spine formation or if it acts as a 'synaptic marker' to promote synapse elimination. I hypothesized that NR3A suppresses NMDAR activity during early life, a period of tremendous neuronal outgrowth, to prevent premature synaptic strengthening and stabilization.

Proper synapse formation and refinement in the CNS during childhood enable us to perceive and respond to our external environment. Not surprisingly, improper formation or function of these synapses leads to many neurodevelopmental disorders, including autism and schizophrenia (Zoghbi, 2003). The unique properties of NR3A suggest that this subunit is well-positioned to dramatically influence synapse maturation, and the improper expression of this subunit is likely to have profound effects on synaptic connectivity. Whether NR3A plays a role in the pathophysiology of the synaptic disorder, schizophrenia is a key question addressed by this dissertation.

Schizophrenia arises from aberrant development, stabilization, and plasticity of synapses in the human prefrontal cortex (Javitt and Zukin, 1991; Olney et al., 1999; Coyle et al., 2003). Recent data raise the intriguing possibility that NMDAR hypofunction in schizophrenia might arise from an aberrant increase in NR3A expression, as NR3A suppresses NMDAR function and NR3A mRNA transcript levels are significantly elevated in the dorsolateral prefrontal cortex (DLPFC) of schizophrenic patients (Mueller and Meador-Woodruff, 2004). In this same region where NR3A is overexpressed, spine densities are reduced in schizophrenic patients (Glantz and Lewis, 2000, 2001). Because NR3A likely has a strong influence on limiting spine density, I

hypothesized that a lack of the normal developmental downregulation of NR3A could result in significant reductions in spine numbers. NR3A's unique ability to suppress NMDAR function makes it an extremely attractive candidate, both as an endogenous contributor to the NMDAR hypofunction observed in schizophrenia and as a potential target for pharmacological interventions.

Little is known about the physiological importance of having NR3A-containing NMDARs during early development. My experiments are among the first to define the role of NR3A in synapse development and these data along with others are forcing a re-evaluation of the classical view of NMDAR-dependent synapse maturation. Examining the role of NR3A in synaptic stabilization during early postnatal development will be crucial to understanding a key process in the refinement of neural circuits capable of learning and storing memories.

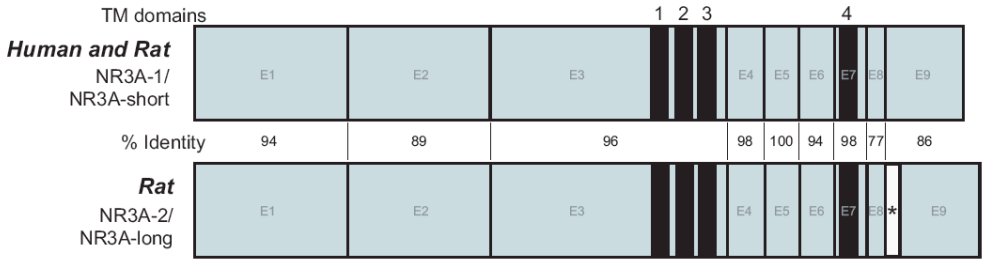


Figure 1.1. Schematic diagram of cDNA structures for human and rat NR3A. Both human and rat have NR3A-1 isoforms, also known as NR3A-short (or -s). Rats also have an NR3A-2 isoform, also known as NR3A-long (or -l) Exons 1–9 appear in blue filled boxes (E1–E9). Predicted transmembrane (TM) domains 1–4 are indicated, and region of highest homology is between TM 1 and 4. Lowest homology is found in the C-terminus, which corresponds to the area following TM4. Location of the alternative splice variant (20-amino acid insert) in rodents is denoted in white with an asterisk (*). Percent identity in exon 9 is between NR3A-short sequences, and does not include the 20-amino acid insert found in rat.

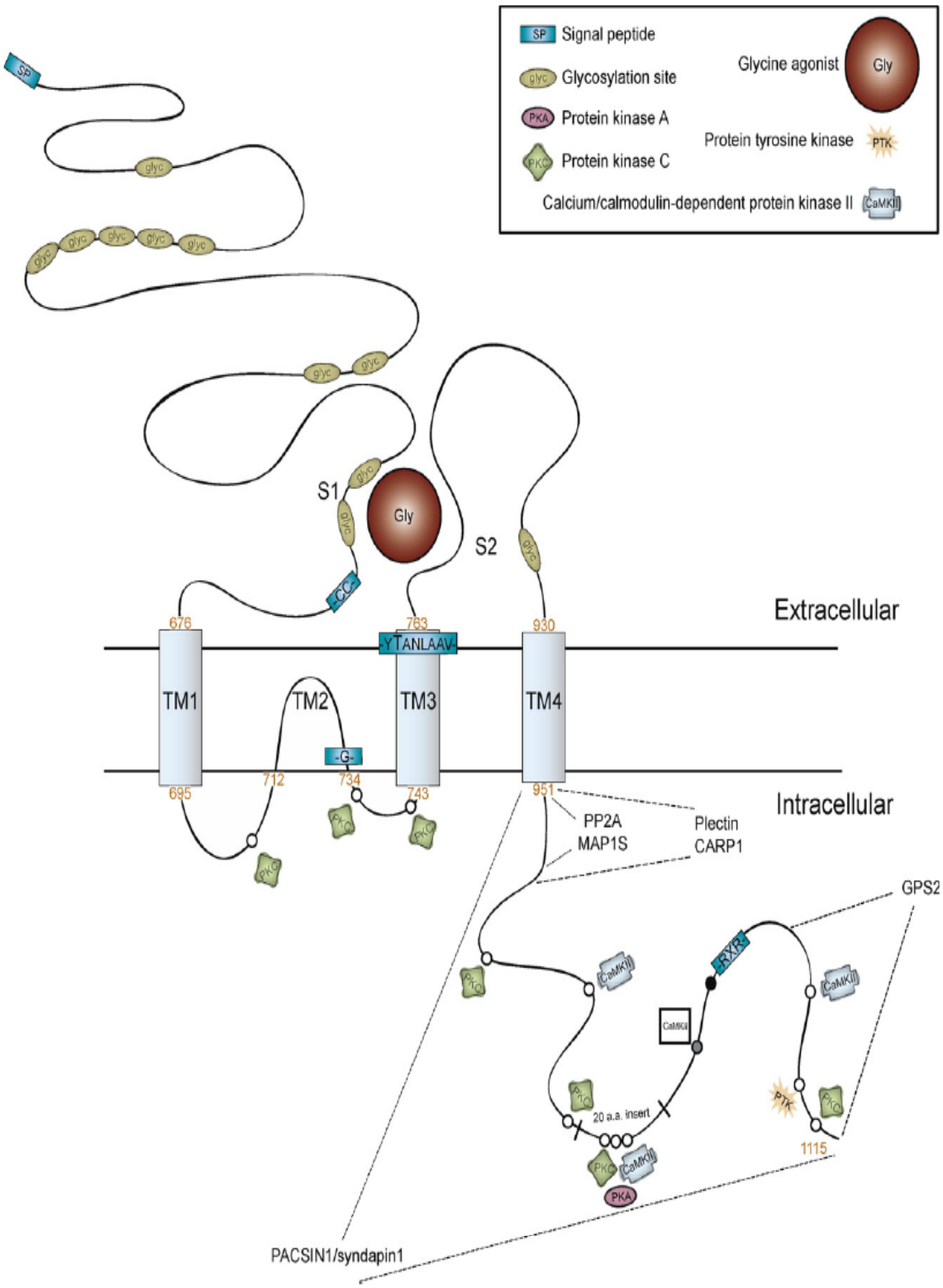


Figure 1.2 Legend appears on the following page.

Figure 1.2 Putative transmembrane topology of rat NR3A. Predicted sites are shown for signal peptide (SP), glycosylation (glyc), phosphorylation (PKC, CaMKII, PTK), and protein binding (indicated by dashed lines for PACSIN1/ syndapin1, MAP1S, PP2A, plectin, CARP1, and GPS2). Sequence motifs (-CC-, -G-, -YTANLAAV-, -RXR-), transmembrane regions (TM1–TM4) and the extracellular ligand-binding domain/glycine binding pocket (S1–S2) are noted. Predicated kinase phosphorylation sites are indicated by open circles (*). A polyproline motif is indicated by a single black circle (*). CaMKII site predicted for human but not rat is indicated within a square symbol adjacent to a gray circle. MAP1S and PP2A binding sites are overlapping and found just intracellular to TM4. TM2 and TM3 segments are thought to form the channel pore. Alternative splicing in rodents but not humans produces a twenty amino acid insert (-SRWRRWTCKTEGDSELSLFP-). This area contains potential phosphorylation sequences for PKA, PKC, and CaMKII. Most sites have been proposed by sequence analysis and are not verified. Amino acid numbers appear in orange. Figure based on sequence data from Andersson et al. (2001).

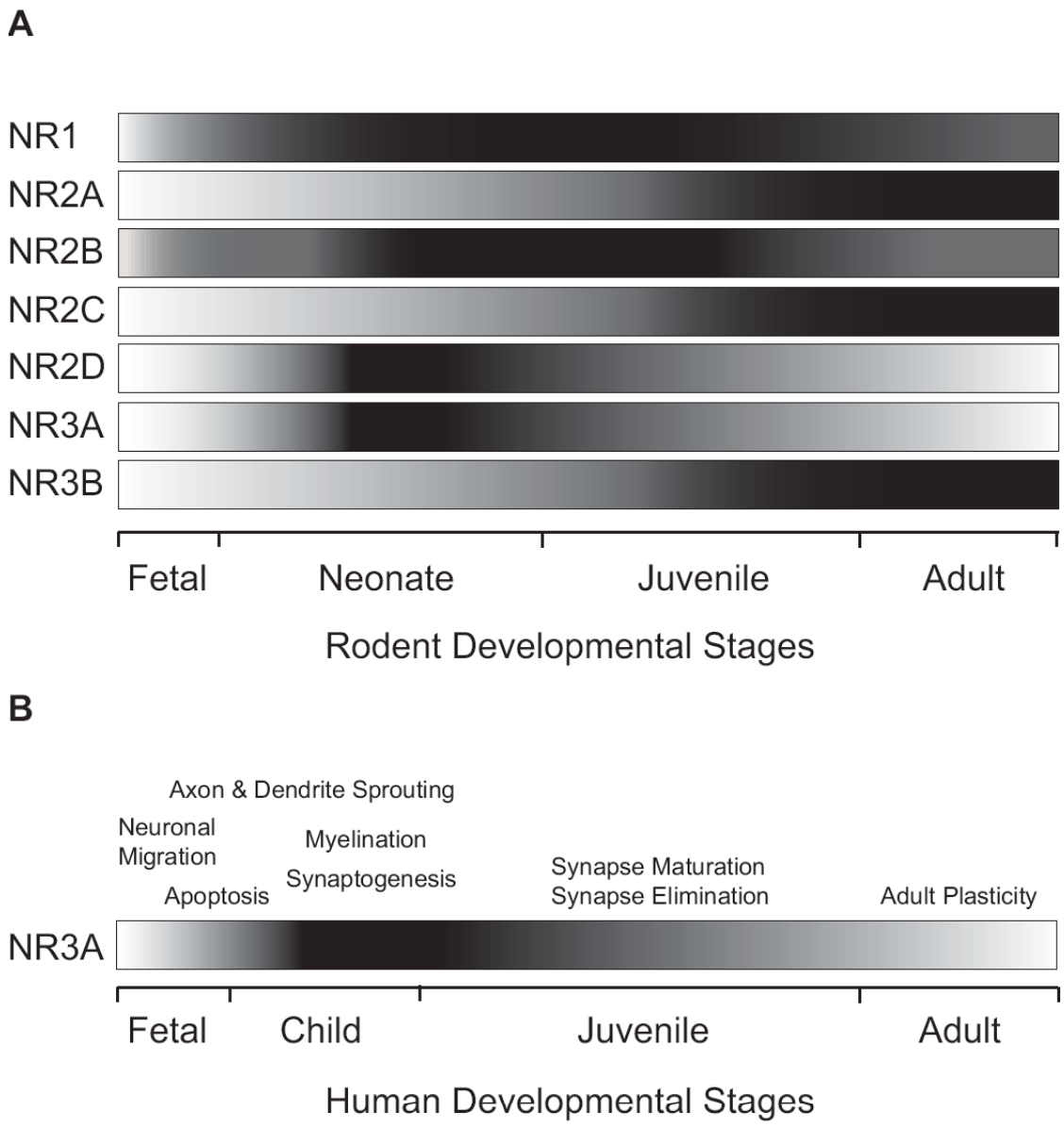


Figure 1.3. (A) Schematic representation of NMDAR subunit expression in the developing rat brain. The gray scale gradient shows the differences of each subunit relative to maximum, with the darkest regions reflecting the strongest expression. NR3A appears to be expressed in similar temporal fashion to NR2D, with subunits peaking between P7 and P14. This is contrasted with NR3B, NR2A, and NR2C, which increase developmentally and peak in the third postnatal week. Adapted with permission from Lujan et al. (2005). (B) Schematic representation of NR3A expression profile and potential roles in the developing human central nervous system. Several key developmental processes in the brain characterize the early postnatal timeframe, when NR3A is maximally expressed: axon and dendrite sprouting, synaptogenesis, myelination, cell death, synapse maturation and elimination (de Graaf-Peters and Hadders-Algra, 2006). The gray scale gradient illustrates the changes in NR3A expression levels, with the darkest regions (child/juvenile stages) reflecting the strongest expression, and low expression seen in thelightest regions (fetal and adult life).

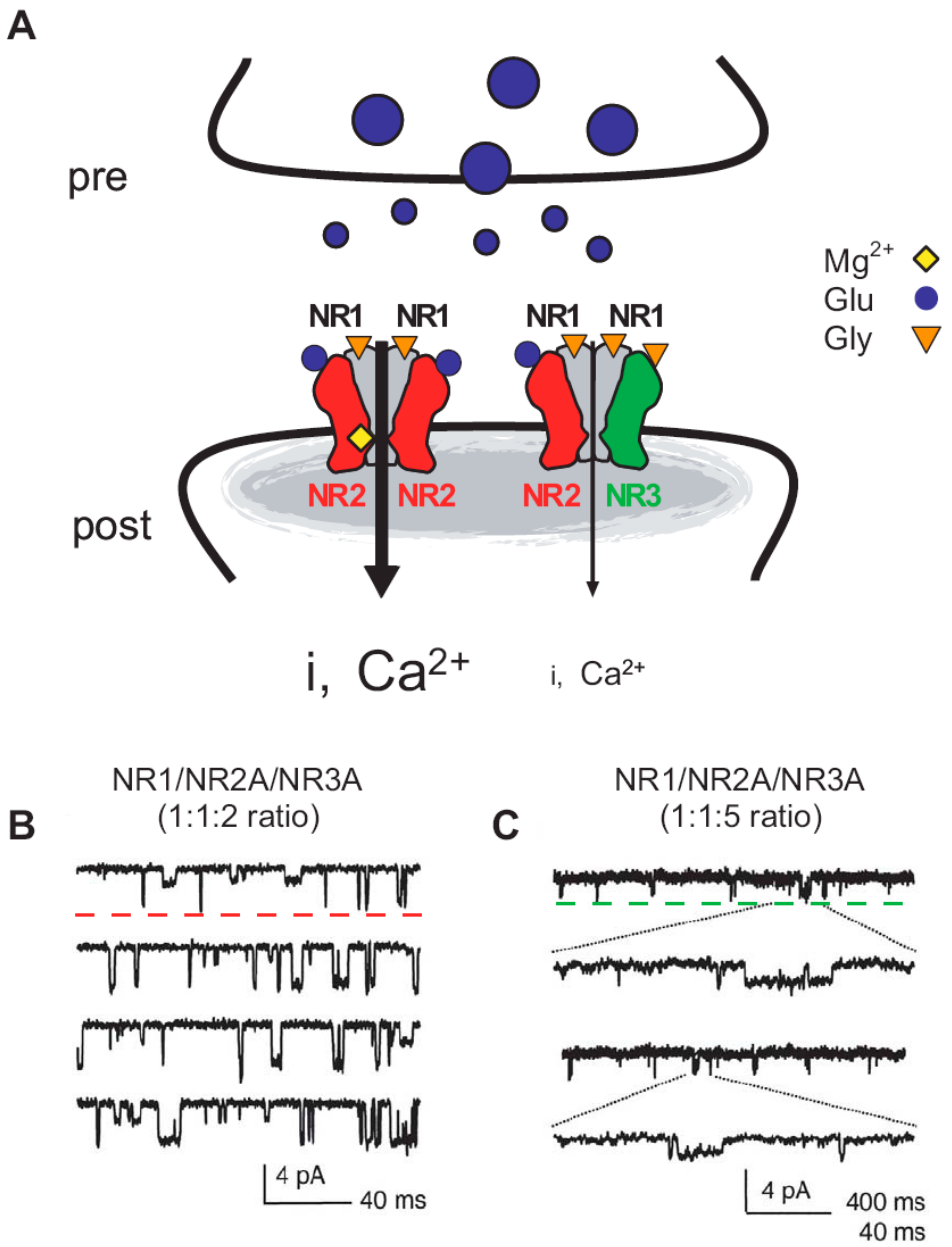


Figure 1.4. The NR3 subfamily decreases NMDAR-mediated neurotransmission. (A) Model demonstrating the influence of the NR3 subfamily on current flux through NMDARs. NMDARs containing the glutamate-binding NR2 subunits (red) are highly permeable to Ca2+ and dependent upon postsynaptic depolarization due to the Mg2+ block. Conversely, NMDARs containing glycine-binding NR3 subunits (green) flux less current ("i"), are less permeable to Ca2+, and are less sensitive to Mg2+ block. This is demonstrated in panels (B) and (C) by single-channel recordings (used with permission from Sasaki et al. (2002). These recordings from outside-out patches are taken from oocytes expressing NR1/NR2A/NR3A from injections of cRNA in a (B) 1:1:2 ratio or (C) 1:1:5 ratio and demonstrate the dominant-negative effects of the NR3 subunits on NMDAR-mediated neurotransmission. Dashed red line in (B) indicates high conductance state of a putative NR1/NR2A-receptor, while dotted green line in (C) indicates low conductance state of a putative NR1/NR2A/NR3Areceptor. Abbreviations: pre, presynaptic bouton; post, postsynaptic spine; Mg2+, magnesium; Glu, glutamate; Gly, glycine; Ca2+, calcium; pA, picoamps; ms, milliseconds

Species	Rodent					Monkey			Human			
Age	Fetal	P0-P3	P4-10	P11-P20	Adult	3 mo	15 mo	Adult	Fetal	0-1yr	1-18yr	Adult
Whole brain	f	a, b	**a, b**	a, b	a, b							
Forebrain		f, h, l	**f, h, l, q**	f, h, l	a, f, h, l, q				q			q
Midbrain	b	a, b, i	a, b, i	a, i, j	a, b, c, i			k				
Hindbrain	b	a, f, i	a, f, i	a, f, i, j	a, c, f, i			k				m
Cortex	b	a, b, i	**a, b, g, i**	a, g, i, j	b, c, i, m	**d**	d	d, k	p, s			m
-EC		a, b	**a, b**	a, b	b, e							
-VC		r	g, r	g, r	e, r							
-FC			g	g, j					d	**d**	d	d, o
Hpc	b	a, b, g, h, i	**a, b, g, i, n**	a, g, i, n	b, c, g, i, j, n			k				
Thalamus	b	a, b	**b, g**	a, b, g, j	b, j			k				m
Str/GP	b, ND	a, ND	a, g	a, g, j	c, j			k				
Amygdala			g	g, j	**j**			k				
Olf. Bulb		a, f, i	a, f, i	a, f, i, j	c, e, f, i							
Spinal Cord	b		**m**		b, m				**m, s**			m

Abbreviations: P, postnatal day; mo, months; yr, year; EC, entorhinal cortex; VC, visual cortex; FC, frontal cortex; Hpc, hippocampus; Str/GP, striatum/globus pallidus; Olf. bulb, olfactory bulb

a, Sucher *et al.*, 1995
b, Ciabarra *et al.*, 1995
c, Goebel and Poosch, 1999
d, Henson *et al.*, 2008
e, Sun *et al.*, 2000
f, Sun *et al.*, 1998
g, Roberts *et al.*, 2009
h, Sasaki *et al.*, 2002
i, Al-Hallaq *et al.*, 2002
j, Wong *et al.*, 2002
k, Mueller and Meador-Woodruff, 2005
l, Perez- Otano *et al.*, 2006
m, Nilsson *et al.*, 2007b
n, Naassila *et al.*, 2002
o, Mueller and Meador-Woodruff, 2004
p, Mueller and Meador-Woodruff, 2003
q, Eriksson *et al.*, 2007b
r, Henson and Philpot, unpublished observations
s, Eriksson *et al.*, 2002

Table 1.1 Developmental profile of NR3A expression patterns in rodent, monkey, and human brain. Consensus results of mRNA and protein expression data are depicted as having high (dark), moderate (gray), weak (light gray), and non-detectable (ND) levels of expression. Boxes with diagonal lines indicate no data available to date. Letters within boxes denote references as matched below. Most studies have been conducted in rodents and reveal a general pattern of low prenatal presence of NR3A that increases dramatically in the first two postnatal weeks, and declines into adulthood. When both isoforms were reported, only data from expression of NR3A-short sequences were considered. Note that this schematic is meant to present a general overview of NR3A expression, but the schematic is unable to depict data reported from specific subregions/cell layers (for these details, see cited manuscripts).

Table 2
NR3A loss-of-function and gain-of-function studies demonstrate reciprocal effects in mutant mice.

Measurement	NR3A-KO	NR3A-OE	References
Spine number and synapse size	↑	↓	Das et al. (1998), Roberts et al. (2009)
Magnitude of NMDAR-mediated currents	↑	↓	Das et al. (1998), Perez-Otano et al. (2001), Sasaki et al. (2002), Tong et al. (2008), Roberts et al. (2009)
NMDAR calcium permeability	↑	↓	Perez-Otano et al. (2001), Sasaki et al. (2002), Sucher et al. (2003), Tong et al. (2008)
Hyperpolarization-mediated block of NMDAR currents by magnesium	↑	↓	Sasaki et al. (2002), Tong et al. (2008), Roberts et al. (2009)
- LTP (3×100 Hz) - LTD (1 Hz; 15 min)	Early developmental onset No change	↓ No change	Roberts et al. (2009)
Cell death after ischemic-hypoxic insults	↑	↓	Nakanishi et al. (2009)
Spatial memory (Morris water maze)	ND	↓	Roberts et al. (2009)
Long-term memory (food preference and object recognition)	ND	↓	Roberts et al. (2009)
Sensorimotor gating (prepulse inhibition to startle)	↑ in males; no change in females	No change	Brody et al. (2005)

Abbreviations: NR3A-KO, NR3A knockout mouse; NR3A-OE, NR3A overexpressor mouse; LTP, long-term potentiation; LTD, long-term depression; Hz, hertz; ND, no data.

