NEURON SPECIFIC GENE 2 (NSG2): A NOVEL REGULATOR OF EXCITATORY NEUROTRANSMISSION VIA AMPA RECEPTOR TRAFFICKING AT A SUBSET OF GLUTAMATERGIC SYNAPSES

Praveen Chander
University of New Mexico

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Praveen Chander

Candidate

Neurosciences

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Jason P. Weick, PhD, Chairperson

Kevin Caldwell, PhD

Angela Wandinger-Ness, PhD

Nora Perrone-Bizzozero, PhD
NEURON SPECIFIC GENE 2 (NSG2): A NOVEL REGULATOR OF EXCITATORY NEUROTRANSMISSION VIA AMPA RECEPTOR TRAFFICKING AT A SUBSET OF GLUTAMATERGIC SYNAPSES

BY

PRAVEEN CHANDER

MS, Biology, Cleveland State University, 2010
MSc, Biotechnology, Bangalore University 2005
BSc, Microbiology, Madras University, 2003

DISSERTATION
Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Biomedical Sciences
The University of New Mexico
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December 2019
DEDICATION

This work is firstly dedicated to, THAT 'source of everything' which I seek to know. It is THAT unknown which has been the root of my strength and the invisible driver of all my endeavors and accomplishments. Secondly, this work would not been possible without the unconditional support of my parents, my wife, my family and friends. It is their patient understanding, sacrifices, nurturing help and life lessons that instilled in me the yearning to strive to learn, to look at any situation in life from several perspectives, and better myself against many odds. Lastly, this work is also dedicated to all the valuable lessons I have learned from the failures in my life. Failures provide harsh but valuable lessons to the ones that dare to rise and rebuild themselves after being repeatedly knocked down.
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Jason hired me when I had lost my job and was on the verge of being deported back to India. Jason and his wife Kate provided me with a place to stay and all their support, both material and emotional to help me get back on my feet in Albuquerque (where I randomly decided to move after watching Breaking Bad). Once I started learning the nuts and bolts of neuroscience and tasted some initial success in my project, he first floated and then more forcibly rekindled in me my almost lost interest in pursuing a PhD. Today, I’m thankful for that because he nurtured me throughout the process towards becoming confident and developing my own independent ideas in an area of science that I had no prior experience or exposure. During the last several years he has evolved into a better mentor and an even better person. This has been a long and memorable journey and I could probably write pages, however, words are but, insufficient to articulate and express my gratitude towards his contribution in my life. I hope to continue collaborating with him in the future and will seek his guidance both professionally and personally. Several members have been through the lab during my stay here and thinking about everyone brings some interesting mix of memories and valuable life lessons learned. But I value the fact that I have been able to build some lasting friendships with some people who spent a longer time in the lab and they were also very instrumental in keeping me honest towards my goals. I would like to thank my
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PhD. Biomedical Sciences, University of New Mexico, 2019

ABSTRACT

Members of the Neuron-Specific Gene family (NSG1-3) play critical roles in excitatory synaptic transmission via regulation of AMPA receptor surface expression within the post-synaptic density (PSD). While NSG1 and NSG3 regulate AMPAR recycling and endocytosis, respectively, the function of NSG2 has remained elusive. Here we undertook a series of studies to assess the role of NSG2 in excitatory synaptic transmission. We found that a portion of NSG2 punctae localized with HOMER1 and surface AMPARs at excitatory synapses and that NSG2 AMPAR subunits in mouse brain. Knockout of NSG2 selectively impaired the frequency of AMPA mEPSCs, while overexpression caused a significant increase in the amplitude of AMPA mEPSCs. Despite the fact that NSG2 is actively trafficked, both static and extended timelapse (3 hr) imaging revealed that NSG2 was stably localized at ~30% of glutamatergic PSDs. Interestingly, this subset appeared largely non-overlapping with NSG1. Furthermore, endogenous NSG2 was associated with greater AMPAR surface expression that appeared to be independent of neuronal activity. Together, these data reveal that NSG2 is an AMPAR-binding protein that is required for normal synapse formation and/or maintenance. In addition, they suggest a novel type of postsynaptic diversity via selective incorporation of NSG1 and NSG2.
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Chapter 1: Introduction
The excitatory synapse and synaptic neurotransmission

The mammalian brain consists of billions of neurons that form precise functional connections with each other at specialized junctions called synapses to give rise to a functional network. Synapses are fundamental units of neurotransmission where presynaptic axons contact postsynaptic dendrites. Glutamate is one of the most prevalent neurotransmitter in the brain and mediates excitatory neurotransmission (Micheva et al., 2010). Presynaptic glutamate is released as a culminating event of an action potential and diffuses across the synaptic cleft and acts on excitatory postsynaptic glutamate receptors. These are primarily the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors (AMPARs) and NMDA (N-methyl-D-aspartic acid) receptors (NMDARs). Glutamate binding allows for an influx of Na\(^+\) ions (AMPAR) and Na\(^+\) and Ca\(^{2+}\) (NMDAR) into the postsynaptic neuron causing an electrical depolarization and thereby, propagation of excitatory synaptic transmission. This transmission is thought to occur specifically within the postsynaptic density (PSD). The ~100nm of membrane immediately adjacent to the PSD is called the perisynaptic region and differs considerably in molecular composition from the more distal extrasynaptic membrane and contains ‘endocytic zones’ which have proteins like clathrin and AP2 (Rácz et al., 2004).

A critical and intriguing property of neuronal synapses is ability to undergo ‘synaptic plasticity’, long-lasting changes in the strength of a synapse in response to altered neuronal activity. It is widely accepted that these changes allow for information storage in response to experience and is the basis for learning and memory (Malenka and Bear, 2004). In the long term, information storage is also facilitated by the plasticity of the functional neural networks which lends itself to modifications by addition of new and/or removal of existing synapses (Yuste and Bonhoeffer, 2001; Chklovskii et al., 2004). It can therefore be appreciated that the fidelity of synaptogenesis is critical for every conceivable function we carry out on a daily basis. Synaptogenesis involves the correct localization of both pre- and postsynaptic proteins. Improper synaptic development or function are major
contributors to pathological states such as various neuropsychiatric (schizophrenia), neurodevelopmental (autism) and neurodegenerative conditions (Alzheimer’s disease) (Javitt, 2004). Therefore, recognizing the molecular underpinnings of synaptic activity and plasticity is of fundamental biological and clinical relevance.

The AMPA Receptors (AMPARs)

The postsynaptic AMPARs are critical mediators of the vast majority of fast excitatory synaptic transmission in the mammalian brain. Further, synaptic strength at glutamatergic synapses is largely determined by the number, composition, and posttranslational modifications (PTM) of AMPARs in the postsynaptic membrane (Malinow and Malenka, 2002; Huganir and Nicoll, 2013). At their most fundamental, AMPARs ligand-gated ion channels that only require the presence of glutamate within their binding domain to gate open (Shepherd and Huganir, 2007). In addition, they are non-specific ion channels which primarily conduct sodium (Na$^+$) and potassium (K$^+$) down their electrochemical gradients. In most cases, glutamatergic transmission causes AMPAR gating, causing depolarization via Na$^+$ influx. Only in very specific cases are AMPARs permeable to Ca$^{2+}$, and can therefore, trigger downstream signaling cascades (Shepherd and Huganir, 2007), but we will not discuss this here as it is of unknown relevance to the current work.

The numbers and the types of AMPARs present at synapses determine the degree of synaptic plasticity at that synapse. For example, during strengthening, synapses are potentiated by addition of AMPARs and during weakening, AMPAR at the synapse are removed by endocytosis. AMPARs are comprised of four subunits (GluA1 through GluA4) that associate into hetero-tetramers. Each tetramer is made of two homomeric dimers to give rise to various combinations of postsynaptic AMPARs. The particular type at a synapse governs the protein trafficking and ionic conductivity characteristics (Shepherd and Huganir, 2007). While GluA1–GluA3 are the most commonly expressed subunits throughout the nervous system, GluA4 expression is more abundant during early development.
(Zhu et al., 2000). In the hippocampal CA1 neurons, the majority of AMPARs are made up of GluA1/GluA2 and GluA2/GluA3 tetramers (Wenthold et al., 1996). Previous studies of AMPAR subunit expression levels suggest that subunit combinations are relatively similar across various brain regions (Lu et al., 2009; Schwenk et al., 2014).

**How could AMPARs mediate information storage in the brain?**

The stoichiometry of AMPAR at a synapse, their association and interactions with other proteins and PTMs allows for a range of probabilities for information coding. This could be the molecular basis for AMPAR dependent synaptic plasticity during learning and memory. AMPARs turnover at the synaptic surface involves three modes of highly regulated protein trafficking events. The first two modes are AMPAR endocytosis and exocytosis which may be homeostatic or activity dependent. The third mode involves exocytosis of AMPAR at an extrasynaptic site followed by lateral diffusion in the plasma membrane. Diffusing AMPARs at the synapse are trapped at the postsynaptic density (PSD) (Malinow and Malenka, 2002; Huganir and Nicoll, 2013). AMPARs surface levels are also regulated by a range of PTMs such as ubiquitination, phosphorylation and palmitoylation. PTMs and an array of protein-protein interactions during cellular signaling events further funnel in to regulate AMPAR synaptic trafficking and anchoring, and ion conductance properties (Lu and Roche, 2012; Huganir and Nicoll, 2013). Together AMPARs are highly dynamic and their synaptic levels which in turn is governed by receptor subunit composition (channel conductance), protein interaction and trafficking and PTMs regulate the balance between synaptic accumulation and removal. While the vast amount of information regarding specific AMPAR trafficking mechanisms is too large to cover here, a number of excellent review articles are available (Anggono and Huganir, 2012; Huganir and Nicoll, 2013; Diering and Huganir, 2018). Here I will lay a general foundation for understanding AMPAR trafficking with a focus on the specific mechanisms pertinent to NSG family members.
Synaptic Plasticity

Synaptic plasticity is widely accepted to be the molecular and cellular correlate of learning and memory. Plasticity involves a change in the equilibrium that regulates synaptic AMPAR accumulation or removal. This experience dependent potentiation or depression of synaptic strength tunes neural circuits and enables coding for learned memories and behaviors (Mayford et al., 2012; Takeuchi et al., 2014). Regulated endocytosis of synaptic AMPAR leading to reduced synaptic strength corresponds to the molecular process of long-term depression (LTD). The opposite effect of regulated exocytosis of AMPARs leads to increased synaptic AMPARs which correlates with increases in synaptic strength. This molecular process is known as long-term potentiation (LTP) (Bredt and Nicoll, 2003; Henley and Wilkinson, 2013). Additionally synapses can also get scaled during homeostatic plasticity. This process involves a cell wide or global network wide adjustment of synaptic AMPARs levels to maintain a homeostatic output from particular neurons during variable levels of neuronal circuit activity (Fernandes and Carvalho, 2016).

**AMPARs in LTP:** One of the best characterized and major form of synaptic strengthening is the NMDAR dependent LTP. This Ca\(^{2+}\) dependent process results in heightened AMPAR numbers in the postsynaptic membrane (Huganir and Nicoll, 2013). Pre-synaptic activity is coupled to Ca\(^{2+}\) influx through the NMDARs leading to a local increase in synaptic Ca\(^{2+}\). Calcium dependent downstream signaling via Ca\(^{2+}\)/Calmodulin dependent protein kinases (CaMKII) eventually leads to phosphorylation of various GluA subunits via PKA. However, phosphorylation of GluA1 appears to be the most critical in promoting AMPAR targeting to the postsynaptic membrane (Barria et al., 1997; Kristensen et al., 2011). Extrasynaptic AMPAR diffusing locally in the plasma membrane near the synapse also appear to supply the increased demand for AMPAR during LTP. This non synaptic AMPAR is replenished by homeostatic exocytosis from an intracellular reservoir of AMPARs in recycling endosomes (Anggono and Huganir, 2012; Huganir and Nicoll, 2013). AMPAR subunit GluA1’s interaction with the scaffold protein 4.1N
via its C-termini promotes its exocytosis from endosomal receptor pools (Lin et al., 2009). In addition to GluA1, AMPAR subunits GluA2 and GluA3 also undergo activity dependent exocytosis to the postsynaptic membrane in the mature hippocampus (Adesnik and Nicoll, 2007). Protein-protein interaction play a key role in GluA2/3 exocytosis. This involves a large molecular complex involving direct and indirect interactions of GluA2/3 with GRIP1/GRIP2, PICK1, KIBRA, Grasp1 and NSG1 (Anggono and Huganir, 2012).

**AMPARs in LTD:** During depression of synaptic strength the balance between AMPAR endo- and exocytosis is shifted towards endocytosis leading to reduced numbers of AMPARs in the postsynaptic membrane (Huganir and Nicoll, 2013). AMPAR endocytosis can take either the Clathrin or Dynamin routes during LTD (Shepherd and Huganir, 2007). In contrast to LTP where GluA1 phosphorylation plays an important role, GluA2 phosphorylation is important in LTD. LTD is suppressed in a mice model carrying a phospho-null mutation, where GluA2 phosphorylation is eliminated (Chung et al., 2000; Steinberg et al., 2006). As mentioned above in the case of LTP, a molecular complex involving GluA2’s interaction with GRIP1/GRIP2 and PICK1 mediates its membrane trafficking. This is demonstrated by the loss of LTD upon genetic ablation of GRIP1/GRIP2 or PICK1 (Chung et al., 2000; Seidenman et al., 2003; Steinberg et al., 2006; Takamiya et al., 2008). Specifically, it was shown that phosphorylation of the C-termini of GluA2 weakens its interactions with GRIP/PICK1 and allows for GluA2 internalization (Chung et al., 2000; Seidenman et al., 2003; Steinberg et al., 2006) While GluA2 has a dominant role in LTD, GluA1 has also been shown to be important. Unlike LTP however, GluA1 de-phosphorylation appears to be more important. A mutation resulting in a continuous phosphorylation of S845 appears to prevent LTD (Lee et al., 2009).

**AMPARs in homeostatic plasticity:** Neurons appear to have a physiological range of activity. Homeostatic plasticity refers to the increase or decrease in the number of postsynaptic AMPARs in order to maintain activity around this set point.
For example, when neurons are subject to hyperactivity by blocking GABA receptors (Bicuculine treatment), it results in scaling down of AMPARs. Similarly, chronic hypoactivity caused by blocking sodium channels (TTX treatment) leads to a homeostatic scaling up resulting in increased number of postsynaptic AMPARs (Turrigiano et al., 1998; Turrigiano, 2008). Similar to LTP and LTD, synaptic strength is altered during homeostatic scaling by changing the abundance of postsynaptic AMPARs, subunit composition, protein interactions, and PTMs (O’Brien et al., 1998). One common method is the induction of homeostatic upscaling via chronic (24-48hr) treatment of cultured neurons with tetrodotoxin (TTX). This causes increases in GluA1 protein expression as well as surface and synaptic levels of AMPARs (Soares et al., 2013; Kim and Ziff, 2014). While the general principles of AMPAR trafficking to or from the membrane appear shared between long term and homeostatic plasticity, the specifics may vary. For example, scaling up of GluA1, like in LTP, involves phosphorylation. More specifically however, LTP involves phosphorylation of GluA1 residue S845. In the case of a mutant where S845 is constitutively phosphorylated, scaling up occurs upon chronic TTX treatment however the GluA1 does not recruit specifically at synapses (Diering et al., 2014; Kim and Ziff, 2014). Similarly, the GluA2 molecular complex with GRIP1/GRIP2 and PICK1 also plays an important role in scaling up (Gainey et al., 2009). For example, shRNA mediated knockdown or blockade of GluA2 functions by the introduction of a competing C termini can block homeostatic scale up. Curiously, in addition to the overlap in the molecular principles adopted to deliver or AMPAR, there also seem to be functional redundancy among the AMPAR subunits. to there appears to be redundancy inherent to homeostatic scaling. For example, homeostatic scaling of GluA1/3 can still be observed when GluA1 is knocked out, however GluA1 surface levels are found to increase upon GluA2/3 knockout (Altimimi and Stellwagen, 2013).

**PDZ domain containing AMPAR interacting proteins**

All of the AMPAR subunits share a consensus (-SVKI) sequence at their C-terminus end. This conserved motif has been shown to facilitate their interaction
with PDZ domain containing proteins (Dong et al., 1997). In this instance, the GRIP family of proteins including GRIP1, GRIP2 and AMPAR-binding protein (ABP) are of particular relevance to NSG proteins (Dong et al., 1997; Srivastava et al., 1998). Interestingly, GRIP not only functions in the PSD but also binds to the motor protein KIF5 and regulates AMPARs during short and long-range trafficking. This is demonstrated by the effects on surface expression of AMPARs upon GRIP1/2 overexpression or knockout. Mice carrying a gain of function mutation in GRIP1 show faster AMPAR recycling and heightened surface expression. On the contrary, decreased surface AMPARs is seen in mice with GRIP1/2 knockout (Mejias et al., 2011; Han et al., 2017). When AMPAR endocytosis is promoted either by an LTD like stimulus or AMPA treatment, internalized AMPARs are tethered and sequestered intracellularly (Daw et al., 2000; Braithwaite et al., 2002; Hirbec et al., 2003). Alternatively, GRIP1 may also regulate AMPAR recycling and insertion back into the membrane (Mao et al., 2010). NMDA stimulation of neurons also leads to GluA2 internalization. Recycling GluA2 back to the membrane followed by its reinsertion was shown to be regulated by GRIP1 (Lu and Ziff, 2005). These examples further exemplify the role of the GRIP proteins in regulating AMPAR trafficking (Hayashi and Huganir, 2004).

The post synaptic density

The PSD of glutamatergic synapses is usually located at the tip of the dendritic spine, a region called the spine head. Dendritic spines are tiny 0.5–2 μm actin rich membrane protrusions. There appears to be a correlation between the size of the spine head with the synaptic strength with larger ones expressing more number of AMPAR (Kasai et al., 2003). The majority of excitatory synapses of principal neurons like the pyramidal neurons of the mammalian hippocampus occur at 1–10 spines/μm of dendrite length. The heterogeneous spines come in various shapes such as ‘thin filopodia’, ‘stubby’ or ‘mushroom’. However these shapes are not fixed and display significant plasticity in size, shape and number depending on the stage of neural development and strength of synapses and also show dynamic motility during development (Kasai et al., 2003; Ethell and
Pasquale, 2005; Tada and Sheng, 2006). Interestingly, subjecting the mice to new experiences leads to sprouting of new dendritic spines (Holtmaat et al., 2006). Glutamate uncaging through photolysis revealed potentiation of AMPAR current at a single spine as a consequence of quantal uncaging which was correlated with an increase in the volume of the spine head (Kasai et al., 2003; Holtmaat et al., 2006).

The identity of the protein complex of the cerebral cortex PSD was first elucidated in the 1990s using a combination of 2D gel electrophoresis and N-terminal peptide sequencing. An initial set of 30 proteins were identified which included two proteins associated with vesicular trafficking, dynamin and N-ethylmaleimide-sensitive factor (Walsh and Kuruc, 1992). Cho et al (1992) showed that the prototypic PSD protein post-synaptic density of 95 kDa (PSD95) enriched in the rat PSD shares sequence similarity with the Drosophila tumor suppressor protein discs-large-1 (dlg) (Cho et al., 1992). Antisera based screening of brain cDNA expression libraries, yeast two hybrid based screening of rat brain cDNA library and the advent of Mass spectrometry (MS) revolutionized protein discovery and led to the identification of hundreds of putative PSD proteins with known or unknown functions (Langnaese et al., 1996; Collins et al., 2006; Yoshimura et al., 2004). These methods allowed for a better understanding of the molecular mechanisms underlying PSD’s role in synaptic plasticity. The PSD proteins can be generally grouped into several functional categories like cytoskeletal and actin related, kinases/phosphatases, receptors and channels, membrane trafficking and motor proteins, cell adhesion, GTPases and regulators, metabolism, scaffolds and translation (Sheng and Kim, 2011).

**Protein Signaling in the PSD**

Signaling molecules and scaffolding molecules taken together as a group make up a large part of the PSD. Among the signaling molecules the most abundant are the kinases which include the CaMKIIα and non-receptor tyrosine kinases. These are followed by phosphatases like the serine/threonine protein phosphatase I and protein tyrosine phosphatases (Sheng and Hoogenraad, 2007).
NMDA receptor stimulation promotes calcium/calmodulin dependent phosphorylation and recruitment of CamKII into the PSD (Bayer et al., 2001) leading to activity dependent potentiation of the PSD via synaptic recruitment of GluA1 (Hayashi et al., 2000). Activity dependent Ca\(^{2+}\) influx through the NMDAR allows for formation of new spines as well as strengthening of existing ones via a process involving a multiprotein signaling complex (Saneyoshi et al., 2008).

**Scaffolding proteins of the PSD**

Scaffolding proteins in the PSD serve as a platform to hold together an enriched array of postsynaptic receptors and cytoplasmic signaling proteins like protein kinases, phosphatases, cell adhesion molecules and GTPases (Sheng and Hoogenraad, 2007). One of the better characterized scaffolding proteins includes Membrane associated guanylate kinases.

