Characterization of NP22 and its Potential Role in NMDA Receptor-Mediated Transmission

by

Moti Gulersen

A thesis submitted in conformity with the requirements for the degree of Master of Science
Pharmacology and Toxicology
University of Toronto

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Pharmacology and Toxicology
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2011

Abstract

N-methyl D-aspartate (NMDA) receptors represent integral signal transducers for excitatory glutamate neurotransmission. While NMDA receptors are critical for synaptic plasticity, the molecular events underlying this process are not fully elucidated. The potential role of NP22, a novel neuronal protein, as a downstream mediator of NMDA receptor function is explored. NP22 protein expression in genetic and pharmacological models of NMDA receptor hypofunction is examined and no significant changes are reported. Characterization of the NP22 protein complex via tandem-affinity and FLAG-purification coupled with mass spectrometry was used and no novel protein interactions are reported. GFP-tagged NP22 colocalization with F-actin decreases in cell processes of transiently transfected HEK293 cells in response to elevated intracellular calcium, while similar colocalization reductions are not seen in stably transfected HEK293 under a comparable treatment regiment. Changes in intracellular calcium affecting NP22 biology can be useful in the ongoing characterization of this novel protein.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-5</td>
<td>(2R)-amino-5-phosphonovaleric acid</td>
</tr>
<tr>
<td>AP-7</td>
<td>2-amino-7-phosphonoheptanoic acid</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca2+/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CK-BB</td>
<td>Creatine kinase brain-type</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-Immunoprecipitation</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DISC1</td>
<td>Disrupted in Schizophrenia 1</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>FACT complex</td>
<td>Facilitates chromatin transcription complex</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphate phosphohydrolase</td>
</tr>
<tr>
<td>HCN-1</td>
<td>Human cortical neuronal cell-1 line</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney cell line</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>hNP22</td>
<td>Human neuronal protein 22</td>
</tr>
<tr>
<td>Kd</td>
<td>Binding dissociation constant</td>
</tr>
<tr>
<td>LCMS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation</td>
</tr>
<tr>
<td>MAP2</td>
<td>Microtubule-associated protein 2</td>
</tr>
<tr>
<td>MK801</td>
<td>Dizocilpine</td>
</tr>
<tr>
<td>MP20</td>
<td><em>Drosophila</em> muscle protein 20</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl D-aspartate</td>
</tr>
<tr>
<td>NP22</td>
<td>Neuronal protein 22</td>
</tr>
<tr>
<td>NP25</td>
<td>Neuronal protein 25</td>
</tr>
<tr>
<td>NR1-KD</td>
<td>N-methyl D-aspartate receptor subunit NR1 knockdown</td>
</tr>
<tr>
<td>PC12</td>
<td>Pheochromocytoma 12</td>
</tr>
<tr>
<td>PCC</td>
<td>Pearson’s correlation coefficient</td>
</tr>
<tr>
<td>PCP</td>
<td>Phencyclidine</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>pIRES</td>
<td>Plasmid containing internal ribosomal entry site</td>
</tr>
<tr>
<td>PSD</td>
<td>Postsynaptic density</td>
</tr>
<tr>
<td>PSD-95</td>
<td>Postsynaptic density scaffolding protein 95</td>
</tr>
<tr>
<td>rNP22</td>
<td>Rat neuronal protein 22</td>
</tr>
<tr>
<td>rNP25</td>
<td>Rat neuronal protein 25</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>Scg10</td>
<td>Superior cervical ganglion 10</td>
</tr>
<tr>
<td>SM22α</td>
<td>Smooth muscle specific protein 22</td>
</tr>
<tr>
<td>SPM</td>
<td>Synaptic plasma membranes</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>TAP</td>
<td>Tandem affinity purification</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor α</td>
</tr>
</tbody>
</table>
Chapter 1
Introduction

Perhaps one of the most fascinating characteristics of the human brain is its plasticity. The manner in which the brain responds to the outside world is not fixed, but rather is shaped by experience, and it continually learns, adapts, recalls, and predicts based on that experience. At the cellular level, experience-dependent plasticity involves the change in the number and strength of connections between neurons, which is critical for learning and memory (Feldman and Knudsen, 1998; Genoux and Montgomery, 2007; Holtmaat and Svoboda, 2009). Modifications in the number and strength of synapses between existing neurons occur through a variety of mechanisms (Lau and Zukin, 2007; Newpher and Ehlers, 2008; Hotulainen and Hoogenraad, 2010; Rebola et al., 2010; Svitkina et al., 2010). One of the most widely studied mechanisms by which synaptic strength is altered is the coordinated activation of glutamate receptors (Yashiro and Philpot, 2008). Glutamate is the principal excitatory neurotransmitter in the brain of mammals (Watkins, 1981), and intense activation of AMPA and NMDA-type glutamate receptors has been shown to result in lasting changes in the excitability of a neuron (Bashir et al., 1991) and in the physical properties of the synapse (Collingridge and Bliss, 1995), the point of contact between two neurons. NMDA receptors have been shown to be essential in many cases for these phenomena, and are thus believed to play a central role in synaptic plasticity (Malenka and Nicoll, 1993; Collingridge et al., 2004).

While it has been well-established that NMDA receptors are critical for synaptic plasticity, the molecular events underlying this process are not fully elucidated. In this thesis, the potential role of NP22 as a downstream mediator of NMDA receptor function is explored. NP22 is a neuron-specific protein that has been shown to bind to cytoskeletal proteins (Mori et al., 2004; de las Heras et al., 2007); however, its role in synapse biology, or in NMDA receptor biology, is not completely understood. This thesis aims to investigate a novel neuronal and cytoskeleton-associated protein, NP22, and the potential link it could have to NMDA receptor signaling and neuronal plasticity.
1 NMDA Receptor Biology

NMDA receptors are glutamate-gated cation channels with high calcium permeability that play a role in several biological processes related to cognition such as learning (Morris et al., 1986; Tsien et al., 1996; Shimizu et al., 2000) and memory formation (Levin et al., 1998; May-Simera and Levin, 2003). Functional expression of the receptor is critical for setting up a correct neuronal wiring scheme during brain development (Cline and Constantine-Paton, 1989; Ikonomidou et al., 1999). Although NMDA receptors are widely implicated in many aspects of neuron function and biology, the focus of this thesis rests on the role of NMDA receptors in molecular events underlying synaptic plasticity. Other significant features of NMDA receptors include their requirement for survival (Li et al., 1994; Forrest et al., 1994) and implication in several disorders such as brain trauma (Parton et al., 2005), ischemic brain damage (Johnston, 2005), seizure (Meldrum, 1994; Mares et al., 2004), neurodegeneration (Hynd et al., 2004), alcohol and drug addiction (Narita et al., 2005; Carpenter-Hyland and Chandler, 2007; Vengeliene et al., 2008 Ma et al., 2009) and schizophrenia (Coyle, 1996; Mohn et al., 1999).

1.1 Receptor Composition, Expression and Ligands

NMDA receptors are members of the ionotrophic glutamate receptor family (Dingledine et al., 1999), activated by simultaneous binding of the excitatory amino acid L-glutamate and the co-agonist glycine (Hirai et al., 1996). They are pharmacologically distinguished from other glutamate receptors by their binding of NMDA, the receptor’s selective agonist discovered by Curtis and Watkins over 30 years ago (Watkins, 1981).

NMDA receptors are heteromeric tetramer assemblies of four subunits derived from three related subtypes: NR1, NR2 and NR3 (Nakanishi et al., 1992; Mori and Mishina, 1995; Seeburg et al., 1995). Presence of the well-characterized functional receptor in mammalian cells requires the co-expression of two obligatory glycine-binding NR1 subunits and two glutamate-binding NR2 subunits assembled around a central, cation-selective pore (Laube et al., 1997). Occasionally, cells expressing NR3, which also binds glycine, can form functional receptors by substituting for one or both NR2 subunits (Chatterton et al., 2002). Multiple receptor isoforms with distinct functional properties arise by alternative splicing of the NR1 gene (Exons 5, 21 and 22) (Sugihara et al., 1992) and from differential expression of NR2 and NR3 subunits (Mori and Mishina, 1995). All three subunit subfamilies share a common membrane topology characterized
by an extracellular amino terminal domain that includes the ligand binding domain, a membrane region containing three transmembrane segments (TM1, 3 and 4), a re-entrant pore loop (M2) that forms the pore-lining region, and a cytoplasmic carboxy tail (Frank, 2011).

Several compounds, both endogenous and therapeutic, can interact with NMDA receptors. There are a number of known agonists and antagonists that can bind the receptor and alter its function (Table 1). Dissociative anesthetic compounds such as phencyclidine (PCP) and dizocilpine (MK-801) are effective blockers of the ion channel associated with the NMDA receptor complex. At low doses, these compounds are psychotomimetic and induce a schizophrenia-like state (Krystal et al., 1994). These antagonists do not compete for ligand binding (non-competitive antagonists), and can only mediate their action if the receptor channel is open to make the binding site accessible (hence they are use-dependent). Some of the most well known antagonists competing for the glutamate-binding site of the NMDA receptor (competitive antagonists) include AP-5 and AP-7, which are also able to produce hallucinogenic effects in both animal models and humans (Willetts et al., 1990). Although NMDA remains as the specific agonist for the receptor, it is less potent than L-glutamate (Varney et al., 1996), the predominant endogenous neurotransmitter that activates the receptor. There are also several natural and synthetic molecules (both endogenous and exogenous) that modulate the receptor such as polyamines, protons, zinc, steroids and redox reagents (Christine et al., 1990; Williams et al., 1991; Low et al., 2000). It is reported that acute concentrations of ethanol (5-100 mM) inhibit NMDA receptor-activated ion currents in cultured mouse hippocampus neurons (Lovinger et al., 1989). While synthetic modulators can be used as experimental tools to study properties of the NMDA receptor and aid in the development of therapeutically useful targets, other endogenous modulators may exert important regulatory effects on the receptor under physiological or pathological conditions (VanDongen, 2009).
<table>
<thead>
<tr>
<th>Agent</th>
<th>Binding Site</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
</tr>
<tr>
<td>NMDA (S)-Glutamic Acid</td>
<td>Glutamate binding site on the NR2 subunit</td>
</tr>
<tr>
<td>Tetrazolylglycine</td>
<td></td>
</tr>
<tr>
<td>Homoquinolinic acid</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>Glycine binding site on the NR1 subunit</td>
</tr>
<tr>
<td>D-Serine</td>
<td></td>
</tr>
<tr>
<td><strong>Antagonists</strong></td>
<td></td>
</tr>
<tr>
<td>(R)-2-amino-5-phosphonopentanoate (R-AP5)</td>
<td>Glutamate binding site on the NR2 subunit</td>
</tr>
<tr>
<td>NVP-AAM077</td>
<td></td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>Glycine binding site on the NR1 subunit</td>
</tr>
<tr>
<td>Phencyclidine (PCP)</td>
<td>Inside the channel of NMDA receptor complex; ion channel pore</td>
</tr>
<tr>
<td>Ketamine</td>
<td></td>
</tr>
<tr>
<td>Dizocilpine (MK-801)</td>
<td></td>
</tr>
<tr>
<td>Memantine</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.1: Pharmacological agents that interact with the NMDA receptor including binding site. Adapted from VanDongen, 2009.

Synaptic NMDA receptors are localized to the post-synaptic density (PSD), a protein-dense region at the membrane of the postsynaptic neuron, where they are structurally organized in a large macromolecular complex composed of receptors, scaffolding proteins, and signal transduction machinery (Husi et al., 2000). Scaffolding proteins link NMDA receptors to downstream signaling molecules, kinases and phosphatases, and other transmembrane proteins (Rebola et al., 2010). Synaptic activity regulates the receptor composition of the PSD and its associated macromolecular complex of proteins, resulting in the dynamic regulation of NMDA receptor trafficking from intracellular compartments to the synaptic membrane (Lau and Zukin, 2007).

1.2 Signal Transduction and Implications in Neuron Biology

Similar to several other neurotransmission mechanisms, postsynaptic NMDA receptor activation relies on the release of L-glutamate from presynaptic terminals that diffuses across the synaptic cleft. With each presynaptic action potential driving the release of the neurotransmitter, glutamate becomes available for receptor binding and activation. However, due to the receptor’s pronounced voltage dependence, glutamate binding only is not sufficient to activate the receptor and cause subsequent ion permeation (Nowak et al., 1984; Mayer et al., 1984; Seeburg et al., 1995). As magnesium ions clog the NMDA receptor pore at resting membrane potentials, a
depolarization of sufficient amplitude and duration is needed to expel the magnesium ions from the pore and allow for ion conductance (Nowak et al., 1984; Mayer et al., 1984). As a result, NMDA receptors were coined ‘molecular coincidence detectors’ (Seeburg et al., 1995), where efficient activation of the receptor requires both strong depolarization and synaptic release of glutamate. Once fully activated, a significant influx of cations, most notably calcium, flows into the postsynaptic neuron and regulate its properties through a variety of signal cascades. Excessive intracellular calcium concentrations, via activation of these intracellular pathways, lead to both physiological and pathological processes (Collingridge et al., 2004). The rise in intracellular calcium directly and indirectly activates kinases such as calcium/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA), mitogen-activated protein kinase (MAPK), and protein phosphatases (calcineurin) (Rebola et al., 2010). Subsequently, changes in synaptogenesis, cytoskeletal synaptic remodeling, and long-lasting changes in synaptic efficacy ensue (Rebola et al., 2010). NMDA receptors work in concert with other ionotropic glutamate receptors such as AMPA receptors to either strengthen synapses and potentiate signal transmission, a process known as long-term potentiation (LTP), or weaken synapse strength such that there is a reduction in transmission efficacy, conversely called long-term depression (LTD). These changes can last for hours or longer and contribute to learning and memory in the human brain (Malenka and Bear, 2004; Perez-Otano and Ehlers, 2005).