CHAPTER 2

NR3A and NR1 in Human Development

Abstract

Subunit composition of NMDA-type glutamate receptors (NMDARs) dictates their function, yet the ontogenic profiles of human NMDAR subunits from gestation to adulthood have not been determined. We examined NMDAR mRNA and protein development in human dorsolateral prefrontal cortex (DLPFC), an area in which NMDARs are critical for higher cognitive processing and NMDAR hypofunction is hypothesized in schizophrenia. Using quantitative RT-PCR and western blotting, we found NR1 expression begins low prenatally, peaks in adolescence, yet remains high throughout life, suggesting lifelong importance of NMDAR function. In contrast, NR3A levels are low during gestation, surge soon after birth, and decline progressively through adolescence and into adulthood. Because NR3A subunits uniquely attenuate NMDAR-mediated currents, limit calcium influx, and suppress dendritic spine formation, high levels during early childhood may be important for regulating neuroprotection and activity-dependent sculpting of synapses. We also examined whether subunit changes underlie reduced NMDAR activity in schizophrenia. Our results reveal normal NR1 and NR3A protein levels in DLPFC from schizophrenic patients, indicating that NMDAR hypofunction is unlikely to be maintained by gross changes in NR3A-containing NMDARs or overall NMDAR numbers. These data provide insights into NMDAR

functions in the developing CNS and will contribute to designing pharmacotherapies for neurological disorders.

Introduction

NMDA-type ionotropic glutamate receptors (NMDARs) are involved in a wide array of biological processes crucial for brain development and function. In addition to modulating neuronal and synapse maturation in development (Perez-Otano and Ehlers, 2004), NMDARs are responsible for short-term and long-term memory storage through mechanisms of synaptic plasticity (Malenka and Nicoll, 1999). For example, many executive functions and working memory tasks require NMDAR activity in the prefrontal cortex (Lisman et al., 1998; Durstewitz and Gabriel, 2007), suggesting that disruption of these receptors could cause profound cognitive deficits (Goldman-Rakic, 1995; Lewis, 1997). Given the diverse roles of NMDARs, it is not surprising that NMDAR dysfunction is thought to underlie several neurological and psychiatric disorders. Accordingly, a major goal in neuroscience is to understand how NMDARs normally change during human development and how this might be altered in disease states.

NMDAR function is dictated by its subunit composition (Monyer et al., 1992). NMDARs are tetramers consisting of essential NR1 subunits in combination with NR2 (A-D) or NR3 (A-B) subunits that provide functional molecular diversity (Perez-Otano et al., 2001; Al-Hallaq et al., 2002; Matsuda et al., 2003). However, unlike the conventional NR1/NR2 receptors, those containing the recently identified NR3 subunits exhibit decreased single-channel conductance, insensitivity to magnesium blockade, and reduced calcium (Ca^{2+}) permeability (Das et al., 1998; Perez-Otano et al., 2001; Chatterton et al., 2002; Sasaki et al., 2002). Because Ca^{2+} influx is responsible for many forms of synaptic

plasticity (Malenka and Bear, 2004) and excitotoxic cell death (Choi, 1988), the inclusion of NR3 subunits into NMDARs is likely to critically regulate the properties of NMDAR-mediated plasticity and may also serve a neuroprotective role.

Developmental regulation of human NMDAR subunits is poorly understood. Such knowledge is important for the rational design of pharmacotherapies for diseases involving NMDAR subunit dysfunction, such as neurodegenerative conditions, stroke, epilepsy, neuropathic pain, and schizophrenia (reviewed in (Cull-Candy et al., 2001; Waxman and Lynch, 2005; Kristiansen et al., 2007). For example, existing treatments for schizophrenia are very limited in their specificity and efficacy (Ross et al., 2006). This disease is thought to arise from decreased NMDAR activity that disrupts normal synaptic connectivity and plasticity, especially within the dorsolateral prefrontal cortex (DLPFC) (Javitt and Zukin, 1991; Olney et al., 1999; Lewis and Levitt, 2002; Coyle et al., 2003; Frankle et al., 2003). While there is little consistent evidence that overall NR1 and NR2 levels differ between schizophrenic patients and controls (Kristiansen et al., 2007), a recent study suggests that NR3A may be elevated within the schizophrenic DLPFC (Mueller and Meador-Woodruff, 2004). Therefore, NR3A's unique ability to suppress NMDAR function makes it an extremely attractive candidate both as an endogenous contributor to the NMDAR hypofunction observed in schizophrenia and as a potential target for pharmacological interventions.

To reveal how NMDAR subunit composition changes through the course of brain development, and to begin to understand whether NMDAR subunits are altered in schizophrenia, we used postmortem tissue to examine NMDAR subunits, NR3A and NR1, during normal human DLPFC development and in the DLPFC of schizophrenic

patients. Additionally, we demonstrate the effects of antipsychotic medications on NMDAR subunits. To our knowledge, we provide the first systematic evaluation of the normal developmental profiles of NMDAR expression in maturation of the human prefrontal cortex from mid-gestation to early adulthood.

Materials and Methods

Postmortem Human Samples

Postmortem human tissue samples were de-identified to protect personal health information. Samples from the developmental cohort contained uniform 1 cm coronal sections of prefrontal cortex (Brodmann areas 9/46) from 45 subjects ranging in age from 18 weeks gestation through 25 years (Table 1). Frozen tissue was obtained from the NICHD-University of Maryland Brain and Tissue Bank for Developmental Disorders under contracts N01-HD-4-3368 and N01-HD-4-3383. Individuals died from non-CNS causes and had no known history of substance abuse or major psychiatric disorders. *A priori*, samples were divided into 7 age groups established in a previous study (Glantz et al., 2007): prenatal (n=6), birth to 12 months of age (n=5), one to five years (n=6), six to ten years (n=6), eleven to fifteen years (n=11), sixteen to twenty years (n=4), and twenty-one to twenty-five years (n=7). The schizophrenia cohort consisted of Brodmann area 9 tissue samples from 35 subjects (control n=20; schizophrenia n=15) obtained from the Harvard Brain Tissue Resource Center (McLean, MA; supported in part by PHS grant number R24 MH068855), with ages ranging from 21 to 80^{+} years (Table 2). To protect personal health information, exact ages were used for statistical purposes, but only age ranges of subjects can be published. Prefrontal cortex samples in the schizophrenia and control groups were group-matched for age, gender, ethnicity, side-of-brain, brain pH,

and postmortem interval (PMI). All fresh frozen tissue blocks from the UMD and Harvard collections were chipped from larger frozen cortical slabs and consisted primarily of gray matter with small amounts of underlying white matter. The samples from the UMD tissue collection were harvested as 1 cm coronal slabs. Cuts were made with guidance from a neuroanatomical atlas to select Brodmann areas 9 and 46, and immediately frozen at -80°C. Cytoarchitectonic localizations of Brodmann areas 9 and 46 were ascertained using Nissl staining of sections cut immediately adjacent to tissue blocks used for this study (see (Glantz et al., 2007) for details). Samples of Brodmann area 9 from the Harvard tissue were obtained through uniform dissection from the superior prefrontal gyrus according to the Brodmann map. All tissue was stored frozen at -80°C until use. Tissue pH was measured as described (Salimi et al., 2008) and samples with pH < 5.8 were excluded from analysis. This study was approved by the Biomedical Institutional Review Board of the University of North Carolina at Chapel Hill.

Animal Use

The animal use in this study was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. All rodents were maintained and sacrificed according to protocol guidelines.

Postmortem Stability of NMDAR Proteins

Eighteen C57BL/6 mice at age postnatal day 10 (P10) were divided into three equal groups, anesthetized with a lethal dose of sodium pentobarbital, and decapitated upon disappearance of corneal reflexes. In the first group, frontal cortex was dissected and immediately frozen at -80°C (PMI = 0 hrs). To approximate the human postmortem interval and simulate morgue conditions, heads of the second and third groups were kept

at room temperature for 6 hrs, then at 4°C for 6 hrs and 18 hrs, respectively, after which time frontal cortices were dissected (PMI = 12 hrs and PMI = 24 hrs) and frozen at -80°C until use in immunoblotting analysis (Jarskog et al., 2004).

Antipsychotic Treatment

Sub-chronic drug treatments were administered to thirty-six singly housed, male Sprague–Dawley rats (150–200g, Charles River) as described previously (Jarskog et al., 2007). Briefly, animals received daily intraperitoneal injections of haloperidol (1mg/kg/day, n=12), clozapine (10 mg/kg/day, n=12), or saline (0.9%, n=12) for 4 weeks. One hour after the final dose, rats were killed and anterior right medial frontal cortices were dissected. All tissue was kept frozen at -80°C until use in immunoblotting analysis.

RNA Extraction, cDNA Preparation, and Quantitative RT-PCR

Total RNA was extracted from pulverized human developmental PFC tissue samples using the RNeasy Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RNA concentrations and quality (mean RNA Integrity Number = 8.75 ± 1.0) were determined using Series II RNA 6000 Nano Assay in the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). RNA was reverse transcribed using the High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Relative expression levels of NMDAR mRNAs were measured by quantitative RT-PCR using pre-validated assays for NR1, NR3A, and GUSB (β-glucuronidase, which served as the endogenous control) (Applied Biosystems Assays-on-Demand: Hs00609557_m1 for NR1, Hs00370290_m1 for NR3A, and 4333767F for GUSB). All probes had FAM reporter dye and MGB quencher. The NR1 primer-probe combination spanned exon boundary 1-2, covering all eight splice variants,

and the NR3A assay covered exon boundary 3-4. Pilot experiments using cDNA dilutions were used to define the dynamic range of each assay. GUSB was selected from several candidate reference genes as the endogenous control most closely matching in expression level to the target NMDAR genes. Reactions were run in a 384-well plate format on an ABI 7900HT Fast RT-PCR sequence detection system (Applied Biosystems). Each 20 μl reaction contained 9 μl diluted cDNA (NR1: 6.25ng; NR3A: 12.5 ng; GUSB: 6.25ng or 12.5ng), 0.9 μM each primer, 0.25 μM probe, and 10 μl TaqMan Universal PCR 2X MasterMix with AmpErase UNG (Applied Biosystems) with MGB/TAMRA 5' endonuclease and ROX passive reference dye. PCR cycle parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 60 sec. Each examined mRNA was quantified from a single plate and C_T values were within the linear range of the standard curve. All samples were loaded in triplicates. Reverse transcriptase was omitted from control reactions for each tissue sample to verify the absence of amplified genomic DNA. The same threshold and baseline were used for all samples. Three of the 45 human brain samples did not meet our inclusion criteria for the mRNA quantification and were thus omitted from analysis.

Cortical Brain Extracts

Homogenates were prepared from postmortem human, monkey, mouse, and rat frontal cortices. Tissues were homogenized (1:10, w/v) on ice for 30 sec (PowerGen 125, Fisher Scientific, Pittsburgh, PA) and sonicated for 10 sec at 10 mV (Sonic Dismembrator 60, Fisher Scientific) in ice-cold 50 mM Tris-HCl buffer (pH 7.4) with 0.6 M NaCl, 0.2% Triton X-100, 1 mM benzamidine, 0.1 mM benzethonium chloride, and 0.1 mM PMSF (Sigma, St Louis, MO). Samples were cleared of debris by centrifugation

at 4°C for 15 min at 15,000 x *g* and supernatants were assayed for total protein in triplicate using the bicinchoninic acid method (Micro BCA Protein Assay Kit, Pierce Chemical, Rockford, IL). Aliquots were stored at -80°C until use.

Immunoblotting

Equal amounts of total protein (25-50 μg) were heated for 10 min at 70°C in sample buffer and applied to 10-lane 4-12% gradient NuPAGE Novex tris-glycine mini-gels (Invitrogen, Carlsbad, CA), along with molecular weight markers, a pooled sample homogenate, and a mouse frontal cortex sample as positive control. Coded samples were loaded randomly onto gels to preserve the integrity of blinded, unbiased data analyses. Samples were resolved by SDS-PAGE at 125V for two hrs. Separated proteins were then electrophoretically transferred (Bio-Rad, Hercules, CA) to nitrocellulose membranes (Millipore, Billerica, MA) at 25V overnight at 4°C. After eliminating nonspecific protein binding to membranes with blocking buffer (Odyssey, LI-COR, Lincoln, NE) for 1 hr at room temperature, membranes were cut into three strips with the aid of molecular size markers. Because the Odyssey Imaging System uses two near-infrared channels to detect fluorescent signals, two proteins can be simultaneously probed on each blot strip. Thus, six proteins were able to be detected from the same gel (upper blot strip, NR2B/NR2A; middle, NR3A/NR1; lower, β-tubulin/GAPDH). This approach allowed us to analyze multiple proteins while avoiding complications arising from stripping and re-probing membranes. The following primary antibodies were incubated overnight at 4°C in 1:1 blocking buffer:phosphate-buffered saline (PBS), at optimized concentrations: rabbit anti-pan NR1 (#SC9058, 1:6000, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-NR1 (#556308, 1:5000, BD/Pharmingen, San Jose, CA), rabbit anti-NR2A

(#SC9056, 1:500, Santa Cruz), goat anti-NR2B (#SC1469, 1:10,000, Santa Cruz), mouse anti-NR3A (#MAB5388, 1:1000, Chemicon, Temecula, CA), rabbit anti-NR3A (#07-356, 1:500, Millipore/Upstate), goat anti-GAPDH (#IMG3073, 1:40,000, Imgenex, San Diego, CA), and mouse anti-β-tubulin (#MAB3408, 1:100,000, Millipore/Chemicon). Membranes were washed extensively in PBS-0.1% Tween 20 and then incubated at room temperature for 1 hr (shielded from light) with appropriate secondary antibodies diluted in 1:1 blocking buffer/PBS-0.1% Tween 20: Alexa Fluor 680-labeled anti-goat IgG or anti-mouse IgG (1:5000, Molecular Probes/Invitrogen), and IRDye 800-labeled anti-rabbit IgG (1:3000, Rockland Immunochemicals, Gilbertsville, PA). Membranes were washed and fluorescent signals measured directly on the Odyssey Infrared Imaging System (LI-COR). Band density analysis was performed using Odyssey software (v2.1) supplied by the manufacturer. Antibody specificities for the following have been previously established: rabbit anti-pan NR1 (Fernandez-Monreal et al., 2004; Miyamoto et al., 2005; Offenhauser et al., 2006; Talbot et al., 2006; Li et al., 2007), mouse anti-NR1 (Siegel et al., 1994; Siegel et al., 1995; Wood et al., 1995; Lack et al., 2005; Stepulak et al., 2005; Perez-Otano et al., 2006; Welch et al., 2007), rabbit anti-NR3A (Ishihama and Turman, 2006), rabbit anti-NR2A (Miyamoto et al., 2005; Yashiro et al., 2005), and goat anti-NR2B (May et al., 2004; Pawlak et al., 2005; Yashiro et al., 2005; Czaja et al., 2006). Mouse anti-NR3 antibody produces no band at the expected molecular weight (~130 kD) in NR3A knockout versus wild-type cortical homogenates (data not shown). Antibody signal intensities were collected from three separate experiments for each sample. A standard curve of increasing protein amounts of pooled sample was

immunoblotted for each protein to ensure a linear relationship between increasing total protein amounts and density of the respective bands (data not shown).

Data Collection and Statistical Analyses

To assess mRNA levels for each human target gene, GUSB was used as the internal reference gene and relative quantities were obtained by the ΔΔCT method using Sequence Detection Software (SDS v.2.2.2, Applied Biosystems). Western blot experiments measured antibody signal intensities in postmortem tissue, interleaving control and experimental conditions where appropriate. A pooled sample was run on each gel and the band density of each experimental sample was measured relative to this reference standard to permit inter-gel comparisons. Samples were run in triplicate experiments, and individual antibody values for each sample were averaged.

Developmental age group or diagnosis group means were calculated from individual averages. Group means were normalized either to the mean level of expression for the maximally-expressing age group (developmental studies) or to the control group (schizophrenia and antipsychotic drug studies). For multiple group comparisons, one-way analyses of variance (ANOVAs) were performed, followed by between-group comparisons with Tukey-Kramer tests. Unpaired Student's t-tests were used to compare diagnosis between schizophrenic subjects and normal controls with NMDAR proteins as variables. Two-way ANOVAs were performed post hoc to probe interactions between diagnosis and sex. All levels of significance represent two-tailed values. Statistical analyses were conducted using Graphpad Instat (San Diego, CA) and SAS (Cary, NC). Developmental brain tissues binned into seven groups based on age were not significantly different with regard to pH or sex; however, significant differences

were observed between the groups due to PMI (ANOVA, $F_{(6,38)}= 8.11$, $p < 0.0001$) and storage time (ANOVA, $F_{(6,38)}= 8.08$, $p < 0.0001$) (see Table 2.1). Therefore, we included PMI and storage time in secondary ANCOVA analyses with these variables as covariates. While brain tissues from normal controls and schizophrenic patients had no significant differences between groups with regard to age, pH or PMI, a significant difference was detected between the groups due to storage time. No secondary analyses were performed for the schizophrenia study.

Results

NR1 and NR3A, but not NR2A and NR2B, protein levels can be studied effectively in postmortem tissue

A difficulty of studying human tissue is that protein degradation occurs during the postmortem interval (PMI) after death and prior to tissue preservation. Therefore, it is necessary to assess how PMI affects antibodies' recognition of target sites. To test this, we used a mouse model to mimic the decay of human tissue under simulated morgue conditions (Jarskog et al., 2004). Protein integrity was measured by immunoblotting of cortical homogenates run in triplicate **(Figure 2.1)**. Antibodies directed against NMDA receptor subunits NR3A (125kD), NR2A (165kD), and NR2B (170kD), as well as loading controls, β-tubulin (55kD) and GAPDH (36kD), all detected single bands at the appropriate molecular weights, consistent with predicted sizes. The double band for NR1 (116kD) may be the result of splice variants, deglycosylation, or proteolytic cleavage products (Monyer et al., 1992; Brose et al., 1993; Sheng et al., 1994; Zukin and Bennett, 1995; Luo et al., 1996).

Immunoblotting revealed that NR1 and NR3A proteins displayed modest and predictable decreases in band densities, with no main effects noted by ANOVA (NR1, n=4/group, $F_{(2,9)}$=3.01, p=0.10; NR3A, n=4/group, $F_{(2,9)}$=2.89, p=0.11). Moderate protein degradation was also observed for the loading controls, although the decrease in β-tubulin reached statistical significance (β-tubulin, n=4/group, $F_{(2,9)}$=6.27, p=0.02; GAPDH, n=2/group, $F_{(2,3)}$=7.06, p=0.07). Unlike NR1 and NR3A, however, degradation of both NR2A and NR2B subunits was substantial and progressive, with total decreases in signal intensities of 69% and 75%, respectively. ANOVA demonstrated that there was a main effect of PMI on NR2A and NR2B levels (NR2A, n=4, $F_{(2,9)}$=18.91, p<0.001; NR2B, n=4, $F_{(2,9)}$=30.74, p<0.0001), and post hoc analyses revealed that protein levels were significantly reduced at both 12 and 24 hr PMI compared to baseline (PMI=0) values. This effect of postmortem interval on NR2A and NR2B is consistent with the rapid proteolysis of NR2 subunits soon after death in human tissue (Wang et al., 2000), and is also consistent with difficulties in reliably measuring NR2A and NR2B levels in rhesus monkeys (O'Connor et al., 2006) and in human tissue (current study, data not shown). For these reasons, we performed no further analyses of NR2A and NR2B in human tissue. Furthermore, to minimize problems arising from studying tissue taken after a long PMI, we limited our analysis to tissue with a relatively low PMI (mean PMI ~ 13 and 21 hours for the developmental and schizophrenia studies, respectively).

Our mouse data indicate that NR1 and NR3A subunits exhibit relatively high postmortem stability (>65%) for at least 24 hours, suggesting that they are also likely to be more stable in human tissue than NR2 subunits. Importantly, our pilot experiments demonstrated that in both mouse and human tissue, the NR1 and NR3A antibodies

recognized bands consistent with predicted sizes at 116kD and ~130kD, respectively. This indicates that these antibodies similarly recognize mouse and human NMDAR subunit homologues at their expected molecular weights. Assuming the postmortem stability of these proteins is similar in mice and humans, our data indicate that the NR1 and NR3A antibodies used in this study are appropriate to study these NMDAR subunits in human postmortem tissue.

In addition to PMI, another important consideration in western blotting of postmortem tissue is accurate protein band measurement. Typically, signal intensity for the protein of interest is measured relative to an in-lane reference, a loading control such as GAPDH or β-tubulin. However, as both of these common proteins varied considerably in their expression levels across development (data not shown), they were inappropriate for our studies. To overcome this limitation, we instead standardized protein levels of each sample to a homogenate pool that was included on every gel as a reference (see Methods), and allowed for inter-gel comparisons. Other studies have found similar methods to be reliable alternatives for quantification (Quinlan et al., 1999; Folkerth et al., 2004; Haynes et al., 2005; Murphy et al., 2005; Glantz et al., 2007; Salimi et al., 2008). GAPDH and β-tubulin normalizations were then used only for confirmatory analyses of adult samples in the schizophrenia study **(Supplementary Figures S2.4 and S2.5)**, which had similar levels of these loading control proteins across groups. Thus, unless otherwise noted, all analyses were performed on protein levels standardized to the pooled sample that was run on each gel. Moreover, protein data from each sample was averaged from three independent western blotting experiments, as this further eliminated the possibility of loading errors.

Developmental expression of NR3A peaks in early childhood in human DLPFC

Studies in rodents indicate that expression of the non-conventional NMDAR subunit, NR3A, is upregulated soon after birth, peaks during early postnatal life (around postnatal day 7; P7), then decreases through the subsequent weeks in many regions of the brain (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Sun et al., 1998; Al-Hallaq et al., 2002; Sasaki et al., 2002; Wong et al., 2002; Ishihama and Turman, 2006; Perez-Otano et al., 2006). We hypothesized that NR3A expression in humans is also upregulated early in development and downregulated in adolescence. To test this, we determined NR3A transcript levels by quantitative RT-PCR of postmortem human tissue from individuals aged 18 wks gestation to 25 years (see **Table 2.1** and Methods). Our studies focused on the DLPFC, an area implicated in higher cognitive function, and in which NMDAR hypofunction has been hypothesized in the pathophysiology of schizophrenia.