**Membrane associated Guanylate Kinases (MAGUKs):** One of the most studied and best characterized family of scaffolding proteins are the MAGUKs. The PSD95 family of MAGUK proteins and have been implicated in diverse aspects of synaptic plasticity and structural integrity of the PSD. The family includes four proteins, PSD-95, PSD-93, SAP102, and SAP97 that share highly conserved structural domains (Sheng and Hoogenraad, 2007). Their protein interaction domains help in integrating external cues from cell surface receptors or channels and adhesion molecules with intracellular signals and cytoskeletal components. The prototypic MAGUK PSD95 was one of the first identified constituents of the PSD from rat brain (Cho et al., 1992), and its relevance in sustaining the 3D architecture of the PSDs became apparent with the EM tomography based observation that RNAi mediated PSD95 knockdown led to the loss of entire patches of PSD (Chen et al., 2011). The importance of MAGUKs in anchoring AMPAR and NMDAR in the PSD stems from a study by Chen and colleagues (2011) involving chained knockdown of PSD-95, PSD-93 and SAP102. EM tomography results from this study show a significant reduction in the size of PSDs and a disintegration of the molecular fabric that organizes these glutamate receptors (Chen et al., 2011). Parallel
electrophysiology experiments showed that triple MAGUK knockdown significantly diminished AMPA and NMDA currents and gave rise to silent synapses (Chen et al., 2015). Immunogold electron microscopy studies showed that the number of AMPAR at a PSD has a linear correlation with the size of the PSD. 75% of the synapses from adult rat Schaffer collateral-comissural synapses contained both NMDAR and AMPAR and the size of these synapses were correlated to the number of AMPAR. Silent synapses which did not have AMPAR but only NMDA receptors were significantly smaller in size. This heterogeneity in synapse size and AMPAR was not observable in mossy fiber synapses (Takumi et al., 1999). Curiously, when Lu and colleagues (2013) knocked out AMPAR from the CA1 synapses using Cre-Lox recombination in Gria1-3/− Grin1/− mice, they did not observe any morphological differences in the CA1 synapses despite not having any glutamatergic transmission (Lu et al., 2013). These observations indicate that while the size of the synapse is an indicator of synapse strength, the AMPAR complement may only have a small contribution to of synapse size. In the case of AMPAR, PSD95 indirectly interacts with AMPAR via the Transmembrane AMPA regulatory protein (TARP), stargazing (stg) (Chen et al., 2000). A deletion of the PDZ-binding domain in stg disrupts hippocampal synapses resulting in a decrease in AMPA mini EPSC amplitude and frequency in hippocampal neurons (Chen et al., 2000). Considerable redundancy in functions is observed among the various MAGUK family members. While overexpression of PSD95 increased both the number and size of synapses evident in the increase amplitude and frequency of AMPA mEPSCs (El-Husseini et al., 2000; Stein et al., 2003), genetically inactive, truncated PSD95 did not alter AMPAR synaptic transmission (Migaud et al., 1998)). Interestingly, RNA interference mediated knockdown of PSD95 in cultured hippocampal neurons resulted in a 42% reduction in GluA2 localization in dendritic spines compared to control and a significant reduction AMPA mini EPSC frequency but curiously no change in the amplitude (Levy et al., 2015). Similarly, AMPAR EPSC recordings from hippocampal slices expressing the shRNA for PSD95 showed a 51% reduction in amplitude compared to scrambled shRNA (Elias et al., 2006). Similar effects were observed for acute knockdown of PSD93
with a 48% reduction in AMPAR EPSC amplitude in hippocampal slices compared to control (Elias et al., 2006). In order to overcome the functional redundancy and definitively identify the functional contribution of each MAGUK family member in synaptic transmission Elias and colleagues (2006) generated a PSD93/95 double KO mouse. AMPAR current recordings from hippocampal slices from this mouse also showed a 55% reduction in EPSC amplitude and corresponded with a significant reduction of GluA1 and GluA2 receptors in a PSD enriched fraction (Elias et al., 2006). While each of PSD95 and PSD93 resulted in an ~50% reduction in evoked AMPA EPSC amplitude, the combined knockout also resulting in a 50% reduction was indicative of compensation from another MAGUK family member. This was indeed the case, as an shRNA mediated knockdown of SAP102 in hippocampal slices from PSD93/PSD95 double KO mouse further decreased the amplitude of AMPA EPSC to ~80% of WT and the PSD fractions of double KO mice were also enriched in SAP102 (Elias et al., 2006). These studies suggest that MAGUKs are not only compensate for the functions of a missing family member as indicated by studies in the germ line KO mice but are critical for targeting nearly all glutamate receptors (AMPAR and NMDARs) to the synapse.

The Neuron Specific Gene (NSG) family: Candidates for AMPAR trafficking

The NSG family of orthologous genes encode for the three closely related neuron-enriched endosomal proteins, NSG1 (also NEEP21), NSG2 (also HMP19/Neuronal Vesicle Trafficking Associated/Protein P19) and NSG3 (also Calcyon/Calyp). NSG1 and NSG2 were originally discovered through cDNA library based screens for genes expressed in embryonic rat brains (Saberan-Djoneidi et al., 1995; Saberan-Djoneidi et al., 1998). NSG3 was identified through a yeast two-hybrid screening of the human brain cDNA library (Lezcano et al., 2000). Evidence from previous research points to the evolution of the NSG gene family (also NEEP21/Calyp/p19 family) as important vesicular trafficking mediators in neurons during early vertebrate speciation, as flies and worms don’t express NSG proteins. Northern blots demonstrated enrichment of NSG transcripts in the brain (Saberan-
Djoneidi et al., 1995; Saberan-Djoneidi et al., 1998; Lezcano et al., 2000). The tripartite family of proteins are thus highly enriched in the central nervous system and show very little expression in other tissues, although some expression is seen in testes. NSG1 and NSG2 are highly developmentally regulated with peak expression shown to be around Embryonic day (E) 17 and heightened expression up to the first postnatal week (Saberan-Djoneidi et al., 1995; Saberan-Djoneidi et al., 1998).

**Molecular Structure of NSG Family Members**

All NSG family proteins have a single TM domain and a highly conserved 47 amino acids residue immediately adjoining the TM region on the C-terminus. Outside of this unique motif, sequence comparison studies suggest a greater conservation at the N-terminus region and more divergence at the C-termini. NSG1 and NSG3 have previously been shown to carry out distinct roles in intracellular vesicular trafficking including in post synaptic AMPA receptor endocytosis, recycling, and degradation. Co-immunoprecipitation studies showed that NSG1 specifically bound the GluA1 and GluA2 subunits and regulated the recycling of AMPA receptors at mature excitatory glutamatergic synapses (Steiner et al., 2002; Steiner et al., 2005), transcytosis of adhesion protein L1CAM to axons (Yap et al., 2008) and processing of beta-amyloid precursor protein (Norstrom et al., 2010). In contrast, NSG3 was shown to be involved in activity dependent clathrin mediated endocytosis (CME) of AMPA receptors at excitatory synapses (Xiao et al., 2006a; Davidson et al., 2009a) and proteolytic cleavage of Neuregulin 1 (Yin et al., 2015). Together, NSG family proteins sub serve roles in neuron development, synaptic function, and neurodegeneration.

**Cellular localization and trafficking dynamics**

The subcellular localization of NSG1, NSG2 and NSG3 has been examined in neurons, using both the light microscope and at high resolution using the electron microscope. Immunofluorescence assays revealed that these proteins are enriched in the trans-Golgi network (TGN) and distributed as puncta within neurons.
However, while NSG1 showed a predominantly somatodendritic vesicular localization (Steiner et al., 2002), NSG3 was distributed throughout the neurons including in the axons (Xiao et al., 2006). In electron micrographs NSG1 and NSG3 proteins appear within endosomal vesicles and interestingly both within and at the base of dendritic spines. While NSG1 appears to be localized within multivesicular bodies and also near the PSD, NSG3 is usually along the wall of the dendritic spine lateral to the PSDs (Xiao et al., 2006; Utvik et al., 2009). Steiner and colleagues (2002) used immunocytochemical labeling and found that NSG1 was localized in Rab5+ early endosomes (EE’s), in Rab4/5+ sorting endosomes. A smaller subset of NSG1 localized in Rab11+ recycling endosomes (REs)(Steiner et al., 2002). Curiously, they did not present any quantitative data and was mostly qualitative. More recently, Yap and colleagues (2017), have shown that both NSG1 and NSG2 are co-localized in EEA1+ and Rab5+ EEs. Overexpression of the constitutively active Rab5 (Rab5-Q79L) led to the accumulation of both NSG1 and NSG2 in enlarged somatic and dendritic endosomes due to excessive fusion of endosomes. In contrast, overexpression of the dominant negative Rab5 (Rab5-S34N) resulted in a loss of NSG1 and NSG2 punctate endosomal staining resulting in diffuse staining in the transfected cell. Consistent with earlier findings both NSG1 and NSG2 were found to minimally localize to Rab11+ and Transferrin+ (Tf) REs. Finally, in contrast to previous reports suggesting that NSG1 may occupy a unique set of neuron specific endosomes, they have shown that a significant subset of NSG1 and NSG2 are trafficked from Rab5+ EEs to Rab7+ late endosomes (LEs) and eventually targeted for degradation in LAMP1+ lysosomes. Overexpression of the dominant negative (Rab7-T22N) in cultured neurons resulted in increased NSG1 and NSG2 staining intensities compared to untransfected neurons. Similar accumulation of NSG1 and NSG2 was observed upon shRNA mediated knockdown of Rab7 (Yap et al., 2017). These findings of NSG1 and NSG2 entering LEs after endocytosis through Rab5 EEs and not recycling via Rab11+ REs are intriguing in light of previous reports implicating NSG1 as a critical mediator of recycling post synaptic AMPA receptors. NSG3 has previously been shown to
localize in EEA1+/Rab5+ EEs consistent with its role in activity-induced endocytosis of postsynaptic AMPA receptor (Xiao et al., 2006; Davidson et al., 2009). However, more recently NSG3 has also been shown to be localized and trafficked in Rab7+ LEs and LAMP1+ lysosome related organelles (LROs) in neuronal axons (Shi et al., 2018). Interestingly, all proteins have also been shown to be expressed at the cell surface. NSG3 was shown to be expressed in the surface of HEK293 cells in unstimulated condition. Surface expression was mediated by cytoskeletal proteins as blocking microtubule polymerization with Nocadazole reduced surface NSG3 (sNSG3) expression. Pharmacological perturbation of intracellular calcium levels also impacted sNSG3 expression, for example sNSG3 was increased by treatment with Thapsigargin that elevates intracellular calcium levels and KN62 dependent block of CAMKII activity reduced sNSG3 (Ali and Bergson, 2003). NSG1 and NSG2 have also recently been shown to be expressed on the surface of neurons (Yap et al., 2017).

Live cell imaging studies have further revealed the dynamic shuttling of NSG proteins within cells. These movements are thought to be facilitated by microtubule associated motor proteins. Yap and colleagues (2017) carried out simultaneous live imaging of both NSG1-mcherry and NSG2-GFP in cultured neurons and showed ~60% co-localization and co-trafficking of both proteins in the stationary and motile endosomal population. The motile puncta displayed dynamic antero- and retrograde motion along dendrites and a small proportion of NSG puncta reversed direction (Yap et al., 2017). Further, they showed that surface NSG1 and NSG2 first get endocytosed and co-traffic within Rab5+ EE compartment which then are transferred into Rab7+ LE compartment (Yap et al., 2017). Similarly, NSG3 vesicles have also been shown to be dynamically trafficked in HEK 293, SH-SY5Y and Neural cells. For example, TIRFM imaging of HEK 293 cells overexpressing mVenus-NSG3 was used to confirm its cell surface localization. Interestingly, sNSG3 displayed spontaneous lateral and vertical movements even on the cell surface (Ali and Bergson, 2003; Shi et al., 2017). Live cell imaging of endocytosis of sNSG3 was confirmed by treating surface proteins with WGA-Alexa Fluor (AF-594), which binds to plasma membrane proteins and
remains bound even during cellular uptake. NSG3 and WGA were co-trafficked to the Golgi apparatus. Further it also showed that mVenus-NSG3 puncta showed dynamic bidirectional movement within dendrites for variable distances as seen with NSG1 and NSG2. The movement was also shown to be temperature sensitive and could be arrested by transitioning the cells during imaging from 37°C to 15°C. Movement could also be arrested by treating the cells with the microtubule polymerization inhibitor nocodazole demonstrating that NSG3 vesicular transport is microtubule dependent (Shi et al., 2017). Thus NSG family of proteins appear to be critical for a variety of intracellular vesicular trafficking events including at excitatory synapses.

**Endosomal Trafficking and interacting adapters**

NSG1 and NSG3 have been shown interact and/or associate with a unique complement of proteins that determine their unique roles in endosomal trafficking. Steiner and colleagues first identified NSG1 as a molecule that co-purified with the SNARE protein Syntaxin13 (Stx13) from early postnatal rat brain (Steiner et al., 2005a). SNARE proteins have demonstrated roles in endosomal vesicle fusion and docking. Stx13 has been shown to be associated with REs and play an important role in regulating the recycling of Tfn receptors and neurite outgrowth (Prekeris et al., 1998; Hirling et al., 2000). Steiner and colleagues (2005) also demonstrated that NSG1 binds to the Glutamate Receptor Interacting Protein 1 (GRIP1) via a C-termini binding domain (aa 129-164). NSG1’s interaction with Stx13 is thus facilitated by a molecular complex involving NSG1/GRIP1 associated with the bridging molecule GRIP1-associated protein 1 (GRASP1) which is associated with REs (Hoogenraad et al., 2010). Several proteomic approaches including yeast two hybrid and mass spectrometry based approaches have identified the protein interactions partners of the C-terminus of NSG3. Most interacting partners appear to facilitate NSG3’s role in CME, for example its binding with the clathrin light chain regulates clathrin assembly. The purified NSG3 C-terminus appears to promote clathrin self-assembly *in vitro* (Xiao et al., 2006). However, there is significant reduction in steady state levels of assembled clathrin *in vivo* in a NSG3 KO mouse.
model suggesting that NSG3 clathrin light chain interactions are critical for clathrin coated vesicle formation in the brain. NSG3 C-terminus also stably interacts with the μ subunits of the clathrin adapter proteins AP-1, AP-2 and AP-3 (Muthusamy et al., 2012). These adapter proteins have been implicated in various endosomal trafficking functions ranging from sequestering cell surface cargo destined for internalization to facilitating forward trafficking from TGN to REs and reverse trafficking from EEs to LEs and Lysosomes (Bonifacino and Traub, 2003; Newell-Litwa et al., 2007)

**NSG1 and NSG3 have unique trafficking roles**

The complementary and unique vesicle trafficking roles of NSG1 and NSG3 is clearly demonstrated in the case of Tfn, which localizes to the somatodendritic regions in neurons (West et al., 1997). Tfn which is internalized through CME can be recycled via one of the following kinetically distinct trafficking routes: A) the faster route involving the Rab4/Rab5+ sorting endosomes (SEs) or, B) the slower Rab4/Rab11+ REs. Tfn adopts the faster route demonstrated by the fact that treating the cells with wortmannin results in Tfn accumulation in enlarged Rab4/Rab5+ vesicles consequently resulting in a significant delay in Tfn recycling (Sheff et al., 1999; Trischler et al., 1999). In contrast, treatment with brefeldin A increases tubulation of Rab4/Rab11+ REs which however, do not significantly accumulate Tfn and only cause a minor delay in Tfn recycling (Klausner et al., 1992; Sönnichsen et al., 2000). While anti-sense mediated NSG1 knock down delays recycling of internalized Tfn. Overexpression alters both Tfn recycling and internalization (Steiner et al., 2002). Comparatively, NSG3 KO and overexpression only alter Tfn internalization but not recycling (Xiao et al., 2006). Other examples of NSG1 and NSG3’s role in recycling and endocytosis emerges from the study on the G-protein coupled Neurotensin receptor 1 (NTS1R) and Dopamine receptor D1 (D1DR). NTS1R and NTS2R have independent trafficking pathways. While NTS1R gets sorted to the degradative lysosomes after endocytosis through the EE pathway, the NTS2R recycles back to the plasma membrane through both the recycling pathways mentioned above for Tfn. Debaigt and colleagues (2004)
showed that overexpression of NSG1 in COS7 cells causes NTS1R to resort to a recycling pathway. One likely explanation could be that excess NSG1 redirects the trafficking of NTS1R from a degradative fate to a recycling fate through one of the recycling pathways similar to the effects of NSG1 overexpression on Tfn (Debaigt et al., 2004). The opposite effect of increased degradation of internalized NTS1R is seen upon knockdown of NSG1. In the case of D1DR, Ha CM and colleagues (2012) showed that NSG3 forms a ternary complex with the synaptic scaffolding protein PSD95 and D1DR. Phosphorylation of the Ser169 residue of NSG3 as a consequence of activation of the protein kinase C (PKC) with a phorbol myristate acetate combined with the D1DR agonist SK81297 not only resulted in recruitment of NSG3 at the plasma membrane and increased interaction with PSD95 but also increased internalization of the D1DR. This effect on D1DR internalization was nullified when the NSG3 phospho-deficient mutant S169A was used (Ha et al., 2012a). These observations are strongly suggestive of NSG1’s role in the RE pathway and opposed to NSG3 which has been shown to be a critical regulator of endocytosis.

**NSG proteins in AMPA receptor trafficking**

A logical question that emerges from the observation that NSG1 and NSG3 are involved in various aspects of vesicular trafficking and localize in excitatory spine synapses is whether the NSG family of proteins are involved in regulating excitatory synaptic transmission and synaptic plasticity. Evidence from previous studies involving perturbation of the levels of NSG1 and NSG3 in cells support their role in post-synaptic AMPA receptor trafficking and synaptic plasticity. NMDA/TTX and AMPA stimulation enhances internalization and recycling of AMPA receptors (Ehlers, 2000; Lee et al., 2004). However, NMDA stimulation alone without TTX induces sorting to the degradation pathway (Lee et al., 2004). Davidson and colleagues (2009) showed that cultured neurons from a NSG3 KO mouse show impaired AMPA receptor subunit GluA1 and GluA2 internalization upon simulation of LTD like conditions using AMPA or NMDA treatment compared to cultured neurons from wild-type mouse. Similarly, mEPSC recordings from
NSG3 KO cultures did not indicate any difference in basal glutamatergic transmission (Davidson et al., 2009). There were also no differences between the two groups in AMPA/NMDA ratio, AMPA and NMDA-dependent I/V relationships or pre-synaptic neurotransmitter release probability. EPSC recordings from acute hippocampal slices of NSG3 KO mouse also showed a deficit in the internalization of AMPA receptors in the CA1 cells upon induction of LTD using Low Frequency Stimulus (LFS) of the Schaffer Collaterals, again suggesting an impairment in the AMPA receptor internalization (Davidson et al., 2009). This deficit could also be mimicked by titrating a peptide containing the Caly CLC-binding domain (GST-Cal123–155) into wild-type CA1 neurons via the patch pipette. The defects in AMPA receptor internalization upon LFS simulated LTD could be rescued by overexpressing NSG3 (Davidson et al., 2009). These effects on AMPA receptor internalization are likely modulated indirectly by NSG3’s effects on CME which has previously been shown to be involved in AMPA receptor endocytosis (Kastning et al., 2007).

Results from several previous studies indicate that NSG1 is also an important player in the AMPA receptor recycling. A predicted role of NSG1 in AMPA receptor recruitment and/or subunit exchange at synapses during postnatal development and during synaptogenesis stems from the fact that NSG1 mRNA (Saberan-Djoneidi et al., 1998) and protein (Steiner et al., 2002) is highly expressed between the first weeks post conception to the first postnatal week. This is when AMPA receptors are recruited to hippocampal synapses with the proportion of NMDAR + AMPAR - synapses decreasing to give rise to NMDAR + AMPAR + synapses. This is also accompanied by a switch of GluA4 receptor subunits to GluA4 (Diering and Huganir, 2018). Concrete evidence of NSG1 involvement in AMPA receptor trafficking emerged from work from the Hirling group. While anti-sense mediated down-regulation of NSG1 retarded recycling of GluA1 and GluA2 (to a smaller extent) after NMDA application, internalized AMPA receptors which feeds into the RE pathway resulted in significant co-localization between NSG1 and GluA2. Stimulation that fed into the degradative pathway did not show this. Anti-sense mediated NSG1 knockdown or
a purified C-terminal fragment of NSG1 (aa129-164) served as a dominant negative affected both AMPA mEPSC and evoked AMPA EPSC. The effect of decreased mEPSC amplitude was specific and did not affect NMDA receptors or pre-synaptic release properties. Further, a high frequency stimulus (HFS) paradigm for inducing LTP in organotypic hippocampal slices failed to induce the same extent of potentiation of AMPA EPSC as WT when the patch pipette included either the anti-sense NSG1 or a purified C-terminal fragment of NSG1 (aa129-164) which served as a dominant negative. Finally, AMPA current amplitude did not increase in NSG1 knock down neurons following inhibition of CME using peptide D15 (Alberi et al., 2005). Together, these effects suggest NSG1 promotes both constitutive and activity-dependent AMPA receptor recycling. Probing for a potential molecular mechanism for NSG1’s regulation of AMPA receptor recycling led to the identification of the regulated multi-molecular complex involving interactions between NEEP21, GRIP1, GluR2 and syntaxin 13 (Steiner et al., 2005). GRIP1, a type II PDZ protein localizes to both the plasma membrane and intracellular compartments and importantly in dendritic spines. NSG1 was shown to bind GRIP1 via the C-terminal domain that contains PDZ repeat 7 in GRIP1. On the other hand AMPA receptor subunit GluA2 interacts with the GRIP1 PDZ repeats 4 and 5. Competitive blockade of the interaction between NSG1 and GRIP1 by transfecting neurons with an NSG1 GRIP1 binding domain (aa129-164) prevented GluA2 recycling under both unstimulated and stimulation to promote AMPA receptor internalization. Curiously, this effect was found to be specific for GluA2 and baseline surface expression or recycling of GluA1 was not affected. Further, Steiner and colleagues, used co-immunoprecipitation and found enhanced complex formation of GRIP1/GluR2 and NSG1 under AMPA receptor recycling stimulation, while it is decreased under conditions that stimulate AMPA receptor degradation. Finally, Kulangara and colleagues (2007) discovered a novel kinase associated with NSG1 that phosphorylates GRIP1. They showed that the phosphorylating activity of the unknown kinase was targeted to Ser 917 and was regulated by NMDA and AMPA treatments. The model that emerged from this study suggests that while dephosphorylated GRIP1 associates with NSG1 thus
facilitating its interaction with GluA2 in endosomes, rephosphorylation of GRIP1 destabilizes its interaction with NSG1 thus allowing GluA2 to recycle back to the plasma membrane (Kulangara et al., 2007).