More specifically, NMDA receptors are integral to regulating the actin-signaling pathways responsible for spine remodeling, another functional change that leads to formation and elimination of synapses (Hotulainan and Hoogenraad, 2010; Svitkina et al., 2010). The functional changes that take place in spines, small dendritic protrusions at excitatory synapses, are integral to mediating synaptic plasticity (Bourne and Harris, 2008). Spines are responsible for compartmentalizing postsynaptic signaling pathways and controlling the diffusion of postsynaptic molecules (Nimchinsky et al., 2002, Newpher and Ehlers, 2009). A major player that regulates spine formation, elimination and dynamics is the actin cytoskeleton, responsible for organizing the postsynaptic density and anchoring postsynaptic receptors (Hotulainan and Hoogenraad, 2010; Svitkina et al., 2010). When accounting for changes in spine morphology, fluorescence resonance energy transfer studies show a dynamic shift in equilibrium between globular and filamentous actin as a result of synaptic stimulation and/or depression (Okamoto et al., 2004). LTP induction shifts the equilibrium in favour of filamentous actin, resulting in a rise
in spine actin filaments and spine volume, whereas LTD induction shifts the equilibrium in favour of globular actin, reducing spine actin filaments and density. Thus, the treadmilling of actin pools drives the rapid reorganization of spines, regulating their structure and plasticity (Okamoto et al., 2004; Hotulainen and Hoogenraad, 2010). Furthermore, increases in spine volume are believed to result in a greater contribution of that spine towards downstream signaling events and transmission of membrane depolarization (Kasai et al., 2010).

NMDA receptors mediate changes in the actin cytoskeleton of spines through a variety of ways. The influx of calcium into postsynaptic neurons modulates the activity of actin binding proteins such as CaMKIIβ (Lisman et al., 2002) and gelsolin (Nag et al., 2009). Also, direct interactions of NMDA receptors with actin-binding and actin-regulating proteins such as CaMKII (Raveendran et al., 2009), α-actinin (Wyszynski et al., 1997) and myosin regulatory light chain (Bajaj et al., 2009) has been reported. The Rho family of GTPases are also important in regulating changes in the actin cytoskeleton (Ethell and Pasquale, 2005; Tada and Sheng, 2006). RhoA, Rac and Cdc42 are Rho GTPases that have been extensively studied in regulating actin dynamics. Activation of RhoA and Cdc42 are dependent on NMDA receptors and CaMKII, ultimately affecting actin filament and spine stabilization (Rex et al., 2009; Hotulainen et al., 2009). Together, NMDA receptor signaling and cytoskeletal modifications are believed to contribute to changes in neuron biology responsible for synaptic transmission and plasticity.

1.3 Animal Model of NMDA Receptor Hypofunction

Reducing the function of receptors in vivo is a popular strategy employed when characterizing the effects of a neurotransmitter system. Through use of these genetic models, there is an ongoing effort to understand the physiological consequences of sustained NMDA receptor hypofunction in constrast to acute pharmacological models of NMDA receptor antagonism. Mice completely deficient in NR1 or NR2B protein cannot be used because they die perinatally, providing evidence that NMDA receptor signaling is essential for an animal to survive (Li et al., 1994; Forrest et al., 1994). Mohn and colleagues (1999) generated a mouse model of NMDA receptor hypofunction wherein homozygous mutant mice express 5%-10% of the normal level of NR1. The hypomorphic mutation was carried out using a targeting vector with a neomycin resistance gene that integrated into intron 20 of the NR1 locus (Fig. 1.1). The resulting effects not only include decreases in NR1 mRNA and protein, but quantitative radioligand binding
shows an approximate 90% reduction in functional NMDA receptors (Fig. 1.2). The NR1 hypomorphic, or knockdown, mice (NR1-KD), represent a viable model of NMDA receptor hypofunction, and are used in our studies to investigate the role of NP22 in NMDA receptor biology.

**Figure 1.1.** Hypomorphic mutation of Grin1 reduces NMDA receptor subunit NR1 message and protein. In wild type mice (A), Grin1 is transcribed and alternatively spliced. NR1 subunits are produced in excess of NR2 subunits, which limit the amount of functional receptor that can be produced. In NR1-KD (B), the presence of neo leads to the production of transcripts that are truncated due to the polyadenylation signal of the neo gene. For those transcripts that are full length, proper splicing of the message removes neo and produces functional NR1 subunits. NR1 levels are reduced and are no longer in excess of NR2 subunits, producing fewer functional NMDA receptors. Although mutation of NR1 leads to global reductions in NMDA receptors, the deficit is most apparent in brain regions where NMDA receptors are normally highest. Reprinted with permission from author (Ramsey, 2009).
Quantitative analysis of reduced NMDA receptor levels in NR1-KD model. Radioligand binding of [3H]MK-801 to membrane homogenates from the prefrontal cortex of WT and NR1-KD mice. A competition binding curve was generated using 2 nM [3H]MK-801 and increasing concentrations of MK-801 to calculate the Kd and Bmax of MK-801 binding. The Kd for both the wild-type and mutant mouse is 2 nM. The Bmax is 0.12 pmol/mg for the NR1-KD and 1.2 pmol/mg for wild-type mice (n = 3 for each genotype). Reprinted with permission from author (Mohn et al., 1999).

With the notion that NMDA receptor signaling has been implicated in mediating spine synaptic plasticity and regulating spine density, Ramsey et al. (2011) hypothesized that reduced NMDA receptor function in the NR1-KD mice would alter the biochemical composition of striatal medium spiny neurons leading to structural changes in postsynaptic spines. The striatum represented a logical brain region to investigate changes in spine density because of the amount of medium spiny neurons it contains. Approximately 95% of neurons in the striatum are medium spiny neurons, which have highly dense spinous dendrites covered by glutamatergic afferents (Nicola et al., 2000; Surmeier et al., 2007). Thus, biochemical preparations of the striatum would contain synaptic protein concentrations from a nearly homogenous neuronal population. They discovered that the NMDA receptor deficient mice show an age-dependent deficit in synaptic spine density, with density reductions reaching 18% in 6-week old mice as opposed to the no change observed in 2-week old mice (Ramsey et al., 2011). Further proteomic investigations revealed synaptic reductions in 14-3-3ε and DISC1 (Ramsey et al., 2011), both proteins implicated in processes regulating neurite outgrowth and spine density (Toyo-oka et al., 2003; Ikeda et al., 2008; Kvajo et al., 2008; Brandon et al., 2009; Hayashi-Takagi et al., 2010). Subchronic pharmacological antagonism of NMDA receptors via MK-801 confirmed the decreases in spine density and synaptic DISC1 (Ramsey et al., 2011). These studies indicate that
NMDA receptor dysfunction leads to alterations in synaptic spine density; given the role of actin-binding proteins in the process of spine remodeling, it is possible that these reductions in spine density are due to changes in the function of actin-binding or actin-regulating proteins.

1.4 Clinical Significance: Implications in Disease States

Due to the importance of NMDA receptor function in the human brain, it is not surprising to find that NMDA receptors have been implicated in several neurological disorders such as Alzheimer’s disease, Parkinson’s disease and schizophrenia (VanDongen, 2009). Alzheimer’s disease (AD), signified by the accumulation of β-amyloid peptide (Hardy and Selkoe, 2002; Price et al., 1998), is characterized by impairments in memory and cognition (Kim and Tsai, 2009). Evidence suggests a glutamatergic synaptic dysfunction in early stages of the disease as observations in biopsied and postmortem AD brains reported losses of synaptic spines in cortical neurons (Lacor et al., 2007). Also, analyses of postmortem AD brains have shown altered NMDA receptor subunit expression (Hynd et al., 2001). Currently, memantine, a noncompetitive NMDA receptor antagonist, has been approved for treatment of AD (Lipton, 2005). Parkinson’s disease (PD) is characterized by degeneration of dopaminergic neurons projecting from the substantia nigra to the striatum in the brain. NMDA receptors have been implicated in the disease as studies have shown effective reduction of PD neuropathology via NMDA receptor antagonists ifenprodil and CP-101,606 in experimental models and humans (Uitti et al., 1996; Loschmann et al., 2004). NMDA receptor subcellular localization and synaptic function are reportedly altered in animal models of PD as well (Gardoni and Di Luca, 2006).

The involvement of NMDA receptor signaling in schizophrenia has emerged as a counterpart to the well-known dopamine hypothesis for the etiology of the disease (Mohn et al., 1999). Schizophrenia is characterized by cognitive impairments, positive symptoms such as hallucinations, delusions and paranoia and/or negative symptoms such as social withdrawal and impaired attention. The glutamate dysfunction hypothesis stemmed from the observation that reduced NMDA receptor function, via use of NMDA receptor antagonists phencyclidine (PCP) and ketamine, closely mimics the disease by producing several of its symptoms (Luby et al., 1959; Lahti et al., 1995). In the animal model of NMDA receptor hypofunction mentioned earlier, mice expressing 5% of normal levels of NR1 displayed behavioural abnormalities correlated with schizophrenia and were reversed by treatment with commonly used
antipsychotics such as haloperidol or clozapine (Mohn et al., 1999). Additionally, altered expression levels of all NMDA receptor subunits have been reported in postmortem tissues of schizophrenic patients (Mueller and Meador-Woodruff, 2004).

NMDA receptors have also been implicated in alcohol addiction and ethanol-associated phenotypes such as tolerance, dependence, withdrawal, craving and relapse (Trujillo and Akil, 1995; Krystal et al., 2003). NMDA receptors were identified as a major target of ethanol after it was discovered that ethanol inhibits NMDA receptor-mediated excitatory postsynaptic potentials/currents in many brain regions such as the hippocampus (Lovinger et al., 1989; Morrisett et al., 1991; Kolb et al., 2005), cortex (Wirkner et al., 2000; Li et al., 2002), amygdala, nucleus accumbens (Nie et al., 1994; Maldve et al., 2002) and dorsal striatum (Popp et al., 1998; Yin et al., 2007; Wang et al., 2007). The mechanism by which ethanol inhibits NMDA receptor function is still under investigation although it has been reported that ethanol decreases open channel probability and mean open time of NMDA receptors in single channel recordings of cultured cortical neurons (Wright et al., 1996). A unique observation regarding ethanol-induced NMDA receptor inhibition is that neuronal preparations exposed to ethanol for 24 hours or longer followed by withdrawal have shown NMDA receptor channel hyperactivation (Esol, 2006). This is due, in part, to the compensatory upregulation of NMDA receptor subunit mRNA (Hu et al., 1996; Kumari and Ticku, 1998) and protein (Follesa and Ticku, 1996). Thus, mammals who undergo chronic ethanol inhibition of NMDA receptors can experience withdrawal seizures as a result of heightened NMDA receptor activation (Hendricson et al., 2007; Hughes, 2009).

2 NP22/NP25/Transgelin-3: A Neuronal Protein

Amidst the search for renin-like cDNA clones in the rat brain over 15 years ago, Choong-Chin Liew and collaborators (1994) at the University of Toronto discovered a novel neuronal protein. Neuronal Protein 25 (NP25), designated after its predicted molecular mass of 24,710 daltons, had been isolated as a protein that was widely and differentially expressed by neuronal subpopulations. The nucleic acid sequence of NP25 cDNA revealed a single open reading frame encoding the 219 amino acid residues that represented a typical globular protein and had conserved sequences among mice and humans. While its function in vitro was not elucidated at the time, a search through protein databases indicated that NP25 shared significant amino acid
sequence homology to the smooth muscle calponin family of proteins. From this initial study came further efforts to understand the biological role of the protein, which was eventually renamed Transgelin-3 based on sequence homology, but has been alternately referred to as NP22 and NP25 in the literature (Ren et al., 1994).

2.1 NP22 Expression and Localization

Seven years after rat NP25 was discovered, the protein re-emerged in the literature with a different name, NP22. Li Fan and collaborators (2001) at the University of Queensland in Australia were employing PCR-based differential display to search for differentially expressed genes in post-mortem brains of human alcoholics. Targeting the superior frontal cortex and primary motor cortex, a cDNA fragment of 1367 base pairs was retrieved and submitted to GENBANK for identification. The sequence shared high homology with the existing human NP25 sequence previously submitted but differed in one critical base. The new sequence, named human NP22 to reflect the size and homology of the human gene product, had one less thymidine resulting in an earlier STOP codon in the open reading frame and a deduced protein of 199 amino acids instead of 282. It was first reported that the neural-specific human protein had a calculated molecular mass of 22 469.5 Da with charged amino acids, a hydrophilic profile, putative actin-binding and phosphorylation sites and two possible E-F hand-like calcium-binding sites. The sequence’s homology with calponin and SM22α suggested a possible interaction of hNP22 with the cytoskeleton. What was more intriguing was its elevated mRNA expression in the frontal cortex of alcoholic cases. Chronic alcohol exposure results in pathological changes in several brain regions and it was hypothesized that hNP22 may be a mediator in the regulatory signal transduction pathways that would follow because of its calcium-binding, potential cytoskeleton- and PKC-interacting characteristics; all of which had been associated with ethanol exposure (Fan et al., 2001).

While initial expression mRNA studies were intriguing, the Wilce lab in Australia continued to characterize NP22 by investigating its protein expression profile in the brains of post-mortem human alcoholics and rats treated with ethanol vapor. Using their own hNP22 antibody, western blot analysis showed hNP22 protein levels were significantly elevated in the post-mortem frontal cortex of human alcoholics. Immunohistochemical studies confirmed the increased NP22 expression in the frontal cortex and also displayed differential increased NP22 expression in
layers (CA3 and 4 only) of the hippocampus of alcoholics. With the function of the protein still unclear, immunochemical data also revealed that NP22 expression was restricted to the cytoplasm and processes of neurons in the prefrontal cortex and hippocampus and was not shown in any of the brain regions’ glial cells (Depaz et al., 2003). Furthermore, subsequent rat brain profiling yielded more clues about NP22 and its potential function. Not only were rat brain NP22 mRNA and protein expression levels altered by chronic alcohol exposure and withdrawal in a region-selective manner, colocalization of rNP22 with several cytoskeletal proteins were also reported. These proteins included tubulin, microtubule-associated protein 2 (MAP2), F-actin and tau. This provided further evidence that it was possible NP22 expression changes along with its colocalization patterns with cytoskeletal proteins may be indicative of a signal cascade mediating changes in neuronal biology in response to prolonged ethanol exposure (Depaz et al., 2005).