Consistent with previous rodent studies, we found highly regulated expression of NR3A during prenatal and postnatal cortical development for both mRNA and protein levels **(Figure 2.2)**. NR3A mRNA expression was shown to be significantly regulated across the various age groups (ANOVA: $F_{(6, 35)} = 8.60$, $p<0.0001$). Post hoc analyses indicate that NR3A transcript levels are significantly reduced prenatally compared to the 0-1 yr group ($p<0.0001$), the 1-5 yrs group ($p<0.05$), and the 11-15 yrs group ($p<0.01$). Furthermore we observed a statistically significant decline in NR3A from the peak expression of NR3A mRNA (0-1 yr group) compared to the 1-5 yrs group ($p<0.02$), the 6-10 yrs group ($p<0.001$), the 11-15 yrs group ($p<0.02$), the 16-20 yrs group ($p<0.01$), and the 21-25 yrs group ($p<0.0001$). These data demonstrate that NR3A transcription is

weak embryonically, increases dramatically in the first year of life, and then declines progressively into adulthood. NR3A mRNA expression levels increased 10-fold from the fetal samples as compared to the age group with peak expression. In a secondary analysis, we performed an ANCOVA covarying PMI and storage time across the different age groups (ANOVA: $F_{(6, 33)} = 6.84$, $p<0.0001$). The results were largely consistent with the previous analysis run without covariates, as post hoc tests indicated that fetal tissue was significantly reduced compared to the same older age groups as described above ($p<0.05$). Moreover, the peak expression of NR3A mRNA (0-1 yrs) was significantly greater than that at 16-20 yrs ($p<0.02$) and 21-25 yrs ($p<0.01$). Unlike the primary analysis, peak expression compared to 1-5 yrs, 6-10 yrs, and 11-15 yrs age groups was not significant.

Strikingly, developmental changes in NR3A protein abundance were qualitatively similar to that of NR3A mRNA levels. After low prenatal expression, a sharp postnatal increase in band densities was followed by a progressive reduction in NR3A protein levels through childhood and adolescence **(Figure 2.2)**. A one-way ANOVA detected a significant effect of age on NR3A expression ($F_{(6,38)}=5.60$, $p<0.001$). Subsequent Tukey-Kramer post hoc tests revealed that prenatal and young adult (ages 21-25 yrs) tissues expressed significantly less NR3A than infants aged 0-1 yr ($p<0.001$ and $p<0.01$, respectively). Also, prenatal values differed significantly from age groups 1-5 and 6-10 (both $p<0.05$). The results of an ANCOVA covarying PMI and storage time across the different age groups (ANOVA: $F_{(6, 36)} = 4.72$, $p<0.002$) and post hoc tests were consistent with the previous analysis, indicating that even with PMI and storage time

taken into account, the developmental differences we observed were robust and still significant.

To examine NMDAR expression changes in another relevant model system where postmortem protein decay was not a potential confound, we immunoblotted NMDAR subunit proteins in developing postnatal macaque temporal cortex **(Supplementary Figure S2.1A)**. Postmortem frozen cortical brain tissue from 17 rhesus monkeys (*Macaca mulatta*) ages 3 months to 8 years were obtained from D.A. Lewis (University of Pittsburgh). Although we were unable to acquire fetal macaque tissue, our findings in monkeys show similar developmental changes as those observed in human postnatal NR3A protein expression **(Figure 2.2)**, with high expression in infancy that tapers off into adulthood.

NR1 levels are relatively high in the developing human DLPFC

As the obligatory subunit of the NMDA-type ionotropic glutamate receptor (Monyer et al., 1992; Perez-Otano et al., 2001; Matsuda et al., 2003), NR1 serves as an accurate gauge of the total number of NMDARs. Transcript and protein levels of NR1 have been reported in various brain regions in humans (Zhong et al., 1995; Akbarian et al., 1996; Scherzer et al., 1998; Law et al., 2003; Clinton et al., 2006; Kristiansen et al., 2006) and in rodents (Monyer et al., 1994; Laurie et al., 1997; Goebel and Poosch, 1999; Prybylowski and Wolfe, 2000; Sun et al., 2000; Ritter et al., 2002; Babb et al., 2005). However, to our knowledge, no study has examined NR1 expression over development in human prefrontal cortex.

To determine the normal developmental profile of NR1 mRNA transcript and protein levels in the DLPFC, we again turned to quantitative RT-PCR and

immunoblotting **(Figure 2.3)**. NR1 mRNA expression was shown to be significantly regulated across the various ages (ANOVA: $F_{(6, 35)} = 8.26$, $p < 0.0001$). As in the NR3A qPCR assays, there was a 10-fold increase in NR1 mRNA expression from prenatal to maximal postnatal levels. Post hoc analyses revealed that in the group with the lowest mRNA content (fetal samples), NR1 was significantly ($p < 0.001$) reduced compared to samples from the age group in which transcript levels were maximal, the 11-15 yrs group. Furthermore, samples from the 0-1 yr group ($p < 0.01$), the 1-5 yrs group ($p < 0.05$), and the 6-10 yrs group ($p < 0.05$) also were significantly reduced compared to the 11-15 yrs group. In a secondary analysis, we performed an ANCOVA covarying PMI and storage time across the binned age groups (ANOVA: $F_{(6, 33)} = 6.73$, $p < 0.0001$). As with the NR3A secondary analyses, these results were consistent with the primary analysis as well as subsequent post hoc analyses, suggesting that PMI and storage time were independent from the developmental differences we observed.

Western blot analyses of frontal cortical homogenates prepared from the developmental samples demonstrate robust expression of NR1. Consistent with NR1 mRNA, protein levels are low prenatally and rise gradually to early adolescence and then decline modestly into adulthood **(Figure 2.3)**. Protein expression of NR1 was found to be significantly regulated across developmental groups (ANOVA: $F_{(6, 38)} = 3.98$, $p < 0.005$). Post hoc tests indicate that the prenatal group was significantly ($p < 0.01$) reduced compared to the 11-15 yrs group, and that there is a significant reduction ($p < 0.02$) of NR1 protein levels when comparing the 11-15 yrs group to the 21-25 yrs group. This reduction in adult tissue was not observed with NR1 mRNA, suggesting that protein expression may be regulated differently in maturity. An ANCOVA covarying

PMI and storage time across the different age groups found that NR1 mRNA expression was significantly regulated across developmental groups (ANOVA: $F_{(6, 36)} = 2.86$, $p<0.05$). However, post hoc tests revealed that, unlike the previous analysis, fetal tissue failed to differ significantly from the 11-15 yrs group. The observed significant ($p<0.01$) decline in protein levels comparing the 11-15 yrs group to the 21-25 yrs group was maintained when including PMI and storage time as covariates.

In a parallel study of cortical development, we probed fresh-frozen postmortem tissue from developing postnatal macaque cortex for NR1 protein (generous gift from D.A. Lewis, University of Pittsburgh) **(Supplementary Figure S2.1B)**. Although not statistically significant, developmental expression levels of NR1 in monkeys appeared similar to that in humans, with high levels exhibited until a drop during late puberty.

Because NR1 is essential for all functional NMDARs (Monyer et al., 1992; Perez-Otano et al., 2001), the ratio of NR3A to NR1 provides an estimate for the proportion of NR3A-containing receptors out of the total pool of NMDARs **(Supplementary Figure S2.2)**. By this measure, the NR3A/NR1 mRNA ratio was shown to be significantly regulated across the various age groups (ANOVA: $F_{(6, 35)} = 14.66$, $p<0.0001$). Post hoc analyses indicate that peak NR3A/NR1 ratio of transcript levels (0-1 year of age) were significantly different from all other age groups. NR3A/NR1 levels are significantly reduced prenatally compared to the 0-1 yr group ($p<0.0001$). Furthermore we observed a statistically significant decline in NR3A/NR1 ratio from peak expression (0-1 yr group) compared to the all other age groups ($p<0.0001$). In a secondary analysis, we performed an ANCOVA with PMI and storage time as covariates across the different age groups

(ANOVA: $F_{(6, 33)} = 13.09$, $p<0.0001$). The results of this analysis were consistent with the previous analysis run without covariates.

The ratio of NR3A/NR1 protein similarly changed over development **(Supplementary Figure S2.2)**. A one-way ANOVA detected a significant effect of age on NR3A/NR1 ($F_{(6,38)}=7.81$, $p<0.0001$). Subsequent Tukey-Kramer post hoc tests revealed that prenatal tissue had a significantly lower NR3A/NR1 ratio than infants aged 0-1 yr and ages 1-5 ($p<0.0001$ and $p<0.01$, respectively). Furthermore, the maximum ratio of NR3A/NR1 (0-1 yr) differed significantly from all other age groups ($p<0.01$) except 1-5 yrs. From an ANCOVA covarying PMI and storage time across the different age groups (ANOVA: $F_{(6, 36)} = 7.27$, $p<0.0001$) our results were largely consistent with the previous analysis, as well as subsequent post hoc analyses. Of the differences we observed, fetal expression was no longer significantly different from the 1-5 yrs age group and maximal expression did not differ substantially from the 6-10 yrs age group. Overall, this impressive age-dependent regulation of NR3A/NR1 mRNA and protein levels underscores how vital the understanding of the ontogeny of these subunits will be to investigations of how their dysregulation contributes to neurodevelopmental disorders, such as schizophrenia or bipolar disorder (Mueller and Meador-Woodruff, 2004).

NR1 and NR3A protein levels are unchanged in schizophrenic DLPFC

A prominent theory of schizophrenia suggests that the disease may arise from NMDAR hypofunction in the prefrontal cortex (Javitt and Zukin, 1991; Tamminga, 1998; Weickert and Weinberger, 1998; Olney et al., 1999; Lewis and Levitt, 2002; Coyle et al., 2003; Frankle et al., 2003). However, studies examining NR1 levels in schizophrenia have found conflicting results (reviewed in (Kristiansen et al., 2007)), suggesting that

possible changes in NR1 levels need further evaluation. To test whether protein expression of NR1 is abnormal in the schizophrenic DLPFC compared to tissue from comparison subjects **(Table 2.2)**, we probed immunoblots of human tissue homogenates to measure NR1 protein **(Figure 2.4)**. A two-way ANOVA (diagnosis x sex) revealed no significant main effects on NR1 expression (diagnosis $F_{1,31)}$=2.68, p=0.11; sex $F_{(1,31)}$=0.08; p=0.78). However, there was an interesting trend for an interaction between diagnosis and sex ($F_{(1,31)}$=3.32, p=0.08) **(Supplementary Figure S2.3)**, indicating that it might be worthwhile for future studies to investigate sex differences in NR1 expression in schizophrenic and control subjects.

To our knowledge, the NR3A subunit has been evaluated for a role in schizophrenia in only one study, which demonstrated that NR3A mRNA levels are significantly increased by 32% within subregions of the DLPFC in schizophrenic patients (Mueller and Meador-Woodruff, 2004). We hypothesized that schizophrenia could arise in part because of a failure of NR3A to downregulate during development. Because NR3A suppresses calcium entry and NMDAR-mediated currents, higher than normal NR3A levels would be expected to cause NMDAR hypofunction. Thus, we sought to determine whether NR3A levels were increased at the protein level in the DLPFC of the schizophrenic brain **(Figure 2.4)**. Contrary to our hypothesis, there was no main effect of diagnosis by a two-way ANOVA ($F_{(1,31)}$=0.53, p=0.47), indicating that NR3A levels are similar in control and schizophrenic DLPFC. However, there was a significant main effect of sex ($F_{(1,31)}$=4.21, p<0.05) (Supplementary Fig. S3), suggesting that NR3A expression in the DLPFC is lower in females compared to males. Storage time and PMI were not statistically controlled because no significant differences were observed

between men and women regarding these variables. Although these findings are intriguing, limited tissue availability precluded our ability to further explore the possible gender-related regulation of NR3A. To demonstrate that the lack of effect of NR1 and NR3A on schizophrenia was not a consequence of our standardization procedure, we also show similar results by standardizing band densities to loading controls, β-tubulin and GAPDH (see **Supplementary Figures S2.4 and S2.5**).

Antipsychotic drugs fail to alter NMDAR subunit expression

Antipsychotic drugs are standard treatments for schizophrenic patients, primarily producing direct antagonistic effects on the dopamine and serotonin systems. For example, haloperidol, a typical antipsychotic, is a potent dopamine D2-like receptor antagonist, whereas clozapine, an atypical antipsychotic, blocks not only D2 but also serotonin receptors. Many drugs also interact directly with the glutamate system, binding to NMDARs (Ilyin et al., 1996; Gallagher et al., 1998) and affecting NMDAR expression and activity (Ossowska et al., 1999; Leveque et al., 2000; Ossowska et al., 2000; Schmitt et al., 2003; Bressan et al., 2005; O'Connor et al., 2006). It was therefore important that we examine how antipsychotics affect the expression of NMDAR subunits.

To evaluate the possibility that differences in NMDAR subunits between controls and schizophrenics might have been "normalized" by antipsychotic drug usage, we measured NMDAR subunit proteins in frontal cortical tissue from sub-chronic drug-treated rats (Jarskog et al., 2007) **(Figure 2.5)**. Adult rats were injected for four weeks with saline, haloperidol, or clozapine, and immunoblotting was used to establish protein levels from cortical homogenates. In our study, the drug treatments failed to modify NMDAR subunit levels, as indicated by ANOVA (**Figure 2.5**; $0.29 < F_{(2,32)} < 1.16$, $p > 0.32$

for all subunits), suggesting that antipsychotic treatments are unlikely to have affected NR1 and NR3A protein levels observed in schizophrenic patients.

Discussion

To our knowledge, this report provides the first evidence, in any region of the human brain, for age-dependent differences in NMDAR expression spanning the range from gestation to early adulthood. We performed quantitative analyses of NMDAR subunit mRNA and protein in postmortem human brain sections from the DLPFC. Specifically, we demonstrate (1) robust developmental regulation of NR3A and moderate developmental regulation of NR1, (2) close associations in abundance of NR3A and NR1 transcript and protein levels, and (3) strong parallels to previous findings in developing cortex in other mammalian systems. Our results show that NR3A levels are low prenatally, surge after birth, and then decrease progressively into adulthood. These data indicate that NR3A serves a prominent role in the development of the prefrontal cortex soon after birth, and its role is likely less prominent prenatally and in adulthood. In contrast, NR1 levels rise from prenatal levels and vary only modestly over development, supporting a lifelong importance of NMDAR-dependent functions, including many forms of learning and memory. The defined expression patterns of these particular subunits will increase our understanding of NMDAR-mediated processes during ontogeny, will aid studies of NMDAR dysfunction, and will guide the rational design of subunit-specific NMDAR pharmacotherapies for neurological disorders.

Importance of age-dependent changes in NMDAR subunits to normal human development

NMDAR subunits exhibit remarkable heterogeneity of expression. The significance of this molecular diversity is poorly understood in humans, in part due to a lack of knowledge of how these subunits change during development. Compared to the rich literature describing developmental regulation of NMDAR subunits in animal models, human studies have been largely limited to investigations at the mRNA level (Law et al., 2003). Studies measuring mRNA must be interpreted cautiously because protein and mRNA levels are not always well correlated (Luo et al., 1996; Philpot et al., 2001b). Thus, we felt it essential to accurately describe both NMDAR mRNA and protein levels in the human brain. By using quantitative RT-PCR (Mimmack et al., 2004) and infrared immunoblot imaging, we obtained highly sensitive detection of mRNA and protein levels from postmortem tissues. Additionally, the samples were binned into developmental age groups based on models of cortical development, which was a hypothesis-driven measure to enable us to overlay the data onto findings from prior investigations (Glantz et al., 2007; Salimi et al., 2008).

While NR1 is obligatory for NMDAR function, the specific properties of NMDARs are shaped by the combination of NR1 with NR2 and/or NR3 subunits. NR3A is the most recently described NMDAR subunit, and consequently its influence on NMDAR properties is less well-defined compared to the NR2 subunits. However, interest in this unique subunit has grown recently with exciting observations that it acts in a novel, dominant-negative manner to reduce calcium influx and the unitary conductance of NMDAR currents (Ciabarra et al., 1995; Sucher et al., 1995; Perez-Otano et al., 2001; Sasaki et al., 2002; Matsuda et al., 2003), thereby suppressing NMDAR function. Here we show that in human prefrontal cortex NR3A levels peak during early childhood and

then decrease into adulthood. Animal studies have established a similar developmental pattern of NR3A expression (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Sun et al., 1998; Al-Hallaq et al., 2002; Sasaki et al., 2002; Wong et al., 2002; Ishihama and Turman, 2006; Perez-Otano et al., 2006), suggesting that this might be a general feature of mammalian brain development.

What functions might high levels of NR3A have during early development? Based on *in vitro* and *in vivo* studies in animal models, at least six non-mutually exclusive possibilities exist for the function of NR3A during DLPFC development. (1) Given that genetic deletion of NR3A in mice increases spine density (Das et al., 1998), NR3A may regulate the formation or loss of dendritic spines, the major sites of excitatory synapses. Indeed, massive spinogenesis and synaptogenesis in infancy and early childhood, as well as synapse elimination in adolescence, have been demonstrated in both human (Huttenlocher, 1979; Bourgeois et al., 1994; Huttenlocher and Dabholkar, 1997; de Graaf-Peters and Hadders-Algra, 2006; Glantz et al., 2007) and non-human primate prefrontal cortex (Rakic et al., 1986; Bourgeois et al., 1994; Anderson et al., 1995; Gonzalez-Burgos et al., 2007). (2) Because NR3A limits NMDAR-mediated calcium entry and its expression is elevated during a period of intense programmed cell death, NR3A is positioned to actively influence apoptosis by attenuating calcium-mediated excitotoxicity (Lipton and Nakanishi, 1999). On the other hand, since NMDAR antagonism can also lead to cell death (Ikonomidou et al., 1999), excessive NR3A levels could actually promote apoptosis. Thus, the relative balance of NR3A expression might serve to control which cells are targeted for cell death versus survival. (3) Calcium is a critical mediator of both long-term depression and potentiation, which are thought to be

mechanistic substrates for learning and memory (Malenka and Bear, 2004). NR3A-dependent control of calcium entry would be expected to dramatically shape the properties of NMDAR-mediated plasticity, which could be revealed in future studies through mutant mice that either lack or overexpress NR3A (Das et al., 1998; Sucher et al., 2003; Brody et al., 2005). (4) Exciting new data demonstrate that NR3A-containing receptors appear to undergo rapid endocytosis that is regulated in an activity-dependent manner by PACSIN1/syndapin1 (Perez-Otano et al., 2006). Thus, NR3A may be important for clearing immature synaptic NMDARs so that they can be replaced by more mature receptors. (5) NR3A specifically forms a signaling complex with PP2A (Chan and Sucher, 2001), a phosphatase that can dephosphorylate NR1 subunits on serine 897. NR3A may indirectly modulate NMDAR function through this interaction, and thus provide bidirectional control of synaptic activity. (6) Uniquely, NR3A-containing receptors lack strong blockade by magnesium at hyperpolarized potentials (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Al-Hallaq et al., 2002; Sasaki et al., 2002). Thus, while speculative, a novel role for NR3A-containing NMDARs during early life might be to support synaptic transmission at 'silent synapses' before there is an activity-dependent mobilization of AMPA receptors to the synapse during maturation (Durand et al., 1996; Wu et al., 1996; Isaac et al., 1997; Rumpel et al., 1998; Zhu et al., 2000; Plitzko et al., 2001).

The observation that expression levels of the essential NR1 subunit increase progressively from prenatal stages and remain relatively high throughout life is perhaps not surprising, as NMDAR function has been implicated in a variety of vital functions, including learning and memory, neuronal migration, synapse stabilization, pain

perception, and neuronal cell death (Komuro and Rakic, 1993) and reviewed in (Malenka and Nicoll, 1999; Cull-Candy and Leszkiewicz, 2004). These data are consistent with that seen in other regions of the human brain (Law et al., 2003) and observations in animal models (Watanabe et al., 1992; Luo et al., 1996; Laurie et al., 1997; Chen et al., 2000; Sans et al., 2000; Ritter et al., 2002; Awobuluyi et al., 2003; Ontl et al., 2004; Petralia et al., 2005). NMDAR levels may also be particularly high in the DLPFC, as the prefrontal cortex may contain the highest concentration of NMDARs in the cortex (Scherzer et al., 1998). Notably, NMDAR-mediated functions are apparently crucial even for embryonic life, because genetic deletion of NR1 in mice leads to neonatal lethality (Forrest et al., 1994).

Whereas we observed clear developmental regulation of NR1 mRNA indicating a low prenatal level of expression compared to later developmental age groups, we observed a more modest increase in NR1 protein levels when comparing prenatal to maximum expression (11-15 yrs). However, when we performed a secondary analysis using PMI and storage time as covariates, this difference in protein levels failed to reach significance. Due to the modest developmental regulation of NR1 compared to other NMDAR subunits, such as NR3A, the detection of subtle differences in NR1 protein expression levels over development will require closer examination in future studies. We largely observed similar trends in mRNA and protein expression in both NR3A and NR1. We did, however, observe one inconsistency. Interestingly, whereas NR1 transcript levels remain high in adult prefrontal cortex, protein levels drop significantly. Similar discrepancies between mRNA and protein levels have been noted and may result from

translational inefficiency, or increased rates of protein degradation (Awobuluyi et al., 2003; VanDongen and VanDongen, 2004).

The abundant NR1 protein levels observed during childhood are present during a period of extensive development of neuronal processes and formation of synaptic connections (Webb et al., 2001; de Graaf-Peters and Hadders-Algra, 2006). Why does NMDAR expression peak around early adolescence in the DLPFC? The elevated NR1 levels may provide a molecular substrate for robust synaptic plasticity in cortical regions at this time. The intriguing reductions in NR1 protein expression after puberty parallel the loss of synapses observed in human prefrontal cortex, and are consistent with a role for NMDARs in synaptic pruning or elimination in humans (Huttenlocher and Dabholkar, 1997; Glantz et al., 2007) and in non-human primates (Bourgeois et al., 1994; Gonzalez-Burgos et al., 2007). Additionally, this might also be associated with critical period closure for many forms of NMDAR-mediated plasticity (Malenka and Bear, 2004).

Currently little is known about NR3A in humans, but the high level of homology between human and rodent NR3A (93%) (Andersson et al., 2001; Eriksson et al., 2002) and the strong overall relationship between NMDAR subunit mRNA and protein levels found in this study suggest its function is likely similar between mammalian species. To observe NMDAR developmental changes without the issues of postmortem degradation, we ran a parallel study in cortical tissue from rhesus macaque, a close genetic relative of humans (Gibbs et al., 2007), and found broad similarities to the human studies **(Supplementary Figure S2.1)**. Most importantly, the early peak in NR3A protein expression that tapers into maturity coincides with the developmental loss of NR3A in humans and rodents. Thus, despite the unavoidable caveats associated with studying

human postmortem tissue, the similarities in the combined human transcript and protein data and monkey protein results produced from this study strengthen our conclusions and support the view that NMDAR subunits are similarly modified across development in rodents, non-human primates, and humans.