**Alternate trafficking pathways and protein interactions**
Yap and colleagues (2008), showed that NSG1 is involved in transcytosis (trafficking back from the somatodendritic region into axons) of the cell adhesion molecule L1/neuron-glia cell adhesion molecule (L1/NgCam) (Yap et al., 2008). While L1/NgCam is typically enriched in the axon, it accumulates in the somatodendritic region of neurons when NSG1 is knocked down consequently resulting in less accumulation of L1/NgCam in axons. Live cell imaging studies revealed that L1/NgCam transiently co-localizes with NSG1+/Tfn⁺ stationary vesicles prior to transitioning into motile NSG1+/Tfn⁻ vesicles. Their data suggests that NSG1 facilitates sorting of axonal cargo via transcytosis and diverts cargos from a degradative pathway. Similarly NSG3 is also involved in axonal cargo transport. NSG3 has been shown to interact with the μ3 subunit of AP-3 (Muthusamy et al., 2012). AP-3 increases sorting of cargos from somatodendritic Tfn⁺ endosomes to axons (Newell-Litwa et al., 2007). In NSG3 KO mice, significant deficits are observed in the targeting of two AP-3 cargos phosphoinositol-4-phosphate kinase IIα and zinc transporter 3 in the mossy fibers of the dentate gyrus (Salazar et al., 2004; Seong et al., 2005).

Thus, the preponderance of evidence points to an important contribution of the NSG family in various aspects of protein trafficking and processes involved in neuronal development and function. Critically, NSG family members NSG1 and NSG3 have been shown to mediate vesicular trafficking events that serve to regulate AMPAR turnover at the cell surface during both basal and activity dependent synaptic transmission. Curiously, the role of NSG2, the founding member of the family based on evolutionary gene ontology, remains unknown. We discovered NSG2 as one among the highest expressed genes in a high throughput microarray analysis during heightened synaptogenesis. Incidentally, NSG2’s expression profile closely resembled the expression profile for AMPAR encoding
genes Gria1 and Gria2 and hypothesized that NSG2 may have a role in regulating AMPAR trafficking at synapses during neural development (Figure 1).

**HYPOTHESIS:**

*Figure 1: Diagrammatic summary of hypothesized role of NSG2 in PSDs.* (Left) Summary of previous research showing NSG3 (Yellow) involved in endocytosis of AMPAR (blue/red) from the cell surface via early endosomal vesicles (EE) and NSG1 (magenta) sorting of internalized AMPARs into either the lysosomes (Lys) or recycling endosomes (RE). Based on developmental expression, that NSG2 (cyan) primarily affects trafficking of AMPARs at nascent synapses (right), but may also function at more mature synapses (left) in the final secretory process to promote surface expression.
Chapter 2: Neuron-Specific Gene 2 (NSG2) encodes an AMPA receptor interacting protein that modulates excitatory neurotransmission

Introduction

As mentioned, the function of NSG2 (also termed Neuronal Vesicle Trafficking-Associated 2) remains unknown. However, based on the function of other NSG family members (NSG1 and NSG3) (Muthusamy et al., 2009; Norstrom et al., 2010; Lasiecka et al., 2014), we can make some hypotheses as to how NSG2 affects neuronal excitability. One major function attributed to both NSG1 and NSG3 is the regulated trafficking of the AMPA-type glutamate receptors (AMPARs) at postsynaptic densities (PSDs) during neuronal activity that leads to changes in synaptic efficacy (reviewed in (Yap and Winckler, 2012; Muthusamy et al., 2015). For instance, Calcyon is found in RAB5+/EEA1+ early endosomes, interacts with AP2 and AP3, and is critical for clathrin-mediated endocytosis of AMPARs (Xiao et al., 2006; Muthusamy et al., 2012; Muthusamy et al., 2015). Knockout of Calcyon impairs long term depression (LTD)-mediated AMPAR endocytosis, while overexpression reduces GluA1 and GluA2 surface expression and impairs performance on a fear extinction paradigm (Xiao et al., 2006; Kruusmägi et al., 2007; Davidson et al., 2009; Vazdarjanova et al., 2011). NSG1, on the other hand, is colocalized to a limited extent with early endosome marker RAB5 and recycling endosome marker RAB11 (Steiner et al., 2002; Yap et al., 2017). Overexpression of the carboxy (C)-terminal tail of NSG1 acts as a dominant negative and prevents recycling of GluA1/2-containing AMPARs following NMDA-mediated internalization (Alberi et al., 2005; Steiner et al., 2005). Together these proteins play complementary roles in regulating internalization (Calcyon) and recycling (NSG1) to promote AMPAR sorting between intracellular compartments and the plasma membrane during synaptic plasticity at established synapses.

NSG2 is expressed widely throughout the brain (Saberan-Djoneidi et al., 1995) with significant overlapping expression but some degree of divergence with NSG1 (Barford et al., 2017); the expression of NSG3 remains unknown. At the cellular
level, NSG2 is found extensively localized to the trans-Golgi network as well as in dendritic endosomes, and was recently found to be expressed transiently at the cell surface, similar to NSG1 (Yap et al., 2017). Interestingly, NSG2 also contains a secretogranin III domain as well as an atypical EGF-like motif (Saberan-Djoneidi et al., 1995), making it a good candidate for regulating signaling and exocytosis. We therefore hypothesized that NSG2 is a novel AMPAR binding protein that complements the NSG3 (endocytosis) and NSG1 (recycling) vesicular trafficking functions and thus regulates AMPAR surface recycling/expression.

Interestingly however, human NSG2 shares only 50% sequence homology with NSG1 and a 30% sequence homology with Calcyon at the amino acid level (Muthusamy et al., 2009), and therefore likely has unexpected roles in synaptic development and plasticity. NSG2 is one of the most highly expressed transcripts and proteins during human neural differentiation and synapse formation (Kang et al., 2011; Miller et al., 2014; Barford et al., 2017; Murillo et al., 2017). Thus, in addition to AMPAR exocytosis, the predominant expression of NSG2 during early periods of development (Saberan-Djoneidi et al., 1995; Barford et al., 2017) led us to consider whether it may function during early periods of synaptogenesis.

Here we report that NSG2 is a critical regulator of neuronal AMPAR surface expression in hippocampal cultures during early periods of neuronal development. In agreement with previous reports (Yap et al., 2017), we found NSG2 strongly localized to the trans-Golgi network as well as in a punctate pattern selectively in developing MAP2+ dendrites but excluded from axons. In addition, we now uncover that a significant proportion of NSG2 punctae co-localized with synaptic markers including AMPAR subunits. Co-immunoprecipitation and capillary immuno-electrophoresis experiments demonstrated a physical interaction with both GluA1 and GluA2. Overexpression and CRISPR-mediated knockout of NSG2 altered AMPAR surface expression as well as AMPAR-mediated postsynaptic currents. These data are the first to demonstrate a role for NSG2 in the regulation of AMPAR surface expression and implicate NSG2 as a potential partner of AMPARs in regulated endosomal trafficking in developing dendrites.
Materials and Methods

Cell culture, Transfection and Transduction: HEK293T cells and Neuro-2a cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 1x Penicillin/Streptomycin (Pen/Strep; Thermo Fisher Scientific, Waltham, MA). Cells at 90% confluence were passaged every 3 days using 0.05% Trypsin (Thermo Fisher Scientific). HEK293T cells were transfected using the calcium phosphate method with 10µg plasmid DNA. Neuro-2a cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific) as per manufacturer’s recommendation. Hippocampal pyramidal neurons were derived from P0-P1 C57Bl/6J pups (The Jackson Laboratory, Bar Harbor, ME) of either sex. Briefly, brains were isolated, and the hippocampus was dissected in ice-cold HBSS solution (Sigma, St. Louis, MO) supplemented with 20% FBS and NaHCO3 (4.2mM), HEPES (1mM; Sigma), pH 7.4. Dissected hippocampi were digested for 10 min with 0.25% Trypsin (Thermo Fisher Scientific). Tissue was washed and dissociated using fire polished Pasteur pipettes of decreasing diameter in ice cold HBSS containing DNase (1500 U; Sigma). The cells were pelleted, resuspended in plating media and plated at a density of 4-5×10⁵ cells/12-mm coverslip (Electron Microscopy Sciences, Hatfield, PA) coated with poly-Ornithine (0.1mg/ml; Sigma; Cat. #4638) and laminin (5µg/ml; Thermo Fisher Scientific). Cells were allowed to adhere for 20 min before addition of 0.5ml of plating media containing Neurobasal supplemented with 1X B27, 2mM Glutamax, 0.5mg/ml Pen/Strep and 10% FBS (all from Thermo Fisher Scientific) for the first 24h. Serum was eliminated from this media after 24h and again replaced after 48h supplemented with 4µM cytosine 1-β-d-arabinofuranoside (Ara-C; Sigma). Neurons were fed by replacing half the volume of spent media with fresh media without serum or Ara-C every week. All animal procedures were performed in accordance with author’s university animal care committee’s regulations. Neurons on coverslips were transduced with lentivirus (MOI 2 - 3) on day 4 in culture for CRISPR studies or day 7 in culture for NSG2 overexpression studies and assayed at the days indicated in results. Human pluripotent stem cells (hPSC; line WA09) were maintained in mTesR (Stemcell
technologies, Vancouver, Canada) and passaged using 1U/ml Dispase (Stemcell technologies) as per manufacturer’s recommendation once the cells reached 80% confluence. hPSC-derived neurons (hPSNs) were differentiated from one six well plate of hPSCs. Briefly, the cells were lifted using Dispase and allowed to grow in suspension in a T75 flask for 4 days in mTeSR. From Day5 to Day20, cells were grown in Neural Induction Media (DMEM/F12; Sigma) supplemented with 1x concentrations of N2, Glutamax and Pen/Strep (Thermo Fisher Scientific) and 2µg/ml heparin (Sigma) to derive neurospheres with 2/3rd volume of media replaced every other day. On Day 21, 8 to 10 neurospheres were plated down/12mm glass coverslip in a 24 well dish coated with poly-ornithine and laminin as described above and allowed to attach for a minimum of 4h before feeding with 0.5 ml Neural differentiation media (50:50 mixture of Neurobasal:DMEM/F12 supplemented with 1x concentrations of N2, Glutamax, Pen/Strep and 10ng/ml of cAMP [Sigma], BDNF and GDNF [Peprotech, Rocky Hill. NJ] and 100mM ascorbic acid [Sigma]). Half volume of media was replaced every other day until the assayed days mentioned in the Results section.

**Plasmids and Lentivirus production:** Episomal vectors encoding mCherry or NSG2-mCherry (NSG2-mC) were generated in house by cloning either mCherry or the open reading frame of human NSG2 (NM_015980.4) in frame with mCherry into the pCS2+ mammalian expression plasmid (kind gift from Bill Bement, Univ. of Wisconsin). The NSG1-GFP plasmid was a kind gift from Bettina Winckler (Univ. of Virginia). The pSIN REP5-GFP-GluA2(R) was purchased from Addgene (Addgene, Cambridge,MA; Cat. # 24005) and the YFP-GluA1 was previously described (Sharma et al., 2006). Lentiviral constructs were generated by cloning either mCherry or NSG2-mC downstream of the Synapsin-1 promoter in the pFCK plasmid (Dittgen et al., 2004). CRISPR/Cas9 guide RNAs (gRNAs) targeting mouse NSG2 (NM_008741.4) were generated using the CRSPR design webtool ([https://zlab.bio/guide-design-resources](https://zlab.bio/guide-design-resources)). gRNAs (MsNSG2#1: 5'-GCGTGTGATGAGAGGGACGGTC-3' and MsNSG2#2: 5'-CGTCCCTCTCAGGACGACGGC-3') were cloned into pL-Crispr.EFS.GFP
(Addgene, Cat. # 57818) into the BsmBI site as previously suggested (Heckl et al., 2014). Lentivirus were produced in HEK293T cells using Calcium phosphate transfection of a 15µg total mixture of lentiviral DNA and packaging plasmids psPax2 and pMD2.G (gifts from Didier Trono, Addgene, Cat. # 12260 and Cat. # 12259 respectively) at a ratio of 3:2:1 for lentivirus production. 36-48h post transfection lentivirus containing media was harvested and concentrated using Lenti-X concentrator (Clontech, Mountain view, CA) as per manufacturer’s recommendations. Pellets were resuspended in ice cold DMEM, aliquoted, and frozen at −80°C till use.

**Cell and Whole brain lysates** - HEK293T cells were lysed 48h post transfection by incubating cells for 30min in ice-cold lysis buffer containing (in mM): 50 Tris base, 150 NaCl, 1 EDTA, 1% Triton X-100 and 1x protease inhibitor cocktail (Thermo Fisher Scientific), pH 7.4. Whole brain homogenate was prepared by harvesting mouse brains from P0 pups and subjecting them to a brief ice-cold PBS wash. Brains were then homogenized by sonication (5 x 10sec) in ice cold lysis buffer using a sonic dismembrator (Fisher Scientific, model F60) with output power set at 1. Protein amounts in the supernatant was quantified using the BCA assay (Thermo Fisher Scientific) and was aliquoted and frozen at -80°C until use after cold centrifugation at 10,000 x g for 15 min.

**Co-immunoprecipitation** - For AMPAR co-immunoprecipitation 2mg total protein was incubated with 4µg of either mouse anti-GluA1 (Clone RH95; Millipore, Billerica, MA), mouse anti-GluA2 (Clone 6C4; Millipore), or control mouse IgG1 (Clone G3A1; Cell Signaling technology, Danvers, MA) antibodies overnight at 4 °C, with gentle rocking. Immune complexes were precipitated for 2h at 4°C using 20µl protein A/G agarose beads (Santa Cruz Biotechnology). For NSG2-mC co-immunoprecipitation, 1.5mg total protein was incubated with either 50µl anti-RFP mAb-agarose (MBL intl. corp., Woburn, MA) or RFP-Trap_M (ChromoTek Inc., Hauppauge, NY) according to manufacturer’s instructions. Agarose beads from the above reactions were washed and denatured in 50µl 1% SDS and 20µl was
loaded on an SDS-PAGE gel for Western Blotting or 2µl of the supernatant was subject to capillary electrophoresis.

**Western Blotting and Capillary Electrophoresis** - SDS denatured proteins were processed via SDS-PAGE and transferred to FL-PVDF membranes (Li-COR, Lincoln, NE) for traditional immunoblotting. For Western blotting primary antibodies used were rabbit anti-NSG2 (1:500; Abcam, Cambridge, MA), mouse anti-beta-actin (1:5000; Clone AC-15; Thermo Fisher Scientific) and mouse anti-GAPDH (1:500; Clone 6C5; Thermo Fisher Scientific). Secondary antibodies used for detection of primary antibodies were goat anti-mouse 800CW (1:15,000; LiCOR) and goat anti-rabbit 680RD (1:15,000; LiCOR) and blots were scanned using the Odyssey infrared imager and acquired on the Image Studio Lite software suite (Version 3.1, LiCOR). Capillary electrophoresis was performed on the fully automated Wes system (ProteinSimple, San Jose, CA) following the manufacturer’s recommendations. Briefly, 0.5µg protein lysate (Input lanes) or 2µl protein (IP lanes) were mixed with 2µl of the 5x Master mix containing SDS and DTT. The samples and protein standard were boiled at 95°C for 5min. The samples were dispensed into microplates containing blocking buffer, primary and secondary antibody and wash buffer in independent wells for sequential processing. The plate was briefly spun and loaded into the instrument for electrophoretic separation of proteins in capillary tubes containing a 12-250kDa separation matrix. The chemiluminescence based electrophoretogram was auto generated and digitally-rendered bands shown in Figure 4 were generated from the chemiluminescent peaks using the Compass software (ProteinSimple). For the Wes, primary antibodies were rabbit anti-NSG2 (1:300), mouse anti-GluA1 (1:100) and mouse anti-GluA2 (1:50). HRP-conjugated secondary anti-mouse and anti-rabbit antibodies were used at the predefined concentrations provided by the manufacturer.

**T7 Endonuclease I assay** – Neuro-2a cells were transfected with either the control or CRISPR NSG2 KO gRNA plasmids and allowed to express the
constructs for 48-60h. The cells were then harvested and a purified GFP+ cells population was obtained by flow cytometry. The genomic DNA from the GFP+ cell population was extracted using a commercially available kit (Zymo Research, Irvine, CA). 100ng of genomic DNA was used as a template for PCR amplification of a fragment surrounding the putative gRNA cleavage site using the following primer pair: Fwd 5’-TCCCCGGACAATGGGAATCA TG-3’ and Rev 5’-GTGGCTGGAAGAATGAAAGGAT-3’. Amplicons were then subjected to a single cycle of denaturation and renaturation to generate heteroduplex molecules containing mismatches which could be recognized and cleaved using the T7 endonuclease I enzyme (New England Biolabs, Ipswich, MA). The products of the reaction were resolved on a 2% agarose gel containing 1x gel red stain (Biotium, Fremont, CA) and imaged on a gel documentation system (Biorad, Hercules, CA). The relative band intensities of the cut fragments to the uncut fragment were used to calculate the gRNA-mediated cleavage efficiency.

**Immunocytochemistry** – Neurons on coverslips were fixed with 4% paraformaldehyde/4% sucrose for 15min, rinsed 3 times for 5min in phosphate-buffered saline (PBS; Sigma), and permeabilized using 0.2% Triton for 10min (except when staining for surface GluA1 and GluA2). Cells were blocked with 10% donkey serum in PBS for 1h, followed by an overnight incubation of primary antibody in 5% donkey serum at 4°C. Primary antibodies consisted of rabbit anti-NSG2 (1:500; Abcam), goat anti-NSG1 (1:400; Everest Biotech, Oxford, UK), mouse anti-GFAP (1:1000; Neuromab, Davis, CA), chicken anti-βIII-tubulin (1:500; Millipore), rabbit anti-Homer1 (1:1000; Synaptic Systems, GmbH, Goettingen, Germany), guinea pig anti-Homer1 (1:200; Synaptic Systems), mouse anti-Synapsin-1 (1:2000; Synaptic Systems), chicken anti-MAP2 (1:5000; Biolegend, San Diego, CA), mouse anti-SMI312 (1:1000; Biolegend), mouse anti-PSD95 (Clone 7E3, 1:100; Thermo Fisher Scientific), and antibodies targeting the amino(N)-terminus of GluA1 and GluA2 (1:100) (see “coimmunoprecipitation” section). Following primary antibody incubation cells were washed thrice with PBS and incubated for 1h with secondary antibody in 5% donkey serum. Conjugated
secondary antibodies used were: DyLight 488, 550 and 647 (1:1000; Thermo Fisher Scientific), donkey anti-guinea pig CF555 and goat anti-chicken CF647 (both at 1:500; Sigma). Cells were washed with PBS and then in some cases treated with DAPI (1:10,000 in PBS; Thermo Fisher Scientific), followed by 3 washes with 1X PBS, and mounted on superfrost slides on Fluoromount-G as an anti-quenching reagent (Southern Biotech, Birmingham, AL). In our hands the GluA1 antibody described above did not yield much success with hPSNs. Therefore, we carried out live staining for surface GluA1 on hPSNs with a custom developed rabbit anti-GluA1 targeting the extracellular epitope of GluA1 (1:200; a kind gift from Dr. Matthew Kennedy, UC Denver). The cells were incubated live with the antibody for 15min before three brief PBS washes and fixation with 4% PFA. The post fixation treatments and secondary antibody incubations were same as described above.