Considering the potential function of hNP22 in the regulation of cytoskeletal architecture and the reported morphological abnormalities seen in neurons from individuals with schizophrenia, the expression of NP22 in several affected brain regions of postmortem schizophrenic brains was also investigated (Ito et al., 2005). The regions of interest included the anterior cingulate cortex, hippocampus and prefrontal cortex; all have been implicated with abnormal morphological changes in the disease (Bogerts et al., 1985; Jeste and Lohr, 1989; Benes et al., 1991; Benes et al., 1992; Selemon et al., 1998; Crespo-Facorro et al., 2000). Using immunohistochemistry, no changes of hNP22 expression were identified in the prefrontal cortex and hippocampus of postmortem schizophrenic brains compared to controls (Ito et al., 2005). However, in layer V and VI of the anterior cingulate cortex, hNP22 expression was significantly reduced compared to controls (Ito et al., 2005). The results suggested that lowered levels of hNP22 may be associated with the disturbed neural connectivity seen in the anterior cingulate cortex pathologies of the schizophrenic brain (Ito et al., 2005).

Most recently, Pape et al. (2008) characterized NP22 mRNA and protein expression in the chick embryo neural tube (spinal cord, dorsal root ganglion and sympathetic ganglia) via in situ hybridization and immunohistochemistry. Early onset of mRNA expression was identified in parallel to expression of NeuroM and Scg10, early markers of neuron development and differentiated neurons. Thus, NP22 represented a protein expressed at the very onset of neuron differentiation in all neuronal lineages analyzed, suggesting another potential role for the protein (Pape et al., 2008).
Although several rather primitive hypotheses have been suggested, NP22 had emerged as a protein that required further studies to be conducted in order to learn more about the significance of its’ protein motifs and potential role in modifying cytoskeletal architecture and neuron biology.

2.2 Significance of Protein Motifs

To date, NP22 has been identified as a member of the calponin protein family due to the presence of a highly conserved calponin homology domain. Calponin proteins, mediating their actions through control of smooth muscle contraction, have been implicated in direct interactions with the actin and microtubule cytoskeleton and regulatory functions through Rho-signalling; lending to their involvement in cell migration, neurite extension and neuronal plasticity (Mori et al., 2003; Pape et al., 2008). The calponin homology domain is found in a number of actin binding proteins including filamin, α-actinin, dystrophin and utrophin, which are essential for regulation of cytoskeleton organization and cell shape (Gimona et al., 2002). As previously mentioned, NP22 shares high sequence homology with other calponin family proteins such as calponin, smooth muscle specific protein 22 (SM22α) and Drosophila muscle protein 20 (MP20) (Ren et al., 1994).

With regards to protein topology, NP22 contains a number of signal protein motifs including the aforementioned calponin homology domain, two EF hand motifs, a putative actin-binding site and two potential PKC phosphorylation sites (Fig. 1.3) (de las Heras et al., 2007). Characterized by a helix-loop-helix structural blueprint found in several calcium-binding proteins, the EF hand motif allows for protein-ligand binding via calcium coordination (Nelson et al., 2002). The consensus PKC phosphorylation sites are also highly conserved among the calponin family of proteins. Activated by intracellular increases in diacylglycerol or calcium, the PKC family of protein kinases has been implicated in signal cascades regulating actin dynamics and cell growth (Squire et al., 2008).
Figure 1.3. Schematic representation of motifs present in human NP22. NP22 is a protein spanning 199 amino acids and includes a number of signal protein motifs (from left to right): two EF hand like calcium binding sites, putative actin-binding site, calponin homology domain containing two protein kinase C phosphorylation sites. Adapted from de las Heras et al., 2007.

2.3 Investigating Functional Role via Potential Interactions

Several investigators have attempted to elucidate the function of NP22 through various in vivo and in vitro studies. Kenji Mori and collaborators (2004) investigated NP22 expression in the human neuroblastoma cell line, SK-N-SH. After initially showing strong colocalization of NP22 with the actin cytoskeleton via immunocytochemistry, co-sedimentation experiments using actin and either NP22 or GST revealed an interaction between actin and NP22 (Mori et al., 2004). Furthermore, with the use of an indirect immunofluorescence technique, they provided strong intracellular FRET signals between fluorophores bound to NP22 and actin fibers, indicating that both proteins were in close proximity in situ (Mori et al., 2004). Regulation of actin dynamics is crucial for precise neuronal development as several neural differentiation processes, such as neurite extension, involve the dynamic reorganization of actin filaments (Furnish et al., 2001).

Providing further evidence of NP22’s interaction with cytoskeletal proteins and its ability to mediate changes in cell shape through such interactions was the continuing goal of the Wilce lab in Australia. While investigating NP22’s ability to induce cytoskeletal changes, overexpression of the protein in Chinese hamster ovary (CHO) cells resulted in de novo process formation that was even enhanced upon treatment with the actin depolymerising agent, cytochalasin D. Additionally, there was also colocalization of NP22 with actin and α-tubulin in the processes formed (de las Heras et al., 2007). However, unlike what Mori et al. (2004) were able to show, column immunoprecipitation studies with adult human brain lysates and hNP22 transfected CHO cells revealed no association between NP22 and actin. Instead, an NP22 and α-tubulin interaction was confirmed (de las Heras et al., 2007).
Mutated forms of hNP22 constructs were also generated and transfected into CHO cells to investigate the resulting effect on cell morphology (de las Heras et al., 2007). Even though actin binding with NP22 was not reported, deletion of the putative actin-binding site in hNP22 still interfered with the protein’s ability to induce process formation in cytochalasin D treated CHO cells as fewer process were observed (de las Heras et al., 2007). Mutations of putative phosphorylation sites of hNP22 also affected cellular morphology. CHO cells expressing serine-specific phosphorylated hNP22 showed the same morphological changes as cells transfected with wild-type hNP22 (de las Heras et al., 2007). However, CHO cells expressing mutated phosphorylation sites were unable to form and extend processes following cytochalasin D exposure (de las Heras et al., 2007). Although it is clear that actin plays a role in NP22’s ability to form processes in CHO cells, the lack of column immunoprecipitation evidence to support the interaction seems controversial. However, it is possible that phosphorylation of the protein could be altering its association with cytoskeletal proteins. The immunoprecipitation assay in the study did not control for the protein’s phosphorylation state, such that in a case where NP22 can only bind actin when phosphorylated, affinity experiments may have masked the interaction. The α-tubulin association reported is significant because NP22 was still able to enhance process formation in CHO cells when the actin network was disrupted with cytochalasin D (de las Heras et al., 2007). This is consistent with the fact that both actin filaments and microtubules are major elements in the cytoskeleton that control neuronal process formation and morphology (Svitkana et al., 2010).

Having already demonstrated colocalization of NP22 and proteins of the cytoskeleton as well as its ability to induce process formation in vitro, Depaz and Wilce (2006) hypothesized that NP22 could be important in neuronal development. Their study showed significant increases in NP22 mRNA expression during postnatal brain development days 4 and 10 and remained elevated until day 24 (Depaz and Wilce, 2006). NP22 protein expression, showing positive levels at even the embryonic stage, was also elevated during postnatal neuronal development, increasing significantly at days 4 and 16 after birth (Depaz and Wilce, 2006). Interestingly, synapses are formed and dendritic spines become more complex between days 6 and 25 of rat brain development (Depaz and Wilce, 2006). The elevated levels of NP22 shown during this period suggest a potential role it has in neurite, synapse and spine formation. This provides more
encouraging evidence as NP22 has already been shown to associate with cytoskeletal proteins and induce process formation in transfected CHO cells (de las Heras et al., 2007).

Building on the ability of NP22 to induce process formation in CHO cells, Pape and collaborators (2008) investigated the function of NP22 in two different primary neuron culture models, chick sensory dorsal root ganglion (DRG) and E7 sympathetic neurons as well as the PC12 cell line. For the first time, both loss of function and gain of function approaches were used. NP22 overexpression increased neurite length in sensory DRG neurons and PC12 cells but decreased neurite length in sympathetic neurons (Pape et al., 2008). Conversely, siRNA knockdown of NP22 decreased neurite length in sensory DRG neurons and PC12 cells but increased neurite length in sympathetic neurons (Pape et al., 2008). The opposing effects of NP22 overexpression and knock-down seen in different cell types may be related to a level dependent action of NP22: NP22 transfections enhanced neurite growth in cells expressing endogenously low levels of NP22 but inhibited neurite growth in cells expressing endogenously high levels of NP22. Conversely, NP22 knock-down enhanced neurite growth in cells expressing endogenously high levels of NP22 but inhibited neurite growth in cells expressing endogenously low levels of NP22 (Pape et al., 2008). Therefore, it appears that there is an optimal level of NP22 expression that maximizes neurite outgrowth; if levels fall below or above the optimal level, neurite growth and extension is decreased.

Colocalization studies on the primary neuron cultures and PC12 cells were also performed via confocal microscopy to investigate if the biological effects of NP22 are mediated by interactions with the actin cytoskeleton. No clear evidence for colocalization of NP22 and F-actin was observed in the cytoplasm or submembranous cortex of the primary neuron cell cultures. Consistent with the idea that the actin-binding activity of NP22 may be inhibited by PKC phosphorylation, the same lack of clear colocalization was still seen in cells treated with PKC inhibitor bisindolylmaleimide II. In contrast, colocalization of NP22 and F-actin was observed in short cellular processes most likely representing filopodia in PC12 cells transiently transfected with rNP25 vectors (Pape et al., 2008).
3 Experimental Approach: Methods to Identify Protein Interactions

Knowledge and characterization of protein-protein interactions is important to unveil the molecular basis and mechanisms in most biological processes. In turn, by determining interaction partners of novel proteins, the possible functions of these new proteins may be inferred. Several important processes such as DNA replication, transcription, translation, protein degradation and signal transduction employ the use of highly efficient protein networks (Ramisetty and Washburn, 2011). Establishing the molecular interactions forming the basis of these regulatory networks continues to be a major goal for molecular biologists in the post-genomic era. In fact, since aberrant protein-protein interactions can lead to human disease and cancer, modulation of protein-protein binding not only represents an emerging therapeutic regimen but protein interaction targets also provide us with a new class of attractive targets for drug development (Kaake et al., 2010). There are several methodologies used to study protein-protein interactions in a system such as the yeast two-hybrid system, bioluminescence resonance energy transfer, in vitro binding assays, fluorescence imaging and co-immunoprecipitation (Miernyk and Thelen, 2008). The sensitivity and efficiency of tandem affinity purification coupled with mass spectrometry (MS), however, has made this emerging high-throughput biochemical strategy a method of choice for investigating protein interaction networks (Kaake et al., 2010; Volkel et al., 2010).

3.1 Purifying Protein Complexes: Tandem Affinity and FLAG Purification, Yeast Two-Hybrid and Co-Immunoprecipitation

One biochemical approach developed to purify protein complexes in vitro is based on the use of an antibody that targets a known protein believed to be a member of a larger, potentially unknown, protein complex. The method, termed co-immunoprecipitation (Co-IP), ‘pulls down’ the protein of interest along with possible associated proteins that bind to it (Miernyk and Thelen, 2008). Co-IP has been used to study receptor-ligand interactions, enzyme-substrate interactions and interactions of subunits within a protein complex (Honda et al., 1997; Shi et al., 2000). Identifying members of the protein complex via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining, autoradiography or Western blot analysis is typically used (Miernyk and Thelen, 2008). Recently, mass spectrometry has emerged as a
common strategy used to discern novel binding partners (Gingras et al., 2007; Wepf et al., 2009). Regardless, each interaction must be verified by repeating the same pull-down strategy with the targeted antibody of the novel interacting protein. Western blot analysis of the purified protein complex using both interacting proteins’ antibodies can also be done. These classical purification procedures have been used to purify large complexes such as the human spliceosome (Dziembowski and Seraphin, 2004).

Representing one of the first genetic approaches to identifying protein-protein interactions, two-hybrid screening in yeast was developed over 20 years ago (Fields and Song, 1989) and has evolved as a powerful high-throughput tool for protein interaction studies (Bruckner et al., 2009). The technique is based on utilizing transcription factors that regulate essential nutrients in yeast to monitor protein-protein interactions. Two interacting proteins of interest, each fused to two separate domains of a transcription factor, are called bait and prey; when these interactors come into proximity, they reconstitute a functional transcription factor that subsequently drives gene expression. In yeast cells, functional transcription factors drive transcription of essential nutrients needed for their survival. This poses a useful selection mechanism as yeast cells showing a positive interaction would survive on media lacking the essential nutrient used as the reporter gene (Bruckner et al., 2009). On an even larger scale, genome-wide screens for interactors of given baits can be implemented using whole cDNA libraries. Because of its relative inexpensiveness, versatility and ease of implementation, yeast two-hybrid rapidly became the system of choice for detecting protein-protein interactions (Bruckner et al., 2009). The procedure has been automated for studies of interactions from bacteriophage T7 (Bartel et al., 1996), Saccharomyces cerevisiae (Ito et al., 2001), Drosophila melanogaster (Formstecher et al., 2005), Caenorhabditis elegans (Obrdlik et al., 2004) and humans (Rual et al., 2005). Yeast two-hybrid screening has reported a remarkable 5,600+ protein interactions in yeast, and almost 6,000 for humans, establishing extensive networks that represent almost half of all protein interaction data available in various databases (Bruckner et al., 2009).