We initially sought to characterize all the predominant subunits in postnatal cortex: NR1, NR2A, NR2B, and NR3A (Watanabe et al., 1993; Monyer et al., 1994; Sheng et al., 1994; Stocca and Vicini, 1998). Because all proteins are subject to decay after death, we first established our ability to quantify NMDAR subunit levels in postmortem tissue using immunoblotting (see Methods). While > 65% of NR1 and NR3A remained intact up to a 24 hr PMI, less than 50% of the NR2A and NR2B levels could be detected. This was consistent with our difficulty, and that of others, in reliably measuring NR2A and NR2B levels in human and non-human primate tissue (Wang et al., 2000; O'Connor et al., 2006). While we observed rapid decay of NR2A and NR2B with these particular antibodies (see Methods), protein degradation may differ among epitopes or different tissue preparation techniques. As such, antibodies or approaches may exist that are better suited for detecting NR2A and NR2B levels in tissue with long PMIs (see (Murphy et al., 2005; Kristiansen et al., 2006). Due to our difficulties in detecting NR2A and NR2B levels, we focused on NR1 and NR3A.

Relevance of NR1 and NR3A expression to schizophrenia

Because NMDAR hypofunction has been hypothesized to underlie some of the cognitive deficits observed in schizophrenia (Javitt and Zukin, 1991; Jentsch and Roth, 1999; Olney et al., 1999; Lewis and Levitt, 2002; Coyle et al., 2003; Frankle et al., 2003), we asked whether this could be due to alterations in NMDAR subunit proteins within the

DLPFC. Reports of subunit modifications in schizophrenia are largely conflicting (reviewed in (Kristiansen et al., 2007)). Our data reach the same conclusions as Kristiansen and colleagues (Kristiansen et al., 2006), indicating that NR1 protein levels are unchanged in the schizophrenic brain. Subtle changes in NR1 splice-variant expression (Prybylowski and Wolfe, 2000; Magnusson et al., 2005) might account for alterations of the subunit, but may not be detectable in our system.

Recent data raised the intriguing possibility that NMDAR hypofunction in schizophrenia might arise from an aberrant increase in NR3A expression, as NR3A suppresses NMDAR function and NR3A mRNA transcript levels are significantly elevated in the DLPFC of schizophrenic patients (Mueller and Meador-Woodruff, 2004). In this same region where NR3A is overexpressed, spine densities are reduced in schizophrenic patients (Glantz and Lewis, 2000). Because NR3A likely has a strong influence on limiting spine density (Das et al., 1998), a lack of the normal developmental downregulation of NR3A could result in significant reductions in spine numbers. Therefore, it is appealing to hypothesize that NR3A levels are elevated in schizophrenics, as this could explain both the NMDAR hypofunction and the reductions in dendritic spine density that have been observed.

However, our data provide the first evidence that schizophrenia is not associated with a gross change in NR3A at the protein level within the DLPFC. Given that NR3A expression is normally very low in the adult brain (this study), and that subregion- and lamina-specific differences in the expression of NR3A exist (Mueller and Meador-Woodruff, 2004; Bendel et al., 2005; Mueller and Meador-Woodruff, 2005), we cannot rule out the possibility that there may be subtle or region-specific differences in NR3A

proteins that our methods were unable to detect. A more selective analysis of synaptic membranes from different regions and/or laminae have the potential to reveal significant differences in receptor protein expression in the schizophrenic brain, if such differences exist.

Even in the absence of differences in adult NR3A protein levels, other possibilities for NMDAR hypofunction involving NR3A may exist. Genetic variants of NR3A could provide a molecular substrate for abnormal NR3A function (Gallinat et al., 2007), particularly relevant for prefrontal information processing. As schizophrenia is considered a neurodevelopmental disorder, a transient increase of NR3A during development could disturb the normal formation of cortical circuits, yet not be apparent in the adult brain (Lewis, 1997). Because antipsychotic drugs can alter NMDAR subunit expression in a regional-specific manner (Fitzgerald et al., 1995; Hanaoka et al., 2003; Schmitt et al., 2003; O'Connor et al., 2006), we explored the possibility that drug treatments might account for the normal levels of NR3A and NR1 we found in schizophrenic patients. After modeling the effects of sub-chronic clozapine and haloperidol exposure in rodent frontal cortex, we found that NMDAR subunit expression is unchanged, consistent with previous observations (Hanaoka et al., 2003). Although we cannot preclude the possibility that different antipsychotic treatment regimens might alter NMDAR protein levels, our data suggest that normal NR1 and NR3A protein levels in the DLPFC of schizophrenic patients are unlikely to be consequences of antipsychotic treatments. This suggests either that overall NMDAR hypofunction in schizophrenia is not related to widespread changes in NMDAR proteins or that such deviations fail to be maintained throughout life in this brain region. We argue that the NMDAR hypofunction

observed in the disease is not maintained by gross differences in total NMDAR number or the proportion of NR3A-containing NMDARs, although there may be more subtle laminar or regional effects.

In light of reports of gender influences in normal NMDAR expression, as well as in schizophrenia, we examined possible gender differences in NR1 and NR3A. We observed marginal significance for sex-based differences suggesting that females have significantly less NR3A and a trend for less NR1 than their male counterparts. The meaning of these results in human DLPFC is unknown. However, there is a precedent for gender-specific differences in NR1 expression within other regions of the brain (Shi and Schlenker, 2002; Ontl et al., 2004), suggesting that NR1 levels are differentially regulated by sex hormones (Gazzaley et al., 1996; McEwen, 2002). Like NR1, NR3A levels might also be regulated by sex hormones, such as estrogen. Indeed, genetic deletion of NR3A impairs prepulse inhibition, a measure of sensorimotor gating, in male but not female mice (Brody et al., 2005). This finding suggests that estrogen might normally downregulate NR3A levels, thus mirroring effects of genetic NR3A deletion in mice. Such an interpretation would be consistent with our observation that NR3A, and possibly NR1, levels are lower in the DLPFC of females compared to males. It might also explain the observation that there is a sex-specific difference in prepulse inhibition in normal human subjects (Swerdlow et al., 1993). Interestingly, schizophrenics are known to exhibit deficits in prepulse inhibition to startle (reviewed in (Braff et al., 2001b)), suggesting a potential link between sex-specific NMDAR subunit expression and the schizophrenic condition. While we stress the preliminary nature of these gender-related observations, the possibility for reduced expression of NR3A in females warrants more

intense scrutiny, as it could provide a biological basis to explain why female schizophrenic patients tend to manifest symptoms at a later age, are more responsive to medications, and exhibit less severe symptoms than males (Castle and Murray, 1991).

While we attempted to carefully address the confounding factors that are inherent to all human postmortem studies, one of the main limitations to such investigations is the relatively small numbers of postmortem brain samples available. However, even after considering such factors as PMI, pH, sex, ethnicity, and storage time on the quality of the mRNA and proteins studied, we still found significant developmental changes in NMDAR subunits, suggesting the increases and decreases in expression patterns are due to robust developmental regulation. The observed statistical differences in developmental NMDAR subunit levels likely represent the most dramatic and consistent age-dependent differences in subunit levels. Analyses of additional tissue might reveal more subtle developmental effects. It is important to note that the current study represents the first developmental analysis of NMDA receptors in human cortex from gestation to adulthood. Developmental studies in human brain of key regulatory systems – including glutamate – are needed to advance our understanding of normal brain development and provide insight into the pathophysiology of neurodevelopmental disorders such as schizophrenia.

In summary, we report the developmental and schizophrenia profiles of NMDA receptor subunits, NR1 and NR3A, in human prefrontal cortex. The data are consistent with lifelong functional roles for NR1 and a particularly important role during early human brain development for NR3A. Our results also suggest that there are no gross differences in NR1 and NR3A protein levels between schizophrenic and control DLPFC.

Collectively, this study will be relevant in understanding subunit functions in key NMDAR-mediated processes of ontogeny, such as the formation and refinement of cortical circuits. Additionally, these results contribute to understanding subunit roles in disorders of NMDAR dysfunction, such as the basis for NMDAR hypofunction in schizophrenia, as well as subtype-specific targeting in drug development.

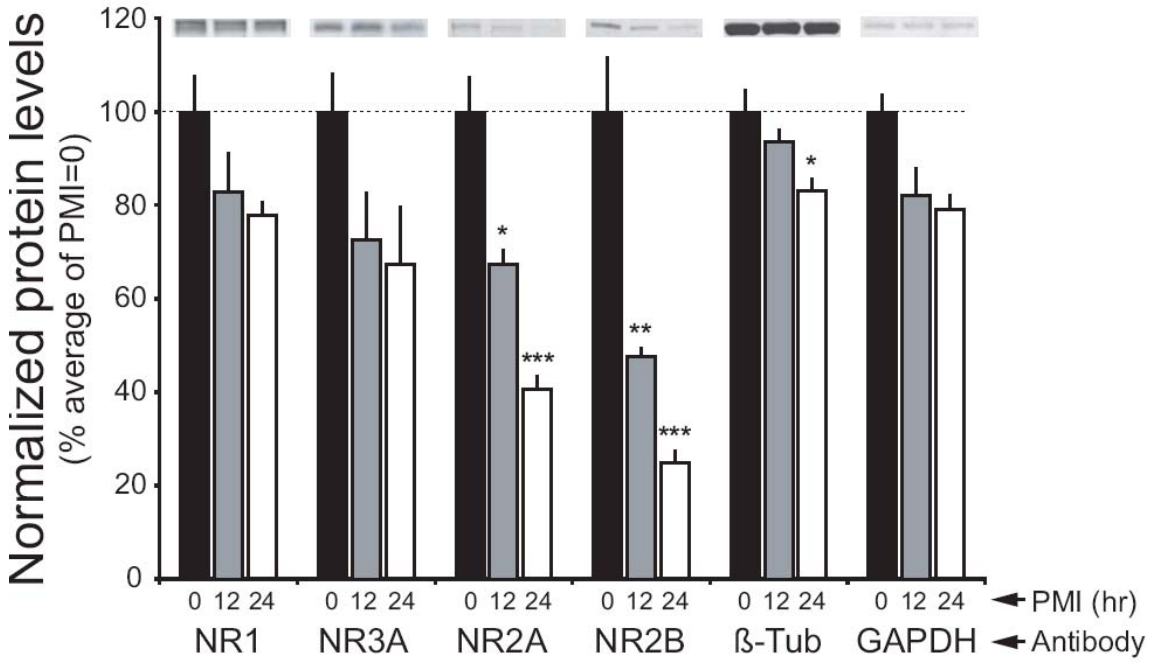


Figure 2.1: Protein stability of NMDA receptor subunits (NR1, NR3A, NR2A, and NR2B) and loading controls (b-Tubulin and GAPDH) in postmortem mouse frontal cortex, normalized to the average of PMI 5 0 (n 5 4/group except n 5 2/group for GAPDH). Data are presented as means ± standard error of the mean. Significance from PMI 5 0 h: *P \ 0.05, **P \ 0.01, ***P \ 0.001. Representative immunoblots are shown in addition to averaged data.

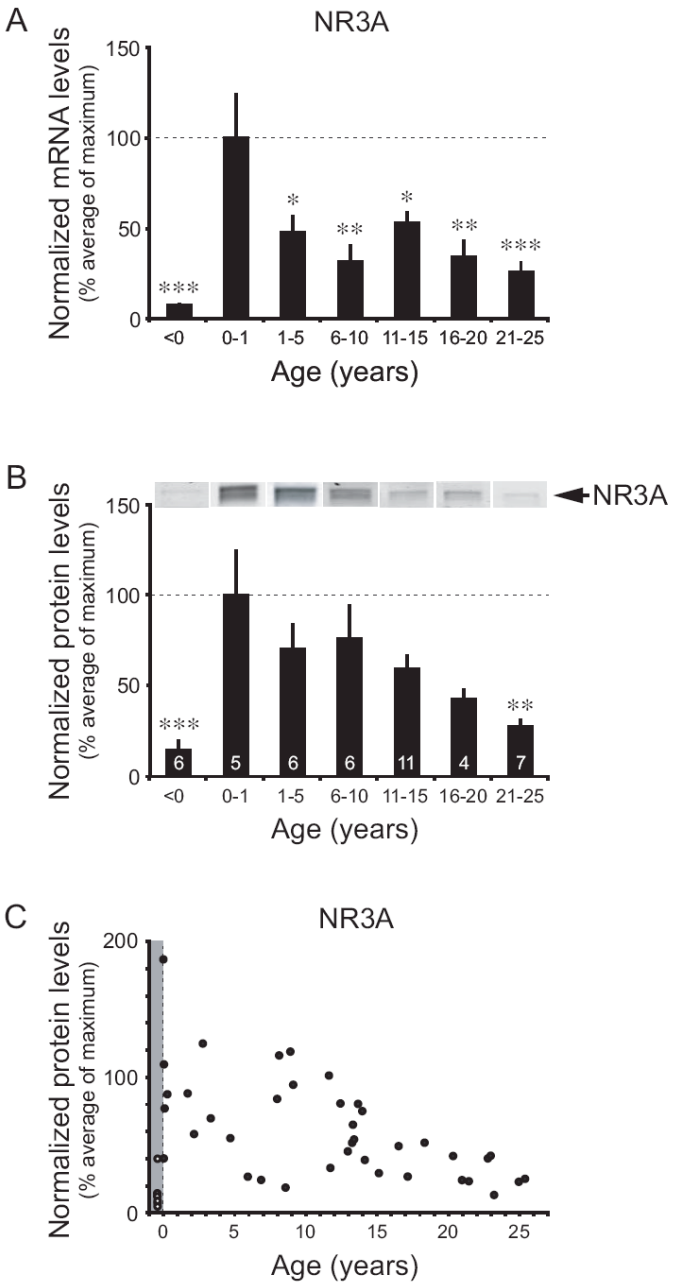


Figure 2.2. NR3A mRNA and protein expression surges in DLPFC after birth and then declines progressively. Quantification of NR3A levels, normalized to the average value in the age group where NR3A expression was maximal (0–1 year). (A) Normalized and averaged NR3A transcript levels binned into developmental age groups. (B) Normalized and averaged NR3A protein expression data. Values within bars represent sample sizes. Representative immunoblots are shown in addition to averaged data. Data are presented as means ± standard error of the mean. Significance from age group 0–1 year: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (C) Scatter plot depicting NR3A expression over development. The dashed line and gray shading divide prenatal and postnatal tissue. Open circles and closed circles represent prenatal and postnatal tissue, respectively. Some points are obscured by overlying points with similar values.

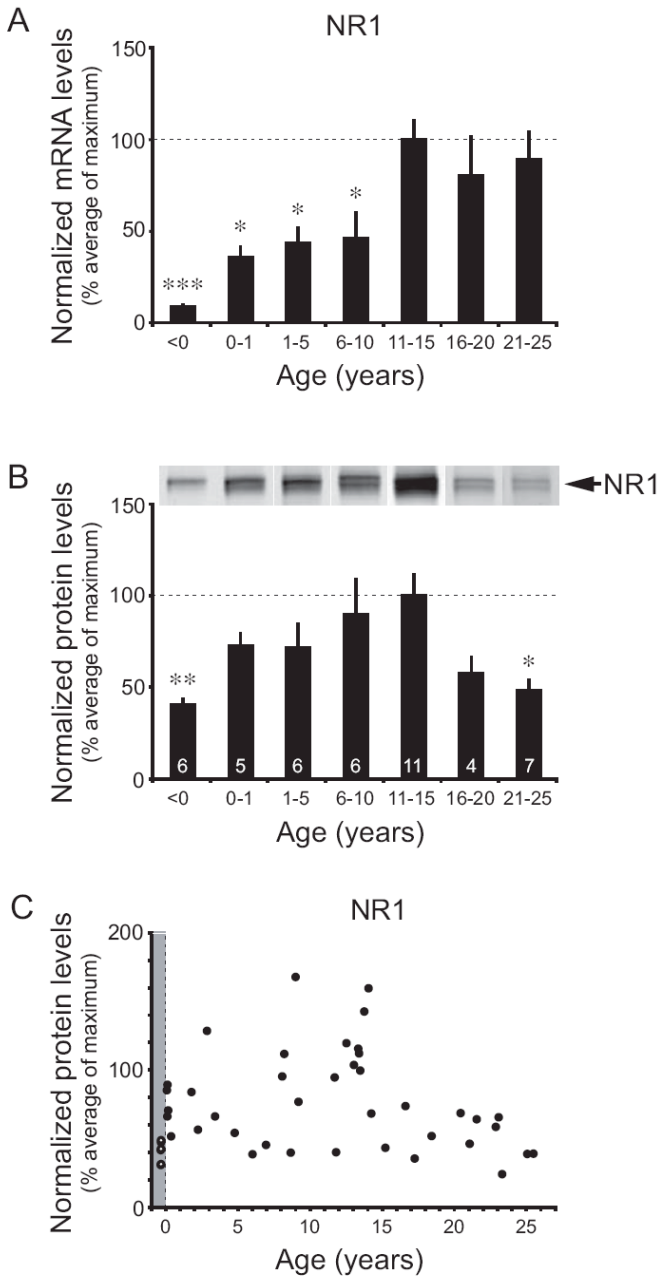


Figure 2.3. NR1 levels in human DLPFC change modestly over development. (A) Normalized and averaged NR1 mRNA expression levels. (B) Normalized and averaged NR1 protein expression data. Data are presented as means ± standard error of the mean. Values within bars represent sample sizes. Representative immunoblots are shown in addition to averaged data. Significance from age group 11–15 years: *p<0.05, **p<0.01, ***p<0.001. (C) Scatter plot depicting NR1 levels over human development. The dashed line and gray shading divide prenatal and postnatal tissue. Open circles and closed circles represent prenatal and postnatal tissue, respectively. Some points are obscured by overlying symbols with similar values.

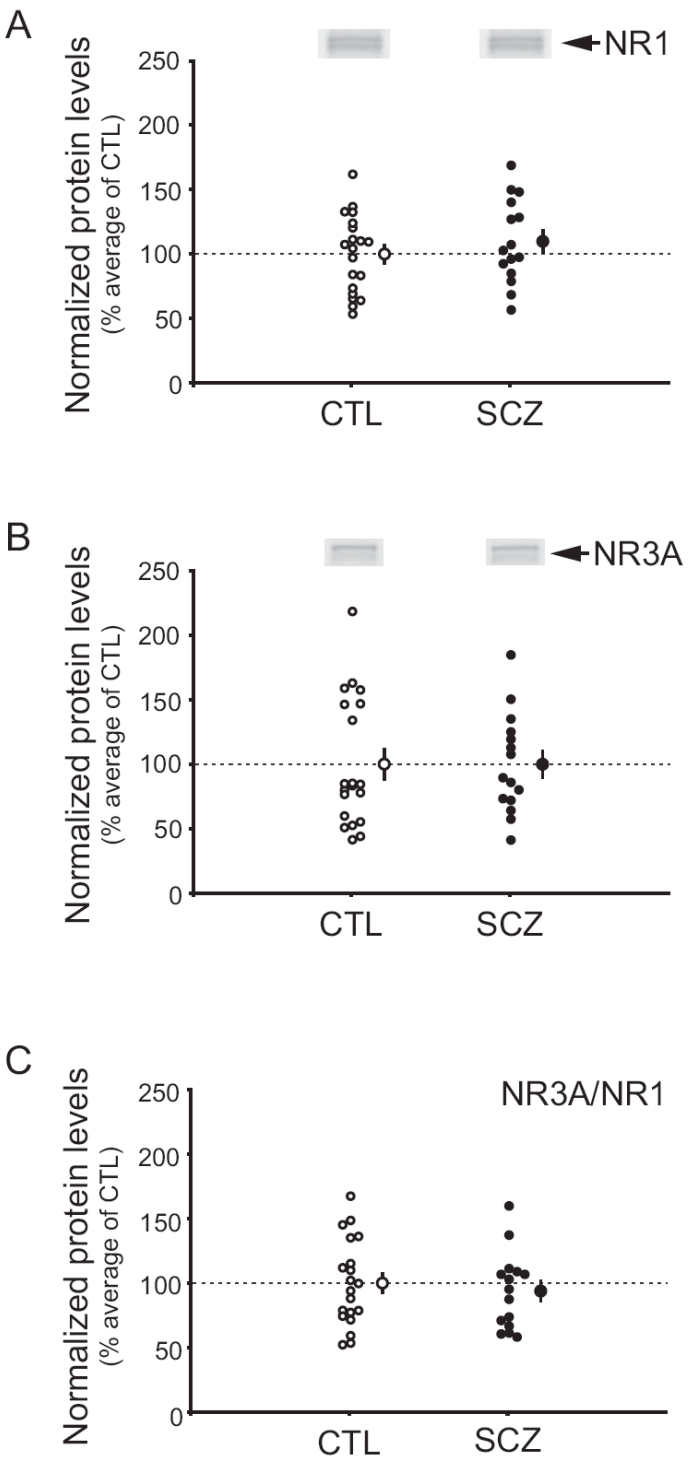


Figure 2.4. NMDAR subunit protein levels in control and schizophrenic subjects. Scatter plots showing (A) NR1, (B) NR3A, and (C) NR3A/NR1. Small circles represent data from individuals; large circles represent group means ± standard error of the mean. Representative immunoblots are shown in addition to averaged data. CTL, control (open circles); SCZ, schizophrenia (filled circles).

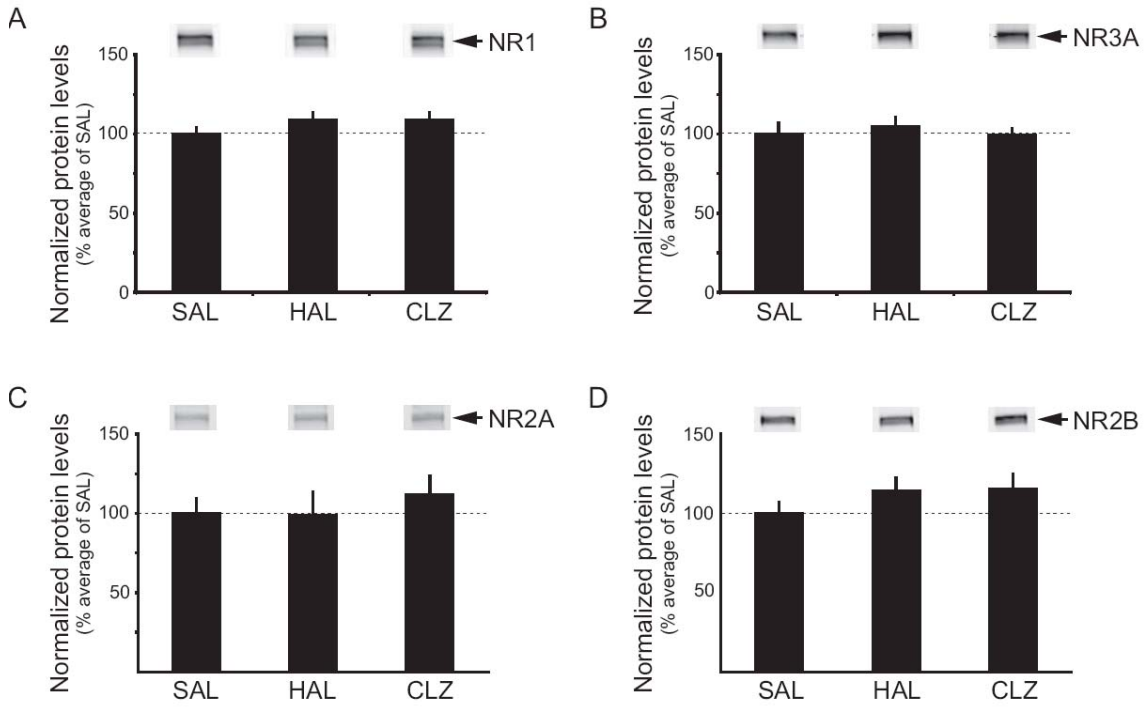


Figure 2.5. Antipsychotic drugs fail to alter NMDAR subunit levels, shown normalized to saline control values. Bar graphs depicting quantification of (A) NR1, (B) NR3A, (C) NR2A, and (D) NR2B in frontal cortical tissue from antipsychotic-treated rats. Data are presented as means ± standard error of the mean. SAL, saline; HAL, haloperidol; and CLZ, clozapine.