**Confocal Imaging and Analysis** - Confocal z-stacks were acquired on the Zeiss LSM800 airyscan confocal microscope using the 63x/1.40NA Oil objective. Sequential frame acquisition was set to acquire an average of 10 planes per stack at 16bit and a minimum of 1024x1024 resolution. Channel gain settings were optimally adjusted to minimize saturation of punctae and were maintained across experimental groups. Unmodified images were utilized for all analyses and linear scaling was applied on images only for presentation purposes using Zen Black 2.3 ([https://www.zeiss.com/microscopy/us/downloads/zen.html](https://www.zeiss.com/microscopy/us/downloads/zen.html)). Fluorescent signal colocalization on the single planes from a stack and quantification of punctae number was performed using the colocalization plugin for ImageJ ComDet version 0.3.4 ([https://github.com/ekatrukha/ComDet](https://github.com/ekatrukha/ComDet)) as previously described (Esteves da Silva et al., 2015). Punctae integrated fluorescence intensity over area measurements were performed with ImageJ 1.48v ([https://imagej.nih.gov/ij/](https://imagej.nih.gov/ij/)). For each experimental group an average of greater than 200µm dendrite was quantified for 8 to 10 images per experiment and repeated for a total of three biological replicates.
**Electrophysiology** - Whole cell patch-clamp recordings were performed as previously described (Weick et al., 2013) with minor modifications. The extracellular solution was a modified Hanks’ balanced salt solution (HBSS) that contained (in mM): 140 NaCl, 3 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 15 HEPES, and 23 glucose, pH 7.4, 300 mOsm. Recording pipettes with resistances of 3 to 5 MΩ were filled with an intracellular recording solution containing the following (in mM): 121 K-gluconate, 20 KCl, 2 MgCl$_2$, 10 EGTA, 10 HEPES acid, 2 Mg$^{2+}$-ATP, 0.2 Na$^+$-GTP, pH 7.2, 290 mOsm. Pharmacological antagonists picrotoxin (50μM; Tocris, Bristol, UK), TTX (1μM; Tocris), and AP5 (25μM; Sigma) were bath applied in the external solution. Each experiment consisted of 10 to 13 cells per group and experiments were repeated three times on different cultures at the same time points indicated in the results. Neurons were visualized using an Olympus Optical BX51WI microscope (Olympus Corp., Tokyo, Japan) with differential interference contrast optics at 40x. Recordings were obtained using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA), filtered at 4 kHz and sampled at 100 kHz using a Digidata 1322A analog-to-digital converter (Molecular Devices). Whole-cell capacitance was fully compensated but series resistance was not compensated. Access resistance was monitored before and after recordings, and cells with resistances greater than 20 MΩ at either point were discarded from analyses. Miniature excitatory postsynaptic currents (mEPSCs) were measured using a holding potential of -70mV while outward potassium currents were elicited by a voltage-step protocol from -50mV to +50mV, and all recordings were performed at 32°C. Step protocols were used to verify the lack of inward sodium current in all cells used for subsequent analysis. Data were stored on a computer hard disk and postsynaptic currents (PSCs) were analyzed using MiniAnalysis software (Synaptosoft, Fort Lee, NJ) while potassium currents were analyzed using Clampfit v. 10.0 (Molecular devices). The cells utilized for mEPSC recordings for NSG2 knockout (control GFP and CRISPR NSG2 KO) were injected with Lucifer yellow (10mM; Thermo Fisher Scientific) included in the internal solution. After recordings cells were immediately fixed and stained for NSG2 as described.
above (see methods; immunocytochemistry). Only cells showing a lack of NSG2 (indicating NSG2 KO) were included for subsequent analysis.

**Statistical Analysis** - Student's \( t \)-tests were used to determine whether mean differences between groups (e.g., control mCherry vs. NSG2-mC) were significant and were considered significant a priori if \( p < 0.05 \). The number of samples used for statistical analyses (\( n \)) refers to the number of cells assayed per group accumulated from three independent biological replicates for all imaging-based assays and electrophysiology experiments. For analysis of synaptic and NSG2 punctae, \( n \)'s are still reported by the criteria above but at least 1500 total punctae were included across cells/replicates. Data are reported as Mean ± Standard Error of Mean (S.E.M).

**Results:**

**NSG2 is localized to postsynaptic densities of excitatory synapses**

NSG2 is one of the most highly upregulated and abundant mRNAs during early neuronal development across multiple species, including human (Kang et al., 2011; Miller et al., 2014; Barford et al., 2017; Murillo et al., 2017). To determine whether NSG2 *protein* was similarly upregulated, we first used differentiation of cortical neurons derived from human pluripotent stem cells (hPSCs) and assayed for NSG2 using an antibody previously shown to detect the rodent protein (Barford et al., 2017). Robust NSG2 expression was observed in 98% of the \( \beta_{\text{III}} \)-Tubulin\(^+\) neurons analyzed (\( n=151/154 \) neurons from 5 independent experiments) at days 30 and 50 in culture (Figure 2-1, A-B). These timepoints correspond to periods prior to and following the formation of functional synapses in hPSC-derived neurons (hPSNs) (Refer to Figures in Appendix A-D), respectively. NSG2 was not found in neuroepithelial cells at Day30 that were PAX6\(^+\)/MAP2\(^-\) (Figure 2-1, C), suggesting its upregulation following the final mitotic division. Similarly, NSG2 was detected by 4 days *in vitro* (DIV4) in cultured mouse hippocampal neurons (Figure 2A), an early timepoint prior to the creation of functional synapses (Grabrucker et
al., 2009). NSG2 remained robustly expressed in all hippocampal neurons examined (n=391/391; from six independent cultures) at all time points assayed up to DIV30 (Figure 2-2). Similar to previous reports, NSG2 was found robustly aggregated in perinuclear regions consistent with localization to the Golgi apparatus (Saberan-Djoneidi et al., 1995) as well as in distinct punctae throughout βIII-tubulin+ neurites (Figure 2A and yellow box magnified in Figure 2B, blue; arrows; Figure 2-1). At DIV4, NSG2 was absent from GFAP+ astrocytes in regions that did not have overlapping βIII-tubulin+ neurites (Figure 2A and yellow box magnified in Figure 2B, cyan; arrowheads). This was also evident in cultures of human neurons and glia at Day50 (Figure 2-3, A), supporting the neuron-specific nature of NSG2 across species. At DIV14 in mouse neurons, the number of NSG2 punctae were dramatically increased as neuronal arbors became more elaborate (Figure 2C). At this time point, NSG2 punctae were restricted to the somatodendritic compartment as MAP2+ dendrites showed robust staining (Figure 2C and yellow box magnified in Figure 2D, Figure 2-3, B, blue; arrows) whereas SMI-312+ axons were devoid of NSG2 (Figure 2C and yellow box magnified in 1D, Figure 2-3, B, cyan; arrowheads). Together, these data support the neuron-specific nature of NSG2 and show its punctate localization restricted to somatodendritic arbors of human and mouse neurons. In contrast to previous reports suggesting that NSG2 protein is nearly absent in brain following early postnatal development (Saberan-Djoneidi et al., 1995), Barford et al. recently showed that expression of NSG2 was maintained in cortex up to the period of adolescence (P16) but was absent from cerebellum in adults (P60; (Barford et al., 2017)).

Due to sequence homology between NSG family members we determined whether the antibody used for immunostaining was specific for NSG2. Western blot analysis was used to validate the specificity of the NSG2 antibody, which revealed a band of approximately 47kDa in lysates taken from HEK293T cells overexpressing NSG2 (19kDa) linked to the mCherry (28kDa) fluorophore (NSG2-mC; Figure 2E, upper panel, middle lane). However, no bands were detected in
Figure 2 (Also Figure 2-1, 2-2 and 2-3): A subset of NSG2 punctae localize to postsynaptic densities of excitatory synapses. (A) Representative confocal images of primary hippocampal neurons (DIV4) illustrate a perinuclear as well as punctate NSG2 expression pattern (magenta; arrows) in β III-Tubulin+ neurons (blue). No NSG2 expression was observed in GFAP+ astrocytes (cyan; arrowheads). The punctate expression pattern of NSG2 was found in all neurons tested up to DIV30 (Figure 2-1). (B) magnified image of yellow box in the Merge panel of A. (C) Immunofluorescent images illustrate NSG2 (magenta; arrows) punctae exclusively in MAP2+ (blue) dendrites and absence of NSG2 punctae in SMI312+ axons (cyan; arrowheads) at DIV14. (D) magnified image of yellow box in the Merge panel of C. (E) Representative western blot image demonstrating the specificity of the NSG2 antibody in detecting NSG2 (NSG2-mC; top panel, ~48kDa band; middle lane) but not the closely-related protein NSG1 (NSG1-GFP; top panel, ~48kDa). Bottom panel shows the β-actin loading control. (F) Representative western blot image demonstrating the expression profile of the NSG2 from mouse whole brain lysate during development (top panel, ~19kDa band). While GAPDH was used as a loading control (middle panel) it shows variability across development. The blot was also stained with Coomassie (bottom panel) to demonstrate equal protein loading. (G) Confocal images showing endogenous NSG2 (left panels) and overexpressed NSG2-mC (right panels) colocalized with HOMER1+ punctae (arrowheads; HOMER1 panel and Merge). Endogenous NSG2 and NSG2-mC were frequently found adjacent to Synapsin1+ punctae (e.g., leftmost arrowheads). Individual panels for HOMER1+ (white) and SYN1+ (cyan) are shown for clarity. The colocalization profile of NSG2 or NSG2-mC (magenta trace) with HOMER1 (white trace) for the indicated punctae are indicated in the bottom panels. Some NSG2 punctae were not found colocalized to either HOMER1 or SYN1 punctae (arrow). (H) Quantification of colocalization of Homer1 with either endogenous NSG2 or NSG2-mC. Bars represent mean ± S.E.M for n=10 for each group; *p=0.024. Scale bar represents 10μm (A-B), 2μm (B, D and G; left panels) or 4μm (G; right panels).
**Figure 2-1 (Supports Figure 2): NSG2 is robustly expressed in human pluripotent stem cell-derived neurons (hPSNs).** (A) Representative confocal immunofluorescence images of Day30 hPSNs showing the expression and distribution of NSG2 (magenta; arrows) in βIII-Tubulin+ neurons (cyan). DAPI+ nuclei are indicated in white. (B) Representative confocal images of Day50 hPSNs showing the expression and distribution of NSG2 (magenta; arrows) in βIII-Tubulin+ neurons (cyan). DAPI+ nuclei are indicated in white. (C) Representative confocal images of hPSNs illustrate NSG2 (magenta; arrows) expression restricted to MAP2+ (blue) neurons and absent from Pax6+ neural progenitors in Day30 hPSN cultures. Nuclear Pax6+ (cyan; arrowheads) colocalized with DAPI+ nuclei (white; arrowheads). Scale bars represent 10μm.
Figure 2-2 (Supports Figure 2): NSG2 remains expressed in mouse hippocampal neurons at DIV30. Representative confocal immunofluorescence images of primary hippocampal neurons (DIV30) showing the expression and distribution of NSG2 (magenta; arrow) in βIII-Tubulin+ neurons (cyan). DAPI* nuclei are indicated in white. Scale bars represent 5μm.

Figure 2-3 (Supports Figure 2): NSG2 expression in hPSNs is neuron-specific and somatodendritic. (A) Representative confocal images of hPSNs illustrate NSG2 expression is perinuclear (magenta; arrows) and localized to punctae distributed in MAP2* (blue) dendrites; NSG2 punctae are absent in GFAP* astrocytic processes (cyan; arrowheads) at Day50. Far right panel shows magnified image of yellow box in 'Merge'. (B) Representative confocal images of hPSNs illustrate NSG2 punctae (magenta; arrows) exclusively in MAP2* (blue) dendrites but absent from SMI312* axons (cyan; arrowheads) at Day50. Far right panel shows magnified image of yellow box in 'Merge'. DAPI* nuclei are indicated in white. Scale bars represent 10μm.
lysates taken from HEK293T cells overexpressing NSG1 (21kDa) linked to GFP (27kDa; Figure 2E, upper panel, right lane) or untransfected HEK293T cell lysates (Figure 2E, upper panel, left lane). β-actin served as a loading control (Figure 2E, lower panel). Further, to determine the expression profile of endogenous NSG2 protein over time we performed western blot of mouse whole brain lysates. We found that while NSG2 has its greatest expression during the perinatal period and is gradually downregulated thereafter, it continues to be expressed even in adult animals (P60; Figure 2F) consistent with recent studies (Barford et al., 2017).

Due to the punctate nature of NSG2 expression in dendrites, we determined whether NSG2 localized to synapses. In DIV14-16 hippocampal neurons a portion of endogenous NSG2 (endo-NSG2) punctae was independent of both presynaptic Synapsin-1 (SYN1) and post-synaptic HOMER1 (Figure 2G, arrow), whereas strong synaptic localization was observed for a subset of endo-NSG2 punctae (Figure 2G, left panels, arrowheads). Quantification of colocalization showed that approximately 30% of HOMER1 punctae colocalized with NSG2. By comparison, NSG2 punctae were frequently found adjacent to, but not colocalized with SYN1 punctae (Figure 2G, left arrowhead). In fact, some endo-NSG2 punctae were found colocalized with HOMER1 that were not adjacent to SYN1 punctae (Figure 2G, left panels, far right arrowhead). Similar results were found with overexpressed NSG2-mC (Figure 2G, right panels), indicating this construct behaves similarly to the endo-NSG2 protein. Interestingly, the percentage of HOMER1 punctae that colocalized with NSG2-mC was significantly increased compared to that for endo-NSG2 (Figure 2H; endo-NSG2: 29.2% ± 2.5; NSG2-mC: 37.0% ± 2.0; *p = 0.024). This happened despite the fact that the total densities of HOMER1 punctae for control and NSG2-mC were not significantly different (mCherry: 126 ± 7 punctae/100µm; NSG2-mC: 133 ± 10 punctae/100µm; p = 0.6). Thus, the increased colocalization between NSG2-mC and HOMER1 is likely a result of NSG2-mC entering HOMER1+ synapses that are not occupied by endo-NSG2. Taken together these data suggest that NSG2 is present in a subset of excitatory synapses, and is likely restricted to the postsynaptic density rather than presynaptic terminals. Furthermore, overexpressed NSG2-mC was not sufficient
to drive formation of new HOMER1⁺ PSDs, but was able to enter new sites where endo-NSG2 was likely absent.

**NSG2 interacts with AMPAR subunits GluA1 and GluA2**

To determine whether NSG2 colocalizes with functional AMPAR-containing synapses, we used surface labeling with antibodies directed toward the extracellular N-termini of the GluA1 and GluA2 subunits. Figure 3A-B shows representative confocal images of neurons (left panels) as well as individual neurites (right panels) from boxed regions. Results for both GluA1 and GluA2 revealed robust colocalization with individual NSG2 punctae (Figure 3A-B, right panel arrowheads). Pooled data revealed that approximately 40% of NSG2 punctae colocalized with either the GluA1 (Figure 3A, C; NSG2/GluA1: 40.1% ± 1.6) or GluA2 subunits (Figure 3B, D; NSG2/GluA2: 41.0% ± 1.5). Reciprocally, over 45% of GluA1 and GluA2 surface labeled punctae colocalized with NSG2, suggesting that nearly half of all AMPAR-containing postsynaptic densities contained NSG2 protein at DIV15 (Figure 3C-D; GluA1/NSG2: 46.2% ± 1.2; GluA2/NSG2: 48.0% ± 0.9). It is noteworthy that the percent of colocalization between AMPAR subunits and NSG2 was slightly greater than that of HOMER1/NSG2, indicating that NSG2 is somewhat selective for AMPAR-containing PSDs. We additionally verified that overexpressed NSG2-mC colocalized with human GluA1 subunits in hPSNs at Day50. While these data revealed significant colocalization of NSG2 with surface GluA1 in hPSNs (Figure 3-1), the inconsistent maturational state of individual hPSNs (Johnson et al., 2007) makes characterization of NSG2 function more difficult. Thus, all future experiments were conducted on cultured mouse hippocampal neurons.

We next determined whether NSG2 physically associates with AMPAR subunits GluA1 and GluA2. We first performed co-immunoprecipitation (Co-IP) from HEK293T cells co-expressing the fusion proteins NSG2-mC (approx. 45kDa) and either YFP-GluA1 or GFP-GluA2 (approx. 128kDa and 125kDa, respectively). Using an anti-mCherry monoclonal antibody we immunoprecipitated NSG2-mC and probed for YFP-GluA1 and GFP-GluA2 using antibodies specific for each
Figure 3 (Also Figure 3-1): NSG2 colocalizes with AMPAR subunits GluA1 and GluA2. (A-B) Representative confocal images of primary hippocampal neurons at DIV15 showing perinuclear and punctate NSG2 (magenta) in MAP2+ (blue) neurites along with surface expressed GluA1 punctae (A, cyan), and GluA2 punctae (B, cyan). Separated color panels for individual markers from the boxed regions have been magnified for clarity (right panels). Lower right panels in A-B show the colocalization profile for NSG2 (magenta)/GluA1 (cyan, panel A) and NSG2 (magenta)/GluA2 (cyan, panel B) across the indicated punctae (arrowheads). (C-D) Quantification of colocalization for NSG2 with surface GluA1 punctae (6423 NSG2 and 5392 GluA1 puncta from 10 neurons) and NSG2 with surface GluA2 punctae (6874 NSG2 and 5963 GluA2 puncta from 9 neurons) respectively from 3 independent cultures. Scale bars represent 10µm (A, B; left panels) and 2µm (A, B; right panels).

Figure 4A shows data from a capillary immunoelectrophoresis that demonstrates NSG2-mC was able to Co-IP both GluA1 and GluA2 independently (lanes marked IP: mCherry) when the two proteins were co-expressed in HEK293T cells. An IgG control antibody did not IP either NSG2 or AMPAR subunits.
demonstrating the specificity of the reaction (Figure 4A, IP: IgG); the heavy chain of the control mouse IgG antibody is detected by the anti-mouse secondary antibody used to detect GluA1 and 2. To further validate our findings we used the traditional Co-IP/SDS-PAGE to probe for a NSG2-mC and GluA2 interaction (Figure 4-1, middle lane). In this case, 24h incubation of lysates taken from

**Figure 3-1 (Supports Figure 2): NSG2 colocalizes with AMPAR subunits GluA1 in hPSNs.** Representative confocal images of hPSNs at Day50 overexpressing NSG2-mC showing perinuclear and punctate NSG2 (magenta) in MAP2+ (blue) dendrites along with surface expressed GluA1 punctae (cyan). Separate magnified panels for each marker indicated by the boxed region are provided for clarity (right panels). Colocalized NSG2 punctae (magenta) and surface GluA1 (cyan) are indicated by arrows while non-colocalized proteins are indicated by arrowheads. Scale bars represent 10µm (left panel) and 2µm (right panels).

HEK293T cells that expressed either protein separately showed significantly reduced Co-IP, suggesting that their co-expression in a cellular context may be necessary to drive NSG2-GluA2 interactions (Figure 4-1, right lane). We also performed the complementary experiment to verify the interaction of overexpressed proteins. Immunoprecipitation of either GluA1 or GluA2 using antibodies previously described (Schwenk et al., 2014) could Co-IP both overexpressed NSG2 (Figure 4B, arrow) as well as endogenous NSG2 (Figure 4C, arrow). In this case we used whole cell lysates from HEK293T cells co-expressing either GFP-GluA2 or YFP-GluA1 with unlabeled NSG2 (approx. 19kDa) to demonstrate that non-specific interactions between the fluorophores played no role in the interactions. In Figure 4B, the lanes marked IP: GluA1 and IP: GluA2
Figure 4 (Also Figure 4-1): NSG2 interacts with AMPAR subunits GluA1 and GluA2. (A) Co-Immunoprecipitation (Co-IP) of overexpressed, fluorophore-conjugated proteins. Representative digitized western blots from the automated western blot system (see methods) showing that using the anti-RFP antibody to pull down NSG2-mC (IP: RFP, arrow) could Co-IP both YFP-GluA1 and GFP-GluA2 (IP: mCherry, arrowhead). A control IgG antibody did not IP nor detect any target proteins (IP: IgG); the heavy chain of mouse and anti-RFP was detected by the anti-mouse secondary antibody used to detect mouse anti-GluA1/2. NSG2-mC (arrow), YFP-GluA1 and GFP-GluA2 (arrowhead) were detected in the input (Input). Traditional western blotting techniques were performed to confirm that GluA2 co-immunoprecipitates with NSG2-mC (Figure 3-1). Samples were taken from HEK293T cells co-expressing NSG2-mC and either YFP-GluA1 or GFP-GluA2. (B) Immunoprecipitation of overexpressed, unlabeled NSG2 with fluorophore-conjugated proteins with antibodies directed toward endogenous protein epitopes. Anti-GluA1 and Anti-GluA2 antibodies immunoprecipitated YFP-GluA1 and GFP-GluA2 respectively (IP: GluA1 and IP: GluA2, arrowhead). Unlabeled NSG2 (arrow) coexpressed in HEK293T cells was also detected using these same conditions, confirming the Co-ip; control IgG did not IP nor detect any target proteins (IP: IgG). NSG2, YFP-GluA1 and GFP-GluA2 are detected in the input (Input; GluA1; GluA2, arrowhead; NSG2, arrow). Lower bands in NSG2 lanes of control IgG pull down conditions is thought to be non-specific is found in all similar reaction conditions and migrates faster than NSG2. (C) Immunoprecipitation from in vivo samples (whole brain lysate of P0 mouse). Anti-GluA1 and Anti-GluA2 antibodies immunoprecipitated endogenous GluA1 and GluA2 (IP: GluA1 and IP: GluA2, arrowhead) as well as endogenous NSG2 (arrow). All target proteins were detected in the input lanes (Input; GluA1 and GluA2, arrowhead; NSG2, arrow). Control mouse IgG antibody did not pull down GluA1, GluA2, or NSG2 (IP: IgG). As expected, heavy chain and light chains from antibodies used for pull down were detected in most Co-IP conditions (all "IP" lanes).
Figure 4-1 (Supports Figure 4): NSG2 and GluA2 physically interact. Representative western blot image showing Co-IP of NSG2-mC and GluA2 by anti-RFP in lysates from HEK293T cells co-expressing both proteins (Co-IP RFP Trap; NSG2-mC, red bands; GFP-GluA2, green bands). The specificity of antibodies is confirmed by the absence of bands in lysates from untransfected cells (Untransfected) and presence of bands of expected size in the input lane (Input coexpression lysate). Anti-RFP was unable to Co-IP NSG2-mC GluA2 when the two proteins were overexpressed in different HEK293T cultures, and lysates were incubated together and then subject to immunoprecipitation (In vitro incubation).

indicates that both GluA1 and GluA2 were able to Co-IP NSG2 (lower band, arrow). Finally, we asked whether the interaction between NSG2 and GluA1 and GluA2 subunits of AMPARs occurs in vivo. Utilizing whole brain protein lysates from P0 mouse we found that immunoprecipitation of either GluA1 or GluA2 AMPAR subunits pulled down endo-NSG2 (Figure 4C; lanes marked IP: GluA1 and IP: GluA2). Non-specific mouse IgG antibody did not pull down either AMPARs or NSG2 (Figure 4A, 4B, 4C; lanes marked IP: IgG). Heavy and light chains of control IgG are present similar to heavy chain in Figures 4A-B; lowest bands in “IP: IgG” group are likely non-specific as they appear in all groups and are of lower molecular weight compared to NSG2.