Recently, the coupling of tandem affinity purification (TAP) with quantitative mass spectrometry has emerged as a successful procedure to study protein-protein interactions (Volkel et al., 2010; Williamson and Sutcliffe, 2010). In efforts to reduce nonspecific binding often found in immunoprecipitates generated from traditional methods, TAP utilizes two rounds of purification in tandem. In TAP, protein A IgG-binding motifs and calmodulin-binding peptide (CBP) affinity
tags are genetically fused in frame with a protein of interest. Subsequent rounds of purification are performed using protein A and calmodulin matrices to elute and isolate the tagged protein along with its potential interacting proteins (Angers, 2008; Kaake et al., 2010; Volkel et al., 2010). The additional round of purification provides stringency as it minimizes the carryover of unwanted contaminating proteins. Following TAP, mass spectrometry is used to study the composition of the protein complex isolated. TAP with mass spectrometry has been used efficiently in mammalian cells to characterize cytoplasmic protein networks involved in tumour necrosis factor alpha (TNF-α)/nuclear factor kappa b (NF-kB) or Wnt/β-catenin signaling pathways (Bouwmeester et al., 2004; Major et al., 2007). Application of TAP with mass spectrometry has also been used in vivo, characterizing novel interacting partners of protein complexes in different tissues of transgenic mice; a valuable tool that could uncover interactions not previously identified in vitro (Angrand et al., 2006). In our study, we use a modified version of TAP developed by our collaborator, Stephane Angers at the University of Toronto, to characterize the potential interacting partners of NP22 in human embryonic kidney cells.

Similar to all other affinity purification methods however, TAP does have limitations. A major problem arises with the possibility of contaminating proteins being pulled down during complex purification. These protein types include abundant cellular proteins such as cytoskeletal proteins, translation factors, and molecular chaperones (Gavin et al., 2002). When pulled down, it becomes difficult to interpret whether these abundant proteins represent true endogenous partners or rather artificial associations induced by cell lysis. Hence, any deduced interaction must be confirmed with multiple binding assays. Furthermore, the addition of tag, whether large or small, can interfere with the protein’s properties and may cause changes in complex stability or composition (Dziembowski and Seraphin, 2004). Due to the stringent purification conditions, it’s also possible that transient interactions or ones occurring only in specific physiological states may not be detected as a second round of purification might disrupt a potentially important protein interaction that could have been detected after only one round (Dziembowski and Seraphin, 2004; Volkel et al., 2010). In efforts to circumvent this limitation, a FLAG-tag purification procedure has been developed (Einhauer and Jungbauer, 2001). The peptide tag, much smaller in weight compared to the TAP tag, is selectively recognized by a monoclonal antibody and the method utilizes one round of purification rather than two. Hence the tandem affinity purification is desirable to reduce artifacts by minimizing contamination of non-specific
binding proteins, whereas the FLAG purification is desirable to prevent disruption of native conformation of the target protein.

3.2 Colocalization via Immunofluorescence

Fluorescent microscopy has also emerged as a valuable tool that has been used to study the potential function of a protein *in vitro*. One of the most common applications of this technique compares the subcellular distributions of two fluorescently labeled molecules (Dunn *et al*., 2011). More specifically, studying whether a protein of interest colocalizes with a marker of a particular organelle could be useful in characterizing the protein. After establishing a conclusion on what intracellular moiety the protein colocalizes with in an intact cell, pharmacological treatments could be tested to identify whether changes in colocalization ensue. Moderate changes in colocalization can be difficult to interpret with the human eye, therefore methods to quantify colocalization are important for identifying any significant changes.

A useful quantification method was developed based on a formula first postulated by Karl Pearson in 1896 (Pearson, 1896; Manders *et al*., 1992). The statistic used to quantify colocalization, entitled the Pearson’s correlation coefficient (PCC), was modified for use in fluorescence microscopy (Manders *et al*., 1992). PCC measures the pixel-by-pixel covariance in the signal levels of two fluorescent images by subtracting the mean intensity values from each pixel’s intensity value across the entire image (Dunn *et al*., 2011). Using the mean intensity values proves to be advantageous for the PCC as it becomes independent of background signal (Dunn *et al*., 2011). PCC can also be measured without any form of image preprocessing, making it a simple and relatively easy method to employ (Dunn *et al*., 2011). PCC values range from -1 to 1, where -1 is denoted for two images whose fluorescence intensities are inversely related to one another and 1 is denoted for two images whose fluorescence intensity are linearly related. The intermediate values are more difficult to interpret; however, isolating a region of interest reduces error and using comparative studies circumvents this limitation (Dunn *et al*., 2011). We employ this strategy to learn more about the function of NP22 and its potential role in altering cell morphology through cytoskeletal remodeling.
4 Overview

4.1 Hypothesis

The biology of NP22 is altered by NMDA receptor signaling.

4.2 Rationale

There are several observations in the scientific literature that point towards the potential link between NP22 biology and NMDA receptor signaling. The reported differences in NP22 protein expression seen in the frontal cortex and striatum of postmortem alcoholics and ethanol treated rats, as well as anterior cingulate cortex of postmortem schizophrenics, suggest that alterations in NMDA receptor function can affect NP22 protein levels. Also, reported interactions of NP22 with actin and α-tubulin \textit{in vitro} and \textit{in vivo}, as well as its reported function in neurite outgrowth \textit{in vivo}, suggest that NP22 may be a novel protein recruited in the molecular machinery that is utilized by NMDA receptor signaling during synaptic cytoskeletal remodeling and plasticity.

4.3 Objectives

4.3.1 To investigate whether pharmacological or genetic alterations of NMDA receptor function influence NP22 biology.

Reductions in NMDA receptor signaling may result in changes in NP22 levels or subcellular localization. Utilizing western blot analysis, NP22 protein levels will be characterized in total and synaptic fractions of striatum and cortex in an animal model of NMDA receptor hypofunction (NR1-KD). Also, NP22 protein levels will be characterized in total and synaptic fractions of striatum in a pharmacological model of NMDA receptor hypofunction (subchronic MK801 treatment).

4.3.2 To characterize NP22 associated proteins.

Identification of proteins that interact with NP22 may suggest functional links with NMDA receptor signaling. To further elucidate the function of NP22, we investigate which proteins interact with NP22 using tandem affinity and FLAG-tag purification.
4.3.3 To determine if there are changes in colocalization of NP22 and filamentous actin in response to changes in intracellular calcium.

While it has been reported that NP22 colocalizes with F-actin *in vitro*, we wanted to investigate whether there are changes in colocalization when intracellular calcium increases. HEK293 cells transiently or stably transfected with GFP-tagged NP22 are used as our model system, allowing for visualization under confocal microscopy. Thapsigargin and ionomycin are used as pharmacological agents that induce intracellular calcium increases and changes in colocalization will be assessed quantitatively by calculating the Pearson’s correlation coefficient for two different regions of interest (cell body and cell processes).
Chapter 2
Materials and Methods

1 DNA Constructs

1.1 TAP-tagged NP22

The pGLUE-NP22 tandem-affinity purification plasmid was obtained from our collaborator, Dr. Stephane Angers (University of Toronto). It consists of pIRE-puro (Clontech, 631605) as the backbone vector containing human NP22 with a dual affinity tag (streptavidin and calmodulin) inserted at the N terminus. NP22 cDNA sequence was amplified by PCR from a human brain cDNA library and cloned in the multiple-cloning site (MCS) of the backbone vector using the restriction enzymes AscI (New England Biolabs) and NotI (New England Biolabs).

1.2 FLAG-NP22 and VENUS-NP22

The FLAG-NP22 and VENUS-NP22 plasmids were generated by excising NP22 from pGLUE-NP22 using the restriction enzymes AscI (New England Biolabs) and NotI (New England Biolabs) and ligating it into similarly digested pIRESpuroFLAG-cdc211 and pIRESpuroVENUS-cdc211 plasmids (Clontech) obtained from Dr. Stephane Angers (University of Toronto). Vector constructs were developed and adapted from the original pIRES-puro (Clontech, 631605) backbone vector. Both vector constructs were verified by DNA sequencing (TCAG, Hospital for Sick Children).

2 Animals

NR1 knockdown (KD) mice were generated by a hypomorphic mutation of Grin1 using a targeted insertion of a neomycin cassette into intron 17 of the gene as described in Mohn et al. (1999). Experimental mice were the F1 generation of an intercross between C57BL/6J Grin1 (NR1-KD) heterozygotes with 129X1Sv/J Grin1 (NR1-KD) heterozygotes. Mice were housed in the Division of Comparative Medicine (DCM) facility at the University of Toronto on a 12-hour light/dark cycle (7 am to 7 pm) and were given access to food and water ad libitum. Mice were housed in accordance with University of Toronto Animal Care and Use Policies.
3 Cell Culture and Transfections

All tissue culture reagents were obtained from Sigma unless otherwise stated. Human embryonic kidney (HEK) 293 (Genlantis) and Neuro-2a (ATCC) cells were cultured in high glucose (4500 mg/L) Dulbecco’s Modified Eagles Medium (DMEM) (GIBCO) supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 I.U./mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere containing 5% CO2 at 37°C. HEK and Neuro-2a cells stably expressing TAP-tagged NP22, FLAG-NP22 or VENUS-NP22 were cultured in the same media and supplements with 2 µg/mL of puromycin (Bioshop) for selection. Cells at 50% confluency were transfected using polyethylenimine (PEI) (name of company). For transient transfections, DNA and PEI (3 µL of 1 mg/mL PEI in water for every 1 µg DNA) were added to 500 µL of serum- and supplement-free DMEM and incubated at room temperature for 20 minutes with intermittent flicking of the tube. Cell media was changed to DMEM supplemented with 2.5% (v/v) FBS, L-glutamine, penicillin and streptomycin before adding the DNA and PEI mix. Twenty-four hours after transfection, cell media was changed back to DMEM supplemented with 10% (v/v) FBS, L-glutamine, penicillin and streptomycin for cell growth to resume at a normal rate. Forty-eight hours after transfection, cells were treated with either NMDA (Sigma), ionomycin (Sigma) or thapsigargin (Sigma) before fixing and subsequent immunofluorescent staining (see below). Stable transfections to generate polyclonal cell lines were performed using the same protocol; however, 48 hours after transfection, cells were split into selection using DMEM supplemented with 10% (v/v), 2 mM L-glutamine, 100 I.U./mL penicillin, 100 µg/mL streptomycin and 2 µg/mL puromycin. For stable transfections yielding individual clonal cell lines, the transfection protocol and selection media were the same; however, cells were plated at low density to isolate clonal cell lines, which were expanded by subsequent trypsinization and growth in 96-well, 48-well, 24-well and 6-well plates. To assess protein expression, transfected cell lines were harvested by trypsinization, centrifuged at 300xg, and lysed in either RIPA lysis buffer (25 mM Tris HCL pH = 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate) or TAP lysis buffer (10% glycerol, 50 mM HEPES-KOH pH 8.0, 100 mM KCL, 2 mM EDTA, 0.1% NP-40, 2 mM DTT) containing protease inhibitors (0.25 mM PMSF, 1.5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 0.1 mg/mL benzamidine, all inhibitors from Bioshop). Protein concentration in harvested cell extracts was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific).
4 Western Blotting

Protein extracts from brain tissue and from cultured cells were suspended in 1X Laemmli buffer and separated on either 10% or 12.5% polyacrylamide gels, then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% (w/v) milk powder in phosphate buffered saline containing 0.1% (v/v) Tween 20 (Sigma) for 30 minutes at room temperature. Primary antibody incubations were performed using the same blocking solution overnight at 4°C, and the following antibodies were used: goat anti-NP22 (1:1000, Santa Cruz), rabbit anti-HA (1:1000, Roche), goat anti-brain creatine kinase (1:1000, Santa Cruz), mouse anti-drebrin (1:1000, Abcam), mouse anti-GAPDH (1:3000, Abcam) and mouse anti-FLAG (1:1000, Sigma). Secondary antibody incubations were performed in the same blocking buffer for one hour at room temperature using species-appropriate secondary antibodies conjugated to infrared dye (Invitrogen). Antibody binding was detected by Li-Cor Odyssey infrared imaging, which contains a larger linear range for protein quantification compared to the traditional film-based exposure method. Quantification of immunoblot labeling was performed with ImageJ (NIH) software and protein levels were normalized to GAPDH.

5 Solubilization Assay

Cell culture dishes (100 mm) containing HEK293 cells stably expressing TAP-tagged NP22 were washed once with PBS. Cells were collected after treatment with 0.05% trypsin and pelleted with short centrifugation (500xg, 10 min). Pelleted cells were resuspended with either 1 mL of RIPA lysis buffer (25 mM Tris HCL pH = 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate), CHAPS lysis buffer (25 mM Tris HCL pH = 7.6, 150 mM NaCl, 1% NP-40, 1% CHAPS, 0.1% sodium dodecyl sulfate) or TAP lysis buffer (10% glycerol, 50 mM HEPES-KOH pH 8.0, 100 mM KCL, 2 mM EDTA, 0.1% NP-40, 2 mM DTT) containing protease inhibitors (0.25 mM PMSF, 1.5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 0.1 mg/mL benzamidine) and agitated at 4°C for 30 minutes. HEK293 cells stably expressing TAP-tagged NP22 were also treated with 2 µM cytochalasin D (Bioshop), collected as described above, resuspended with 1 mL of TAP lysis buffer containing protease inhibitors and agitated at 4°C for 30 minutes. Cell pellets in varying lysis conditions were independently centrifuged at 21,130xg for 15 minutes at 4°C. Supernatants were transferred into new 1.5 mL eppendorf tubes and cell pellets were resuspended in 100 µL of RIPA lysis buffer,
CHAPS lysis buffer or TAP lysis buffer. Insoluble and soluble fractions from each lysis/treatment condition were subjected to western blot analysis (described above). Protein concentration in harvested cell extracts was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific).