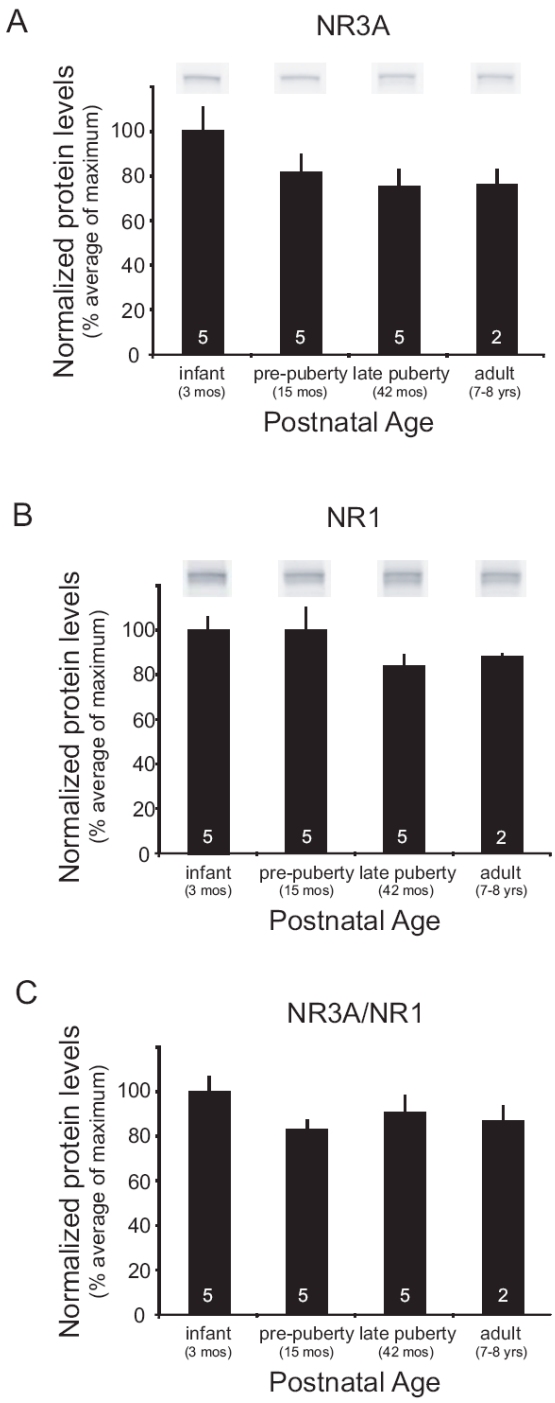


Figure S2.1: NMDAR subunit protein levels in postnatal monkey temporal cortex. (A) Quantification of NR3A, normalized to the average value in the age group where NR3A expression was maximal (3 months) and (B) quantitative analysis of NR1 protein levels, normalized to the average value in the age group where NR1 expression was maximal (3 months). Averaged data binned into developmental age groups. Representative immunoblots are shown in addition to averaged data. Data are presented as means ± SEM. Values within bars represent sample sizes.

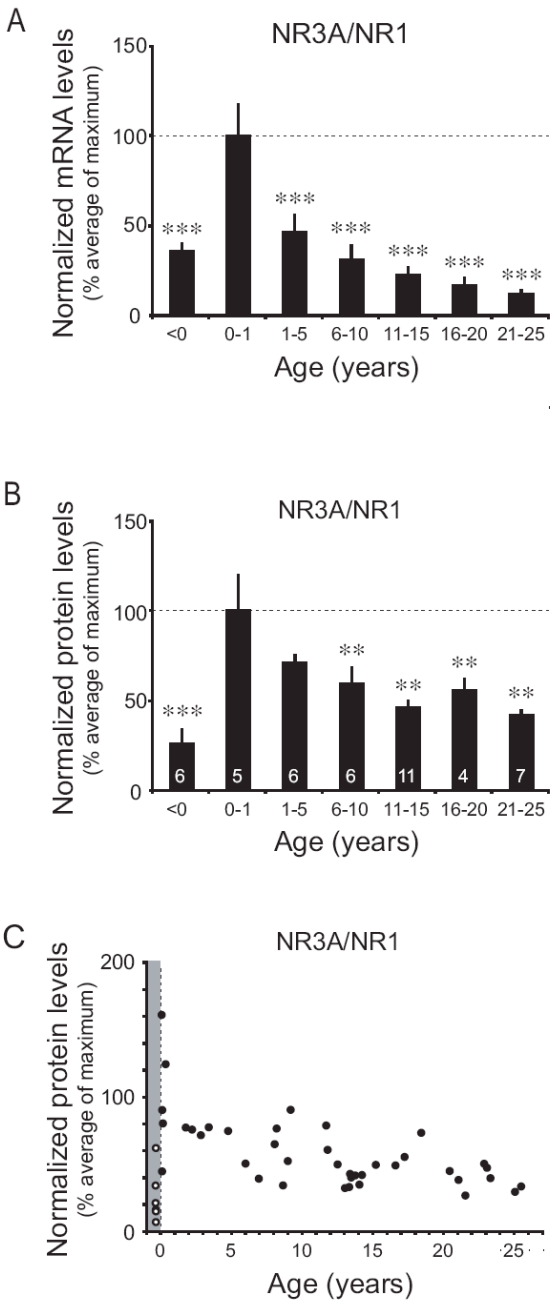


Figure S2.2: NR3A/NR1 ratios mirror NR3A expression over development. (A) Normalized and averaged quantification of NR3A/NR1 transcript levels. (B) Normalized and averaged quantification of NR3A/NR1 protein expression data binned into developmental age groups. Values within bars represent sample sizes. Data are presented as means ± SEM. Significance from age group 0-1yr: *p<0.05, **p<0.01, ***p<0.001. (C) Scatter plot depicting NR3A/NR1 protein ratios over development. The dashed line and gray shading divide prenatal and postnatal tissue. Open circles and closed circles represent prenatal and postnatal tissue, respectively. One point is obscured by an overlying point with similar value.

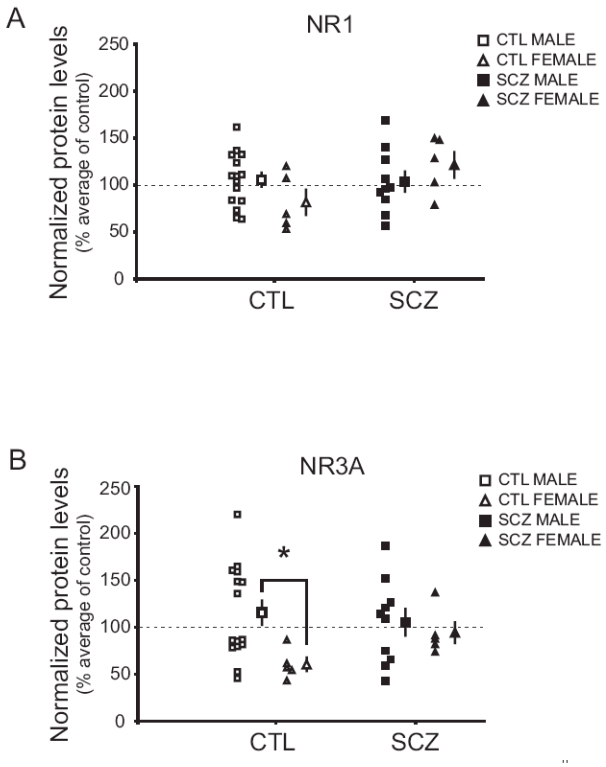


Figure S2.3: NMDAR subunit protein levels in male (squares) and female (triangles) control and schizophrenic subjects. Scatter plots showing (A) NR1 and (B) NR3A expression. Small symbols represent data from individuals, large symbols represent group means ± SEM. CTL=control (open symbols); SCZ=schizophrenia (filled symbols). * $p < 0.05$.

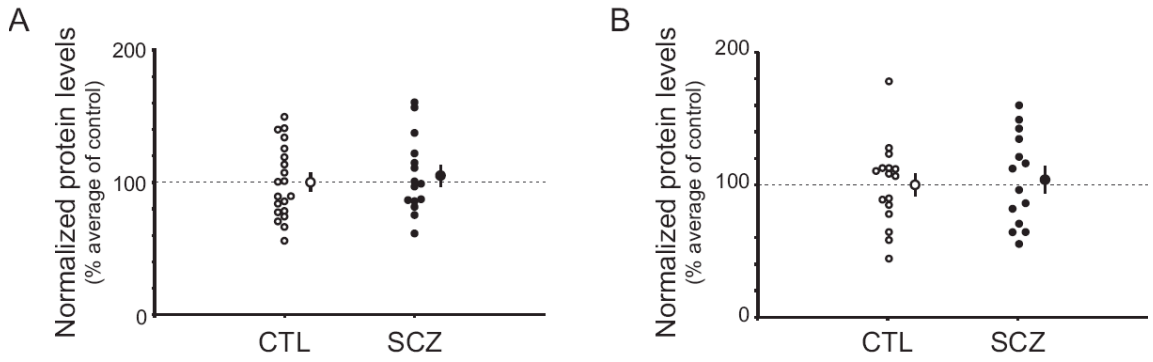


Figure S2.4: Scatter plots depicting NR1 protein levels normalized to (A) β-tubulin and (B) GAPDH loading controls, relative to normal control subjects. Small symbols represent data from individuals, large symbols represent group means ± SEM. CTL=control (open circles); SCZ=schizophrenia (filled circles).

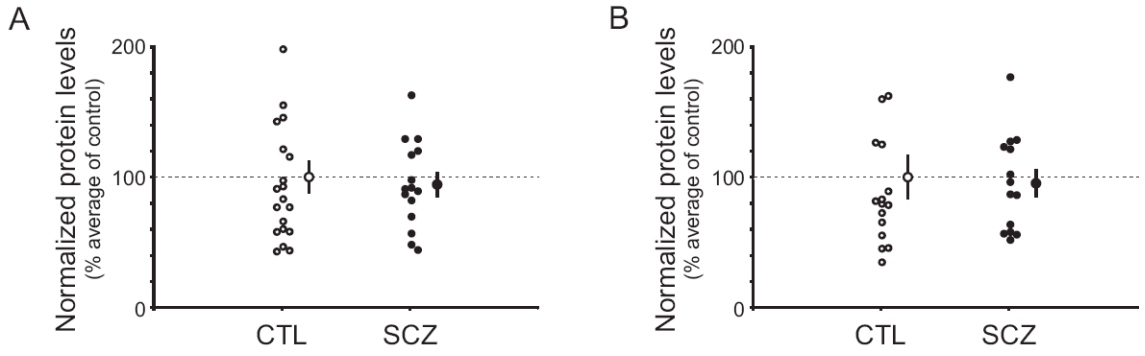


Figure S2.5: Scatter plots depicting NR3A protein levels normalized to (A) β-tubulin and (B) GAPDH loading controls, relative to normal control subjects. Small symbols represent data from individuals, large symbols represent group means ± SEM. CTL=control (open circles); SCZ=schizophrenia (filled circles).

Tables 2.1 and 2.2

Table 1
Developmental study demographics

Age group (years)	Age (years)	Subjects	PMI (h)	pH	Storage time (years)	Race	Sex
<0	−0.41 ± 0.0	6	1.0 ± 0.0	6.1 ± 0.2	13.6 ± 0.8	1 C, 5 AA	3 M, 3 F
0–1	0.1 ± 0.1	5	7 ± 3.2	6.3 ± 0.4	10.3 ± 2.9	2 C, 3 AA	3 M, 2 F
1–5	3.4 ± 1.6	6	15.2 ± 4.8	6.1 ± 0.3	6.1 ± 1.1	2 C, 4 AA	2 M, 4 F
6–10	8.3 ± 0.8	6	19.8 ± 9.8	6.3 ± 0.3	5.0 ± 0.8	3 C, 3 AA	2 M, 4 F
11–15	13.3 ± 1.0	11	18.3 ± 6.1	6.4 ± 0.3	7.3 ± 3.7	5 C, 3 AA, 3 U	9 M, 2 F
16–20	18.1 ± 1.7	4	14.8 ± 5.4	6.1 ± 0.1	9.3 ± 4.0	1 C, 3 AA	3 M, 1 F
21–25	23.1 ± 1.6	7	14.7 ± 5.9	6.2 ± 0.2	6.5 ± 1.5	2 C, 4 AA	6 M, 1 F

Note: Characteristics of normal subjects. Means are reported as ±standard deviation. C, Caucasian; AA, African-American; U, unknown; M, male; and F, female.

Table 2
Schizophrenia study demographics

Diagnosis	Subjects	Age (years)	PMI (h)	pH	Storage time (years)	Race	Sex
Control	20	56.7 ± 18.3	21.2 ± 6.0	6.4 ± 0.3	6.1 ± 1.5	9 C, 11 U	15 M, 5 F
Schizophrenia	15	54.3 ± 17.0	21.7 ± 5.1	6.4 ± 0.3	7.8 ± 1.2	14 C, 1 U	10 M, 5 F

Note: Characteristics of normal control and schizophrenic subjects. Means are reported as ±standard deviation. C, Caucasian; AA, African-American; U, unknown; M, male; and F, female.

CHAPTER 3

Modulation of excitatory synapse development by NR3A

Abstract

The balance between synapse stabilization and elimination is highly sensitive to developmental changes in the complement of synaptic proteins. The repertoire of synaptic proteins, specifically including glutamate receptors, ultimately determines whether synapses will be strengthened or weakened by plasticity mechanisms of long-term potentiation and depression. The molecular composition of NMDA-type glutamate receptors (NMDARs) changes over development, much of which is driven by sensory experience. The transition from immature to mature NMDAR phenotypes in synapse maturation has been recognized, but the contributions of the NR3A subunit in this process have only begun to be considered. Although removing endogenous NR3A accelerates synapse maturation and overproduction of NR3A limits synapse potentiation and stabilization, neither the subcellular localization of NR3A-containing receptors at peak expression levels, nor the consequences of their deletion has been rigorously established. Using biochemical fractionation and quantitative immunoblotting, we have characterized the synaptic presence of NR3A and examined the distribution of a subset of proteins involved in synaptic maturation. Our data support a model whereby NR3A negatively regulates the expression of proteins involved in excitatory neurotransmission,

synaptogenesis, and spine growth. The developmental loss of NR3A may thus guide the maturation and stabilization of excitatory synapses.

Introduction

Activation of the NMDA-type glutamate receptor (NMDAR) is crucial for synaptic strengthening and weakening (Malenka and Bear, 2004), which occur primarily in early brain development (Engert and Bonhoeffer, 1999; Matsuzaki et al., 2004). NMDARs are ionotropic channels that form through assembly of NR2 (A-D) and NR3 (A-B) subunits with the obligatory NR1 dimer (Furukawa et al., 2005). Most research in the mouse forebrain has concentrated on the canonical subtypes, NR2A and NR2B. Recent reports, however, have focused on how the inclusion of NR3 subunits reduces currents, and lowers both calcium permeability and sensitivity to magnesium block (Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Matsuda et al., 2002), resulting in suppression of NMDAR activity.

Developing synapses undergo alterations in glutamate receptor content. In early postnatal life, synaptic activity and sensory experience induce modifications in the PSD by promoting the transition between 'immature' and 'mature' forms of NMDARs (NR2B to NR2A), accompanied by the synaptic incorporation of AMPA receptors (AMPARs). These changes regulate the stabilization of NMDARs in the PSD, the subsequent decline in plasticity of the synapse, and the associated spine growth with maturation (Perez-Otano and Ehlers, 2004).

Removal/replacement of juvenile NR3A-containing NMDARs is involved in experience-driven synapse maturation (Roberts et al., 2009), yet the molecular mechanisms of this transient expression are unknown. Aberrant synaptic changes in both

NR3A-null and NR3A-overexpressing mice suggest this subunit influences synaptogenesis. NR3A knockout mice exhibit a striking increase in the number of dendritic spines on pyramidal neurons (Das et al., 1998), while NR3A-overexpressors have reduced synapse density (Roberts et al., 2009), indicating that NR3A plays a key role in spine and synapse formation and elimination (Henson et al.).

To biochemically characterize the synaptic presence of NR3A and the effects that loss of NR3A has on forebrain content and intracellular distribution of other synapse-associated proteins, we performed subcellular fractionation and quantitative immunoblot analyses in wild-type and NR3A KO mice. Our subcellular characterization of NR3A provides evidence of its PSD-centric localization in early postnatal development that shifts to a more diffuse distribution in mature animals. Accordingly, this thorough assessment of the levels of synaptic NR3A over development and the synaptic proteins associated with NR3A-containing NMDARs during peak expression will be crucial to our understanding of the biochemical events that accompany synapse maturation and elimination.

Materials and Methods

Animal use

Animal use in this study was approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill. All rodents were maintained and sacrificed according to protocol guidelines.

Tissue collection

Brains were removed from NR3A KO/WT mice aged postnatal (P) days 8, 16, and >40. Forebrain tissue was rapidly dissected into dry ice and stored at -80°C until use.

Subcellular fractionation

One to three forebrains from each genotype, NR3A KO or WT, were pooled for each experiment. Biochemical fractions were prepared essentially as described (Yashiro et al., 2005). Briefly, brains were dounce-homogenized in HEPES-buffered sucrose (4 mM HEPES, 0.32 M sucrose, pH 7.4) and lysed in hypo-osmotic solution. Synaptosomal fractions were isolated by density centrifugation (150,000 x *g* for 2 h) using a gradient consisting of 0.32, 0.8, 1.0, and 1.2 M sucrose in 4 mM HEPES, pH 7.4. Synaptic plasma membrane fractions (SPM) were resuspended in 0.5% Triton X-100-containing buffer and centrifuged to obtain postsynaptic density (PSD) fractions. Complete protease inhibitors (Roche Applied Science, Germany), and phosphatase inhibitor mixtures 1 and 2 (Sigma-Aldrich, St Louis, MO) were added to all buffers. Procedures were performed on ice and/or in a cold room and fractions were stored at -80°C. Protein concentrations were determined by MicroBCA Assay (Pierce Chemical, Rockford, IL). Fractions of interest in this study were comprised of the following cellular components: PNS, whole homogenate, postnuclear supernatant; SPM, synaptic plasma membranes, complex of presynaptic active zone and postsynaptic densities with some extrasynaptic membranes attached; PSD, postsynaptic density isolated from detergent-extracted synaptic junctions.

Quantitative immunoblotting

Increasing amounts (1 -15 μg) of total protein from each fraction were loaded in wells of 4-12% or 8% tris-glycine NuPage gels (Invitrogen), resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Blotting (Bio-Rad) and Odyssey system imaging and quantitation (LI-COR) were carried out following manufacturers' protocols. The following antibodies were used at optimized concentrations: goat anti-NR1 (SC1467,

0.01μg/ml, Santa Cruz Biotechnology), rabbit anti-NR3A (07-356, 2μg/ml, Millipore), mouse anti-PSD-95 (MAB1596, 1μg/ml, Chemicon) mouse anti-synaptophysin (S-5768, 0.5μg/ml, Sigma-Aldrich), mouse anti-β tubulin (MAB 3408, 10μg/ml, Chemicon), Alexa Fluor 680-labeled anti-goat IgG (#A21084, Invitrogen), Alexa Fluor 680-labeled anti-mouse IgG (#A21058, Invitrogen), and IRDye 800-labeled anti-rabbit IgG (#611-732-127, Rockland Immunochemicals). All immunoblots were repeated at least once with similar results.

Data Analysis

Calculations of signal intensity per microgram protein were determined from multiple wells on each gel for each target antigen and then averaged across multiple gels. Fraction means per genotype were then either presented as immunoreactive units/μg protein or normalized to mean control values and expressed as % of control or % of maximum ± SEM. Statistical evaluations were performed using two-tailed student's *t*-tests and significance placed at $p < 0.05$. For multiple group comparisons, one-way analyses of variance (ANOVAs) were performed, followed by between-group comparisons with Tukey-Kramer tests. Statistical analyses were conducted using Graphpad Instat (San Diego, CA).

Results

We used biochemical fractionation and quantitative immunoblotting to characterize the subcellular localization of NR3A and to analyze how the presence or absence of NR3A alters synaptic protein expression over development in the mouse forebrain.

Biochemical enrichment of synaptic proteins by subcellular fractionation

We performed subcellular fractionation to selectively enrich forebrain homogenates for synaptic complexes and to isolate purified PSDs **(Figure 3.1a)**. This was then followed by immunoblot analysis of fractions containing cellular molecules of interest. To demonstrate the quality of the biochemical enrichments from the whole homogenate fraction (PNS, postnatal supernatant), each preparation was assessed by immunoblotting for changes in specific markers: absence of membrane proteins (e.g. glutamate receptors) in the cytosolic fraction; absence of presynaptic protein, synaptophysin, in the PSD; absence of postsynaptic density protein, PSD-95, from the Triton-soluble fraction; and progressive enrichment of NMDAR subunit, NR1, and PSD-95 from the initial homogenate (PNS) to the PSD. These indicators provide assessments of the relative purity of the isolated PSD fractions **(Figure 3.1b)**. Representative western blots show NR1, PSD-95, and synaptophysin expression with the amount of protein loaded in each lane. Because the PSD fractions are so highly enriched, protein loading is a two-fold reduction over the previous fractions in order to avoid saturation and to be able to accurately see differences.