NSG2 expression affects AMPAR currents
Since NSG2 colocalizes with and is found in a complex with AMPAR subunits GluA1 and GluA2, we next asked if manipulating NSG2 protein levels
would alter AMPAR surface expression as well as excitatory synaptic transmission. Two CRISPR/Cas9 guide RNAs (gRNAs) were designed using the MIT CRISPR design tool to target the mouse NSG2 locus. A T7 endonuclease cleavage assay determined that both gRNA#1 and gRNA#2 created indels in the NSG2 locus in mouse Neuro-2a neuroblastoma cells. However, the efficiency (Figure 5-3C) of gRNA#2 was significantly greater than gRNA#1, so this one was used for all experiments in primary hippocampal neurons. Transduction of control CRISPR virus and NSG2 gRNA was performed at DIV4 and neurons were analyzed at DIV16-18. Figure 5A (upper panels) shows robust NSG2 punctae (arrowheads) in neurons transduced with control CRISPR lentivirus. In contrast, neurons transduced with the CRISPR gRNA targeting NSG2 resulted in a complete knockout of NSG2 (Figure 5A, lower panels; NSG2 KO; arrow). Note that no effect on NSG2 expression was observed in adjacent, non-transduced neurons (Figure 5A, lower panels, arrowhead). Interestingly, the quantification of NSG2 KO in neurons transduced with NSG2 CRISPR virus revealed that approximately 70% of neurons (n=38/55, from 3 independent cultures) had a complete KO. To confirm specificity of CRISPR targeting, we analyzed NSG1 expression in neurons that expressed NSG2 gRNA and showed complete KO of NSG2 protein. These neurons showed no significant difference in the density of NSG1 punctae compared to neurons expressing control CRISPR constructs (Figure 5-3B; control: 346.3 ± 137.75 punctae/100μm; NSG2 KO: 354.3 ± 134.26 punctae/100μm; p = 0.97). To determine the effect of NSG2 KO on synaptic transmission we performed whole-cell patch clamp recordings on GFP^+ neurons that received the CRISPR-NSG2 KO virus. To eliminate possible contamination from the small proportion of neurons that did not show NSG2 KO, we used Lucifer Yellow injections followed by post-hoc analysis of KO efficiency by immunocytochemistry to correlate NSG2 levels with physiological recording data (Figure 5-1; only cells with undetectable NSG2 signal were used for analysis). Figure 5B shows representative traces of voltage-clamp recordings from neurons expressing control CRISPR (black) or NSG2 KO CRISPR virus (green). Compared to control neurons, NSG2 KO neurons showed a significant decrease in the frequency of miniature excitatory
Figure 5 (Also Figure 5-1, 5-2 and 5-3): Knockout of NSG2 decreases mEPSC frequency. (A) Representative confocal images of primary hippocampal neurons at DIV15 showing robust NSG2 (Magenta) in MAP2+ (blue) neurons transduced with control CRISPR GFP lentivirus (cyan; top panels) whereas neurons transduced with CRISPR KO NSG2 lentivirus (cyan, bottom panels) show the absence of NSG2 (arrow; bottom panels); NSG2 (magenta) is present in an adjacent neuron not transduced with the CRISPR KO NSG2 lentivirus in the same field (arrowhead; bottom panels). (B) Representative traces from whole cell patch clamp recordings from neurons expressing either control CRISPR GFP (upper trace, black) or CRISPR KO NSG2 (bottom trace, green). Averaged mEPSCs from both control (black, n=10) and NSG2 KO (green, n=13) are shown to the right. (C) Pooled data revealed a significant decrease in mEPSC frequency in neurons expressing NSG2 KO compared to cells expressing control CRISPR GFP (**p = 0.001). The amplitude of mEPSCs was not significantly different between groups (p = 0.34). Data in the KO group were derived only from neurons devoid of NSG2 confirmed by post-recording immunostaining of Lucifer yellow injected neurons (Figure 4-1). NSG2 KO did not alter outward potassium I/V relationship (Figure 4-2). (D) Quantification of presynaptic marker Synapsin1+ punctae (control, n=10; NSG2 KO, n=10; p = 0.46) and post synaptic marker PSD95+ punctae (control, n=9; NSG2 KO, n=11; p = 0.18). (E) Representative confocal images illustrate PSD95 immunofluorescence (cyan) in neurons expressing CRISPR KO NSG2 (right panels) or controls CRISPR GFP (left panels). GFP expression from both groups (top panels) is presented in grayscale for clarity. Quantification revealed a significant reduction in PSD95 fluorescence intensity in neurons expressing NSG2 KO (n=11) compared to controls (n=9; *p = 0.029). (F) Pooled data show that the number of surface GluA1+ punctae (left; control, n=10 and NSG2 KO, n=9; p = 0.86) and surface GluA2+ punctae (right; Control, n=10 and NSG2 KO, n=9; p = 0.18) remained unchanged between groups. Bars represent mean ± S.E.M. Scale bars represent 10µm in (A) and 1µm (E).
Figure 5-1 (Supports Figure 5): Lucifer Yellow injections used to verify NSG2 KO in recorded neurons. Representative confocal images of primary hippocampal neurons (DIV15) showing the absence of NSG2 (magenta, arrow) in MAP2+ neurons (blue) transduced with CRISPR KO-NSG2 lentivirus (cyan). Lucifer Yellow was injected into neurons expressing CRISPR KO-NSG2 during whole cell patch clamp experiments (Inset: Luc. Yellow; Figure 4). NSG2 (magenta) was present in MAP2+ neurons not expressing CRISPR KO-NSG2 (arrowhead). Scale bars represent 10μm.

Figure 5-2 (Supports Figure 5): Alterations in NSG2 levels does not affect voltage-gated potassium currents. (A-B) Current-voltage relationship plots illustrating that neither NSG2-KO (A, open circles) nor NSG2-mC overexpression (B, open circles) caused a significant change to transient potassium current elicited by voltage steps compared to controls (A-B, black squares). (C-D) Representative traces from plots in panels A-B in response to voltage steps from -50mV to +50mV (step size, 10mV).
Figure 5-3 (Supports Figure 5): Knockout of NSG2 does not alter NSG1 levels. (A) Representative confocal images of primary hippocampal neurons (DIV15) showing the presence of NSG1 (white, arrowhead) in MAP2* (blue) neurons from GFP* cells transduced with either CRISPR control (cyan; top panels) or CRISPR KO-NSG2 lentivirus (cyan; bottom panels). Middle panels illustrate the presence of NSG2 (magenta, arrow) in MAP2* neurons (blue) transduced with CRISPR control (top panels) and absence of NSG2 (arrow) in MAP2* neurons (blue) transduced with CRISPR KO-NSG2 lentivirus (cyan). Scale bars represent 10 µm. (B) Pooled data show that the density of NSG1 punctae remained unchanged between groups (Bars represent mean ± S.E.M, n=10 per group; p = 0.97). (C) Representative agarose gel showing digested fragments from mouse NSG2 DNA amplicon (arrow) were observed in cells that received either gRNA#1 or gRNA#2 + T7, but not in control or without T7.
postsynaptic currents (mEPSCs), with no change in amplitude (Figure 4B-C; Frequency; control: 12.41 ± 2.31 Hz; NSG2 KO: 4.07 ± 0.68 Hz; **p = 0.001; Amplitude; control: 16.71 ± 4.10 pA; NSG2 KO: 15.84 ± 4.47 pA; p = 0.34). These effects were specific to synaptic currents as voltage-gated potassium currents remained unchanged (Figure 5-2). To probe the mechanism of how NSG2 might affect mEPSCs, we performed immunocytochemical labeling of multiple synaptic proteins. NSG2 KO did not affect the density of presynaptic SYN1+ nor postsynaptic PSD95+ punctae along neurites (Figure 5D; SYN1; control: 93 ± 8 punctae/100μm; NSG2 KO: 79 ± 7 punctae/100μm; p = 0.46; PSD95; control: 97 ± 7 punctae/100μm; NSG2 KO: 80 ± 9 punctae/100μm; p = 0.18). Interestingly, the fluorescence intensity of PSD95+ punctae was significantly decreased in NSG2 KO neurons compared to controls (Figure 5E; control: 3.70 ± 0.45 A.U.; NSG2 KO: 2.57 ± 0.28 A.U.; *p = 0.04). This occurred without a corresponding change in the density of GluA1- and GluA2-containing punctae along neurites (Figure 4F; surface GluA1+; control: 86 ± 8 punctae/100μm; NSG2 KO: 83 ± 12 punctae/100μm; p = 0.86 and surface GluA2+; control: 94 ± 7 punctae/100μm; NSG2 KO: 74 ± 12 punctae/100μm; p = 0.18). Together, these data indicate that the reduction in mEPSC frequency due to loss of NSG2 may be caused by a relatively subtle effect on postsynaptic scaffolding rather than a failure of AMPAR exocytosis.

To test whether NSG2 can augment postsynaptic targeting of AMPARs we used overexpression of NSG2-mC, which shows nearly identical synaptic targeting as endo-NSG2 (Figure 2D). Figure 6A shows representative traces of voltage-clamp recordings from neurons overexpressing mCherry (black) alone or NSG2-mC (red). Pooled data revealed that neurons expressing NSG2-mC showed a significant increase in mEPSC amplitude compared to those expressing mCherry alone (Figure 6A, B; mCherry: 21.61 ± 1.43 pA; NSG2-mC: 30.22 ± 3.07 pA; **p = 0.01). In contrast to NSG2 KO, no significant difference was observed for mEPSC frequency upon NSG2 overexpression (Figure 6B; mCherry: 12.88 ± 2.91 Hz; NSG-mC: 17.66 ± 4.12 Hz; p = 0.35). Immunocytochemical analyses revealed that the intensity of GluA1 and GluA2 staining showed a trend toward an increase but that did not reach significance (Figure 6C, E; Fluorescence Intensity of GluA1;
mCherry: 1.00 ± 0.38 A.U.; NSG2-mC: 1.63 ± 0.54 A.U.; p = 0.36 and GluA2; mCherry: 1.00 ± 0.11 A.U.; NSG2-mC: 1.32 ± 0.21 A.U.; p = 0.19). Interestingly, while the number of surface GluA1+ punctae remained unchanged (Figure 6C; mCherry: 92 ± 10 punctae/100μm; NSG2-mC: 97 ± 8 punctae/100μm; p = 0.58), the number of GluA2+ punctae was significantly increased upon NSG2-mC overexpression (Figure 6E; mCherry: 94 ± 6 punctae/100μm; NSG2-mC: 123 ± 10 punctae/100μm; *p = 0.02). These data suggest that NSG2 is sufficient to drive increased surface expression of AMPARs into existing functional synapses, and potentially to a small proportion of previously silent synapses.

**Figure 6: Overexpression of NSG2 increases mEPSC amplitude.** (A) Representative traces from whole cell patch clamp recordings from neurons expressing either mCherry alone (upper trace, black) or NSG2-mC (bottom trace, red). Averaged mEPSCs from both control (black, n=11) and NSG2-mC (red, n=10) are shown to the right. (B) Pooled data illustrate that neurons expressing NSG2-mC showed a significant increase in mEPSC amplitude compared to controls (**p = 0.01) whereas the frequency of mEPSCs was not different between groups (p = 0.35). (C) Neither GluA1+ punctae number (p = 0.58) nor Relative Fluorescence Intensity (p = 0.36) were significantly different between groups (mCherry vs NSG2-mC). (D) Representative confocal images showing neurons expressing either mCherry alone (left panels) or NSG2-mC (right panels) stained for MAP2 (magenta) and surface GluA2 (cyan). Individual GluA2 and MAP2 expression from both groups (top two panels) are presented in grayscale for clarity. (E) Pooled data revealed the number of GluA2+ punctae was significantly increased in neurons expressing NSG2-mC (n=10) compared to controls (n=10; *p = 0.02) while Relative Fluorescence Intensity remained unchanged (p = 0.19). Bars represent mean ± S.E.M; A.U. = Arbitrary units. Scale bars represent 2 μm.
Conclusion and Discussion

While critical roles in endosomal trafficking and synaptic plasticity have been established for NSG1 and NSG3, no functional role for NSG2 has previously been demonstrated. Here we present evidence that NSG2 is a novel modulator of excitatory synaptic function. We found NSG2 displays a punctate pattern of expression that is restricted from axons but found robustly throughout somatodendritic arbors. Approximately forty percent of all postsynaptic densities that contained surface expressed AMPAR subunits also displayed colocalized NSG2 punctae. Reciprocal immunoprecipitation assays revealed that NSG2 interacts with both GluA1 and GluA2 AMPAR subunits in vitro and in vivo. CRISPR/Cas9-mediated knockout of NSG2 in primary hippocampal neurons resulted in a significantly reduced frequency of AMPAR-mediated mEPSCs, in the absence of a reduction in total surface expressed AMPAR levels. Surprisingly, overexpression of NSG2 caused a significant increase in the amplitude of AMPAR-mediated mEPSCs that was coincident with increased surface expression of the AMPAR subunit GluA2, but did not affect frequency as in the knock out condition. Together, these data suggest NSG2 is a novel player in excitatory postsynaptic function potentially via trafficking of AMPAR and/or associated cargo proteins (see below and Figure 7).

While it remains unclear as to how NSG2 modulates excitatory synaptic function specifically, the preponderance of evidence points to the regulation of AMPAR surface expression via secretory trafficking. This is supported by our current findings in conjunction with a number of findings for other NSG family members. For instance, NSG3 facilitates clathrin-mediated endocytosis of AMPARs during agonist stimulated activity that causes synaptic depression (Xiao et al., 2006b; Davidson et al., 2009b). Overexpression of NSG3 was shown to cause internalization of GluA1 and GluA2, decreasing surface expression levels of AMPARs. Furthermore, chronic upregulation of NSG3 in a transgenic mice was shown to impair executive cognitive function (Xiao et al., 2006b; Kruusmägi et al., 2007; Davidson et al., 2009b; Vazdarjanova et al., 2011), likely via alterations in AMPAR trafficking and altered long-term depression like processes.
Figure 7: Summary of important findings from Chapter 2. (A) NSG2 (cyan) localizes as distinct punctae at or near postsynaptic regions in what are presumed to be vesicular compartments. NSG2 interacts with AMPARs (dark blue/red) and helps deliver them to the surface of the cell either within dendritic spines (upper left), or in perisynaptic regions (lower right). (B) AMPAR surface delivery is revealed via average AMPA mEPSCs under basal conditions (left) which significantly increases upon NSG2 overexpression (right).

(Myers et al., 2006; Kim et al., 2007). In contrast, NSG1 has been shown to regulate the rate of AMPAR receptor recycling within the post-synaptic density (Steiner et al., 2005b; Utvik et al., 2009). While no studies report the functional consequences of full-length NSG1 overexpression, inhibition of NSG1 function via
a dominant negative peptide prevented constitutive recycling of GluA1/2-containing AMPARs following NMDA-mediated internalization (Alberi et al., 2005b; Steiner et al., 2005a). Similarly, antisense-mediated suppression of NSG1 function severely impaired the amplitude of evoked potentials in organotypic hippocampal slices (Alberi et al., 2005a). However, it is not clear whether the observed effect on amplitude was a consequence of a reduction in the number of AMPARs within individual synapses, or a reduction in the number of synapses that contained AMPARs. For example, Levy et al., demonstrate that the effect of changing levels of the MAGUK family of scaffolding proteins, specifically knockdown of PSD95 causes a reduction in AMPAR mEPSC frequency in dissociated hippocampal neurons by increasing the number of silent synapses, however without affecting mEPSC amplitudes (Levy et al., 2015). Thus, one explanation for our observations could be that in NSG2 KO neurons, a subset of synapses that would have contained NSG2 showed reduced or altered AMPAR localization that our methods were not sensitive enough to detect via immunocytochemistry. In this case, these synapses demonstrated increased failure rates or sub-threshold events which resulted in a decrease in mEPSC frequency. However, the absence of an effect on AMPAR surface expression suggests either subtle changes in AMPAR localization or an alternate mechanism. We cannot rule out a role of NSG2 on presynaptic function, although manipulation of NSG1 and NSG3 does not alter presynaptic release (Alberi et al., 2005; Davidson et al., 2009), and NSG2 is solely found in the post-synaptic density (Figures 2 and 3). While future studies are needed to elucidate the mechanistic details of how NSG2 is involved in regulating AMPAR trafficking, our data support a complementary role of NSG2 with that other NSG family members.

One mechanism likely involves secretion of AMPARs via Post-Golgi endosomal vesicles that may contain individual or combinations of NSG family members depending on function. NSG2 was originally proposed to have a role in secretory trafficking for the following reasons: 1) it is a single-pass transmembrane protein enriched in the Golgi apparatus and post-Golgi vesicular compartments, 2) its presence in the P3 subcellular fraction of whole brain ultracentrifugation and, 3)
it contains a secretogranin III-like domain in its carboxy-terminus (Saberan-Djoneidi et al., 1995). Recent evidence from Yap and colleagues confirmed the secretory capacity of NSG2. Blocking endocytosis using a dominant negative Rab5 resulted in accumulation of NSG2 on the surface of hippocampal neurons (Yap et al., 2017). Here, we found that surface GluA2 levels and consequently AMPAR mEPSC amplitude were significantly increased upon NSG2 overexpression, suggestive of a link between its role in the secretory pathway and surface AMPAR levels. It is especially intriguing that NSG2 has an asymmetric synaptic distribution, being present in approximately forty percent of excitatory synapses. As mentioned, data from this and previous studies show that inhibiting either NSG1 or NSG2 significantly, but not completely, inhibits synaptic transmission. Thus, it will be critical to determine whether other NSG family members occupy the same, or divergent populations of synapses, and whether the effects of their inhibition are additive. Furthermore, understanding their temporal or activity-dependence will be critical. For instance, do NSG family members mark specific subsets of synapses, or do NSG proteins transiently visit most or all synapses during development or during particular types of activity? Future investigations to answer these questions should reveal previously unappreciated functional differences between individual excitatory synapses across multiple brain regions.
Chapter 3: NSG2 is stably incorporated into a subset of synapses

Introduction

As mentioned, NSG1 and NSG3 are established regulators of AMPAR trafficking at PSDs through multiple endo-lysosomal compartments, and have recently been shown to localize to the plasma membrane (Yap et al., 2017). Experiments in Chapter 2 discovered that NSG2 is also an AMPAR-binding protein that regulates network excitability under basal conditions. Most interestingly, static analysis of NSG2 localization found it in a minority of synapses (30-40%) that colocalize with surface AMPARs. Assuming that NSG2 exerts the same function across synapses, the greatest effect of NSG2 KO on mEPSC frequency should be proportional to the number of synapses that contain NSG2; (i.e. 30-40%). However, constitutive knockout of NSG2 reduced mEPSCs frequency by greater than 60% (Chapter 2, Figure 5). This apparent discrepancy is difficult to reconcile and requires further investigation.

Previously published research describing NSG family member functions provide some insight into one possible mechanism. Yap and colleagues used cultured hippocampal neurons at DIV 9 and simultaneously imaged NSG1 and NSG2 tagged to different fluorescent proteins. They found that both NSG1 and NSG2 are motile proteins that were trafficked in both antero- and retrograde directions in dendrites (Yap et al., 2017). Interestingly, they were largely trafficked together, and quantified data suggested ~60% colocalization. However, this general finding raises an interesting question about how NSG proteins affect their function at synapses. It is possible that NSG proteins traffic between synapses to influence a larger fraction of synapses than their steady state localization would suggest. Alternatively, NSG2 could be targeted to a minority of synapses that carry a disproportionate amount of synaptic activity.

Support for both hypotheses can be found in previous studies. In support of the trafficking hypothesis, previous research has demonstrated the internalization of AMPARs via EEA+/EEs (Parkinson and Hanley, 2018), recycling of AMPARs during LTP via RAB4/11+ vesicles (Park et al., 2004; Hoogenraad and van der
Sluijs, 2010; Esteves da Silva et al., 2015; Hiester et al., 2018), and degradation via RAB7+ LEs and LAMP1+ lysosomes (Ehlers, 2000). Interestingly, recent studies have even demonstrated LAMP1+ vesicles are trafficked between synapses in an activity-dependent manner (Goo et al., 2017; Padamsey et al., 2017). NSG1 and NSG2 have been found co-localized with several different types of endolysosomal vesicles including EEA1, Rab4/5, Rab7, Rab11, and Lamp1, and NSG2 localized to ~37% of the synaptic fraction (PSD95+) that also contained LAMP1 (Figure 8A-B).