6 Tandem Affinity Purification (TAP) for Direct LCMS/MS

The purification of TAP-tagged proteins for direct analysis by LCMS/MS was adapted from Dr. Stephane Angers (University of Toronto) and modified to solubilize and purify our cytoskeleton-associated protein. Original efforts followed the protocol described in Angers 2008; however, this method did not properly solubilize NP22 protein. Therefore the following modified procedure was employed. Cell culture dishes (150 mm) containing HEK293 or Neuro-2A cells stably expressing TAP-tagged NP22 were washed once with PBS. Cells were collected after treatment with 0.5% trypsin and pelleted with short centrifugation (500xg, 10 min). Pelleted cells were resuspended with 5 mL of cold 0.32 M sucrose in 4 mM HEPES containing protease inhibitors (0.25 mM PMSF, 1.5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 0.1 mg/mL benzamide) and homogenized using a motor driven glass-teflon homogenizer. Homogenate samples were centrifuged at 900xg for 10 minutes at 4°C. The supernatant was collected and transferred to a new tube containing an equal volume of 2x cold TAP lysis buffer and protease inhibitors (10% glycerol, 50 mM HEPES-KOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, 0.25 mM PMSF, 1.5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 0.1 mg/mL benzamide); pelleted nuclear fraction was discarded. Protein lysate was then incubated on shaker at 4°C for an additional 30 minutes to ensure complete lysis. The lysate was incubated with 20 µL of packed Streptavidin Sepharose High Performance beads (Amersham) overnight at 4°C with gentle rocking. After incubation, beads were spun down with low speed centrifugation (500xg, 10 min, 4°C) and washed 3 times with 1 mL of cold TAP lysis buffer followed by 2 times with 1 mL of cold calmodulin binding buffer (10 mM 2-mercaptoethanol, 50 mM HEPES-NaOH pH 8.0, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 0.1% NP-40, 2 mM CaCl2) containing protease inhibitors. Protein complexes were eluted with streptavidin elution buffer (500 µL of calmodulin binding buffer containing 50 mM D-Biotin (Sigma), 20 µL of 1M NaOH to bring pH to 7.5-8.0) in three fractions, two of 200 µL.
followed by one of 100 μL, on ice for 5 minutes each with intermittent flicking of the tube to keep the beads resuspended. The combined elution samples were incubated with 20 μL of packed Calmodulin Sepharose 4B beads (Amersham), 300 μL of cold calmodulin binding buffer and 5 μL of 1M CaCl₂ for 90 minutes at 4°C with gentle rocking to initiate another round of purification. After incubation, beads were spun down with low speed centrifugation (500xg, 10 min, 4°C) and washed 3 times with 20 volumes of cold calmodulin binding buffer followed by 3 times with 20 volumes calmodulin rinsing buffer (50 mM ammonium bicarbonate pH 8.0, 75 mM NaCl, 1 mM MgOAc, 1 mM Imidazole, 2 mM CaCl₂) containing protease inhibitors. Protein complexes were eluted with calmodulin elution buffer (50 mM ammonium bicarbonate (pH 8.0), 25 mM EGTA) in two 100μL fractions at 37°C for 5 minutes each with intermittent flicking of the tube to keep the beads resuspended. The combined elution samples were lyophilized, resuspended in 50 μL of 50 mM ammonium bicarbonate (pH 7.8) and incubated with 0.75 μg of sequencing-grade trypsin (Promega) overnight at 37°C. After trypsinization, 2 μL of concentrated HCl was added to the digested sample and incubated for 30 minutes at 37°C. The samples were then centrifuged at maximum speed in a microfuge for 20 minutes to remove any residual lipophilic detergent; the soluble fraction was collected and lyophilized without heat to a volume of 100 μL for loading. The resulting peptide mixture was then analyzed by liquid chromatography-tandem mass spectrometry using a LTQ-XL Linear Ion Trap mass spectrometer (Thermo Scientific). The acquired tandem mass spectra were searched against a FASTA file containing the human NCBI sequences using a normalized implementation of SEQUEST running on the Sorcerer platform (Sage-N Research). The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using peptide and protein prophets (Institute of Systems Biology). All steps performed after the final elution were completed by Dr. Stephane Angers and Dr. Avais Daulat (University of Toronto).

7 FLAG Immunoprecipitation for Direct LCMS/MS

The immunoprecipitation of FLAG-tagged proteins for direct analysis by LCMS/MS was adapted from Dr. Stephane Angers (University of Toronto) and modified to solubilize and purify a cytoskeleton-associated protein. Cell culture dishes (150 mm) containing HEK293 cells stably expressing FLAG-tagged NP22 were washed once with PBS. Cells were collected after treatment with 0.5% trypsin and pelleted with short centrifugation (500xg, 10 min). Pelleted cells
were resuspended with 5 mL of cold 0.32 M sucrose in 4 mM HEPES containing protease inhibitors (0.25 mM PMSF, 1.5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 0.1 mg/mL benzamide) and homogenized using a motor driven glass-teflon homogenizer. Homogenate samples were centrifuged at 900xg for 10 minutes at 4°C. The supernatant was collected and transferred to a new tube containing an equal volume of 2x cold TAP lysis buffer and protease inhibitors (10% glycerol, 50 mM HEPES-KOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP-40, 2 mM DTT, 0.25 mM PMSF, 1.5 µg/mL aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 0.1 mg/mL benzamide); pelleted nuclear fraction was discarded. Protein lysate was then incubated on shaker at 4°C for an additional 30 minutes to ensure complete lysis. After lysis, protein content was determined with the Pierce 660nm Protein Assay Kit (Thermo Scientific). 10 mg of each sample were incubated with 20 µL of packed FLAG M2 beads (Sigma) overnight at 4°C on shaker. After incubation, beads were spun down with low speed centrifugation (500xg, 10 min, 4°C) and washed 3 times with cold TAP lysis buffer followed by 3 times with cold 50 mM ammonium bicarbonate (pH 7.8) to remove any residual detergent. Protein complexes were eluted with 500mM ammonium hydroxide (pH 11.0) in three 100 µL fractions at 37°C for 5 minutes each with intermittent flicking of the tube to keep the beads resuspended. The combined elution samples were lyophilized twice: first to remove ammonium hydroxide (pH 7.8) and once again after resuspension with 100 µL of milliQ water. After complete lyophilization, samples were resuspended in 50 µL of 50 mM ammonium bicarbonate and digested with 0.75 µg of sequencing-grade trypsin (Promega) overnight at 37°C. The following day, 1.3 µL of 1M DTT was added to protein digest and samples were incubated for 40 minutes at 50°C to reduce disulfide bonds. After this reduction step, 5.1 µL of 1M iodoacetamide was added to samples and incubated for 20 minutes at room temperature in the dark to prevent reformation of the broken disulfide bonds. The resulting peptide mixture was then analyzed by liquid chromatography-tandem mass spectrometry using a LTQ-XL Linear Ion Trap mass spectrometer (Thermo Scientific). The acquired tandem mass spectra were searched against a FASTA file containing the human NCBI sequences using a normalized implementation of SEQUEST running on the Sorcerer platform (Sage-N Research). The resulting peptide identifications returned by SEQUEST were filtered and assembled into protein identifications using peptide and protein prophets (Institute of Systems Biology). Final reduction, alkylation and loading steps were completed by Dr. Avais Daulat (University of Toronto).
8 Co-Immunoprecipitation

FLAG immunoprecipitation was performed on untransfected HEK293 cells and HEK293 cells stably expressing FLAG-tagged NP22 as described above. 100 µL of total protein extract was removed for positive control. After protein complexes were eluted, final sample was divided into three and subjected to western blot analysis (previously described). Briefly, primary antibody incubations were performed overnight at 4°C and the following were used: goat anti-NP22 (1:1000, Santa Cruz), goat anti-brain creatine kinase (1:1000, Santa Cruz) and mouse anti-drebrin (1:1000, Abcam). Secondary antibody incubations were performed for one hour at room temperature using species-appropriate secondary antibodies conjugated to infrared dye (Invitrogen). Antibody binding was detected by Li-Cor Odyssey infrared imaging.

9 Immunocytochemistry

HEK293 cells transfected either transiently or stably with VENUS-NP22 were plated onto 22-mm coverslips in 6-well plates. After treatment with either 1 µM ionomycin or 1 µM thapsigargin (see below), cells were washed with ice-cold PBS, fixed with 4% (v/v) paraformaldehyde (Sigma) in PBS at room temperature for 20 min, and permeabilized with 0.1% (v/v) Triton X-100 (Sigma), 5% (w/v) fish gelatin (Sigma) in PBS at room temperature for 5 minutes. For fluorescent phalloidin staining, cells were incubated with AlexaFluor 546 phalloidin (Invitrogen) in the dark for 30 minutes at room temperature. Coverslips were mounted on glass slides using Vectashield mounting media with DAPI (Vector Laboratories) and visualized by confocal microscopy (Olympus microscope and F1000 Fluoview software). For antibody and fluorescent phalloidin staining, cells were incubated with blocking solution (PBS/5% (w/v) fish gelatin) for 10 min at room temperature after permeabilization. Cells were then incubated with primary antibody for 45 min in a designed chamber with a humid environment. After primary antibody staining, cells were washed gently with PBS 3x for 10 min each on a slow speed shaker. Secondary antibody incubation, which included AlexaFluor 546 phalloidin at the appropriate dilution, was done at room temperature for 30 minutes and coverslips were washed gently again with PBS 3x for 10 min each before mounting on microscope slides with Vectashield mounting media.
10 In Vitro Pharmacological Treatment Assays

10.1 Thapsigargin Treatment Assay - Transient cell line

HEK293 cells were grown and transiently transfected with VENUS-NP22 according to the PEI transfection protocol. Twenty-four hours after transfection, cells were split using the appropriate dilution onto 22-mm coverslips in 6-well plates. Forty-eight hours after transfection, cells were treated with 1 µM thapsigargin in HEPES-buffered saline solution (140 mM NaCl, 4.7 mM KCl, 10 mM CsCl, 2 mM CaCl₂, 1.13 mM MgCl₂, 10 mM glucose and 10 mM Hepes, pH 7.4) for either 15 or 25 minutes. Control treatments were 1 µM DMSO in HEPES-buffered saline solution for either 15 or 25 minutes. After treatment, cells were fixed with 4% (v/v) paraformaldehyde (Sigma) in PBS at room temperature for 20 min and processed for fluorescent phalloidin staining according to the immunocytochemistry protocol and visualized for staining by confocal microscopy.

10.2 Ionomycin and Thapsigargin Treatment Assay - Stable cell line

HEK293 cells stably transfected with VENUS-NP22 were passaged using the appropriate dilution onto 22-mm coverslips in 6-well plates. Twenty-four hours after plating, cells were treated with either 1 µM ionomycin or 1 µM thapsigargin in HEPES-buffered saline solution for 15 minutes. Control treatments were 1 µM DMSO in HEPES-buffered saline solution for 15 minutes. After treatment, cells were fixed with 4% (v/v) paraformaldehyde (Sigma) in PBS at room temperature for 20 min and processed for fluorescent phalloidin staining according to the immunocytochemistry protocol and visualized for staining by confocal microscopy.

11 Colocalization Analysis

Images obtained from confocal microscope were saved as tagged image file format (TIFF) images and subjected to colocalization analysis. Colocalization analysis was completed using a colocalization plugin downloaded from Wright Cell Imaging Facility (Toronto Western Research Institute) for ImageJ (NIH) software. A selected region of interest (ROI) for an individual cell in the image, either cell body or cell process, was outlined using a freehand selection tool. Once the
ROI was selected for each image, a Pearson’s correlation coefficient was calculated and reported by ImageJ.

12 Synaptic Plasma Membrane (SPM) Preparation

Specific brain regions (striatum or cortex, 2 animals per sample) were dissected from either NR1-KD, wild type, MK-801-treated wild type, or saline-treated wild type mice, and were homogenized using a motor-driven glass-teflon homogenizer in 4 mL of cold 0.32 M sucrose in 4 mM HEPES containing protease inhibitors (0.25 mM PMSF, 1.5 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 0.1 mg/mL benzamide). 100 μL of ‘Total’ protein extract was removed from homogenate before a series of centrifugation steps took place. First, the homogenate was centrifuged at 900xg for 10 minutes at 4°C. The supernatant (S1) was collected and transferred to a new tube, while the pelleted nuclear fraction was discarded. S1 samples were centrifuged at 10,000xg for 15 minutes at 4°C to yield the crude synaptosomal pellet (P2); the supernatant (S2) was discarded. Pellets were washed by re-suspending them in 4 mL of cold 0.32M sucrose in 4 mM HEPES containing protease inhibitors and re-spun at 10,000xg for 15 minutes at 4°C; once again, the supernatant was discarded. The washed synaptosomal pellets (P2’) were lysed via hypotonic shock by resuspension in 4 mL of cold distilled water containing protease inhibitors and homogenized by hand in glass/teflon homogenizers. 16 μL of 1 M HEPES was immediately added to each sample after homogenization to restore molarity to 4mM. Samples were then incubated on shaker at 4°C for 30 minutes to ensure complete lysis. After lysis, synaptosomal fractions were centrifuged at 25,000xg for 20 minutes at 4°C to yield the synaptosomal membrane pellet; the supernatant (S3, crude vesicle fraction) was discarded. Each pellet was resuspended in 1.0 mL of cold 0.32 M sucrose in 4 mM HEPES containing sucrose and was layered separately, using a pasteur pipette, onto discontinuous sucrose gradient columns prepared in ultracentrifuge tubes. Each column contained three layers, from top to bottom, of 3.0 mL 0.8 M, 3 mL 1.0 M and 3.5 mL 1.2 M sucrose in 4 mM HEPES. Gradient tubes were placed in SW41 Ti swing buckets, loaded onto an SW41 Ti rotor and ultracentrifuged at 30,000xrpm (150,000xg) for 2 hours at 4°C. Following centrifugation, each column was removed and punctured with a 1 mL syringe containing an 18G needle to withdraw the synaptic plasma membrane fraction (maximum 1 mL withdrawn), which was located on top of the 1.2 M sucrose gradient layer. Synaptic plasma membrane fractions were placed in 3.5 mL ultra tubes and were adjusted back to 0.32 M sucrose after adding 4 mM HEPES with protease inhibitors.
Samples were subsequently centrifuged at 200,000xg for 30 minutes at 4°C. After the spin, each synaptosomal membrane pellet was resuspended in 100 µL of cold 50 mM HEPES and 2 mM EDTA containing phosphatase inhibitors. Both ‘Total’ and ‘SPM’ samples were diluted 1:1 with Laemmli sample buffer containing 5% 2-mercaptoethanol and stored at -80°C. Protein content was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific) and protein expression was measured using western blot analysis.