Glutamate receptors in forebrain PSDs are developmentally regulated in a subunit-specific manner

For comparative purposes, we defined the developmental expression profiles of the predominant NMDAR and AMPAR subunits present in forebrain fractions from P8, P16, and young adult mice. This not only provided a baseline to which we could directly compare the NR3A data, but also confirmed the reproducibility of this biochemical

fractionation protocol. NR1 is required for the functional expression of all NMDARs (Monyer et al., 1992; Perez-Otano et al., 2001; Matsuda et al., 2003). Our data show that NR1 levels are similar across development within each fraction **(Figure 3.2b:** NR1, ANOVA: PNS, $F_{(2,18)} = 1.636$, $p = 0.2258$; SPM, $F_{(2,19)} = 1.404$, $p = 0.2727$; PSD, $F_{(2,19)} = 0.3277$, $p = 0.7250$), consistent with previous reports (Monyer et al., 1992; Henson et al., 2008). However, NR1 expression is most highly concentrated in PSDs at each age tested (**Figure 3.2b:** NR1 ANOVA: P8, $F_{(2,23)} = 74.691$, $p < 0.0001$; P16, $F_{(2,17)} = 44.507$, $p < 0.0001$; Adult, $F_{(2,16)} = 29.234$, $p < 0.0001$)**.** NR2B expression across development was similar to that of NR1, with levels abundant early postnatally and changing only modestly with age **(Figure 3.2b:** NR2B, ANOVA: PNS, $F_{(2,19)} = 2.304$, $p = 0.1302$; SPM, $F_{(2,19)} = 2.167$, $p = 0.1021$; PSD, $F_{(2,19)} = 1.046$, $p = 0.3730$). NR2B is also highly concentrated in PSDs (**Figure 3.2b:** NR2B ANOVA: P8, $F_{(2,23)} = 32.582$, $p < 0.0001$; P16, $F_{(2,17)} = 77.483$, $p < 0.0001$; Adult, $F_{(2,17)} = 31.964$, $p < 0.0001$)**.** In contrast, western blot analyses of forebrain fractions demonstrate that NR2A levels are very low during early development, and increase dramatically over the first several weeks of life. The robust developmental increases in NR2A levels within each fraction, suggest this subunit is significantly regulated across developmental ages **(Figure 3.2b:** NR2A, ANOVA: PNS, $F_{(2,18)} = 12.544$, $p = 0.0005$; SPM, $F_{(2,19)} = 53.279$, $p < 0.0001$; PSD, $F_{(2,18)} = 28.588$, $p < 0.0001$). This developmental shift in subunit composition has been well-characterized, as it alters the ability of synapses to strengthen and weaken in response to electrical activity and neurotransmitter binding. Ratios of NR2A/NR2B also increase with development in PSD fractions **(Supplemental Figure 3.1a:** NR2A/NR2B, ANOVA: PSD, $F_{(2,18)} = 21.994$, $p < 0.0001$**)**. This is likely due to the profound increase in NR2A-containing

receptors, rather than a decrease in NR2B subunits, resulting in the activity-dependent shortening of synaptic currents. Fold-enrichment quantification shows that NR1, NR2A, and NR2B are all highly enriched in the synaptic compared to whole homogenate fractions **(Figure 3.2b:** NR1 ANOVA: P8, $F_{(2,23)} = 51.082$, $p < 0.0001$; P16, $F_{(2,17)} = 100.08$, $p < 0.0001$; Adult, $F_{(2,14)} = 41.656$, $p < 0.0001$; NR2A ANOVA: P8, $F_{(2,22)} = 19.036$, $p < 0.0001$; P16, $F_{(2,14)} = 14.218$, $p = 0.0007$; Adult, $F_{(2,17)} = 29.004$, $p < 0.0001$; NR2B ANOVA: P8, $F_{(2,23)} = 41.095$, $p < 0.0001$; P16, $F_{(2,17)} = 21.855$, $p < 0.0001$; Adult, $F_{(2,17)} = 19.139$, $p < 0.0001$**),** undergoing 7- to 14-fold increases in protein levels. The high concentration of these subunits at the postsynaptic density is consistent with previous reports, and is responsible for NMDAR function in synaptic plasticity.

High levels of NR3A are associated with membrane fractions and concentrated in PSDs

To begin to understand how NR3A affects synapse maturation and elimination, we first needed to know to which subcellular compartments NR3A is targeted in neurons at the peak of its expression. Like the NR2 subunits, NR3A expression is developmentally regulated. However, its profile is unique, being highly expressed in early postnatal life and downregulating sharply into adulthood in humans, monkeys, and rodents suggesting this is a common feature of brain development and that the function of NR3A is similar between mammalian species (Henson et al.).

We determined the subcellular localization of NR3A at P8 in mouse forebrain as being prominently expressed in most biochemical compartments, such as light membrane and synaptic plasma membrane fractions that include intracellular organelles (e.g. microsomes, endosomes, Golgi, endoplasmic reticulum), and synaptic junctions **(Figure**

3.2a), consistent with previous data from juvenile/adult rats (Perez-Otano et al., 2006). The presence of NR3A in membrane fractions suggests that these receptors may be mobile, as has been suggested of NMDARs in general (Tovar and Westbrook, 2002). Because the subcellular distribution of NMDARs determines receptor properties, further probing by quantitative analysis revealed that during its peak of expression, NR3A levels are highest in PSD fractions compared to whole homogenate PNS fractions (total receptor protein) from which it was derived **(Figure 3.2b**, ANOVA: P8, $F_{(2,23)} = 9.000$, $p = 0.0015$; P16, $F_{(2,16)} = 2.682$, $p = 0.1033$; Adult, $F_{(2,15)} = 3.662$, $p = 0.055$). This demonstrates that NR3A is enriched in PSD fractions at P8 only. However, this enrichment is weak when compared to other NMDAR subunits **(Figure 3.2c,** ANOVA: $F_{(3,30)} = 17.818$, $p < 0.0001$**),** which is consistent with previous data from juvenile rats (Perez-Otano et al., 2006). The low level of PSD concentration suggests that NR3A-containing receptors are less effectively targeted to the synapse. This is expected given that NR3A has no PDZ-domain binding motifs in its C-terminus to permit stable anchoring to the PSD, like NR2 subunits (Eriksson et al., 2007b).

Developmental loss of NR3A from glutamatergic postsynaptic densities

To determine how the subcellular expression of NR3A changes over development, we quantified whole homogenate and synaptic fractions taken from P8, P16, and young adult mice and found that levels of NR3A decrease in all fractions with age **(Figure 3.2b,** ANOVA: PNS, $F_{(2,17)} = 16.09$, $p = 0.0002$; SPM, $F_{(2,19)} = 5.341$, $p = 0.0159$; PSD, $F_{(2,18)} = 19.777$, $p < 0.0001$). To further explore this, we then compared the PSD levels of NR3A over development. Quantification of the percent in protein fractions

confirmed there is a developmental loss of NR3A from the PSD **(Figure 3.2d,** $P16_{\%\ of\ maximum} = 70.8 \pm 8.4$, $p < 0.05$; $Ad_{\%\ of\ maximum} = 32.9 \pm 3.1$, $p < 0.0001$). The fractionation process removes synaptic membranes and active zones from the insoluble PSDs (SPM → PSD, see **Figure 3.1a**). These data suggest the decline in postsynaptic NR3A levels is possibly a shifting of the balance of NR3A to the general synaptic plasma membranes fraction. These data indicate that NR3A-containing NMDARs undergo an age-related shift in their subcellular localization. Thus, this is consistent with the idea that NR3A moves from a synaptic to a peri-/extrasynaptic location with development. These data suggest that NR3A has a synaptic role in early postnatal development that may serve to limit synaptic plasticity, but that role may change with age. This is also in line with data showing early onset of LTP in the NR3A KO mouse, as well as decreased LTP and memory consolidation that are linked to the prolonged synaptic presence of NR3A in mutant mice (Roberts et al., 2009). By limiting synapse potentiation, NR3A may have effects on synapse size, strength, and long-term memory.

Synapse maturation markers, NR1, NR2A and GluR1, are enhanced in young NR3A KOs

Our recent study (Roberts et al., 2009) showed that NMDAR subunit abundance in synaptic fractions is significantly increased in P8 NR3A knockouts, while total NR1 expression is unchanged. This suggests that the genetic deletion of NR3A enhances synaptic concentration, and that stabilization of NMDARs, one of the initial events driving synapse maturation, occurs earlier in the absence of NR3A. We reasoned that if NR3A negatively regulates NR1 localization and numbers at synaptic junctions, other glutamate receptor subunits might also be involved. For further examination by

subcellular fractionation of tissue both containing and lacking NR3A, we selected subunits that are predominantly expressed in developing mouse forebrain: NR2A, NR2B, and GluR1. Western blots using an anti-NR3A antibody revealed a band of the expected size, which was absent in controls.

Immunoblot analysis of synaptic membranes revealed that, like NR1 (Roberts et al., 2009) **(Figure 3.3a**, $NR1_{\%\ of\ control} = 133.5 \pm 12.3$, n = 9, $p < 0.05$**)**, PSD levels of synapse maturation markers are enhanced in P8 NR3A KO compared to WT mice for NR2A **(Figure 3.3a**, $NR2A_{\%\ of\ control} = 150.4 \pm 16.3$, n = 7-8, $p < 0.05$**)** and GluR1**(Figure 3.3a**; $GluR1_{\%\ of\ control} = 142.4 \pm 12.9$, n = 8, $p < 0.05$**)**. No differences were observed in total receptor protein (PNS fractions, data not shown). Both NR2A and GluR1 expression levels are normally very low at this age **(Figure 3.2b,** $NR2A_{\%\ of\ maximum} = 12.69 \pm 2.02$, **and Supplemental Figures 3.1a,** $GluR1_{\%\ of\ maximum} = 13.49 \pm 1.95$**)**, and their increased levels in mutant PSDs indicate that the loss of NR3A promotes the early concentrations of both subunits. These data also suggest that NR3A inhibits synaptic expression of AMPARs as well as NMDARs. Because the NR2B subunit is also highly expressed in immature forebrains during the early postnatal period, we examined whether any changes occurred for NR2B subunit expression in the NR3A KO forebrain at 8, 16, and 40-55 days of age postnatal. This effect of selective upregulation of NR1 and NR2A is specific to these subunits, because we observed no differences in NR2B levels **(Supplemental Figure 3.2,** $NR2B_{\%\ of\ control} = 100.6 \pm 9.3$, n = 8-9, $p < 0.05$**)**. Altogether, these data suggest that increased spine density observed in the KO mice results from exuberant spinogenesis that is unrestrained in the absence of NR3A and the data support

the notion of NR3A as a negative regulator of glutamate receptor surface expression in the maturation and stabilization of excitatory synapses.

Early onset of synaptic glutamate receptor concentration may be transient

To investigate whether the nature of the precocious concentration of NMDARs in NR3A KO mice was transient or sustained, we next examined mice at P16 and P40-55, the period during and after which synapse refinements are normally completed. Interestingly, for NR1 levels we find no difference between KO and WT by P16 **(Figure 3.3b,** % of control = 97.25 ± 8.78, n = 6, p = 0.8449**)**, the time when endogenous NR3A levels are normally declining. This phenotype was still evident in more mature mice **(Figure 3.3c,** % of control = 104.64 ± 14.7, n = 6, p = 0.8278**)**, which would be consistent for a modulatory role of NR3A in the narrow temporal window of its intense expression. However, although not statistically significant, NR2A and GluR1 expression still trended towards elevated levels in both P16 **(Figure 3.3b,** NR2A, % of control = 129.83 ± 19.85, n = 6, p = 0.28; GluR1, % of control = 144.05 ± 19.38, n = 6, p = 0.08**)** and young adult mice **(Figure 3.3c,** NR2A, % of control = 134.43 ± 18.72, n = 6, p = 0.13; GluR1, % of control = 130.93 ± 21.71, n = 6, p = 0.24**)**, an observation that may result from the prolonged effects of dysregulated programming in the absence of NR3A. The remarkably similar phenotypes in NMDAR and AMPAR subunits in immature NR3A KOs, along with the strong presence of NR3A proteins in the developing but not adult rodent brain, support a model in which NR3A negatively regulates glutamate receptor expression during normal postnatal CNS development and helps to mediate developmental synapse elimination and maturation. Additionally, the slow return of

NR2A and GluR1 to control expression levels may point to prolonged, unchecked potentiation that is normally restrained by NR3A-NMDARs. Further experiments need to be conducted to address this issue of whether or not glutamate receptor proteins remain elevated in mature NR3A KOs or if they return to control levels.

Discussion

Synapses in many CNS regions undergo significant remodeling during postnatal brain development, although the molecules responsible for the elimination of inappropriate synapses and the maintenance and strengthening of appropriate connections are still largely unknown. In this study, we sought to understand how the NMDAR subunit, NR3A, influences excitatory synapse development by defining the subcellular localization of NR3A over development as well as the composition of proteins modulated by NR3A during synapse refinement.

NR3A-containing receptors in the rodent CNS are most abundant during the second postnatal week (Henson et al.). Our fractionation data localized NR3A peak protein expression predominantly to excitatory PSDs. We also demonstrated a loss of NR3A from PSDs over time, suggesting there is an age-dependent shift in the subcellular distribution of this subunit. These data are consistent with the idea that NR3A is targeted to PSDs early in postnatal life, and it moves from a synaptic to a peri-/extrasynaptic location over time. NR3A's age-dependent translocation from immature detergent-insoluble structures to other membrane regions would be in line with juvenile and adult rat data showing that (1) NR2 subunits were not preferentially localized in PSDs (Al-Hallaq et al., 2007), and (2) NR3A-containing NMDARs showed a uniform distribution

of NR3A at the neuronal surface, reflecting its weak association with the PSD (Perez-Otano et al., 2006). Such a shift would be important for functional properties of the receptor because synaptic activation of NMDARs requires glutamate binding to receptors clustered in the PSD. NMDAR activation outside the synaptic cleft requires an excess of glutamate diffusing from presynaptic release sites. Typically this occurs in mature animals under conditions of high frequency stimulation, glutamate release from astrocytes, or impaired uptake by glutamate transporters (Kohr, 2006). Only high affinity (e.g. glutamate-binding NR2-containing) NMDARs are able to detect such low levels of glutamate. Presynaptic NMDARs also can be functionally activated by ambient glutamate (Larsen, unpublished observations), but it is highly unlikely this receptor subpopulation is involved as they are largely downregulated in adults (Corlew et al., 2007). Furthermore, it is possible that, given its profile of developmental decline, NR3A undergoes a developmental shift from presynaptic to postsynaptic expression mechanisms. However, this scenario also is not likely because the changes in synaptic levels over time suggest NR3A is more abundant in synaptic plasma membranes (which include PSDs) than in PSDs alone. Biochemical analysis of NR3A in the presynaptic active zone (Phillips et al., 2001) will be needed to resolve this issue.

The subunit composition and synaptic localization of NMDARs in the developing forebrain affect channel activity and downstream signaling pathways. NMDAR subtypes are targeted to distinct synaptic or extrasynaptic sites through their interactions with intracellular binding partners, such as membrane-associated guanylate kinases (MAGUKs; e.g. PSD-95 and SAP102). All NR2 subunits contain PDZ binding domains, which are required for PSD attachment through the MAGUKs. Specifically, NR2A-

containing receptors are thought to be delivered to the developing synapse by a preferential association with the prototypical anchor, PSD-95, while NR2B subtypes are trafficked by SAP102 (Sans et al., 2000; van Zundert et al., 2004). However, this PDZ binding sequence is conspicuously absent in NR3 subunits (Matsuda et al., 2002; Eriksson et al., 2007b), indicating that the synaptic attachment of NR3A-NMDARs would be critically dependent upon NR2A and/or NR2B. PSD associations of NR3A may be involved in activity-dependent changes in the subcellular distribution of NMDARs, as the synaptic removal of NR3A is an activity-dependent process. Recent findings have shown that the regulated targeting of NMDARs to nascent synapses drives the developmental increase in NR2A-containing NMDARs (Philpot et al., 2001a; Barria and Malinow, 2002; Bellone and Nicoll, 2007), and hence the ability of the synapse to mature. The effects that MAGUK-NR2 interactions may have on regulating the targeting, anchoring, and stabilization of NR3A at synaptic sites are unknown.

Recent data (Roberts et al., 2009), and this study) show that the ablation of the NR3A subunit does not reduce, but enriches forebrain contents of other endogenous glutamate receptor subunits, NR1, NR2A, and GluR1. This suggests that NR3A is not required for but is responsible for restricting their expression. These subunit-specific effects did not extend to NR2B, which was unchanged, suggesting that the loss of NR3A does not induce any compensatory expression of NR2B. Furthermore, overexpression of NR3A results in a decrease in spine density and limited LTP in hippocampal neurons, as well as restricted expression on small spines in WT mice. The data clearly indicate that the function of NR3A is likely correlated with changes occurring at glutamatergic

synapses in this timeframe. Importantly, the synaptic inclusion of NR3A negatively regulates NMDAR subunit-specific expression and AMPAR insertion.

In this study, postsynaptic NR1 exhibited higher than normal expression in the NR3A KO, but returned to control levels in the narrow temporal window of only several days, in the same timeframe when endogenous NR3A levels are normally declining. Total NR1 expression was unchanged, suggesting that synaptic concentration and stabilization of NMDARs, one of the initial events driving synapse maturation, also occurs earlier in the absence of NR3A. Thus, lack of NR3A may cause a re-arrangement of subunits and premature stabilization of synapses. Data showing increased spine density in the NR3A KO mice (Das et al., 1998) might then be a result of exuberant spinogenesis.

The moderate increase of postsynaptic NR2A and GluR1 levels in P16 and adult NR3A-null PSDs is not significant, although we do not know if the differences are due to variability among samples or insufficient sample numbers. The data may reflect the loss of inhibitory mechanisms in the absence of NR3A, the relief of the brake in synaptic maturation. Such a scenario would be the outcome if NR3A were responsible for delaying the stabilization of NMDARs and the insertion of AMPARs at the synapse. Moreover, the prolonged absence of NR3A would be expected to cause an increase in the fraction of mature synapses (NMDAR+/AMPAR+), along the lines of NR3A removal triggering the 'unsilencing' of synapses. The early onsets of synaptic NMDAR currents and LTP in NR3A knockout mice (Roberts et al., 2009) are in agreement with this interpretation, although these differences are not maintained in P16 and adult mice. In addition, increased spine densities noted in the NR3A KO also do not return to normal by

adulthood (Das et al., 1998). However, whether or not the overexpression of NR2A and GluR1 remain elevated through adulthood in NR3A KOs will require further experiments. Regarding the inclusive evidence that the synaptic accumulation of NR2A and GluR1 may continue past P16, we cannot rule out the possibility that the total NR1 content was not detected: if a splice variant-specific change in NR1 subunits was precipitated by the loss of NR3A (Smothers and Woodward, 2009), the NR1 antibody we used (domain-specific for the C2 splice variant, NR1-C2) may not have bound the entire antigen available if synaptic NR1 at P16 comprised more than the C2 isoform (Zukin and Bennett, 1995). The limitations of biochemical studies also necessitate additional experiments to elucidate the association between the detergent solubility of glutamate receptors and PSD scaffolding proteins during development. These will then reveal how NR3A acts as a molecular brake by restricting the developmental onset of glutamate receptor expression, which effectively prevents premature stabilization of excitatory synapses.

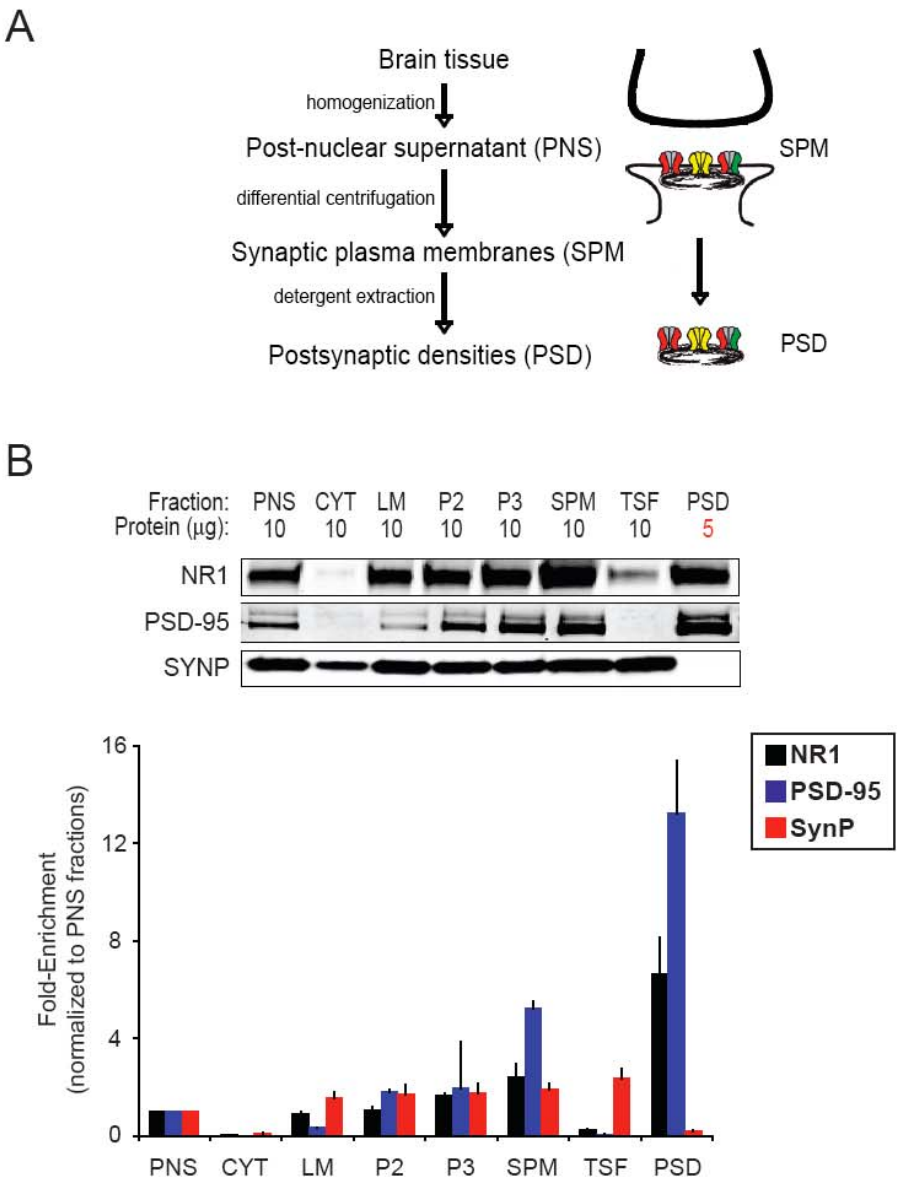


Figure 3.1: Biochemical fractionation of brain tissue demonstrating how subcellular fractionation selectively enriches brain tissue homogenates for postsynaptic membranes. (A) Schematic illustration of the enrichment process. Tissue is homogenized, separated on a sucrose gradient and subjected to a series of centrifugation steps to isolate synaptic junctional complexes (SPM) and purify the postsynaptic densities (PSD). (B) Quantification of biochemical enrichment of synaptic proteins. NR1 and PSD-95 are enriched in membrane fractions and PSDs from P16 forebrains, while presynaptic protein synaptophysin (SynP) is present in all but PSD fractions. PSD-95 is also absent from the TSF, which does not contain PSD. Representative immunoblots show NR1, PSD-95, and SynP expression with 10 or 5 µg protein were loaded in each lane. Quantification of fraction fold-enrichment made with sample values normalized to whole homogenate fractions (PNS). PNS, postnuclear supernatant; CYT, cytosol; LM, light membranes; P2, crude synaptosomes; P3, lysed synaptic membranes; SPM, purified synaptic plasma membranes; TSF, Triton-soluble fraction; PSD, postsynaptic densities.