Figure 8: Localization of NSG2 in LAMP1+ synapses. (A) Representative MAP2* (blue, all panels) dendritic segments from a hippocampal neuron (DIV15) stained for endogenous NSG2 (red, top panel), LAMP1 (white, 2nd panel), PSD95 (green, 3rd panel) and Merge (bottom panel). White arrowheads point to NSG2 puncta that colocalize with PSD95 only, yellow arrowheads point to NSG2 puncta that colocalize with Lamp1 only. White arrows point to the localization of NSG2 at PSDs containing LAMP1 (triple colocalization). Scale bars represent 2μm. (B) Bar graphs showing the pooled data of colocalization of NSG2 with either PSD95 or Lamp1 and PSD95/LAMP1 together (N=10 neurons, at least 5 dendrites from each neurons).
In support of the disproportionate activity-hypothesis, Pak and colleagues (2013) discovered that homeostatic scaling in hippocampal cultures is not uniformly distributed across synapses and cells, but primarily driven by changes in the mossy fiber (MF)-CA3 synapses (Lee et al., 2013). Here we explored these two hypotheses using a combination of static and timelapse imaging. We also performed synaptic analysis of NSG2 localization following various types of stimulation to determine if synaptic localization is dependent on activity. Surprisingly, our data seem to support the stable incorporation of NSG2 into a subset of synapses hypothesis, with alterations in activity causing only slight modifications to NSG2 synaptic localization. Lastly, we performed a cursory examination of which synapses may preferentially incorporate NSG2.

**Materials and Methods**

**Culture and viral transduction:** Primary mouse hippocampal cultures and lentiviral preparation and transduction were performed essentially as described previously in Chapter 2. Neurons meant for live cell imaging were cultured on 25mm glass coverslips and maintained in phenol red free Neurobasal media. pFUW-EFS-PSD95FingR-eGFP was a kind gift from Dr. Don Arnold. For live cell imaging experiments, lentiviral transductions with both NSG2-mCherry and PSD95FingR-eGFP were performed on DIV4-5 neurons prior to synaptogenesis with an MOI of 2-3.

**Live cell imaging:** Virally transduced neurons at DIV16-18 were transferred into a custom built coverslip adapter and placed with phenol red free Neurobasal media into a live imaging environment controlled chamber with a humidified heated stage maintained at 35°C and 5% CO₂. Dual colored live imaging was conducted on an inverted Zeiss LSM880 confocal microscope using 63X water objective (Apochromat 1.2 W). Images from the 488 and 561 channels were acquired every minute for 3h in the sequential bidirectional scan-frame mode with light gate settings to reduce any overlapping fluorescence spectra. (X,Y,Z) co-ordinates were established for 2-3 fields of view which were imaged simultaneously during
a single 3h imaging session from a single coverslip. Imaging was performed from 2-3 coverslips over 3-4 independent cultures.

**KCl induction of plasticity:** Neuronal cultures for KCl induction experiments were used at DIV 15-16. Neuronal media was aspirated and replaced with room temperature Tyrode’s buffer containing (in mM) 128 NaCl, 5 KCl, 1 MgCl2, 2 CaCl2, 4.2 NaHCO3, 20 glucose and 25 HEPES buffer; pH=7.4; mOsm=300) for 5min for neurons to acclimate. This was followed by High K+ stimulation by replacing the regular Tyrode’s buffer with one Tyrode’s containing 50mM K+ (in mM): 78 NaCl, 50 KCl, 1 MgCl2, 2 CaCl2, 4.2 NaHCO3, 20 glucose, and 25 HEPES buffer; pH=7.4; mOsm=300) for 3min. For the 3min timepoint cells were immediately fixed and for the 10 and 20min timepoints the cells were washed once with regular Tyrode’s and allowed to incubate in the buffer for 10 or 20min and subsequently fixed.

**Chemical induction of long-term potentiation (cLTP):** Glycine-stimulation dependent cLTP was performed in DIV 15–17 neurons as previously described by Das and colleagues (Das et al., 2013). Briefly, neurons were incubated at 37°C for 5-10 min in Mg²⁺ free HBSS solution containing (in mM): 124 NaCl, 2 CaCl2, 3 KCl, 10 HEPES, 10 glucose (pH=7.4; mOSM=300). Activity induction was coupled with NMDAR saturation with HBSS supplemented with 20 μM bicuculine and 200 μM glycine for 5 min, and finally HBSS supplemented with only 20 μM bicuculine for 15 min before fixation and imaging.

**Immunocytochemistry and Image Analysis:** Primary neurons were fixed at indicated times and/or treatment conditions using 4% PFA/4% sucrose for 10min and subject to immunostaining as previously described in Chapter 2. Image analysis with ImageJ was also as previously described in Chapter 2.
Results
As mentioned, NSG2 is both developmentally regulated (Chapter 2, Figure 2F) as well as actively trafficked within neurites (Yap et al., 2017). However, Yap et al. (2017) used rat neurons at DIV9 when synapses are still developing and little activity is observed (Basarsky et al., 1994). In contrast, most of our studies are performed in mouse cultures at later timepoints when neurons display more synaptic activity (e.g. DIV14-21; (Basarsky et al., 1994; Grabrucker et al., 2009)). Thus, it is critical to determine whether NSG2 was still actively transported at these timepoints in mouse hippocampal neurons. Figure 9A shows a DIV14 hippocampal neuron expressing NSG2-mCherry that underwent timelapse imaging to examine the dynamic movements of NSG2. Figure 9B (boxed region in 9A) illustrates multiple examples of punctae that moved in the anterograde (magenta) or retrograde (green) direction, or remained stable throughout the imaging period (yellow). Binned data illustrate that close to 80% of NSG2-mCherry punctae moved at rates between 0.2 and 0.6µm/sec, consistent with Kinesin and Dynein-mediated active transport and NSG3 (Ma and Chisholm, 2002; Shi et al., 2017). Some punctae were observed moving at rates as high as 4µm/sec, while a minority of punctae remained stable for the 5 minute imaging session (Figure 9C). Thus, we conclude that at days when synaptic activity is present in hippocampal cultures, the vast majority of NSG2 is still actively transported in neuronal dendrites. To determine whether NSG2 is trafficked between synapses or is relatively stable at a specific subset, we performed extended timelapse imaging of NSG2-mCh and PSD95-FingR-eGFP, which marks relatively mature synapses (Gross et al., 2013). We initially imaged neurons over the course of 3 hours to generate a gross measure of stability/lability. We noticed that many PSD95-FingR punctae were unstable during the 3hr period, with 38.3% (n=75) lost while 30.1% (n=59) were gained throughout the timecourse.
**Figure 9. NSG2-mCherry is actively transported in DIV14 hippocampal neurons.** (A) Merged Differential Interference Contrast (DIC) and Fluorescent images of NSG2-mCherry (red) at the beginning (left panel) and end (right panel) of a 5 minute time series. (B) Kymograph of boxed region in A showing NSG2-mCherry puncta (red) along neurites at timepoint 0 (top) and 300 seconds (bottom). Kymograph illustrates dynamic, bidirectional transport of some NSG2-mCherry punctae as well as a proportion of stationary punctae. (C) Pooled data show that most NSG2-mcherry punctae were actively transported at rates >0.3um/sec, while a smaller proportion of punctae remained stable.
Figure 10. Timelapse imaging reveals synaptic targeting of NSG2 into a subset of hippocampal synapses. (A) Fluorescent images of PSD95-FinGR (green) and NSG2-mCherry (red) at the beginning (upper panels) and end (lower panels) of a 3hr time series. Arrows indicate stable, co-localized punctae. Arrowheads indicate stable PSD95 punctae alone. (B) Pie chart illustrating the proportion of stable and transient interactions.

However, a significant proportion of PSD95-FingR punctae remained stable (n=62, 31.6%), and we quantified those that contained NSG2-mCherry punctae throughout the imaging session. Figure 10A shows images at the outset (T=0) and at the end of the imaging session (T=3hr) illustrating that many PSD95-FingR* synapses were observed that both co-localized with NSG2-mCherry (Figure. 10A, arrowheads), and that were devoid of NSG2-mCherry (Figure. 10A, arrows). Pooled data show that the vast majority of the total PSD95-FingR* synapses that persisted for the imaging period, 63.9% never showed NSG2-mCherry colocalization (orange pie; n=291 punctae across 4 cells) while 33.0% showed stable incorporation of NSG2-mCherry (grey pie). In contrast, a small minority of synapses gained (2.1%, blue pie) or lost NSG2-mCherry (1%, yellow pie).
However, several of the punctae in these last two categories appeared to be transient interactions (n=4/9) lasting only a few frames. It is unknown whether they represent functional interactions (see discussion).

We next asked whether multiple types of plasticity-inducing stimuli could direct endogenous NSG2 to AMPAR-containing PSDs, similar to previous studies of endolysosomal vesicles (Padamsey et al., 2017). First we used the addition of 50mM KCl for 3 minutes to promote neuronal depolarization, a protocol that triggers robust synaptic activity, and downstream activation of multiple kinase cascades and CREB activation (Weick et al., 2003; Molnár, 2011). Figure 11A shows representative images of dendrites from two conditions, unstimulated controls and cells that received a 3 minute 50mM KCl solution followed by 20 min incubation period in unstimulated conditions (see methods). Pooled analysis across multiple conditions demonstrates that 3 minute KCl treatment was sufficient to increase both the number of surface GluA1 punctae and the number of NSG2 punctae in MAP2+ neuronal dendrites (Figure 11B). Punctae density for both GluA1 and NSG2 reached significance at the 10min and 20min timepoints following stimulation, while cells that were fixed immediately following 3min KCl treatment were not statistically different from controls (Figure 11B). It is intriguing that the number of NSG2 punctae were significantly increased by this stimulation as this was not predicted based on previous studies of endolysosomal vesicle trafficking studies.

Second, we examined whether this stimulation paradigm increased the number of NSG2 punctae at synapses. To do so we quantified the percent of surface GluA1 punctae that co-localized with endogenous NSG2 under the same stimulation conditions. Here we noticed a trend toward an increase co-localization, with mean increases of 7.8% and 10.2% in the 10min and 20min incubation groups, respectively (Figure 11C). However, these did not reach statistical significance of p=0.05, likely due to increased variability upon stimulation. Furthermore, co-localization in control conditions was somewhat lower than previously determined, possibly contributing to the appearance of increased synaptic localization of NSG2 upon KCl stimulation. However, it is critical to note
that this apparent increase occurs simultaneously with an increase in surface AMPAR staining, indicating that indeed, at least some new surface GluA1 punctae must incorporate NSG2. Otherwise, NSG2 co-localization levels would be expected to decrease when the numbers of sGluA1 punctae increase if NSG2 levels remained unchanged.

Figure 11: KCl promotes AMPAR surface expression and modestly increased synaptic NSG2 localization. (A) Fluorescent images of NSG2 (red, upper panels), surface GluA1 (green middle panels), and merge with MAP2 (blue, lower panels) in unstimulated conditions (left) and following 3 minute 50mM KCl treatment followed by a 20 minute unstimulated period (right). Arrowheads indicate co-localized punctae while arrows indicated sGluA1 punctae. (B) Pooled data illustrate the number of punctae/um of dendrite under multiple conditions (see methods). (C) Pooled data illustrate the percent of co-localized NSG2 punctae with surface GluA1 punctae under the same stimulation conditions in B.
Next we used chemical method to induce long-term potentiation (cLTP) in cultured neurons (Das et al., 2013). To induce cLTP we treated cells with 200µM Glycine to promote NMDA receptor activation for 5 minutes, which has previously been shown to increase both AMPAR surface expression and increased mEPSC amplitude and frequency (Lu et al., 2001). Figure 12 shows representative confocal images of dendrites stained for both NSG2 and surface GluA1 under control (left) and cLTP-inducing conditions (right). Summarized data illustrates that 5min glycine...
treatment significantly increased both the number (left graph) and intensity (middle graph) of sGluA1 punctae, with magnitudes of approximately two-fold. Interestingly, similar to experiments using KCl treatment, we observed a trend toward increased co-localization of NSG2 and sGluA1 punctae, but again this failed to reach significance. In fact, the greatest proportion of NSG2/GluA1 co-localization observed was similar to that in our published work (~40%; (Chander et al., 2019)). However, also similar to the conclusions from the KCl experiments, the slight increase in co-localization accompanied a highly significant increase in surface AMPARs, suggesting that new synapses did incorporate new NSG2 proteins, despite the fact that the overall proportion remained similar to control conditions. Future experiments are necessary to determine how and whether NSG2 is trafficked to existing synapses or new sGluA1 punctae following these stimulation paradigms.

One major question that arises from these findings is what specific type of synapses preferentially incorporate NSG2. Aside from our work on NSG2 (Chander et al., 2019), the synaptic localization of NSG proteins is unknown. However, based on functional properties and observations from immunostaining (see below) we can make some hypotheses with regard to their spatial segregation. While the majority of hippocampal afferents terminate on PSDs that display similar types of NMDA-dependent forms of postsynaptic plasticity, a major exception are the Mossy fiber (MF) synapses of the dentate granule cells onto Hilar and CA3 pyramidal cells. The postsynaptic structure on the CA3 dendrite consists of elaborate multiheaded spines known as a thorny excrecence (TE) (Amaral and Dent, 1981). Because of its size and position near the soma of CA3 neurons, activation of single mossy fiber synapses can cause spiking in CA3 neurons. Therefore, these are referred to as “detonator” synapses (Urban et al., 2001). This pathway displays pronounced short-term facilitation and a presynaptic form of LTP that is independent of NMDAR activation (Zalutsky and Nicoll, 1990; Nicoll and Schmitz, 2005).
Figure 13: NSG2 is present in large PSDs. (A) Published data illustrating NSG2 colocalized with large surface GluA1 aggregates on proximal dendrites (Chander et al., 2019). (B) AF405-filled neuron (blue) stained for NSG2 (red) shows significant expression in large, multi-headed PSDs (arrowheads) but is absent from some individual spines (arrows). (C) Proof of concept for in vivo injections of viral tdTomato expression (red) and labeling of NSG2 (green, arrowheads) in a hippocampal neuron at P15. Scale bar 10 μm.
We hypothesize that NSG2 plays a predominant role in promoting robust postsynaptic AMPAR surface expression under basal conditions (see Chapter 4), but not plasticity-inducing ones. Interestingly, multi-headed spines are still formed by cultured CA3 neurons and retain their input-specificity from DG neurons (Williams et al., 2011). Thus, as an initial examination of whether NSG2 is present in MF-CA3 neurons we looked for large, multi-headed spines near large cell bodies in mixed hippocampal cultures (Figure 13B). In support of this we observed a preponderance of synaptic NSG2 localization occurred at relatively large PSDs on proximal dendrites (Chapter 2, Figure 3; (Chander et al., 2019) and Figure 13A). In neurons that displayed complex, multi-head spines on proximal dendrites we found that many of these contained NSG2 (Figure 13B, arrowhead), suggesting colocalization with Mossy Fiber synapses. While a previous study showed that MF-CA3 synapses could be identified by colocalization of synaptoporin and/or CDH9 with excitatory synaptic markers, we were unable to replicate these findings. Thus, future studies are necessary to determine whether NSG2 is selectively incorporated into TEs. Finally, we wanted to know if NSG2 punctae get incorporated into firstly, dendritic spines and secondly, into TE like synapses in vivo. For this, we expressed tdTomato in the hippocampal neuron via in vivo lentiviral injection into P0 newborn mouse brain. Mice brains were harvested two week after injection and fixed. 30µm vibratome sectioned brains were stained for endogenous NSG2, followed by imaging. NSG2 punctae (Figure 13C, green, arrowhead) were clearly seen in the tdTomato filled dendritic spines (Figure 13C, magnification of the boxed region). Future studies will aim to decipher if the NSG2 punctae also localize in CA3 mossy fiber (TE) synapses.

**Conclusion and Discussion:**

Here we found that, in contrast to our prediction that NSG2 is trafficked between synapses to affect neuronal excitability, it appears to be stably incorporated into a subset of excitatory PSDs. Furthermore, we showed that alterations in neuronal activity (such as cLTP) have little effect on the distribution of NSG2 at synapses. This is in contrast to many endolysosomal vesicles, which
NSG2 is thought to be present within. Thus, it remains unknown whether NSG2$^+$ vesicles represent a unique subtype of endolysosomal vesicles, or that only a subset of endolysosomal vesicles are trafficked to particular synapses. In this latter case we would predict that NSG2 would prohibit various types of endolysosomes from being trafficked in an activity-dependent manner, but that Rab4/5/Rab7/Lamp1 that lack NSG2 are preferentially able to be recruited to synapses during heightened periods of synaptic activity. Future studies are therefore required to distinguish between these hypotheses (see Discussion in Chapter 5).
Chapter 4: Does NSG2 confer unique functional properties to synapses?

Introduction

A number of factors determine the number of AMPARs and consequently the synaptic strength of a glutamatergic synapses. A number of these factors were detailed previously in Chapter 1 as well as discussed in many reviews (Malinow and Malenka, 2002; Huganir and Nicoll, 2013). It is widely recognized that various forms of synaptic plasticity, either Hebbian (LTP/LTD) or homeostatic scaling, converge on these features of AMPARs (Turrigiano, 2008; Huganir and Nicoll, 2013). The number of AMPARs available to mediate synaptic transmission is regulated by two major processes: 1) lateral diffusion and trapping (Borgdorff and Choquet, 2002; Bats et al., 2007b; Ehlers et al., 2007), and 2) endo- and exocytosis-mediated exchange between the plasma membrane and intracellular stores (Petrini et al., 2009). An additional level of complexity is the alignment of AMPARs with presynaptic active zones into nanocolumns (Tang et al., 2016b), which is critically important for evoked activation of post-synaptic potentials (Raghavachari and Lisman, 2004; Freche et al., 2011; Savtchenko and Rusakov, 2014). While lateral diffusion and trapping are largely responsible for the induction of Hebbian plasticity, membrane trafficking is essential for maintenance (Lledo et al., 1998; Lu et al., 2001; Kopec et al., 2007; Yang et al., 2008). Thus, while mechanistically distinct, these two processes work in concert to permit persistent alterations in synaptic efficacy via AMPAR surface expression.

Previous research has found that NSG1 is critically important for the dynamic shuttling of AMPARs between intracellular compartments and the plasma membrane during synaptic plasticity. For instance, expression of an anti-sense construct or a dominant negative NSG1 peptide reduced AMPAR recycling to the plasma membrane following NMDA stimulation (Alberi et al., 2005; Steiner et al., 2005). This interference also blocked stable induction of LTP (Alberi et al., 2005). Furthermore, NSG1 is expressed in recycling endosomes, and binds to AMPARs and the scaffolding protein GRIP1. Interestingly, this binding appears to be activity-dependent, where NMDA+TTX treatment enhanced GRIP1 binding, but NMDA
alone significantly reduced binding (Steiner et al., 2005). In contrast, NSG2 overexpression significantly increases the amplitude of mEPSCs under basal conditions (Chander et al., 2019). Critically however, while NSG2-mC overexpression caused increases in mEPSC amplitude, it did not significantly alter surface GluA1 intensity or density when a random sample of dendritic arbors were examined (Chander et al., 2019).

Because NSG2 appears to be stably incorporated into a subset (~30%) of synapses, we hypothesized that NSG2 may be critical for promoting AMPAR surface expression at a minority of synapses that carry a disproportionate level of activity in cultured neurons. In support of this idea, one study demonstrated that homeostatic scaling in hippocampal cultures is not uniformly distributed across synapses and cells, but primarily driven by changes in specific types of hippocampal synapses (Lee et al., 2013). In this scenario, our previous immunocytochemical analyses were not sensitive enough to detect changes in surface AMPARs in NSG2-mC overexpressing neurons as we did not distinguish between synapses that contained NSG2 and those that did not. If this is true, targeted analyses of “NSG2 synapses” should reveal significant differences in AMPAR surface expression. Furthermore, if NSG2-containing synapses do show differences in surface AMPARs, alterations in NSG2 expression would be expected to alter a neuron’s ability to undergo various forms of synaptic plasticity. Lastly, if the unique expression pattern of NSG2 extends to other NSG family members (e.g. NSG1), this would have significant implications for interpreting previous data regarding their involvement in synaptic plasticity.

Materials and Methods

Neuronal cultures, Lentivirus Transduction, Immunocytochemistry and Electrophysiology: Primary hippocampal neurons were cultured from P0-P1 mouse pups as previously described in Chapters 2 and 3. NSG2-mC and mCherry lentiviral transduction of neurons were carried out as described previously in Chapter 2. Immunocytochemistry and surface GluA stainings were carried out as
previously described in Chapter 2. Electrophysiological recordings of AMPA mEPSC were carried out as previously described in Chapter 2.

**Induction of Homeostatic plasticity:** TTX based induction of homeostatic plasticity was carried out essentially as described by Lee and colleagues (Lee et al., 2013). Briefly, hippocampal neurons at DIV13-14 were treated with 1µM TTX for 24h prior to recording and AMPA mEPSC were recorded on DIV14-15.

**Image Analysis:** Puncta size and Fluorescence intensity measures for surface GluA1 and GluA2 were determined using Synpanal, a semi-automated software for detection of neuronal punctae as previously described by Danielson and Lee (Danielson and Lee, 2014).