13 Subchronic MK801 Treatment

Microosmotic pumps (Alzet) were prepared to deliver a constant infusion of 0.2 mg/kg/h of MK-801 (Sigma) dissolved in saline solution for two weeks. Wild type males aged 12 weeks were used for the study. Animals were anesthetized with isoflurane and minipumps were implanted subcutaneously at the dorsal region of the neck. Fourteen days after implantation, animals were euthanized for synaptic plasma membrane preparation.
Chapter 3
Results

1 NP22 protein levels in NMDA receptor deficient mice

We hypothesized that NP22, as an actin binding protein with calcium binding motifs, is part of the molecular machinery that connects NMDA receptor signaling to synaptic cytoskeletal remodeling. As such, reductions in NMDA receptor signaling may result in changes in NP22 levels or subcellular localization. To examine the influence of a genetic reduction of NMDA receptor function on NP22 biology, we used genetically engineered mice with global reductions in functional NMDA receptors resulting from hypomorphic mutation of the critical NR1 subunit gene (Mohn et al., 1999). Due to the reported implications of NP22 in neurite outgrowth and interactions with the cytoskeleton (Mori et al., 2004; de las Heras et al., 2007; Pape et al., 2008), we measured NP22 protein levels in both total and synaptic biochemical preparations of two specific brain regions: the striatum and cortex. Synaptic fractions from striatal and cortical preparations of wild type (WT) and NR1 knockdown (NR1-KD) mice were isolated by sucrose density gradient and analyzed by western blot. Due to the reported differences in NP22’s function when phosphorylated (de las Heras et al., 2007), we completed the striatal synaptic preparation in the presence and absence of phosphatase inhibitors. By using this approach we found that NP22 protein expression was unchanged in total levels and synaptic fractions from NR1-KD striatum in the presence and absence of phosphatase inhibitors compared to WT (Fig. 3.1). Also NP22 protein expression was unchanged in total levels and synaptic fractions from NR1-KD cortex compared to WT (Fig. 3.2, phosphate inhibitors were not used). Thus, genetic reductions in NMDA receptor function do not change NP22 protein expression in total and synaptic preparations of striatum and cortex from NR1-KD mice.
Figure 3.1. Synaptic plasma membranes of NR1-KD striatum show no change in NP22 expression. (A) Western blot analysis of total (25 µg protein) and SPM (20 µg protein) preparations from striatum of WT and NR1-KD mice in presence of phosphatase inhibitors. Primary monoclonal antibodies for NP22 (Santa Cruz) and GAPDH (Abcam) detected approximate 22-kDa and 36-kDa proteins respectively. (B) Densitometry analysis revealed that, while there are slight reductions in total NP22 levels, there is no significant difference in total and SPM NP22 protein levels normalized to GAPDH in the striatum of NR1-KD mice compared to WT. (C) Western blot analysis of total (25 µg protein) and SPM (20 µg protein) preparations from striatum of WT and NR1-KD mice in absence of phosphatase inhibitors. Primary monoclonal antibodies for NP22 (Santa Cruz) and GAPDH (Abcam) detected approximate 22-kDa and 36-kDa proteins respectively. (D) Densitometry analysis revealed that, while there are slight reductions in total NP22 levels, there is no significant difference in total and SPM NP22 protein levels normalized to GAPDH in the striatum of NR1-KD mice compared to WT. (*P > 0.05, two tailed t-test)
**Figure 3.2.** Synaptic plasma membranes of NR1-KD cortex show no change in NP22 expression. (A) Western blot analysis of total (25 µg protein) and SPM (20 µg protein) preparations from cortex of WT and NR1-KD mice in absence of phosphatase inhibitors. Primary monoclonal antibodies for NP22 (Santa Cruz) and GAPDH (Abcam) detected approximate 22-kDa and 36-kDa proteins respectively. (B) Densitometry analysis revealed that there is a modest, but not significant, decrease in total NP22 levels and a relatively large, but not significant, increase in SPM NP22 levels normalized to GAPDH in the cortex of NR1-KD mice compared to WT with preparations. (*P > 0.05, two tailed t-test)

## 2 NP22 protein levels in MK801-treated mice

To examine the influence of a pharmacological reduction of NMDA receptor function on NP22 biology, we used WT mice treated subchronically with MK801, a highly selective, noncompetitive NMDA receptor antagonist. A dose of 0.2 mg/kg/h was delivered subcutaneously via osmotic minipump over 2 weeks in adult mice (aged 12 weeks). This dose regimen represents a model of subchronic pharmacological blockade that induces the abnormal behaviours similarly seen in untreated NR1-KD mice (Ramsey et al. 2011). Total and synaptic biochemical preparations from MK801-treated and untreated mice were isolated and analyzed using western blot. By using this approach we found that NP22 protein expression was unchanged in total levels and synaptic fractions from MK801-treated striatum compared to untreated (Fig. 3.3). Thus, subchronic pharmacological blockage of NMDA receptors does not change NP22 protein expression in total and synaptic preparations of striatum of MK801-treated mice.
Figure 3.3. Synaptic plasma membranes from striatum of WT mice subchronically treated with MK801 show no change in NP22 protein level. (A) Western blot analysis of total (25 µg protein) and SPM (20 µg protein) preparations from striatum of saline and MK801-treated (0.2 mg/kg/hr, 14 days) mice in absence of phosphatase inhibitors. Primary monoclonal antibodies for NP22 (Santa Cruz) and GAPDH (Abcam) detected approximate 22-kDa and 36-kDa proteins respectively. (B) Densitometry analysis revealed that there is no significant difference in total and SPM NP22 protein levels normalized to GAPDH in the striatum of MK801-treated mice compared to saline. (n = 6 for each genotype; *P > 0.05, two tailed t-test).

Figure 3.4. MK801 treated mice display increases in locomotion compared to saline treated mice. Mice were placed in a digital activity monitor (Accuscan) for two hours and total distance travelled (cm) was calculated using VersaMax software. Horizontal lines indicate average total distance travelled for each treatment group (n = 9). Each point represents an individual animal in the respective treatment groups.
3 TAP-tag purification of NP22 protein complex

Identification of proteins that interact with NP22 may suggest functional links with NMDA receptor signaling. To further elucidate the function of NP22, we investigated which proteins interact with NP22 using tandem affinity purification (TAP). Because of its stringent conditions, we hypothesized that any candidate proteins uncovered would likely represent true interactions. Our model was a human embryonic kidney (HEK) cell line stably transfected with an NP22 expression vector carrying the dual affinity tag. NP22 protein expression was analyzed using western blot. Initially, there were difficulties solubilizing our protein of interest, and preliminary TAP and mass spectrometry studies did not yield the bait protein. Although the clonal cell lines expressed NP22 (Fig. 3.5), it was only detected in the insoluble fraction of cell lysate. Using lysis buffers that differed in their detergent composition, NP22 expression was detected in total and insoluble fractions of stably transfected HEK cells (Fig. 3.6). CHAPS lysis buffer includes the relatively mild zwitterionic detergent, RIPA lysis buffer contains the nonionic detergent Triton X-100 and the TAP lysis buffer contains NP-40. Because of its reported interaction with actin, we investigated whether use of an actin depolymerizing agent, cytochalasin D, before lysis would solubilize NP22. NP22 expression was still limited to total and insoluble fractions of stably transfected HEK cells treated with cytochalasin D (Fig. 3.6). A modified protein extraction method was used; cells were homogenized in 0.32 M sucrose to gently lyse cells, and debris was cleared with a low spin to pellet only nuclei and not cytoskeletal proteins. This procedure, allowed the large scale purification of NP22 and mass spectrometry to investigate any potential interactors (Fig. 3.7). Using this approach, no interacting proteins with NP22 were found. To investigate whether actin depolymerization would influence protein interactions with NP22, TAP and mass spectrometry analysis on stably transfected HEK cells treated with cytochalasin D was completed. Once again, no interacting proteins with NP22 were found.
**Figure 3.5.** Verification of HEK293 stable cell line expressing transfected TAP-tagged NP22. Western blot analysis with anti-HA antibody reveals four HEK293 clones stably transfected with the approximately 32-kDa TAP-tagged NP22 expression vector (A, B). Negative control = untransfected HEK293 cells (no trans).

**Figure 3.6.** NP22 expression is restricted to the insoluble fraction of HEK293 stable cell lysate. Western blot analysis with anti-HA antibody reveals that NP22 protein is detected in the insoluble fraction (Ins) of HEK293 stable cell lysate after 21,130xg centrifugation. The three lysis buffers used were CHAPS lysis buffer, RIPA lysis buffer and TAP lysis buffer. Use of each buffer and lysis method revealed the same result: NP22 is not found in the soluble fraction (Sol) of HEK293 cell lysate. Treatment of HEK293 cells with cytochalasin D did not improve solubilization of NP22. Negative control was untransfected HEK293 cells seen in the first lane of each blot.
Figure 3.7. Verification of tandem affinity purification from HEK293 cells stably expressing NP22. Western blot analysis with anti-HA antibody revealed TAP-tagged NP22 protein (with tag, ~32 kDa) purification was completed using the TAP method; proteins isolated from elution steps were subsequently used for mass spectrometry analysis. Fractions were taken from each critical step to ensure NP22 protein was not lost. A = total lysate, B = lysate after streptavidin bead incubation, C = streptavidin beads pre-elution with biotin, D = elution from streptavidin beads with biotin, E = streptavidin beads post-elution with biotin, F = supernatant post-calmodulin beads incubation, G = calmodulin beads pre-elution with EGTA, H = elution #1, I = elution #2.

4  FLAG-tag purification of NP22 protein complex

While TAP-tag methods were successful in isolating the NP22 protein, the isolates were remarkably devoid of any other proteins as determined by mass spectrometry. We hypothesized that the calcium-binding motifs of NP22 were affected by the calcium ions required in the TAP method. To avoid this potential confounding factor, we employed an alternate purification method using the FLAG tag. Our model system was a HEK cell line stably transfected with an NP22 vector carrying the FLAG affinity tag (Fig. 3.8). The modified protein extraction approach, with 0.32 M sucrose lysis, was used. FLAG purification was performed on both stably transfected and mock-transfected HEK cells to identify proteins that exhibited non-specific binding to the FLAG tag. Using this approach we found several proteins interacting with NP22 (Table 3.1). After repeating the purification with three separate experiments, two proteins were selected that were hypothesized to be valid interactions.

The two proteins selected were brain-type creatine kinase (CK-BB) and drebrin. Creatine kinases are enzymes that catalyze the reversible conversion of creatine to phosphocreatine, consuming
adenosine triphosphate (ATP) (Beard and Braissant, 2010). Drebrin is an actin-binding protein implicated in the process of neuronal growth (Dun and Chilton, 2010). Co-immunoprecipitation was performed using protein lysates from FLAG pull-down and western blot analysis with antibodies against CK-BB and drebrin. CK-BB and drebrin were not detected in the FLAG pull-down lysate (Fig. 3.9). Thus, NP22 interactions with CK-BB and drebrin may not represent true interactions.

![Figure 3.8](image)

**Figure 3.8.** Verification of HEK293 stable cell line expressing transfected FLAG-tag NP22. Western blot analysis with anti-FLAG antibody reveals five HEK293 clones stably transfected with the approximately 23-kDa FLAG-tagged NP22 expression vector (A, B). Negative control = untransfected HEK293 cells (no trans).

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**Table 3.1.** Peptide identification by mass spectrometry analysis from representative TAP of NP22 in stably transfected HEK293 cells. Proteins are shown in descending orders of percentage sequence coverage of the proteins. The purification method was repeated three times and proteins that appeared in both stable NP22 transfected and untransfected HEK293 experiments were discarded as background.
Figure 3.9. Coimmunoprecipitation does not confirm NP22 interactions with brain-type creatine kinase and drebrin in stably transfected HEK293 cells. Western blot analysis detects FLAG-NP22 (~ 23 kDa), but not brain-type creatine kinase (~ 43 kDa) and drebrin (~ 100 kDa), in stably transfected HEK293 FLAG pull-down. The FLAG purification method was performed and final eluate was split into three for western blot analysis. T = total lysate, P = FLAG pull-down lysate. Negative control was untransfected HEK293 cells and positive control was protein expression in total lysate before FLAG purification. Primary antibodies anti-NP22 (Santa Cruz), anti-creatine brain kinase (Santa Cruz) and anti-drebrin (Abcam) were used.