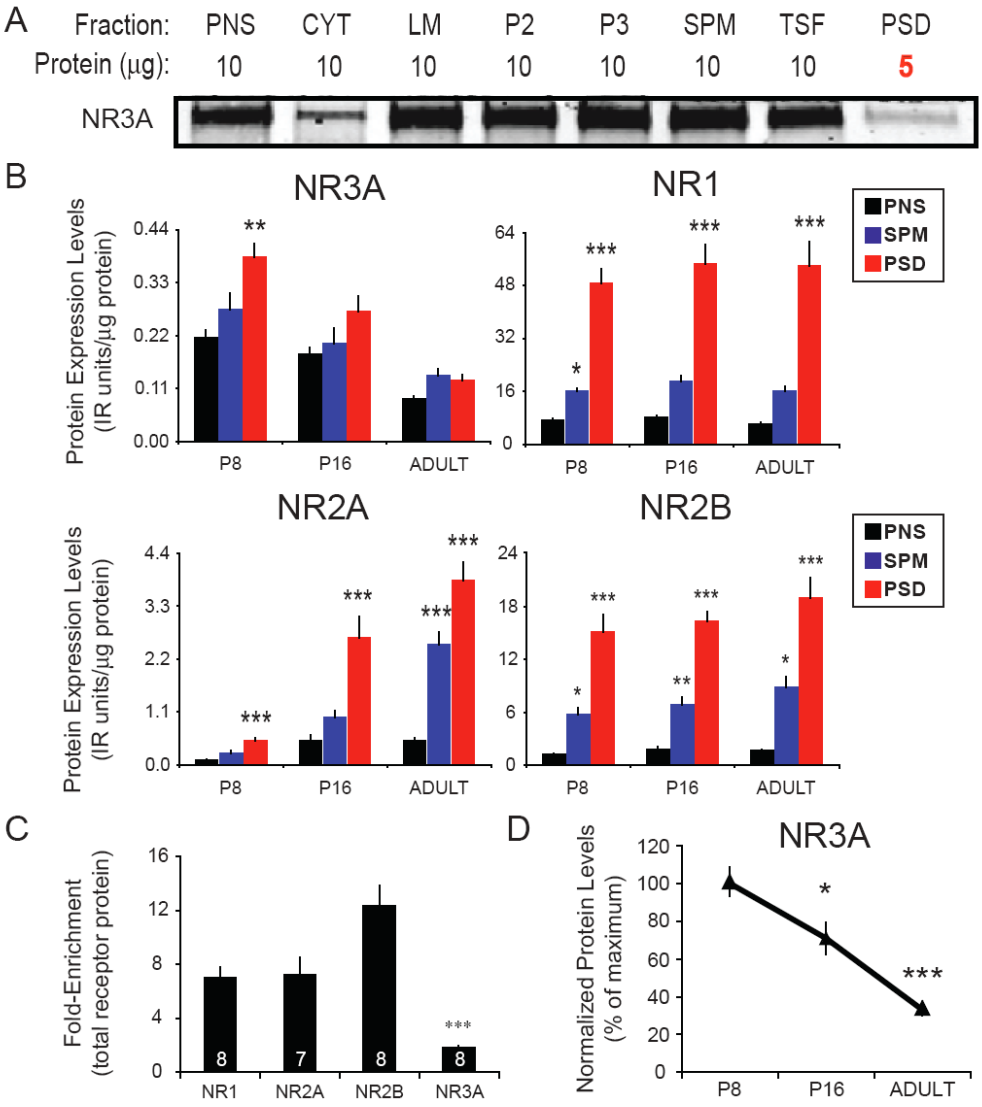


Figure 3.2: Developmental decline of NR3A expression and weak PSD enrichment compared to other NMDAR subunits. Biochemical fractionation and immunoblotting of forebrain fractions reveal NR3A (A) is expressed at P8 in most subcellular compartments, (B) is concentrated in PSDs early but decreases over development in all fractions, (C) is only weakly enhanced compared to PSD enrichment of other NMDAR subunits, and (D) undergoes a developmental decline in PSD expression. Representative immunoblots have 10 or 5 µg protein loaded in each lane. Protein data are averaged means of immunoreactive (IR) values relative to protein loads (µg). Quantitative comparisons of NMDAR subunit enrichment in PSD fractions are normalized to PNS values (whole homogenates, total receptor protein). Sample numbers for (B) and (D), n = 5-9. Values within bars of (C) represent sample numbers. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PNS, postnuclear supernatant CYT, cytosol; LM, light membranes; P2, crude synaptosomes; P3, lysed synaptic membranes; SPM, purified synaptic plasma membranes; TSF, Triton-soluble fraction; PSD, postsynaptic densities.

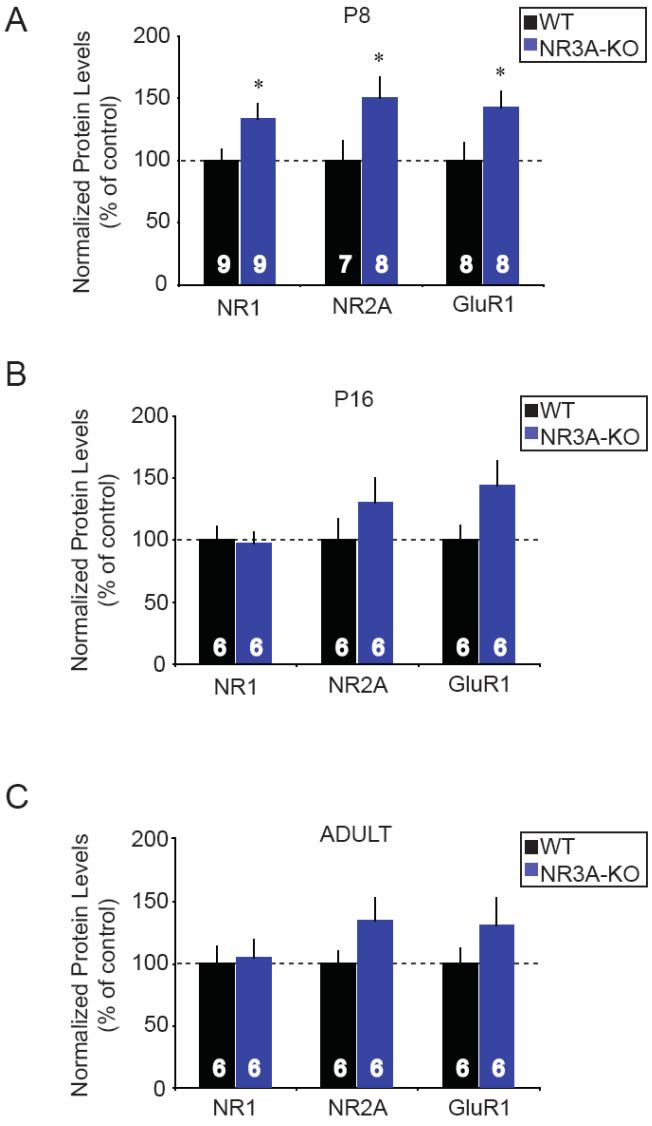
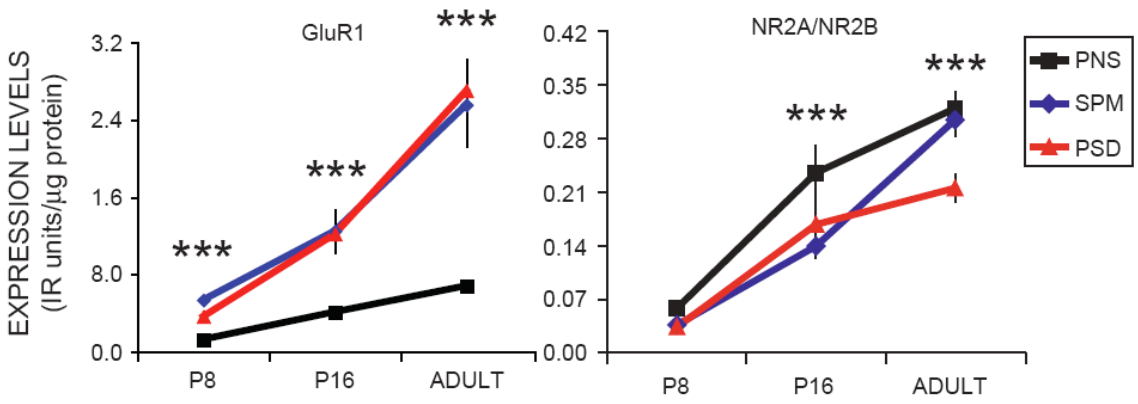
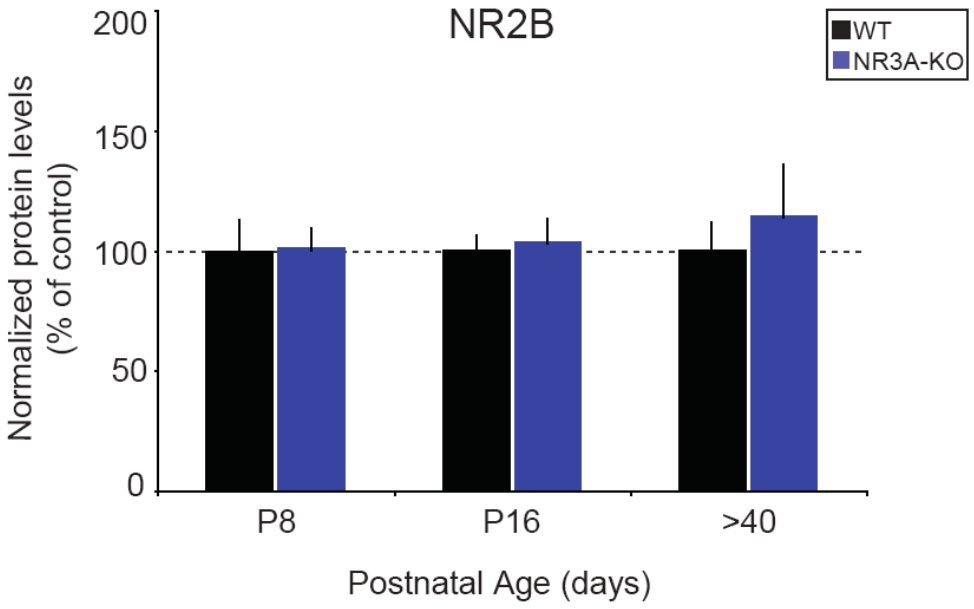


Figure 3.3: NR3A absence allows early onset of glutamate receptor concentration in PSDs that may be transient during synapse maturation. Quantitative immunoblotting of fractions from NR3AKO compared to WT controls show (A) show increased levels of NR1, NR2A, and GluR1 at P8 that may return to normal by (B) P16 and (C) adult ages. Protein data are averaged means of immunoreactive values relative to protein loads (µg). Values within bars indicate sample numbers. * $p < 0.05$.



Supplemental Figure S3.1: GluR1 and ratios of NR2A/NR2B expression levels increase in synaptic fractions at each age tested. Protein data are averaged means of immunoreactive values relative to protein loads (µg). Significance from postnatal day 8. *** $p < 0.001$. PNS, postnatal supernatant fraction; SPM, synaptic plasma membrane fraction; PSD, postsynaptic density fraction.



Supplemental Figure 3.2: NR2B levels are unchanged in the absence of NR3A. Quantitative immunoblotting of NR2B in PSD fractions from NR3AKOs at P8 and P16 show no significant changes between KO and WT levels. Values within bars indicate sample numbers.

CHAPTER 4

Conclusions and Future Directions

Summary of findings

Despite the functional importance of NR3A, its biochemical characterization and regulation at the synaptic level are still largely unknown. This study provides the first systematic characterization of the normal developmental profiles of NMDAR expression in maturation of the (1) mouse forebrain from early postnatal life to adulthood in the presence and absence of NR3A, and (2) human prefrontal cortex from mid-gestation to early adulthood. We defined the subcellular localization of NR3A over development as well as the composition of proteins modulated by NR3A during synapse refinement. Furthermore, we provide the first evidence that schizophrenia is not associated with a gross change in NR3A at the protein level within the human DLPFC. Overall, my work supports a model of NR3A preventing the premature stabilization and promoting the elimination of glutamatergic synapses.

While several groups have reported various aspects of NR3A expression and distribution (Henson et al.), none have performed rigorous assessments of the subcellular localization of NR3A, especially at the peak of its expression, or the consequences of genetic ablation of NR3A. We performed quantitative analyses of NMDAR subunit mRNA and protein in mouse forebrains and postmortem human brain cortex. Specifically, we demonstrated (1) robust developmental downregulation of NR3A, (2)

humans and rodents undergo analogous downregulation of NR3A, (3) synaptic targeting of NR3A declines with age, and (4) lack of NR3A may cause a re-arrangement of synaptic glutamate receptors and inhibit expression of synapse maturation markers. Our results show that NR3A serves a prominent role in the development of the CNS soon after birth, and its role is likely less important prenatally and in adulthood.

Influence of NR3A subunits in the developing brain

NR3A is expressed in the right place at the right time to regulate spine and synapse development. It is unclear, however, if the influence of NR3A is to serve as a 'synaptic brake' to limit synapse/spine formation or if it serves as a 'synaptic marker' to promote the elimination of spines. These two hypotheses are not mutually exclusive and NR3A may influence both processes **(Figure 4.1)**.

Synaptic brake hypothesis

NR3A-containing receptors may act collectively as a synaptic brake. One possibility is that at early stages, when their expression is highest, they limit synapse formation by raising the threshold required for synaptic activity to induce synaptic maturation. Later in development, activity-dependent removal of NR3A by PACSIN1/syndapin1 (Perez-Otano et al., 2006) would relieve this brake and allow the replacement of immature synaptic NMDARs by mature subtypes that are more stably anchored to PSDs. Synapse maturation would then proceed with the recruitment of other synaptic scaffolds and signaling complexes, and the subsequent changes in spine morphology.

Synaptic plasticity mechanisms, such as LTP and LTD, influence the formation and stabilization of synapses and spines (Yuste and Bonhoeffer, 2001) and are critically dependent upon Ca^{2+} entry via NMDARs (Lisman, 1989). The dominant-negative effect of NR3A on current and Ca^{2+} flux has recently been shown to limit synaptic plasticity (Roberts et al., 2009), providing a mechanism for NR3A control of spine and synapse density. It will be interesting to determine whether NR3A knockout mice display changes in synaptic plasticity that are causally related to the changes in spine formation, or *vice versa*.

While Das and coworkers (1998) reported increased spine density and enlarged spines in the cortex of P19 NR3A knockout mice, Tong *et al*. (2008) did not observe a related increase in the frequency or amplitude of AMPAR mEPSC in the same brain region in P10-P13 mice. However, the NMDAR/AMPAR ratio was enhanced, reflecting an increase in the NMDAR component. This sequence of events makes it tempting to speculate that larger NMDAR currents early in development favor enhanced LTP and subsequent increases in spine density/size in NR3A-deficient mice. If true, the early developmental presence of NR3A may serve as a synaptic brake to prevent the premature strengthening of synapses, and thus ensure a well-coordinated strengthening and stabilization of only appropriate synapses in response to experience. Early release of this brake in NR3A-null mutants would lead to enhanced spine formation and maturation of inappropriate synapses.

Synaptic elimination hypothesis

Another hypothesis consistent with the phenotype of NR3A-null mice is that NR3A serves as a tag to label weak synapses for elimination. In this scenario, coordinated or strong synaptic activity could drive NR3A out of the synapse, possibly via activity- and NMDAR-dependent synaptic removal of NR3A. Synapses with weak or uncoordinated activity would retain NR3A and, hence, be targeted for elimination. NR3A may also facilitate the elimination of immature synapses by recruiting the machinery necessary to exclude NMDARs from synapses, one of the steps thought to underlie synapse elimination and spine loss, via its interaction with PACSIN1/syndapin1 (Perez-Otano *et al.*, 2006).

Another possible link between NR3A and spine elimination was suggested by the discovery of a family of proteins termed "takusans" that are upregulated in NR3A-null mice and appear to influence the formation of dendritic spines (Tu et al., 2007). Overexpression of takusans enhances spine density, AMPAR expression, and PSD-95 clustering. Considering that the expression of takusans is reciprocally regulated in development when compared to NR3A subunits, NR3A may foster spine elimination by limiting the synaptic expression of takusan proteins. No direct interaction between these proteins has been observed, and future studies will be needed to investigate any causal relationship between NR3A and takusan expression.

NR3A in disease

Schizophrenia

A prominent theory is that impaired glutamatergic transmission contributes to the pathophysiology of schizophrenia (Olney and Farber, 1995), and several lines of

evidence invoke an important role of NMDAR hypofunction (Coyle *et al.*, 2003; Olney *et al.*, 1999; Tamminga, 1998; Tsai *et al.*, 1998): (1) NMDAR antagonists, such as PCP, exacerbate symptoms in patients with schizophrenia and produce symptoms nearly indistinguishable from schizophrenia in normal individuals (Javitt and Zukin, 1991). Genetic or pharmacological downregulation of NMDAR function in rodents also triggers schizophrenic-related behaviors (Moghaddam and Jackson, 2003; Mohn *et al.*, 1999). (2) NMDAR co-agonists, such as glycine and D-serine, can improve cognitive functioning and alleviate negative symptoms associated with schizophrenia (Goff *et al.*, 1999; Goff *et al.*, 1995; Heresco-Levy *et al.*, 1996; Heresco-Levy *et al.*, 1998; Javitt, 2002; Javitt *et al.*, 1994; Millan, 2005; Tsai *et al.*, 1998). (3) Many candidate schizophrenia genes influence NMDAR signaling and expression (Chowdari *et al.*, 2002; Harrison and Weinberger, 2005; Martucci *et al.*, 2003; Moghaddam, 2003; Straub *et al.*, 2002). For example, neuregulin, the protein product of a major schizophrenia susceptibility gene, alters the surface expression of synaptic NMDARs (Gu *et al.*, 2005; Ozaki *et al.*, 1997; Stefansson *et al.*, 2004). (4) More recently, imaging studies have provided the first *in vivo* evidence for reduced NMDAR binding in medication-free schizophrenic patients (Pilowsky et al., 2006).

Although the mechanistic basis for NMDAR hypofunction in schizophrenia remains unknown, it could be caused by a change in NMDAR subunit composition. To date there is little consensus on alterations in NMDAR subunits that might contribute to the NMDAR hypofunction observed in schizophrenia (Akbarian *et al.*, 1996; Meador-Woodruff and Healy, 2000; Moghaddam, 2003). However, a recent study demonstrated that NR3A mRNA levels are increased within layer 5 in subregions of the dorsolateral

prefrontal cortex in schizophrenics (Mueller and Meador-Woodruff, 2004). This finding raises the possibility that inappropriate expression of NR3A could exacerbate this subunit's unique ability to suppress receptor activity, and underlie NMDAR hypofunction. Consequences of aberrant expression would be predicted to have a larger impact in the mature brain, where NR3A is remarkably downregulated in most brain regions. Four further observations support a role for elevated NR3A in schizophrenia: (1) Schizophrenic brains exhibit decreased phosphorylation of NR1 at serine 897, which can be reversed by antipsychotics (Emamian et al., 2004). Because NR3A subunits form a signaling complex with PP2A, which can dephosphorylate serine 897, overexpression of NR3A could contribute to the reductions in NR1 phosphorylation in schizophrenia (Chan and Sucher, 2001). (2) Alterations in NR3A levels impair prepulse inhibition (Brody et al., 2005), a measure of sensorimotor gating that is also impaired in many schizophrenia patients (Braff et al., 2001a). (3) Spine density is decreased in the DLPFC of schizophrenia patients (Glantz and Lewis, 2000, 2001), a region where NR3A levels are elevated (Mueller and Meador-Woodruff, 2004), and elevated NR3A levels have been shown to decrease spine formation (Roberts et al., 2009). (4) Impaired working memory in schizophrenia could be explained by deficits in NMDAR function, potentially resulting from NR3A overexpression, in layer 5 pyramidal neurons in the DLPFC (Fellous and Sejnowski, 2003; Sanchez-Vives and McCormick, 2000; Sucher *et al.*, 1995; Wong *et al.*, 2002). Although it is now clear that NR3A levels are not changed in a wholesale manner in the schizophrenic DLPFC (Henson et al., 2008), the above data underscore a need to further examine regional changes in NR3A expression, protein interactions, and signaling

in schizophrenics and to evaluate if/how they contribute to the pathophysiology of schizophrenia.

White matter injury

The dogma that NMDARs are expressed only in neurons within the CNS has been overturned, and increasing evidence indicates not only that NMDARs are expressed in glia, but also that glial NMDARs contain the NR3A subunit (Paoletti and Neyton, 2007). Three recent studies highlighted the role of NMDARs in oligodendrocyte damage (Karadottir *et al.*, 2005; Micu *et al.*, 2006; Salter and Fern, 2005), reporting that oligodendrocytes and the myelin sheaths they form are damaged by excitotoxicity in a number of acute and chronic disorders, including ischemic stroke, cerebral palsy, traumatic neural injury, and multiple sclerosis (Matute, 2006; Matute *et al.*, 2007) (**Figure 4.2**). Both Ca^{2+} entry and subsequent oligodendrocyte damage could be attenuated by NMDAR antagonists, indicating that white matter damage was caused at least in part by NMDAR activation (Karadottir *et al.*, 2005; Micu *et al.*, 2006; Salter and Fern, 2005). These studies demonstrated that NMDAR currents in oligodendrocytes failed to show rectification at hyperpolarized potentials due to Mg^{2+} block, mimicking the behavior of NR3A- or NR2C-containing NMDARs. This suggests that these NMDARs are positioned to respond to glutamate activation, even in the absence of strong depolarization. Further, all three studies showed that NR3A levels are high in myelin. Despite the low Ca^{2+} permeability of NR3A-containing NMDARs, high levels of NR3A-containing NMDARs and/or insufficient Ca^{2+} buffering might predispose oligodendrocytes to cell death mediated by elevations of glutamate associated with

ischemic stroke or other excitotoxic events. If so, the development of NR3A-selective antagonists could be of therapeutic benefit for preventing white matter excitotoxic damage, with less severe adverse side effects than broad spectrum NMDAR antagonists.

Diseases of neuronal excitotoxicity

A large number of neurological disorders are associated with increased excitotoxicity, including Huntington's disease (Fan and Raymond, 2007), Parkinson's disease (Bonuccelli and Del Dotto, 2006), Alzheimer's disease (Wenk, 2006), chronic alcohol exposure (Crews *et al.*, 1998; Lovinger, 1993), and neuropathic pain (Dubner and Ruda, 1992). Other disorders associated with excitotoxicity include acute brain injuries such as stroke (Martin et al., 1998), epilepsy (Fujikawa, 2005; Meldrum, 1993), and traumatic injury (Arundine and Tymianski, 2004). Excessive activation of NMDARs is thought to contribute to excitotoxicity because the depolarization-dependent activation of Ca^{2+} entry mediated by NMDARs can more readily induce cell death compared to other forms of glutamatergic activation (Abdrachmanova *et al.*, 2002; Choi, 1992; Rothman and Olney, 1995). While excessive glutamate activity triggers NMDAR-mediated cell death in most of these acute and chronic disorders, disruption of cellular metabolic processes can also cause depolarization-induced activation of NMDARs by shifting ionic balances (Zeevalk and Nicklas, 1992).