**Results**

To determine whether NSG2-containing synapses display unique functional properties we first examined the relative amount of AMPAR surface expression at NSG2-containing synapses compared to those that lacked NSG2. We assessed both the intensity and area of surface GluA2 punctae in DIV14 hippocampal neurons. In cells overexpressing NSG2-mC we found that both fluorescence intensity and surface area of GluA2 punctae were significantly greater in synapses that contained NSG2-mC (Figure 14A-B; red, arrowhead) compared to those lacking NSG2-mC (Figure 14A-B; arrow, Fluorescent Intensity, 388.06±28.83 (AUs) vs 660.16±45.62 (AUs); Puncta area, 0.179±0.007 (µm) vs 0.295±0.011 (µm); N=6 cells/group, 6-10 dendrite segments/group; 1290 NSG2 punctae; 2400 GluA2 punctae). However, we previously showed that overexpression of NSG2-mC caused significant increases in mEPSC amplitude (Chander et al., 2019); Chapter 2, Figure 6), which may be caused by increased levels of surface AMPARs. Thus, we also tested whether surface AMPARs were also disproportionally present at synapses that contained endogenous NSG2. Figure 14C-D shows that synapses that contained endogenous NSG2 (magenta, arrowheads) displayed significantly greater area and fluorescence intensity of
Figure 14: NSG2-containing synapses demonstrate elevated levels of surface AMPARs. (A) Representative confocal images of DIV14 hippocampal neurons expressing NSG2-mCherry (red) and stained for surface GluA2 (green); MAP2 is indicated in blue. (B) Pooled data showed significantly increased surface area and fluorescence intensity of surface GluA2 in synapses that colocalized with NSG2-mCherry (A, arrowhead) compared to regions that did not colocalize with NSG2-mCherry (A, arrow). (C) Representative confocal images of DIV14 hippocampal neurons stained for surface GluA2 (cyan) and endogenous NSG2 (magenta). Dashed lines indicate the dendrite boundary using Alexa-fluor405 cell fill (omitted for clarity). (D) Pooled data showed significantly increased surface area and fluorescence intensity of surface GluA2 in synapses that colocalized with NSG2 (C, arrowheads) compared to NSG2-lacking synapses (C, arrows). For A-D, N=6 cells/group, 6-10 dendrite segments/group. ***p<0.001.

GluA2 punctae (cyan) compared to those that did not (Figure 14C-D; arrow, Fluorescence Intensity, 277.75±77.9 (AUs) vs 465.56±88.09 (AUs); Puncta Area, 0.112±0.009 (µm) vs 0.185±0.024 (µm); N=6 cells/group, 6-10 dendrite segments/group; 2157 NSG2 punctae; 1753 GluA2 punctae). These results suggest that NSG2 promotes increased surface AMPARs at a subset of synapses rather than affecting all synapses equally, in line with timelapse imaging data showing its relatively stable incorporation into a minority of PSDs (Chapter 3,
Figure 10 and 13). Furthermore, these data support the idea that NSG2 may be incorporated into larger synapses, similar to MF-CA3 synapses (Chapter 3) that display unique forms of plasticity. To test this we compared mEPSC amplitude in neurons expressing mCherry with neurons expressing NSG2-mC and that were either left untreated or were treated with TTX (1µM) for 24 hours to induce upscaling (Lee et al., 2013). Interestingly, both TTX-treated cells and cells overexpressing NSG2-mC displayed a significant increase in mEPSC amplitude (Figure 15B, second, third bars, respectively). However, cells expressing NSG2-mC did not show TTX-induced increases in mEPSC amplitude (Figure 15B, fourth bar), suggesting that NSG2 overexpression prevented TTX-mediated scaling. Together these data support the idea that NSG2 promotes alterations in PSD function.

![Figure 15: NSG2 modulates homeostatic synaptic plasticity.](image)

(A) Representative traces from whole cell patch clamp recordings of DIV15 hippocampal neurons expressing either control mCherry (upper trace) or NSG2-mCh (lower trace) either untreated or treated with 1µM TTX for 24hr. (B) Pooled data revealed a significant increase in the mean mEPSC amplitude upon TTX treatment in the mCherry alone group. However, TTX treatment in the NSG2-mCh expressing group did not promote basal increases in mEPSC amplitude (N=10-12/cells/group).

To determine whether NSG1 and NSG2 occupy overlapping or distinct PSDs we quantified the percent that co-localized with PSD95 in DIV21 hippocampal neurons that were filled with Alexa-Fluor 405 (Figure 16A, AF405 signal omitted for clarity). Figure 16A, shows PSD95 punctae that co-localized with
both proteins (arrowhead) as well as PSD95 that primarily colocalized with NSG2 (arrows). Pooled data demonstrate that approximately 17% of PSD95 punctae colocalized with NSG1, ~33% colocalized with NSG2, and both proteins colocalized with PSD95 in only ~5% of synapses (Figure 16B; n=9 cells, 5 dendrites/cell, and greater than 2300 punctae). Thus, the majority of synaptic NSG1 and NSG2 proteins occupy non-overlapping PSD95+ domains. In addition, to determine whether NSG1 could promote increased synaptic efficacy similar to NSG2 under basal conditions, we overexpressed NSG1-mCh and assessed mEPSC amplitude. Figure 16C and D, shows that mEPSC was unchanged compared to cells expressing mCherry alone (Figure 16C; N=10-12 cells/group), suggesting that NSG1 does not affect basal synaptic efficacy in the same manner as NSG2 (Chander et al., 2019).

Figure 16: NSG1 and NSG2 show largely non-overlapping and discrete synaptic localization.
(A) Representative confocal images of DIV18 hippocampal neurons stained for NSG1 (green), NSG2 (white), and PSD95 (red) in Alexa Fluor 405 filled cells (indicated by dashed line). Arrows indicate NSG2/PSD95 colocalization and arrowhead indicates NSG1/NSG2/PSD95 colocalization. (B) Pooled data show the proportion of PSD95 punctae that colocalize with individual or both proteins (N=9 cells, 5 dendrites/cell, and greater than 2300 punctae). (C) Representative traces from whole cell patch clamp recordings of hippocampal neurons expressing either control mCherry (upper trace) or NSG1-mCh (lower trace). Pooled data indicates that NSG1 does not promote basal increases in mEPSC amplitude (n=10-12/cells/group).
To begin to determine the molecular mechanisms for synapse-specific properties we reasoned that particular domains were required for targeting NSG2 to particular synapses. We performed initial mutagenesis on NSG2 to delete the majority of the N-terminus (ΔN-NSG2-mC; amino acids (AA) 2-60 deleted), the transmembrane domain (NSG2-ΔTM-mC; AA 70-91 deleted), or the C-terminus (mC-NSG2-ΔC; AA 101-171 deleted) (Figure 17A-B).

**Figure 17: Mutant NSG2 proteins are deficient in their synaptic localization.** (A) Diagram of conserved (blue) and unique (green) domains, including predicted serine (S) and Threonine (T) phosphorylation sites of NSG1/2. (B) Western blot of full length and mutant NSG2 proteins. (C) Representative confocal images of DIV15 hippocampal neurons expressing NSG2-mC (red, top), NSG2-ΔC (red, middle) or ΔN-NSG2 (red, bottom). Arrowheads indicate colocalization of NSG2 puncta with synaptic marker Homer1 (green). (D) Pooled data illustrate the percent of colocalized NSG2 or NSG2 mutants with Homer1.
Figure 17B shows western blot analysis, where all mutants demonstrate appropriate changes in migration due to reduced molecular weight. Both the ΔN and ΔC mutants expressed in a punctate pattern and were actively trafficked in cells (not shown), suggesting they grossly mimic the full-length protein. However, both mutants displayed significantly lower co-localization with Homer1 compared with full-length NSG2-mC (Figure 17C and D; NSG2-mC: 35.8±4.1%; ΔN-NSG2-mC: 8.5±1.8%; mCh-NSG2-ΔC: 5.8±1.1%, n=8 cells, 41 dendritic segments, >2174 punctae; p<0.05 for each mutant compared to full length NSG2-mC).

Figure 18: NSG2 mutants fail to increase synaptic efficacy. (A) Representative traces from whole cell patch clamp recordings of DIV15 hippocampal neurons expressing either control mCherry, NSG2-mC or NSG2 mutants (as indicated). NSG2-mC expression significantly increased mEPSC amplitude (A) and trended toward an increase in frequency (B). However, mEPSC amplitude and frequency were not different from controls expressing mCherry alone in neurons expressing the ΔN or ΔC mutants (N=11-12 cells/group, **p<0.02).

Interestingly, neither mutant was able to cause increases in mEPSC amplitude like full-length NSG2-mC (Figure 18A), whereas none caused changes to mEPSC frequency (Figure 18B; n=11-12 cells/group).
Conclusion and Discussion

Here we sought to determine if NSG2 localizes to a subset of synapses and alter their functional properties. We found that synapses that contained NSG2 had, on average, larger sGluA2 clusters. This was true for both endogenous NSG2-containing synapses and upon overexpression of NSG2-mCherry (Figure 14 and Summary Figure 19). An interesting question arising from this finding is whether NSG2 localization at these synapses causes the enlargement of these PSDs resulting in larger AMPAR clusters, or whether NSG2 is selectively targeted to these synapses. Future studies are needed to distinguish between the cause-effect relationship between the presence of NSG2 and heightened synaptic efficacy.

Figure 19: Schematic showing that NSG2 localization at synapses correlates with larger AMPAR clusters under basal conditions.

The fact that NSG2 localized to, and functionally affected, a subset of PSDs led us to question whether this was true for other NSG family members. Previously, it was shown by Yap and colleagues, that NSG1 and NSG2 show a ~60% overlap in rat hippocampal neurons at DIV9. While we were able to recapitulate their findings in mouse hippocampal neurons between DIV9-11 (data not shown),
however these data say nothing about their localization at PSDs. NSG1/2 displayed differential localization at PSDs at DIV21, further emphasizing the involvement of differential factors in specific synaptic recruitment of these proteins. Additionally, it also suggests that these proteins may have either redundant functions or work in conjunction early during development and become functionally decoupled later in development. It was surprising to us however, that NSG1 overexpressing neuron did not cause differences in AMPA mEPSC properties compared to control (Figure 16 and Summary Figure 20), especially because NSG1 overexpression has previously been shown to modulate AMPAR recycling (Steiner et al., 2002).

We were curious about how this differential distribution of NSG2 at synapses would play out when we artificially induced homeostatic plasticity shown to modulate a subset of large synapses proximal to the soma. In Chapter 3, we did find some evidence that NSG2 may localize to large multiheaded spines proximal to the soma of dye-filled neurons. Intriguingly, we found that NSG2 overexpressing neurons were not able to upscale AMPA mEPSC amplitude to the same extent as TTX treated control (Figure 15 and Summary Figure 21). Therefore, while TTX treatment of neurons predictably increased AMPA mEPSC amplitude as did NSG2-mC overexpression, TTX-dependent potentiation of AMPA current was limited in NSG2 overexpressing cells. It is possible that NSG2 potentiates synapses to a certain setpoint but does not allow further potentiation of the complement of AMPARs within the PSD. It is clear that synapses could still undergo greater levels of potentiation as TTX-treated cells show greater mean amplitudes than those expressing NSG2. Thus, the presence of NSG2 essentially clamps synapses at a high, but not maximal level of efficacy, and may abrogate the ability of those synapses to undergo further upscaling. Future studies should explore whether this effect of NSG2 affects other types of plasticity such as cLTP, or homeostatic down-scaling.
Figure 20: Schematic showing that overexpression of NSG1 does not lead to higher amplitudes of AMPA mEPSCs unlike NSG2 indicative of a functional difference between NSG1 and NSG2 containing synapses.
Figure 21: Schematic showing that regular synapses undergo TTX dependent homeostatic scaling up which is not able to undergo any further potentiation under NSG2 overexpression conditions.
Chapter 5: Conclusions and Discussion

Here we report the first functional characterization of a novel protein, NSG2. NSG2 mRNA and protein was first discovered in rats (Saberan-Djoneidi et al., 1995). NSG2 mRNA expression data is also available for zebrafish (Muthusamy et al., 2015). Muthusamy and colleagues, previously carried out bioinformatics based analysis of the NSG2 gene and suggested its emergence and evolutionary conservation in vertebrates (Muthusamy et al., 2009). Interestingly, NSG2 expression has previously also been shown to be misregulated in certain types of neurodegeneration (Yamanaka et al., 2014) and developmental disorders like Rett Syndrome (Chahrour et al., 2008). We identified and verified NSG2 expression in mouse and human neurons and showed its localization at a subset of synapses (Chander et al., 2019).

We initially hypothesized that NSG2, the second member of the NSG family (NSG1-3) of proteins would be critical for AMPA receptor exocytosis and surface expression. This hypothesis stemmed from the simple notion that regulated AMPAR trafficking requires endocytosis, recycling, and exocytosis. The first two processes have been shown to be the domains of NSG3 and NSG1, respectively. Thus, whether NSG family members were involved with AMPAR exocytosis remained unknown. While much of our data support the idea that NSG2 does promote AMPAR surface expression, it remains somewhat unclear as to the complex interplay between NSG family members and AMPAR trafficking. This is largely due to the fact that our data revealed unexpected localization and functional properties of both NSG2 and NSG1, which may call into question previous research.

To study NSG2, and the process of synaptogenesis in general, we initially adopted the human pluripotent stem cell-derived neurons as a model system. One of the primary motivation and advantage of using this model was, it represented truly de novo synaptogenesis as opposed to a regenerative synaptogenic system as in primary rodent neural cultures. Additionally, this system offers several advantages – self renewing pluripotent stem cells allows for a potentially unlimited capacity for expansion, ability to mimic and characterize some of the earliest
events during functional neural development, closely follows the timeline of events occurring during embryonic development. When cultured as a monolayer stem cells display unique morphological landmarks during differentiation to neurons. These include downregulation of pluripotency associated marker Oct4 and gain of expression of the neuroepithelial marker Pax6 during the first week of differentiation. This is followed by neural rosette like structures which resemble the closure of the neural tube during embryonic development. The molecular resemblance of this structure to a neural tube is authenticated by a positive staining of the rosette lumen for N-cadherin. βIII-tubulin positive neurons are derived at 3-4 weeks but are electrically inactive and stain poorly with pre-synaptic markers such as Synapsin I. However, in the following week to ten days there is a dramatic increase in the number of Synapsin I positive puncta accompanied by gain of electrical activity. Incidentally, this also corresponds to the time during embryonic development where we may witness twitching in the digits.

We rationalized that investigating the molecular changes occurring during this critical window in neuronal differentiation where neurons go from being immature to action potential firing functional neurons could reveal novel players involved in functional neural development. Microarray gene expression analysis revealed several novel candidate genes. NSG2 however stood out for several reasons, but mainly because it was one among the top ten highly expressed genes among all differentially expressed gene and the highest among novel functionally uncharacterized genes. However, one limitation, which also suggests a possible future direction for this study is that, we only profiled NSG2 in one human cell line. While it would not be a leap to assume NSG2 is expressed in other cell lines, it could have indicated how NSG2 is dysregulated in a disease iPSC line. While our initial characterization of NSG2 cellular localization was carried out in parallel in both human and mouse hippocampal neurons, the human neurons suffered from inherent heterogeneity in terms of cell maturity and variability in synaptogenesis. Therefore, we restricted our synaptic localization and functional studies to rodent neurons.
We demonstrated that NSG2 alters AMPAR currents bidirectionally upon knockout and overexpression. However, while the knockout of NSG2 altered AMPA mEPSC frequency, NSG2 overexpression impacted the AMPA mEPSC amplitude. Unfortunately, at the time of the publication of this thesis we were in the process of procuring NSG2 KO animals and prospective studies could not come to fruition in time to include those results here. Slice electrophysiology on hippocampi from NSG2 KO mouse would have added more weight to our findings about NSG2’s putative involvement in synapse formation/maintenance. While a multitude of published studies have previously shown that in vitro findings translate well in vivo, there are always exceptions. The results of these studies could have better informed us about NSG2 localization at dendritic spines and regulation of excitatory synaptic properties in a neural context which is closer to how it is found in the intact brain.

Traditionally, the frequency is thought of as a correlate of presynaptic function and more reflective of the number of synapses. In contrast the amplitude is reflective of the strength of the synapse and determined by the number and composition of AMPAR at the PSD. While the complex role of NSG2 is not straightforward to deduce from these observations, we can begin to speculate the possible mechanism of NSG2 by drawing some inference from our observation that NSG2 knockout is accompanied by diminished PSD95 fluorescent intensity. Additionally, we can take into consideration some recent studies that serve as a precedent and help reconcile our findings regarding AMPA mEPSC frequency vs amplitude upon NSG2 knockout and overexpression respectively.

The MAGUK hypothesis

NSG2 has been shown to have its highest expression during the period of synapse formation and stabilization during early postnatal periods (Saberan-Djoneidi et al., 1995; Semple et al., 2013; Barford et al., 2017). Alterations in NSG2 levels have similar effects on synaptic transmission as do changes to the MAGUK family of scaffolding proteins. Interestingly, overexpression of either GluA1 or GluA2 alone is not sufficient to produce increases in mEPSC amplitude or
frequency (Hayashi et al., 2000; Sinnen et al., 2017; Watson et al., 2017). However, disrupting AMPAR interactions with PSD95 results in increased AMPAR surface diffusion and localization to extrasynaptic sites (Schnell et al., 2002; Bats et al., 2007). Knockdown of MAGUKs causes a reduction in AMPAR mEPSC frequency in dissociated hippocampal neurons by increasing the number of silent synapses without affecting mEPSC amplitudes (Levy et al., 2015). In contrast, PSD95 overexpression results in increases to AMPAR-mediated mEPSC frequency and amplitude (El-Husseini et al., 2000). These results are similar to our current findings for both NSG2 knockout and overexpression. Interestingly, both the MAGUK knockout and overexpression effects on AMPAR current appear to be developmentally regulated. Simultaneous RNAi-mediated knockdown of MAGUK’s PSD95, PSD93 and SAP-102 altered AMPAR EPSCs only during early development (P7-8) but not in adults (P35) (Levy and Nicoll, 2017). Similarly, significant increases in AMPAR EPSCs were observed only when PSD95 was overexpressed early during development as compared to adults (Levy and Nicoll, 2017). Finally, the NSG2 family member Calcyon has been previously shown to associate with PSD95 (Ha et al., 2012), suggesting a possible conserved function across family members. Unfortunately, our attempts to determine if PSD95 co-immunoprecipitates with NSG2 did not yield any conclusive results (Figure 22).

While the previous section suggests a uniformity of function across MAGUK family members, functional differences in synaptic plasticity are associated with differential expression of individual MAGUK family members (E.g. PSD93, PSD95, and SAP102; (Elias et al., 2008; Sun and Turrigiano, 2011)). For instance, during long-term homeostatic scaling, PSD95 accumulates in highly active PSDs while SAP102 accumulates in PSDs that have reduced activity (Ehlers, 2003). Further, PSD95 and SAP102 are assembled into physically distinct complexes (Elias et al., 2008; Frank et al., 2016), which have been shown to have disparate roles in shaping homeostatic scaling and Hebbian plasticity (Migaud et al., 1998; Elias et al., 2006; Cuthbert et al., 2007; Carlisle et al., 2008), (Sun and Turrigiano, 2011). Interestingly, the number, size and shape of PSD95 and SAP102 complexes within dendritic spines has been shown to correlate with synaptic strength and is altered
Figure 22: Representative Western blots showing inconclusive evidence for NSG2/PSD95 co-immunoprecipitation from HEK293T lysates coexpressing NSG2-mC and PSD95-GFP.

Top: Blot probed with anti-PSD95 and Bottom: Blot probed with NSG2. NSG2/PSD95 are not detected in Lane 2 (HEK lysate – control), Lane 3 (Input + control) in each blot shows the respective bands of correct sizes for PSD95 (top) and NSG2-mC (bottom). Non-specific IgG pull down gives a non-specific smear precluding any useful conclusion (Lane 4; top and bottom). Lane 5 shows IP:GFP and WB:PSD95 (top) and NSG2 (Bottom). It appears that PSD95 is immunoprecipitated, but there is no co-immunoprecipitated NSG2-mC. Similarly, in Lane 6 IP: RFP, WB: NSG2 shows NSG2-mC is immunoprecipitated, however it is inconclusive if PSD95 is co-immunoprecipitated.
bidirectionally during plasticity (Ganeshina et al., 2004; Xu, 2011; Cane et al., 2014). The functional diversity of PSDs based on PSD95/SAP102 expression is thought to provide a mechanism for differential responses to synaptic inputs that determine overall circuit properties (Grant, 2018). Lastly, a recent study that mapped over 1 billion synapses using PSD95-GFP and SAP102-mKO2 transgenic mice identify 37 unique types of synapses based on size, shape, intensity, and co-localization of PSD95 and SAP102 (Zhu et al., 2018). This study revealed a remarkable, previously unknown diversity within the glutamatergic "synaptome". As mentioned, previous reports are highly suggestive of the fact that MAGUK family members are differentially incorporated into PSDs within individual neurons (Zhu et al., 2018). As MAGUKs are known to traffic in post-Golgi vesicles similar to those NSG proteins occupy, it may be the case that individual NSG family members aid in their delivery to, and function within PSDs. While most NSG1/2-containing vesicles appear distinct from those that carry PSD95 based on transport rate (Washbourne, 2015), it is worth examining in the future whether individual MAGUKs occupy overlapping PSDs with NSG proteins.

Taken together, these findings suggest a developmental role for NSG2 where its primary function may be to support the trafficking of essential components for synaptic stabilization and facilitate neurotransmission, rather than on receptor trafficking per se.