5 NP22 colocalization with actin in response to elevated calcium

While it has been reported that NP22 colocalizes with F-actin in vitro, we wanted to investigate whether there are changes in colocalization when intracellular calcium increases. Calcium is the main cation that enters the cell upon NMDA receptor activation; in order to identify a potential
link between NMDA receptor signaling and NP22 biology, we mirrored the rise in intracellular calcium using the pharmacological agent thapsigargin. Thapsigargin is a non-competitive inhibitor of sarco/endoplasmic reticulum calcium ATPase and raises cytosolic calcium concentration by blocking the re-entry of calcium into the sarcoplasmic reticulum and depleting its storage, which further activates calcium channels to allow more to enter the cell (Michelangeli and East, 2011). For our study the model system was a HEK293 cell line transiently transfected with an NP22 vector carrying a fluorescent tag (Venus-YFP), allowing for visualization under confocal microscopy. These cells were treated with thapsigargin for either 15 or 25 minutes. Changes in colocalization in response to thapsigargin treatment were assessed quantitatively by calculating the Pearson’s correlation coefficient for two different regions of interest. The first region of interest was the entire cell body while the other region represented cell processes protruding from the cells. Cell processes were examined because of the reported ability of NP22 to induce process formation in another non-neuronal cell line (de las Heras et al., 2007), and because these processes have been used to model neurite outgrowth and retraction in non-neuronal cells (Wong et al., 2002; de las Heras et al., 2007; Aromolaran et al., 2007).

NP22 colocalized with F-actin in the cell body and cell processes of HEK293 cells; colocalization was detected in cells treated or untreated with thapsigargin, and at both 15 and 25 minute time intervals (Fig. 3.10, 3.11). At both time points of thapsigargin treatment, NP22 colocalization with F-actin in the cell body does not change (Fig. 3.12). However, NP22 colocalization with F-actin in cell processes is significantly reduced in cells treated with thapsigargin for 25 minutes, but not 15 minutes (Fig. 3.12). Thus, quantitatively, thapsigargin treatment for an extended period of 25 minutes significantly reduces the colocalization of NP22 with F-actin in cell processes, and not the cell body, of HEK293 cells. Overall, there is higher degree of colocalization of NP22 with F-actin in the cell processes compared to cell bodies of HEK293 cells independent of thapsigargin treatment.
Figure 3.10. NP22 colocalization with F-actin in the cell body and cell processes of HEK293 cells transiently transfected with NP22 and response to 15 minutes thapsigargin. HEK293 cells were transiently transfected with venus-tagged NP22 and were either untreated or treated with 1 µM thapsigargin 48 hours after transfection for 15 minutes. After fixation, cells were processed for confocal microscopy. F-actin visualization was accomplished through use of AlexaFluor 546 phalloidin (Invitrogen) staining at a 1:500 dilution (B, E). NP22 is expressed in the cell body and cell processes of untreated HEK293 cells (A) and colocalizes with F-actin (C). NP22 expression and colocalization with F-actin is maintained in the cell body and cell processes of HEK293 cells after treatment with thapsigargin for 15 minutes (D, F).
**Figure 3.11.** NP22 colocalization with F-actin in the cell body and cell processes of HEK293 cells transiently transfected with NP22 and response to 25 minutes of thapsigargin treatment. HEK293 cells were transiently transfected with venus-tagged NP22 and were either untreated or treated with 1 µM thapsigargin 48 hours after transfection for 25 minutes. After fixation, cells were processed for confocal microscopy. F-actin visualization was accomplished with AlexaFluor 546 phalloidin staining. (B, E). NP22 is expressed in the cell body and cell processes of untreated HEK293 cells (A) and colocalizes with F-actin (C). NP22 expression and colocalization with F-actin is maintained in the cell body and cell processes of HEK293 cells after treatment with thapsigargin for 25 minutes (D, F).
NP22 colocalization with F-actin is significantly reduced in cell processes of transiently transfected HEK293 cells treated with thapsigargin for 25 minutes. Confocal images were analyzed by ImageJ software to quantify colocalization and determine the Pearson’s correlation coefficient for cell bodies or cell processes of transiently transfected HEK293 cells. (A) NP22 colocalization with F-actin in cell bodies of HEK293 cells does not significantly change (For 15 minutes, n = 11 for NT, 15 for THAPS; For 25 minutes, n = 16 for NT, 16 for THAPS, *P > 0.05, Two-way ANOVA) after both 15 and 25 minutes of thapsigargin treatment. (B) NP22 colocalization with F-actin in cell processes of HEK293 cells does not significantly change (n = 17 for NT, 24 for THAPS, *P > 0.05, Two-way ANOVA) after 15 minutes of thapsigargin treatment. However, after 25 minutes of thapsigargin treatment, NP22 colocalization with F-actin is significantly reduced (n = 16 for NT, 21 for THAPS, *P < 0.0001, Two-way ANOVA) in cell processes. NP22 colocalizes with F-actin to a greater extent in cell processes than cell body of HEK293 cells, as seen by the higher Pearson’s correlation coefficient independent of thapsigargin treatment (A,B).

NP22 colocalization with actin in stably transfected cells

Thapsigargin treatment caused a clear decrease in the colocalization of NP22 with F-actin; however, this was performed in transiently transfected cells with variable expression levels of NP22. It was possible that stable expression of NP22 in a clonal cell line would yield more consistent results in these assays, providing additional information on cell morphology resulting from NP22 expression, and on its colocalization with the cytoskeleton. Thapsigargin was used to induce a rise in extracellular calcium. Another pharmacological agent, ionomycin, was also used to induce a rise in extracellular calcium through a different mechanism. Ionomycin is an ionophore that forms a pore in the cell membrane allowing calcium to flow inside the cell (Dedkova et al., 2000). The model system was a clonal HEK293 cell line stably transfected with an NP22 vector carrying a fluorescent tag (Venus-YFP), allowing for visualization by confocal microscopy. Cells were treated with either thapsigargin or ionomycin treatment for 25 minutes.
The 25-minute time interval was used based on previous findings of reduced colocalization between NP22 and F-actin in transiently transfected HEK293 cells (Fig. 3.12). Changes in colocalization in response to thapsigargin or ionomycin treatment were assessed quantitatively by calculating the Pearson’s correlation coefficient for two different regions of interest. As described previously, the first region of interest is the entire cell body while the other region represents cell processes protruding from the cells.

As with the transient tranfections, NP22 colocalized with F-actin in the cell body and cell processes of stably transfected HEK293 cells (Fig. 3.13). NP22 colocalization with F-actin in the cell body or cell processes did not change in stably transfected HEK293 cells treated with thapsigargin or ionomycin for 25 minutes (Fig. 3.14). Overall, there was a higher degree of colocalization of NP22 with F-actin in the cell processes compared to cell bodies independent of thapsigargin or ionomycin treatment.
Figure 3.13. NP22 colocalizes with F-actin in the cell body and cell processes of HEK293 cells stably expressing NP22 and maintains colocalization after ionomycin and/or thapsigargin treatment. HEK293 cells were transfected with venus-tagged NP22 and stable clones were selected over a two week period under 2 µg/mL puromycin selection. Cells were either untreated or treated with 1 µM thapsigargin or 1 µM ionomycin for 25 minutes. After fixation, cells were processed for confocal microscopy. F-actin visualization was accomplished with AlexaFluor 546 phalloidin. (B, E, H). NP22 is expressed in the cell body and cell processes of untreated transfected HEK293 cells (A) and colocalizes with F-actin (C). NP22 expression and colocalization with F-actin is maintained in the cell body and cell processes of transfected HEK293 cells after treatment with thapsigargin or ionomycin for 15 minutes (D, F, G, I).
Figure 3.14. NP22 colocalization with F-actin does not change in cell bodies or cell processes of stably transfected HEK293 cells treated with thapsigargin or ionomycin. Confocal images were analyzed by ImageJ software to quantify colocalization and determine the Pearson’s correlation coefficient for cell bodies or cell processes of stably transfected HEK293 cells. (A) NP22 colocalization with F-actin in cell bodies does not significantly change with treatment (n = 21 for no treatment (NT), 24 for thapsigargin (THAPS), 18 for ionomycin; *P > 0.05, Two-way ANOVA). (B) NP22 colocalization with F-actin in cell processes of stably transfected HEK293 cells does not significantly change with treatment (n = 19 for NT, 22 for THAPS, 16 for ionomycin; *P > 0.05, Two-way ANOVA). NP22 colocalizes with F-actin to a greater extent in cell processes than cell bodies, as seen by the higher Pearson’s correlation coefficient independent of thapsigargin or ionomycin treatment (A,B).
1 Efforts to establish a link between NMDA receptor signaling and NP22 biology in vivo

Consistent with our efforts to establish a link between NMDA receptor signaling and NP22 biology, we initially studied NP22 biology in vivo. NMDA receptors are integral for synaptic transmission and modifying synapse biology, and calcium influx is the primary intracellular mediator for these events (Collingridge et al., 2004). NP22 is potentially implicated in NMDA receptor biology because it is a protein upregulated in the cortex of post mortem alcoholics (Fan et al., 2001; Depaz et al., 2003) and altered in ethanol-exposed rats (Depaz et al., 2005). Because NP22 contains cytoskeleton- and calcium-binding motifs, and is implicated in neurite outgrowth (Pape et al., 2008), we hypothesized that NP22 protein expression would be altered as a result of functional changes in NMDA receptor signaling. This led us to hypothesize that NP22 protein levels would be altered in synaptic preparations of the striatum and cortex of NMDA receptor deficient NR1-KD mice. These studies are the first to characterize NP22 expression levels in synaptic preparations of these brain regions under conditions of NMDA receptor hypofunction.

These studies characterized the protein levels of NP22 using two models of NMDA receptor hypofunction. A genetic model was utilized in which NMDA receptor levels were reduced to approximately 10% of wild type levels. To complement this genetic model, a pharmacological model of sustained NMDA receptor hypofunction was achieved by continuous infusion of an NMDA receptor antagonist, MK801. Genetic reduction of the receptor affords the opportunity to study the consequences of NMDA receptor dysfunction throughout development, whereas the use of the pharmacological model avoids the developmental period and enables study of the more immediate consequences of NMDA receptor dysfunction in the adult mouse. NR1-KD mice represent a useful genetic model of NMDA receptor hypofunction because of their global reductions of functional receptor levels in the brain that cause behavioural impairments similar to treatment with NMDA receptor antagonists (Mohn et al., 1999). The pharmacological model of subchronic MK801 infusion was chosen because it induces changes in locomotor (increase) and social (decrease) activity that are similar to those of NR1-KD mice (Ramsey et al., 2011).
In the studies using NR1-KD and WT littermates, there were no significant differences in total or synaptic NP22 protein levels in the striatum under two experimental conditions: in the presence or absence of phosphatase inhibitors. Due to the consensus PKC phosphorylation sites that exist in NP22 (de las Heras et al., 2007), it was conceivable that protein levels, particularly in the synaptic fraction, could be affected by the phosphorylation state of NP22. The development of phosphorylation-specific antibodies would be useful to further examine this possibility. In subsequent studies examining the cortex of NR1-KD mice, NP22 protein levels were increased in synaptic fractions, although not to a significant degree. This increase is consistent with the reported upregulation of cortical NP22 protein in postmortem alcoholics (Fan et al., 2001; Depaz et al., 2003) and ethanol treated rats (Depaz et al., 2005), although synaptic levels of the protein were not investigated in those studies. There was also a trend towards a decrease of NP22 in the striatum of NR1-KD mice; in ethanol-treated rats NP22 was also (significantly) decreased (Depaz et al., 2005). NMDA receptor function is inhibited by ethanol (Lovinger et al., 1989), which may mirror the reduced NMDA receptor function seen in NR1-KD mice. However, it should be noted that chronic alcohol use also induces compensatory NMDA receptor upregulation (Follesa and Ticku, 1996; Hu et al., 1996; Kumari and Ticku, 1998).

Although significant changes in protein levels were not detected in adult NR1-KD brain tissues, it is possible that changes might be observed at earlier developmental time periods. Sustained NMDA receptor hypofunction throughout the entire developmental trajectory of NR1-KD mice could cause early changes in NP22 levels during development that could have been corrected by compensatory or adapting changes, restoring NP22 protein levels to normal by adult age. As previously mentioned, NP22 mRNA expression has an early onset in chick neurodevelopment (Pape et al., 2008). In addition, changes in NP22 protein levels affect E5 and E7 sympathetic neuron morphology, suggesting the importance of NP22 function at early stages of development (Pape et al., 2008). Thus, it would be important to investigate whether NP22 protein levels in different brain regions exhibit any type of developmental trajectory (i.e. increased levels at age 2 weeks or 6 weeks) in NR1-KD mice, ultimately leading to the unchanged levels we see in adults compared to WT.

Although several investigators have attempted to characterize NP22 in vivo, the effect of pharmacological intervention, besides alcohol, on NP22 biology in animals had not been studied. Consistent with our efforts to investigate the potential link between NMDA receptor signaling
and NP22, we treated WT mice with MK801 (0.2 mg/kg/hr), an NMDA receptor antagonist, subchronically for two weeks and measured synaptic levels of the protein in the striatum. We hypothesized that NP22 protein levels in synaptic preparations of the striatum would be altered in response to NMDA receptor antagonism. Although our pharmacological model mirrors the genetic model in the sense of chronic receptor hypofunction, it was possible that different effects on NP22 levels might be observed in these two models. For example, the synaptic levels of DISC-1 and 14-3-3ε are differentially regulated by genetic and pharmacological disruption of NMDA receptors (Ramsey et al., 2011). While DISC-1 protein was reduced in synaptic preparations of both models, 14-3-3ε was only reduced in one (NR1-KD), suggesting that proteins could respond differently to sustained or transient reductions in NMDA receptor transmission (Ramsey et al., 2011). However, this differential effect was not observed for NP22; synaptic NP22 levels in the striatum remained unchanged with MK801 treatment, similar to what was observed in NR1-KD mice. Future studies to investigate whether NP22 protein levels change in response to NMDA receptor pharmacological intervention should be conducted, as it is possible that NP22 is sensitive to different types of reduced or enhanced NMDA receptor transmission via varying regulatory mechanisms. Subcutaneous injections of MK801 in WT models on a once- or twice-per-day basis yields an acute model of NMDA receptor antagonism and could affect NP22 biology. Also, NMDA receptor agonism could be tested using acute or subchronic administration of NMDA or other receptor agonists to determine whether changes in NP22 protein levels occur.