The proposed role of NMDAR-mediated excitotoxicity in neuronal disorders has fostered intense research into the therapeutic benefits of NMDAR antagonists, but most efforts have yielded disappointing results due to harmful side effects of the antagonists (Hoyte *et al.*, 2004; Lipton, 2006). Because NMDARs are crucial for so many neuronal

functions, including learning and memory (Bear, 1996), and because too little NMDAR activation can also lead to apoptotic cell death (Ikonomidou *et al.*, 1999), successful blockade of NMDAR-mediated excitotoxicity must carefully attenuate NMDAR functions without eliminating them (Lipton, 2006). NR3A provides an attractive target for accomplishing this subtle manipulation of NMDAR functions. One intriguing idea is that overexpression of NR3A might attenuate NMDAR-mediated cell death by reducing Ca^{2+} permeability of existing NMDARs. However, such an approach would have to carefully titrate the degree of NR3A overexpression, as apoptosis can also be triggered by a dramatic decrease in NMDAR currents (Lipton and Nakanishi, 1999).

A neuroprotective role for NR3A has recently been explored further by Nakanishi and coworkers (2009), who used *in vivo* models of hypoxic-ischemic insults as well as retinal cultures to examine the effects of NR3A on cell death. Using NR3A knockout and transgenic overexpressing mice, these authors have provided convincing evidence that ischemic-induced neuronal damage is extensive in the absence of NR3A, while the presence of NR3A reduces cell loss (**Table 2**). Other recent observations support their findings, namely that retinal ganglion cells are relatively invulnerable to NMDAR-mediated excitotoxicity in contrast to many other neuronal classes (Ullian et al., 2004), and only the retinal cell types with high NR3A content exhibit attenuated calcium responses to NMDA (Nakanishi *et al.*, 2009; Sucher *et al.*, 2003). Consistent with the idea that NR3A might offer neuroprotective benefits, high levels of NR3A expression during early brain development might explain why excitotoxicity is not more prevalent at ages before the maturation of inhibitory circuitry. Perhaps it is not a coincidence that NR3A levels diminish during development (Wong et al., 2002) as inhibition increases

(Coyle and Yamamura, 1976). These tantalizing correlations raise the possibility that exogenous introduction of NR3A might be useful to treat a variety of neurological disorders by preventing inappropriate cell death without producing deleterious side effects.

Mental retardations associated with improper dendritic spine development

Dendritic spines are the major site for excitatory synaptic connections on neurons. Changes in their number, density, and/or shape have been implicated in a number of mental retardations including Fragile X, Rett, and Down syndromes (Carlisle and Kennedy, 2005; Chechlacz and Gleeson, 2003; Fiala *et al.*, 2002; Irwin *et al.*, 2000; Kaufmann and Moser, 2000; Newey *et al.*, 2005), but the molecular basis for these spine abnormalities remains unknown. Activation of NMDARs is required both for the bidirectional changes in synaptic strength thought to underlie learning and memory (Malenka and Bear, 2004) and for the activity-dependent growth or retraction of spines which may help encode enduring changes in synapses (Engert and Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999; Nagerl *et al.*, 2004). Therefore, dysregulation of NMDARs might contribute to abnormalities in spine number or shape that could be prevented (or reverted) by normalizing NMDAR activity. Even if NMDAR dysfunction did not play a causal role, modifying NMDAR function could potentially ameliorate conditions arising from abnormal spine development. Indeed, one of the most striking phenotypes of mice lacking NR3A is an approximate three to five-fold increase in spine density (Das et al., 1998). Studies in mice that lack or overexpress NR3A should help elucidate links between spine abnormalities and deficits in synaptic plasticity and learning, and hence

evaluate if targeted and carefully titrated exogenous modulation of NR3A could be of therapeutic value in certain forms of mental retardation.

Other links of NR3A to clinical issues

NR3A is likely involved in other neurological conditions, yet possible roles for NR3A may have gone unrecognized. NR3A was identified relatively recently, and, because of this, it was not incorporated into many tests, such as gene array analyses, that could have detected its involvement in disease. In addition to the direct and indirect suggestions of NR3A involvement in neurological disorders mentioned above, we list here other possible links of NR3A to disease. (1) Decreased NR3A mRNA and protein levels have been reported in patients with bipolar disorder (Mueller and Meador-Woodruff, 2004). (2) Chronic low-level lead exposure during development is associated with cognitive impairments in young children (Bellinger et al., 1991), and decreased levels of NR3A mRNA are reported in rodent hippocampus after lead exposure (Zhang et al., 2002). (3) Genetic analyses of NR3A in humans have identified a common missense variation (Val362Met) that is associated with a strikingly different prefrontal cortex activation during auditory target processing (Gallinat et al., 2007).

While these are just a few additional examples of how NR3A might be involved in neurological disorders, the recognized involvement of NR3A is likely to increase as disease-related changes in NR3A expression levels, polymorphisms, or function are evaluated. The above studies also provide direct evidence for a functional role for NR3A in the adult human brain, and further support the idea that modulation of NR3A in humans might alter neurological functions. Development of specific agonists and

antagonists of NR3A may thus be of therapeutic value for a variety of neurological disorders. Intriguingly, exposure of rodents to weak magnetic fields alters NR3A mRNA levels, raising the possibility that there may even be non-invasive and non-pharmacological means to modify NR3A levels in humans (Hirai and Yoneda, 2004).

Future Directions

There are a number of intriguing outstanding issues raised by my published data and unpublished observations that can be addressed by future studies. Below I briefly outline several of these issues and propose methods to address them.

Role of NR3A in synapse elimination

The localization of NR3A-containing NMDARs at the synapse needs to be fully elucidated. However, imaging studies of molecular interactions at the synapse have historically been limited by the fact that synapses are incredibly small. Conventional light microscopy methods are unable to magnify individual synapses to a high enough level of axial resolution to accurately distinguish these structures and their morphological features. Array tomography is a powerful imaging technique that significantly improves the spatial resolution of closely apposed synaptic proteins over confocal microscopy (Micheva and Smith, 2007) and allows multiple comparisons in a single experiment with repeated probing of antibodies. I propose that array tomography might offer an approach to gain insights into the precise anatomical localization of NR3A-containing NMDARs, and how these receptors are positioned relative to other synaptic proteins. NR3A-containing NMDARs are thought to be preferentially expressed on small and excluded

from large synapses (Roberts et al., 2009). However, whether or not NR3A-expressing spines are specifically eliminated remains unknown. The three-dimensional renderings produced by array tomography would make it possible to clearly discern changes in NR3A-lacking vs. NR3A-containing synapses and thus to address the issue of a role for NR3A in regulating synapse elimination.

Confirmation of NR1/NR3 excitatory glycine receptors in vivo

Subunit composition defines the functional properties of NMDARs. Conventional NR2-containing NMDARs are activated by glutamate, while NR1/NR3A receptors are thought to bind glycine alone. The high glycine affinity of these receptors (Nilsson *et al.*, 2007a; Yao and Mayer, 2006) suggests that they would be occupied at physiological concentrations of glycine *in vivo*, which may serve to keep neurons in a physiologically depolarized state. However, convincing evidence of the NR1/NR3A diheteromeric combination is lacking. Two methods, electrophysiological recordings and immunoprecipitation, would provide the complementary confirmation necessary to resolve this issue. NR3A-containing receptors in slice recordings would be isolated by pharmacological blockade. This is not a trivial experiment, because not only AMPARs, but also specific NR2 subunits would need to be blocked. If an NR3A-specific electrophysiological signature was detected, the subunit composition of NR3A-NMDARs could then be determined by sequential co-immunoprecipitation on whole brain homogenates to examine specifically whether NR3A co-assembles with NR1 subunits in the absence of NR2. It is possible that the assembly of functional NR1/NR3 receptors is NR1 splice variant-specific (Smothers and Woodward, 2009). After immunodepleting

NR2-containing NMDARs with an NR2 antibody cocktail, the remaining NMDARs would be immunoprecipitated with anti-NR1 antibody and blotted for NR3A. Detection of NR3A after this would confirm that these non-conventional glycine receptors exist *in vivo* and open up a field of research devoted to describing their functional roles.

Sensory experience, critical period, and NR3A

The PACSIN1-mediated removal of NR3A is activity-dependent; however, whether or not NR3A expression is dependent upon sensory experience has not been reported. Using a model of visual deprivation, I conducted pilot experiments with WT mice reared in darkness. The harvested tissue was analyzed biochemically for changes in synaptic NR3A. I found that dark-rearing increased NR3A abundance in PSD fractions, suggesting that NR3A expression may be experience-dependent. Moreover, the downregulation of NR3A expression occurs in parallel with onset of the critical period in the visual cortex (Berardi et al., 2000), suggesting that NR3A's role as a molecular brake in synapse maturation may extend to the refinement of sensory modalities. These issues could be addressed by combining techniques of visual deprivation, electrophysiology and biochemical fractionation

Effects of the NR3A-PP2A association on NMDAR activity

Hypofunction of the NMDA receptor is thought to be associated with schizophrenia. Elevated levels of NR3A could produce a form of NMDAR hypofunction. Schizophrenic brains have decreased phosphorylation of NR1 at serine residue 897, and this can be reversed by antipsychotic medications (Emamian et al.,

2004). PP2A is a phosphatase that can dephosphorylate serine 897 on NR1 subunits, and because NR3A forms a signaling complex with PP2A, overexpression of NR3A could contribute to the changes of NR1 phosphorylation observed in schizophrenia (Chan and Sucher, 2001; Ma and Sucher, 2004). Additionally, PACSIN1 removes NR3A from synapses in an activity-dependent manner, using the same C-terminal binding site as PP2A (Ma and Sucher, 2004; Perez-Otano et al., 2006). PP2A normally binds NR3A and releases this bond upon NMDAR activation, thereby making available the binding site for PACSIN1. While these correlations are intriguing, there are no reports examining the possible links between these molecules and synaptic activity. Li et al. (Li et al., 2009) reported the generation of phophomutant mice at Ser897-NR1. The combination of these NR1 and NR3A mutant mice would provide the tools to parse the signaling roles of NR1, NR3A, PACSIN1 and PP2A.

NR3A regulation of myelination

Until now, gene expression profiling has not been used to identify genes that are differentially expressed at the peak of NR3A expression (P8 in mice). We recently analyzed data from DNA microarrays to define subsets of genes that are reciprocally regulated (overrepresented in the absence of NR3A and underrepresented under conditions of NR3A overproduction) in NR3A mutant mice. Surprisingly, our analysis revealed that myelination genes are prominently represented in the NR3A transcriptome. The tantalizing correlation between NR3A loss and the enrichment of these myelin-associated genes is intriguing, because NR3A levels are high in myelin and NR3A has been implicated in white matter injury (Karadottir et al., 2005; Salter and Fern, 2005;

Micu et al., 2006). These intriguing microarray results could be verified in future experiments by quantitative immunoblotting of proteins in biochemical fractions and possibly immuno-EM. We expect that, consistent with their increased transcript levels in the NR3A KO microarrays, protein expression will also be elevated for proteolipid protein 1 (Plp), myelin basic protein (MBP), contactin-associated protein 1 (CASPR1), CNPase, and neurofascin (Nfasc).

Presynaptic vesicle genes are upregulated in NR3A-null forebrains

To date, little is known about the mechanisms by which presynaptic NMDARs regulate neuronal signaling in the CNS. New evidence suggests NR3A may promote neurotransmitter release in synaptic transmission by tonically activating presynaptic NMDARs in the absence of depolarization (Larsen, unpublished observations). The activity of NR3A-lacking NMDARs is tightly regulated by voltage-sensitivity and the developmental decline of presynaptic NMDAR function parallels the downregulation of NR3A. In light of these findings, we were intrigued to find in DNA microarrays that several synaptic genes that are involved in the activation of vesicular fusion, and the release of neurotransmitter into the synaptic cleft are upregulated in NR3A-null mice. It is possible that NR3A may be involved in presynaptic mechanisms by regulating the vesicle release machinery. NR3A may restrict the expression of these proteins until axon terminals are prepared for the increased activity demands at the synapse during circuit refinement. Future studies are needed to validate the transcript data by examining the protein localization in subcellular compartments of the synaptic vesicle membrane proteins, synaptophysin (Syp), VAMP2 (vesicle-associated membrane protein

2)/synaptobrevin, SNAP-25, and complexin (Cpx1). As all of these molecules are intimately involved in synaptic vesicle release (Sudhof, 2004), their overproduction in mutant mice suggests NR3A signaling is tightly linked to their function. Specific fractionation techniques that isolate the presynapse (Phillips et al., 2001) would be useful in determining the precise subcellular distribution of these proteins and parsing the presynaptic mechanisms of release in NR3A KO mice.

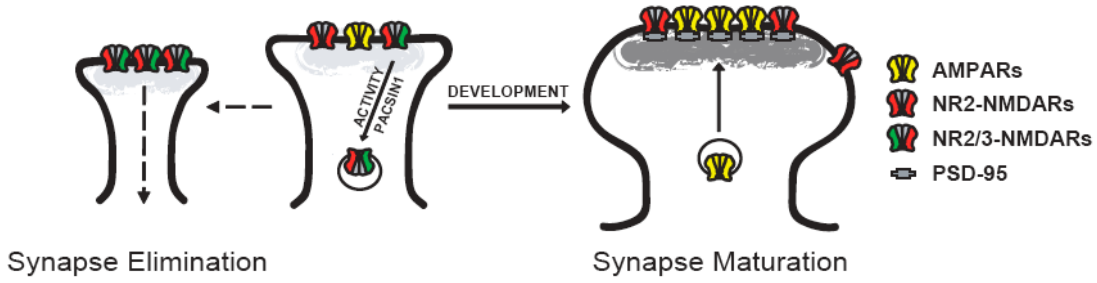


Figure 4.1. Model of the role of NR3A in glutamatergic synapse elimination and maturation. NR3A may act as a molecular brake to prevent premature synapse strengthening. Removal of immature NR3A-containing NMDARs by PACSIN1 may then relieve the 'brake' and allow synapse maturation to proceed, with the insertion of mature NR2-NMDARs, as well as AMPARs, and the morphological spine changes. Expression of NR3A on synapses that receive very little activity and never express AMPARs may mark synapses for elimination.

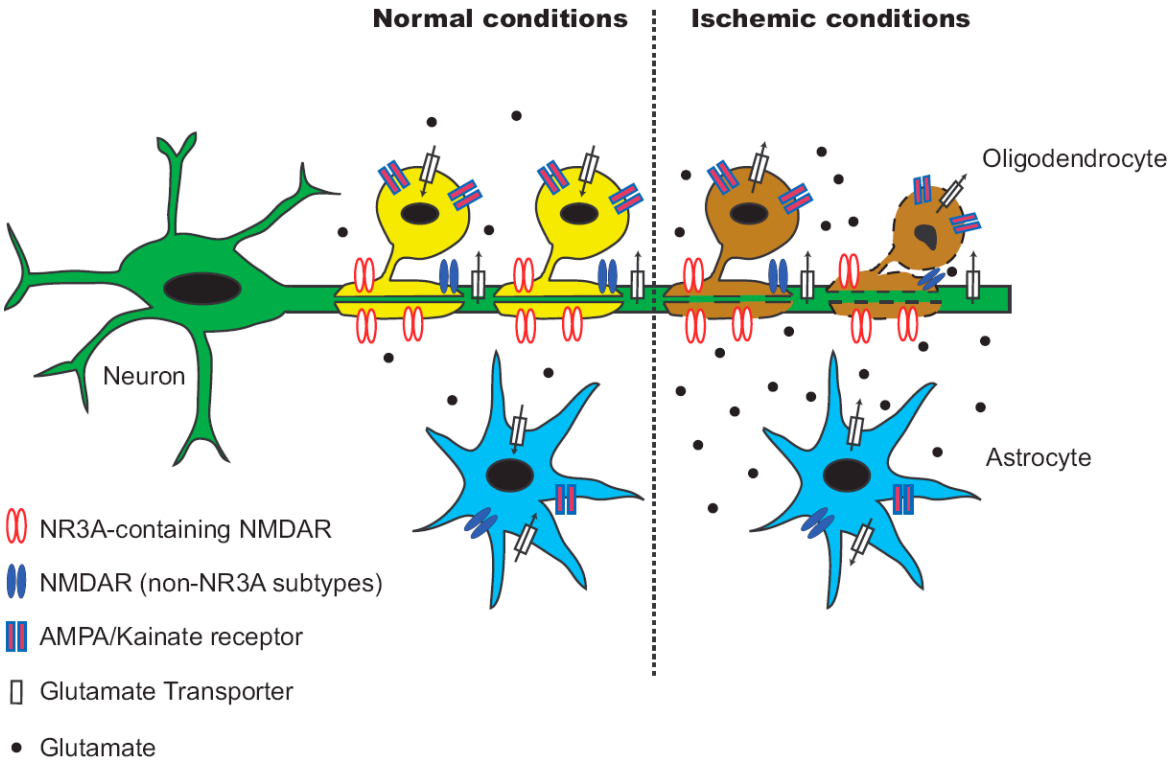


Figure 4.2. NR3A-containing NMDARs may contribute to white matter damage with ischemia. High concentrations of extracellular glutamate caused by reversal of glutamate transport during ischemic conditions can make oligodendrocytes particularly vulnerable to injury. Unlike traditional NMDARs, NR3A-containing receptors may be preferentially activated by glutamate because they are found at high levels in oligodendrocytes and they are insensitive to magnesium block. The resulting calcium influx through NR3A-NMDARs may be sufficient to cause excitotoxic damage to oligodendrocyte processes, ultimately resulting in cell death (indicated by brown color). Modified with permission from Matute (2006).

APPENDIX I

Developmental loss of presynaptic NMDARs parallels the downregulation of NR3A

The notion of presynaptic NMDARs as important modulators of synaptic strengthening and weakening in developmental plasticity has been largely ignored, but is rapidly gaining momentum. NR3A-containing receptors uniquely act to suppress NMDAR-mediated neurotransmission, possibly regulating the synaptic pruning that occurs during this critical developmental timeframe. Because NR3A may play a significant role in the tonic release of glutamate by presynaptic NMDARs (Larsen et al., *Nat Neuro*, in revision), an exciting possibility is that this regulation may occur through the expression of NR3A in presynaptic as opposed to postsynaptic NMDA receptors.

We asked if shifts in subunit composition could explain why the tonic function of preNMDARs during early life is lost later in development (by ~P20 in mouse visual cortex)(Corlew et al., 2007). To determine which subunits have a developmental profile matching that of functional preNMDARs, we quantified protein expression of candidate NMDAR subunits during development of mouse primary visual cortex (V1). Similar to the obligatory NR1 subunit, protein levels of NR2A and NR2B subunits increase with age in V1 (**Figure A.1**). NR2C (Karavanova et al., 2007) and NR3B (Nishi et al., 2001) are not expressed at the right time and regions to contribute to preNMDAR functions in the neocortex. NR2D expression levels did not show a significant developmental change; furthermore, NR2D levels are extremely low in V1 compared to brainstem (**Figure A.2**). In contrast, the expression of NR3A is high early in development and declines

dramatically after the third postnatal week (ANOVA group effect, $p < 0.001$, **Figure A.1**), matching previous observations (Wong et al., 2002; Roberts et al., 2009). Thus, the developmental decrease in NR3A expression matches the loss of preNMDAR functions observed by the third postnatal week (Corlew et al., 2007). This raises the possibility that the NR3A subunit might underlie the tonic activity of preNMDARs.

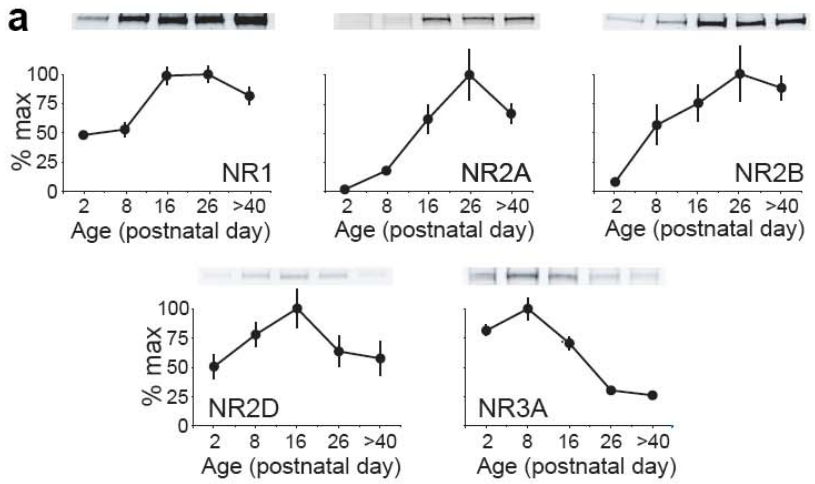


Figure A1: Developmental decline in presynaptic NMDARs parallels the endogenous decrease in NR3A expression. Quantification and representative immunoblots for NMDAR subunits in synaptosomal fractions from developing visual cortex. Protein levels are normalized to actin and presented as percent of maximum expression. Sample sizes are 2-5 for each data point. Each visual cortex sample was pooled from 2-4 mice.

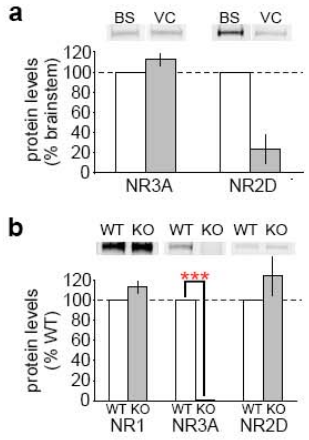


Figure A2: NR2D levels are comparatively low in visual cortex (VC), and these levels are unchanged in NR3A KO mice. (a) Representative blots and quantified protein levels of NR3A and NR2D in P8-P10 visual cortex (n=2) and brainstem (BS) (n=2). NR3A levels are comparable in brainstem and visual cortex, but NR2D levels are minimal in visual cortex compared to brainstem. Protein levels are standardized to an actin loading control and normalized to brainstem values. (b) Representative blots and quantification of NR1 (n = 4 / genotype), NR3A (n =3 / genotype), and NR2D (n = 3 / genotype) protein levels in visual cortex. NR1 and NR2D levels are unchanged in P8 NR3A KO mice compared to WT controls ($p = 0.8$ and $p = 0.6$, respectively, unpaired t-tests), while NR3A was not detected in KO mice ($p << 0.00001$, unpaired t-test). Protein levels in NR3A KO and WT animals are standardized to an actin loading control and expressed as a percentage of WT levels. Error bars are s.e.m. *** $p < 0.00001$.

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