**The PSD nanodomain hypothesis**

Several recent studies showing the presence of nanodomains within PSDs may help explain how alterations in NSG2 levels differentially affect amplitude and frequency of AMPAR-dependent mEPSCs. Sinnen et al. recently used optogenetics to induce AMPAR interactions with PSD95 to recruit AMPAR into postsynaptic densities. They found that forcing additional recruitment of AMPARs to synapses was insufficient to increase AMPAR amplitude, but unexpectedly resulted in increased mEPSC frequency. The authors concluded that AMPAR recruitment at postsynaptic sites facilitated unsilencing of synapses by a novel
mechanism, whereby AMPARs are recruited to PSD95-containing nanodomains at synapses that already contained surface AMPARs rather than to synapses containing only surface NMDA receptors (Sinnen et al., 2017). Additional support for this idea comes from super-resolution imaging studies that have enabled the discovery of PSD95 subdomains within postsynaptic densities that cluster AMPARs (MacGillavry et al., 2013; Nair et al., 2013; Tang et al., 2016).

A potential model for NSG2 function fits with the nanodomain hypothesis, whereby NSG2 is responsible for helping to localize AMPARs into functional PSD95+ nanodomains, but not overall AMPAR exocytosis. This model predicts that NSG2 knockout should result in the loss of AMPARs localized to the PSD95+ nanodomain causing reductions in mEPSC frequency as we observe in Figure 5. Interestingly, NSG2 KO did not cause reductions in immunochemical labeling for surface GluA1/2 subunits. Thus, NSG2 is critical for maintaining AMPAR-mediated mEPSCs possibly through a localization-dependent mechanism, but may not be required for bulk AMPAR exocytosis to the plasma membrane. In the context of this model, it is somewhat difficult to reconcile a lack of effect on mEPSC amplitude upon NSG2 knockout, where loss of NSG2 should result in reduced numbers of AMPARs within functional nanodomains. However, it is possible that the effect of chronic NSG2 KO as performed in our study results in complete elimination AMPARs localized to nanodomains where NSG2 would have been recruited, resulting in silencing. Because NSG2 is restricted to a subset of synapses (Figures 2 and 3), this could allow normal synaptic transmission at synapses where NSG2 is not normally present, resulting in reductions in frequency but not amplitude. The nanodomain model makes different predictions for the effects of NSG2 overexpression, especially given the apparent restriction of NSG2 to a subset of existing synapses. If NSG2-mC is recruited to both silent and functional (AMPAR-containing) synapses equally, we would expect increases in both mEPSC amplitude and frequency. In contrast, if NSG2-mC is primarily recruited to existing functional synapses, overexpression should result in greater recruitment of AMPARs to PSD95 nanodomains that were not saturated with AMPARs, causing increases in mEPSC amplitude but not frequency. While there was a trend toward
increased frequency, the major effect of NSG2 overexpression was to increase mEPSC amplitude (Figure 6). Thus, similar to the knockout findings, the lack of effect on mEPSC frequency may be explained by a restriction of NSG2-mC localization to functional synapses. Curiously, overexpressed NSG2-mC did occupy a somewhat larger proportion of HOMER1⁺ synapses than its endogenous counterpart (Figure 2), however this may not have been sufficient to significantly increase mEPSC frequency due to unsilencing. Future studies are needed to determine whether NSG2 is truly restricted to a subset of already functional synapses and whether it is capable of shifting AMPAR localization between nanodomains to determine the fidelity of this model.

**Contrasting findings on the localization and orientation of NSG proteins**

Steiner et.al., had previously utilized a Triton-X100 based protein digestion assay combined with western blotting to suggest that NSG1 has a Type-I membrane orientation. Their results suggested that NSG1 did not localize to the plasma membrane and the endosomal localized population of NSG1 has a lumenal N-terminus and a cytosolic C-terminus (Steiner et al., 2002). In the same study, they also found that NSG1 co-immunoprecipitated with the SNARE protein Stx13 which is involved in the endosomal recycling pathway and antisense mediated knockdown of NSG1 altered surface recycling of Tf (Steiner et al., 2002). Similarly, in hippocampal neurons, NSG1 was found colocalized with AMPAR subunit GluA2 after NMDA stimulated AMPAR endocytosis and antisense mediated knockdown of NSG1 in hippocampal neurons led to retardation of GluA1 recycling to the cell surface after NMDA stimulation. NSG1 was found strongly colocalized with internalized Tf and Rab4⁺ vesicles in PC12 cells (Steiner et al., 2002). Fluorescent Tf were allowed to be endocytosed in PC12 cells and assayed for their colocalization with NSG1 at 3 min and 15 min. While there was little colocalization at 3 min, a significant proportion of NSG1 localized with Tf after 15 min. The author’s interpret this to mean that NSG1 associates with endosomal vesicles beyond the initial endocytosis and fusion with early endosomes. Tf⁺ compartments
can be both EEs and REs and in order to distinguish if NSG1 colocalized with Tf+ EE or RE, they further treated the cells with either Brefeldin A (BFA) or Wortmanin. BFA caused tubulation of Rab4+/Rab11+ late REs that contained Tf but hardly any NSG1. However NSG1 largely colocalized with wortmanin sensitive enlarged Tf+ compartments that are Rab4+/Rab5+ EEs and intermediary REs (Steiner et al., 2002). Additional immunolabeling experiments in PC12 cells revealed that NSG1 colocalized to a larger extent with Rab4+ REs, to a minor extent with Rab11+ vesicles and wortmanin sensitive endosomes but not with Rab5+/EEA1+ compartments. Surprisingly, NSG1 did not colocalize with lysobisphosphatidic acid (LBPA), a phospholipid enriched in LEs (Steiner et al., 2002). Unfortunately, the colocalization data in this study were not quantified.

However, these findings are quite a contrast to Yap and colleagues (2017) recent demonstration that a significant proportion of NSG1 and NSG2 was found to overlap with Rab7+ LEs and traveling in a retrograde fashion which the authors conclude were likely destined for degradation. Surprisingly, at the developmental time chosen for the assay NSG1 and NSG2 puncta in dendrites showed approximately 60% percent overlapping expression with the remaining 40% not co-localized. Interestingly, smaller proportions of both NSG1 and NSG2 puncta were found in EEA1+ EEs, Rab11+ REs, and Lamp1+ compartments (Yap et al., 2017). Most intriguingly, this analysis accounted for nearly all NSG1+ punctae, but only about 70% of NSG2+ punctae. Thus, it remains to be determined whether there is an additional pool of unique, NSG2-containing vesicles. However, it must be noted that this study focused only on an endocytosed population of vesicles. This is critical because they revealed two properties of NSG1 and NSG2 that are a complete contrast to earlier findings by Steiner et.al. The first is that NSG1/2 are Type-II membrane proteins and have their N-terminus cytosolic and their C-terminus extracellular and the second that the proteins are present on the cell surface. The surface population of NSG1 and NSG2 are detectable with live cell staining under non-permeabilizing conditions with an antibody that targets a C-terminus epitope. Both proteins were shown to accumulate at the plasma membrane when endocytosis is blocked by transfecting neurons with a Rab5-DN
construct. While these findings are somewhat surprising, it is more in line with the finding that NSG3 has previously been shown to be expressed at the cell surface and alter its levels in response to calcium. Moreover, Shi et al., have recently shown that NSG3 associates with motor proteins to coordinates dynamic microtubule dependent trafficking of LysoTracker\textsuperscript{+} late endosome and lysosome related organelles within axons (Shi et al., 2018).

**Lysosome mediated exocytosis hypothesis**

Interestingly, recent evidence suggests that neuronal lysosomes are not only responsible for degrading protein substrates near the soma and in axons, but play a role in synaptic plasticity. The endo-lysosomal system is critical for regulating forward trafficking, recycling, and degradation of integral membrane proteins. It does so through a series of coordinated transitions through various vesicles including EEs, LEs and REs, and finally lysosomes (Hu et al., 2015). Each of these organelles is associated with the expression of specific Rab GTPases, which are used as proxies for the identification of different vesicles. For instance, RAB8 is generally involved in forward trafficking from the trans-Golgi network toward the plasma membrane (Zhen and Stenmark, 2015). Following CME from the membrane, proteins typically enter RAB5\textsuperscript{+} EEs and are then recycled back to the plasma membrane via RAB4/RAB11\textsuperscript{+} REs, or targeted for degradation via RAB7\textsuperscript{+} LEs which will ultimately fuse with LAMP1\textsuperscript{+} Lysosomes for final proteolytic degradation (Hu et al., 2015).

Recent studies have however, started to elucidate the role of lysosomal Ca\textsuperscript{2+} signaling in neurons, especially in postsynaptic Ca\textsuperscript{2+} release and plasticity. Lysosomes are found throughout the dendritic arbour, including a fraction of dendritic spines (Goo et al., 2017; Padamsey et al., 2017). Further, the localization of these organelles is dynamic and activity-dependent, with glutamatergic signaling recruiting lysosomes to the base of spine heads (Goo et al., 2017). Padamsey and colleagues, reported that back propagating action potentials in hippocampal pyramidal neurons elicited Ca\textsuperscript{2+} release from dendritic lysosomes. Remarkably, this release triggered the fusion of the lysosome with the plasma
membrane, resulting in the exocytosis of its luminal contents. Although many lysosomal enzymes are inactive in the pH neutral environment of the extracellular matrix (ECM), some such as Cathepsin B retain their activity (Mort et al., 1984; Linebaugh et al., 1999) and are able to induce extracellular MMP-9 activity to allow ECM remodeling. This process is essential for the maintenance of the functional and structural changes associated with LTP (Wang et al., 2008; Wlodarczyk et al., 2011). Consistent with this model, pharmacological inhibition of lysosomal fusion or Cathepsin B activity, both of which impaired MMP9 activity, prevented long-term maintenance of dendritic spine enlargements induced by Hebbian activity. Moreover, chronic inhibition of lysosomal function or Ca$^{2+}$ signaling, which is implicated in lipid storage disorders, altered spine morphology and reduced spine density (Goo et al., 2017; Padamsey et al., 2017). Of relevance to our study, it was shown that inhibition of lysosome function with Leupeptin results in a significant decrease in mEPSC frequency, without changing amplitude (Goo et al., 2017). Since, the majority of NSG2 is found localized to RAB7$^+$ LEs and LAMP1$^+$ lysosomes (Yap et al., 2017). Our preliminary data (not shown) also suggests that this constitutes a large synaptic fraction. Thus, future studies will focus on understanding the nature of synaptic NSG2-containing vesicles is particularly significant, as neither RAB7$^+$ LEs nor LAMP1$^+$ lysosomes have been shown to be critical for maintaining AMPAR surface expression at PSDs (Hausser and Schlett, 2017).

**Differential synaptic localization of NSG family proteins**

Regardless of whether any of the proposed mechanisms above account for differential trafficking of NSG proteins or their function within synapses, what is clear is that both NSG1 and NSG2 specifically localized to subsets of glutamatergic synapses at any given moment. This suggests a number of different possible roles for NSG family members to affect synaptic function, one of the most significant being the creation of synaptic diversity. Our observations related to the disproportional changes in AMPA mEPSC observed in Chapter 2, and further
described in Chapters 3 and 4, led us to hypothesize that NSG2 may be recruited at specialized synapses harboring relatively higher levels of activity.

Synapse diversity at glutamatergic synapses has been recognized at the laminar level for some time (Yogev and Shen, 2014), and the mechanisms for their development and maintenance are beginning to be uncovered. A host of transynaptic adhesion proteins have been shown to play critical roles in synaptogenesis at excitatory and inhibitory synapses, including neurexins, neuroligins, LRRTMs, SLITRKs, FLRTs, SALMs, and cadherins, to name a few (de Wit and Ghosh, 2016). Many isoforms exist for each of these protein families, and alterantive splicing creates potentially millions or billions of different binding combinations between cis and trans-interacting partners in the synaptic cleft. For the Neurexin-Neuroligin family, RNA-sequencing analysis found over 1,159 alternatively spliced isoforms of Nrxn1α and 1,120 for Nrxn3α alone (Treutlein et al., 2014). It is difficult to imagine a scenario where this immense diverse protein family produces largely redundant functions, leading neuroscientists to consider whether there is an elaborate transsynaptic code that promotes synapse specificity and diversity (Sudhof, 2017).

Abundant examples exist for transsynaptic specificity, but most appear to encode lamina-specific connections rather than truly synapse-specific connections. For instance, Netrin-G2 localizes specifically in the Schaffer collateral axons and connects with the NGL-2 enriched CA1 dendritic spines in the stratum radiatum (Nishimura-Akiyoshi et al., 2007). Interestingly DeNardo and colleagues (2012), found that NGL2 knockdown reduced the synaptic density specifically only in the CA1 proximal dendrites in the stratum radiatum and not in the distal dendrites in the molecular layer (DeNardo et al., 2012). Anderson and colleagues, recently described that LPHN2 is expressed in the dendritic spines of the CA1 hippocampal neurons in the molecular layer, which is involved in forming synapses specifically with the entorhinal cortex inputs. In a LPHN2 KO mouse model, reduced number of spines were observed specifically in the CA1-entorhinal cortex synapses whereas the dendritic spines in the stratum oriens and radiatum were not affected. Interestingly, this region-specific ablation of LPHN2 did cause a homeostatic
increase in the number of CA3-CA1 synapses in stratum radiatum (Anderson et al., 2017). Furthermore, Um and colleagues (2016), found that the synaptic effects of LRRTM2 KO specifically manifested in the dentate granule neurons and not CA1 pyramidal neurons (Um et al., 2016). Similarly, LRRTM4 KO mouse showed a lack of activity dependent trafficking of AMPAR only in the dentate granule neurons and not in the CA1 neurons.

Aside from this work, the synaptic localization of NSG proteins is unknown. However, based on functional properties and our preliminary data we can make some hypotheses wither regard to their spatial segregation. NSG1 and NSG2 have established roles in regulating activity-dependent (Alberi et al., 2005a; Steiner et al., 2005a), and basal activity (Chander et al., 2019), respectively. While the majority of hippocampal afferents terminate on PSDs that display similar types of NMDA-dependent forms of postsynaptic plasticity, a major exception are the Mossy fiber (MF) synapses of the dentate granule cells onto Hilar and CA3 pyramidal cells. The postsynaptic structure on the CA3 dendrite consists of an elaborate multiheaded spine known as a thorny excrescence (TE) (Amaral and Dent, 1981). Because of its size and position near the soma of CA3 neurons, activation of single mossy fiber synapses can cause spiking in CA3 neurons. Therefore, these are referred to as “detonator” synapses (Urban et al., 2001). This pathway displays pronounced short-term facilitation and a presynaptic form of LTP that is independent of NMDAR activation (Zalutsky and Nicoll, 1990; Nicoll and Schmitz, 2005).

We hypothesize that NSG2 plays a predominant role in promoting robust postsynaptic AMPAR surface expression under basal conditions, but perhaps not during plasticity. Interestingly, we did find that many synaptic NSG2 punctae appear to be located on proximal dendrites that grossly resemble MF synapses (Figure 13A and B). Thus, the MF-CA3 synapse is a good candidate for the incorporation of NSG2. Recently, some studies have identified a clear role of a Cadherin type II isoform in synapse specificity. The development of the dentate gyrus and CA3 mossy fiber synapses require Cadherin 9, which is enriched in this region of the hippocampus. Predictably Cadherin 9 KD in vitro specifically reduced...
dentate gyrus – CA3 synapses while the other hippocampal synapses were unaffected. KD of Cadherin 9 in vivo altered the morphology of TEs. The authors suggest that Cadherin 9 present in the pre- and postsynaptic neurons specifically facilitate synapse formation through hemophilic interactions (Williams et al., 2011). Thus, future work should determine whether NSG2 works in conjunction with Cadherin 9 to promote TE formation and function.

In order to ascertain if NSG2 confers unique properties to synapses, another important future direction would be to perform glutamate uncaging at individual synapses harboring NSG2 to compare their excitability with non-NSG2 synapses. One of the drawbacks of this experimental design is that we need to deplete all the NSG2 in the neuron and rely on NSG2 overexpression to discriminate between NSG2+ or NSG- synapse. However if NSG2 overexpression significantly alters synaptic and/or AMPAR properties, it could mask the effect of glutamate uncaging. One way to avoid this confound would be to use ‘rescue expression'; this design would potentially avoid overexpression artifacts while being able to visualize NSG proteins. To do so we could use overexpression of NSG2-mCh in primary hippocampal cultures from NSG2 KO mice. As mentioned, NSG2 is one of the most highly expressed transcripts and proteins in cultured neurons (Floruta et al., 2017; Chander et al., 2019). Thus, we expect overexpression driven by the Synapsin-1 (SYN1) promoter will produce levels similar to endogenous proteins, but which could be tested via quantitative PCR and Western blot.

Another means by which to address the “NSG2 synapse” identification problem would be to identify the synaptic targeting domain(s). As mentioned, our initial deletion of the entire N- or C-terminus significantly reduced synaptic localization of NSG2. Future studies could perform targeted site-directed mutagenesis of the N- and C-termini to determine whether small peptide sequences promote synaptic targeting. Here we could use cultured neurons from NSG2 KO animals that have been transduced with their respective full length and mutant proteins and stained for surface AMPARs to determine what proportion of PSDs are occupied by NSG mutants. If the minimal synaptic Targeting Domain(s)
(TD) can be identified, we could attempt to tag those domains from each protein
to EGFP (EGFP-NSG2TD) in an attempt to target it to “NSG2 synapses” in primary
cultures. Occupation of the same proportion of synapses as full-length protein
would indicate sufficiency of the TD for synaptic targeting.

Lastly, one immediate future direction for this work would be to identify the
protein-protein interactome for NSG2. As mentioned, all NSG family members co-
immunoprecipitate with AMPAR subunits, co-localize at synapses, and regulate
AMPA-mediated synaptic transmission. However, previous studies using mass
spectrometry to examine AMPAR interacting proteins have failed to identify NSG1-3 in rodents (Schwenk et al., 2009; Shanks et al., 2012; Schwenk et al., 2014) and human (Bayes et al., 2011). This may be due to the fact that their interactions are relatively transient during trafficking or that their interactions occur in insoluble membranous fractions. Recently, an affinity purification approach that utilizes a promiscuous E.coli biotinylation enzyme BirAR118G (BioID; (Roux et al., 2012)) has been developed to overcome these limitations. Furthermore, yeast display-based directed evolution yielded a protein with faster kinetics (TurboID; (Branon et al., 2018)) that has been used to identify synaptic protein complexes (Spence et al., 2019). When TurboID is fused to a bait protein expressed in cells (Roux et al., 2012), BirA-dependent covalent biotinylation occurs within 10–50 nm of the bait protein and allows for efficient isolation and analysis of proximal proteins by streptavidin-based affinity purification and mass spectrometry (Kim et al., 2014). Compared to traditional affinity purification methods, BioID reactions occur in situ, enabling the capture of protein complexes, including transient interactions and insoluble proteins from subcellular compartments refractory to biochemical isolation (Yao et al., 2015). Thus, proximity labeling could determine the proteins associated specifically with the synaptic fraction of NSG2 when combined with the proper controls for Golgi, cytoplasmic, and endosomal fractions of NSG2 that may interact with overlapping and non-overlapping protein constituents.

Together, our data reveal for the first time, that NSG2 is an AMPAR-binding
protein that is required for normal synapse formation and/or maintenance. They
also reveal novel functional properties of NSG1 and NSG2, and more significantly,
establish a novel type of synapse diversity based on the incorporation of various NSG proteins.
LIST OF APPENDICES

Appendix A: Differentiation paradigm and timeline of generation of hPSNs from hPSCs.

Appendix B: Derivation of hPSNs from hPSCs is a valid model of synaptogenesis and gives rise to known markers of functional presynaptic development.

Appendix C: Derivation of hPSNs from hPSCs is a valid model of synaptogenesis and gives rise to known markers of functional postsynaptic development.

Appendix D: Derivation of hPSNs from hPSCs led to the identification of several previously uncharacterized genes with putative roles in functional neural maturation.
Appendix A

Differentiation paradigm and timeline of generation of hPSNs from hPSCs (Larsen et al., 2016):

Diagram illustrating the method and time course of neuronal differentiation used in this study. Representative images show cellular morphology at various time points during differentiation; image labeled “immature neurons” was taken from neurons at day 30 and shows βIII-Tubulin, while the image labeled “functional neurons” was taken at day 50 and shows βIII-Tubulin (red) and Synapsin-1 (green). Scale bars indicate 100 μm.
Derivation of hPSNs from hPSCs is a valid model of synaptogenesis and gives rise to known markers of functional presynaptic development: Selected genes shown for representation NRXN1, SNAP25, vGLUT1 and vGAT show upregulation starting from <30 days and attain peak expression around 30-50 days corresponding with functional maturation of hPSNs and synaptogenesis.
Appendix C

Derivation of hPSNs from hPSCs is a valid model of synaptogenesis and gives rise to known markers of functional postsynaptic development:
Selected genes shown for representation \(GRIA1, GRIA2, GRIA2, GABRA2\) and \(GABRB2\) show upregulation starting from <30 days and attain peak expression around 30-50 days corresponding with functional maturation of hPSNs and synaptogenesis.
Appendix D

Previously Uncharacterized

Derivation of hPSNs from hPSCs led to the identification of several previously uncharacterized genes with putative roles in functional neural maturation: *NSG2* was one of the highest expressed novel uncharacterized gene, and its gene expression profile closely paralleled that of *GRIA1* and *GRIA2* (Appendix C)
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