While significant changes in total and synaptic NP22 protein levels were not detected in both models, it is still possible that NP22 function is altered in adults and potentially contributes to the synaptic abnormalities that exist in NR1-KD and MK801 treated mice (Ramsey et al., 2011). Furthermore, western blot analysis may not be sensitive enough to show modest changes, or changes in subcellular localization that can still affect the function of the protein. Thus, it is important to characterize interactions of NP22, as changes in NMDA receptor signaling could alter such interactions and affect the function of NP22 without affecting overall protein levels.

2 The search for novel interacting proteins

Previous studies established that NP22 associates with integral parts of the cytoskeleton, such as actin and tubulin; the purpose of our studies was to determine whether there were other candidate
proteins involved in its complex. This could then lead to more information about the function of NP22 and its potential interacting signaling proteins. The tandem affinity purification method (TAP) has been used to successfully characterize several globular and cytosolic protein complexes (Angers, 2008). This high throughput technology involving the use of mass spectrometry requires a cell line that stably expresses the protein of interest in large quantities. The use of HEK293 cells, as an initial model system, was appropriate because of the cell line’s ease of transfection and ability to produce high levels of the transfected protein. HEK293 cells do not endogenously express NP22, which could have affected the outcome of the purification screens compared to a more neuronal cell line that may endogenously express the protein. However, initial experiments did not yield any candidate interactors. In fact, the bait protein, NP22, was not detected in the analysis. Although the cell line was verified to express NP22, subsequent studies showed that the bait protein was not present in the final elution steps of the purification process.

We determined that, because of its cytoskeleton-associating property, NP22 was retained in the insoluble pellet that is generated after cell lysis and centrifugation to remove cellular debris. Thus, NP22 was not present in the soluble supernatant of proteins that went through the purification method. The use of detergents, whether moderate or harsh, in the lysis buffer is extremely important when attempting to purify protein complexes with use of mass spectrometry. Using harsher detergents can disrupt protein-protein interactions and interfere with mass spectrometry analysis (Annesley, 2003). The use of the mild detergent, NP-40, in the TAP lysis buffer contributed to the inability to solubilize NP22, because NP-40 did not disrupt the association between NP22 and the insoluble cytoskeleton. Our initial thoughts were to increase the strength of the detergent to an acceptable level for mass spectrometry, but the risk of losing protein interactions with NP22 became apparent. Understanding that NP22’s interactions with actin and tubulin were crucial for its function (de las Heras et al., 2007; Pape et al., 2008), we developed a new method to purify NP22 without the use of harsh detergents. Based on our previous method to isolate synaptic proteins from the brain, the HEK293 cells expressing NP22 were lysed with 0.32 M sucrose and low speed centrifugation was performed to pellet nuclei and remove a large majority of the cellular debris. This method ensured that nuclear debris was eliminated while cytoskeletal proteins were preserved in solution. After performing these modifications and performing another round of TAP, mass spectrometry analysis showed our
protein of interest had eluted; however there were surprisingly no interacting proteins. It is important to note that as a result of isolating protein extracts from the large scale procedure, several protein bands were observed in the western blot that shows verification of tandem affinity purification. It is also plausible that the multiple bands arose due to misfolded or post-translationally modified NP22 protein.

One possible explanation for the lack of interactors found using this method was that the conditions for TAP were too stringent and that some of NP22’s interactions with other proteins may have been lost because of the additional round of purification. Another plausible explanation involves the use of calcium in the latter stages of purification; the second round of affinity purification utilizes a calmodulin-binding domain, and calcium is used in the binding of the tagged protein to the calmodulin beads. Since calcium represents a possible regulatory ion in mediating NP22’s interactions with other proteins, the addition of calcium in the purification procedure may interfere with its protein complex. Alternately, interactions may be disrupted in the elution step; NP22 contains EF hand motifs that mediate the proteins interaction with other ligands through calcium coordination (Nelson et al., 2002). By using the calcium-chelating agent EGTA to elute the protein complex from calmodulin beads, removing calcium may disrupt NP22’s interactions with other proteins. Thus, further studies characterizing the role of calcium in regulating NP22 interactions should be conducted. A final possible explanation for the paucity of interactors comes from the size of the TAP tag. The dual affinity TAP tag, weighing approximately 10 kDa (half the size of NP22), may have interfered with NP22’s function such that it could not interact with proteins due to its conformational change.

FLAG-tag purification is an alternate protein complex purification approach that is often utilized. This method requires one purification step, no calcium, and a much smaller tag that is less likely to interfere with protein conformation. We conducted the FLAG-tag purification method on a stable cell line expressing tagged NP22; with each round of purification we also performed the same purification using HEK293 cells that did not express NP22 as a negative control. The FLAG purification was performed three times, and proteins that were present in at least two of the three purifications were considered. Of the candidate proteins, Lamin-B1, histone cluster 1 H1c and FACT complex subunit SPT16 were nuclear associated proteins that were likely to be nonspecific interactions. It is possible that some nuclear proteins contaminated the cytosolic fraction during lysis. NP22 is not a nuclear protein and has not been implicated as such in the
literature (Ren et al., 1994; Fan et al., 2001; Depaz et al., 2003). Furthermore, our collaborator Stephane Angers had reported these proteins as nonspecific interactors in their studies; however, additional control purifications using our lysis method are desirable to further support this conclusion.

Of the short list of candidate proteins, brain-type creatine kinase (CK-BB) and drebrin represented interesting potential interactors based on their repeated interaction in the FLAG-tag pulldown, and their own unique properties. CK-BB represented an interesting potential interaction because of its involvement in energy metabolism (Beard and Braissant, 2010). CK-BB is part of the highly expressed creatine/phosphocreatine/creatine kinase system that allows for the regeneration of ATP acting as an energy buffer (Wallimann et al., 1992). The system plays essential roles in maintenance of membrane potential and ion gradients, calcium homeostasis, neurotransmission and intracellular signaling systems during brain development (Wyss and Kaddurah-Daouk, 2000). We hypothesized that NP22 may recruit CK-BB as an additional energy source when mediating its function of neurite outgrowth and modifying neuron structure. However, co-immunoprecipitation studies did not confirm the proposed interaction.

Drebrin represented an even more compelling candidate protein interaction than CK-BB. Drebrin is a neuron specific F-actin binding protein that is localized at dendritic spines of mature neurons (Hayashi et al., 1996; Aoki et al., 2005). Overexpression of drebrin in fibroblast cells leads to alteration in their cell shape while overexpression in mature cortical neurons causes elongation of their dendritic spines (Hayashi et al., 1999). Drebrin has also been implicated in NMDA receptor signaling as it has been shown that it induces spinous clustering of the PSD scaffold protein, PSD-95, as well as activity-dependent synaptic targeting of NMDA receptors (Takahashi et al., 2006). Drebrin expression is also enhanced within dendritic spines of the hippocampus after LTP induction. We hypothesized that drebrin may act as a chaperone to NP22, providing an indirect association between NMDA receptor mediated synaptic plasticity and NP22 function. However, co-immunoprecipitation studies did not confirm the proposed interaction.

Although both proposed interactions of NP22 with CK-BB and drebrin were not confirmed, it is still possible that CK-BB and drebrin are interacting with phosphorylated forms of NP22, as the co-IP did not control for this possibility. Also, disruption of the cytoskeleton after cell lysis might affect protein binding in the NP22 protein complex. Future studies manipulating changes
in cytoskeleton using actin and tubulin depolymerizing agents, as well as controlling for phosphorylated forms of the NP22 should be conducted. Also, utilizing these purification methods in a more neuronal cell line expressing NP22 could represent a better model. Towards this goal, stably transfected Neuro-2a cells were generated to express tagged forms of NP22; however, expression levels were very low with this cell line, and not sufficient for either purification method. Perhaps pheochromocytoma 12 (PC12) or human cortical neuronal-1 (HCN-1) cells expressing our protein of interest may be more beneficial models. Future studies involving attempts at purifying NP22 complexes in various brain regions of WT and NR1-KD mice should also be conducted.

3 Efforts to establish a link between NMDA receptor signaling and NP22 biology in vitro

Due to NP22’s established role of binding to the cytoskeleton and affecting cell morphology (Mori et al., 2003; de las Heras et al., 2007; Pape et al., 2008), we hypothesized that changes in intracellular calcium would affect NP22 biology in vitro. Increasing intracellular calcium with the use of thapsigargin and ionomycin mirrored a model of NMDA receptor activation in HEK293 cells transfected with NP22. Attempts to transfect HEK293 cells with NP22 and the NMDA receptor subunits and directly treat them with NMDA and glycine were not successful, so treatment with calcium inducers was an alternative approach. It has already been established that NP22 colocalizes with actin in vitro and that mutated forms of the protein resulted in changes in cellular morphology; however, the mechanism has not been fully elucidated (de las Heras et al., 2007). We hypothesized that calcium is a crucial component of NP22’s function and that changes in calcium concentration would alter the colocalization of NP22 with F-actin, ultimately leading to changes in its function. More specifically, because of its function in modifying cell processes, NP22 colocalization with F-actin could change in the cell processes that protrude from HEK293 cells transfected with NP22.

In our transient transfection studies with HEK293 cells, thapsigargin did not induce changes in NP22 and F-actin colocalization in the cell body or cell processes with 15 minutes of treatment. However, at 25 minutes of treatment, there was significantly less colocalization of NP22 and F-actin in the cell processes, as measured by changes in Pearson’s correlation coefficient. Because thapsigargin acts by preventing the reuptake of calcium into intracellular stores (Michelangeli
and East, 2011), it may require additional time to cause a sufficient elevation in intracellular calcium. It appears that the biology of NP22 does change in the presence of increased intracellular calcium for a significant period of time such that a decrease in its colocalization with F-actin could affect its function. This is also consistent with our hypothesis that the involvement of calcium may be disturbing interactions between NP22 and other proteins during TAP. This was evident by the lack of any candidate interacting proteins detected by mass spectrometry analysis after TAP. We were unable to determine, however, whether the intracellular change in calcium affecting colocalization was a reflection of changes in NP22 or F-actin. For example, the reduced colocalization between NP22 and F-actin in the cell process could have been a result of reduced levels of F-actin in the process or reduced levels of NP22. It has already been reported that changes in NMDA receptor signaling do lead to changes in actin dynamics via calcium and signaling cascade activation (Okamoto et al., 2004). Future studies examining this question using live cell imaging may be beneficial in addressing the issue. For example, using fluorescently labeled NP22 and monitoring its mean intensity in cell processes after thapsigargin treatment at different time points may indicate whether colocalization changes are a reflection of changes in NP22. A similar approach can be used with labeling F-actin and tracing its fluorescence at different time points as well.

In the stable transfection studies with HEK293 cells, both thapsigargin and ionomycin were used to induce intracellular calcium increases. Neither treatment, however, significantly altered NP22 colocalization with F-actin in either the cell body or cell processes of transfected HEK293 cells. There were modest reductions detected in cell processes; however, the Pearson’s correlation coefficient may have been a limiting factor in detecting the changes. In general, the protein of interest is produced at lower levels within stable cell lines compared to transient transfections. There were substantially lower expression levels of NP22 in the stable cells as imaged by confocal microscopy; perhaps measurements using the Pearson’s correlation coefficient were not sensitive enough to detect such subtle changes in localization. For example, colocalization coefficients were generated in the range of 0.7-0.8 in the cell processes of transiently transfected cells compared to 0.3-0.45 for stably transfected cells. Thus, it is possible that the reduced baseline pixel intensities that existed in the stable cell images may have made it more difficult for colocalization analysis to detect significant changes. Alternately, NP22 regulation in stable
cells may be different, such that NP22 is insensitive to changes in intracellular calcium in its association with F-actin.

Future investigations should focus on potential cell morphology differences that exist in transiently or stably transfected HEK293 cells as a result of NP22 protein expression. Preliminary qualitative data from confocal images show that stable HEK293 cells had more cell processes protruding from the membrane than untransfected HEK293 cells. To measure morphological changes in the cells as a result of NP22 expression, HEK293 cells (transfected and mock) would be plated at the same cell density and at the same time to allow the cells to adhere and form processes. Process number per cell would be determined with ImageJ or Nikon Elements software. Pharmacological treatment could be used afterwards to determine whether calcium increases alters process number or cell morphology as well. The same approach must be used for stably transfected HEK293 cells, while plating them at the same time as mock cells and monitoring treatment influences. Similar studies could also be used in more neuronal cell lines previously mentioned. Also, using a model of NMDA receptor activation that uses NMDA receptor agonists in cells expressing NP22 would provide valuable information on the role of NMDA receptors in regulating NP22 biology.

4 Clinical implications

Establishing a link between NMDA receptor signaling and NP22 biology was our main objective because we hypothesized that NP22 contributes to the morphological changes seen in synapses as a result of altered NMDA receptor transmission. NP22 has been indirectly implicated in NMDA receptor downstream functions due to its upregulation in post-mortem alcoholic cortex and ethanol-treated rat cortex, and its downregulation in the striatum of ethanol-treated rats (Depaz et al., 2005). Thus, NP22 has been implicated in clinical disease. NP22 may play a role in the morphological or plastic changes that take place after chronic alcohol and withdrawal (Depaz et al., 2003, Depaz et al., 2005). Furthermore, it may be involved in the pathological processes that take place during chronic alcohol exposure such as cortical atrophy (Charness et al., 1989), decreased dendritic arborization and loss of neurons (Kril and Harper, 1989). If accumulating evidence points towards implications of NP22 in synaptic remodeling, it is possible that NP22 may be involved in disease states where spine morphology is altered such as schizophrenia (Garey et al., 1998; Sweet et al., 2009), autism (Bozdagi et al., 2010; Durand et al...)
al., 2011), fragile-X mental retardation (Irwin et al., 2000) and Rett syndrome (Chapleau et al., 2009). These hypotheses need further testing to uncover the molecular mechanisms associated with this complex protein.
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