UNDERSTANDING THE SPATIAL AND FUNCTIONAL RELATIONSHIP BETWEEN CADHERINS AND NEUROLIGINS AT SYNAPSES

by

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ABSTRACT

Cadherins and Neuroligins (NLs) are two of the most extensively studied cell adhesion molecules (CAMs) at synapses and have previously been shown to localize to synapses and exert a key role during their development. Despite this, their spatial and functional relationship with respect to one another has not been studied to date. In the present study, we examine the spatial and functional relationship of cadherin and NL isoforms at glutamatergic and GABAergic synapses in cultured hippocampal neurons. Analysis of the synaptic distribution of N-cadherin and NL1 and NL2 in hippocampal cultures, confirm previous studies demonstrating the enrichment of NL2 at GABAergic synapses and enrichment of NL1 and N-cadherin at glutamatergic synapses. We have also observed subsets of GABAergic synapses that express both N-cadherin and NL2 as well as glutamatergic synapses that only express either NL1 or N-cadherin. These groups of glutmatergic and GABAergic synapses may represent a specific subtype of synapse, or may reflect the differential localization of these adhesion molecules during synapse formation. Moreover, using a combination of overexpression and knockdown analysis we demonstrate that NL1 and N-cadherin promote the formation of synapses, in part, by a common pathway. Indeed, knocking down these proteins individually results in approximately 50% reduction in glutamatergic synapse density with a similar reduction upon combined knockdown. In addition, functional compensation assays demonstrate that NL1 expression can fully rescue synapse loss that is due to knockdown of N-cadherin expression. Furthermore, N-cadherin expression can partially rescue synapse loss that is due to knockdown of NL1 expression. Together this work demonstrates that these two cell adhesion proteins act in concert to regulate excitatory synapse formation. Specifically, we show that N-cadherin acts upstream of NL1 to promote synapse formation and that NL1 is a limiting factor in this pathway.

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LIST OF ABBREVIATIONS

AMPA – α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ARVCF – armadillo repeat protein deleted in velo-cario-facial syndrome

Cad – cadherin

CAM – cell adhesion molecule

CNS – central nervous system

EC – extracellular domain

N-cad – N-cadherin

NL1 – neuroligin-1

NL2 – neuroligin-2

NL3 – neuroligin-3

NMDA – N-methyl-D-aspartic acid

GABA – gamma-aminobutyric acid

HA – haemagglutinin

HBSS – hank's balanced salt solution

HRP – horseradish peroxidase

HEK – human embryonic kidney

LTP – Long-term potentiation

L-LTP – Late long-term potentiation

mEPSC – miniature excitatory postsynaptic current

PBS – phosphate buffered saline

PDZ – PSD-95/Disc-large/ZO-1

PSD-95 – Postsynaptic density protein 95

S-SCAM – Synaptic scaffolding molecule

SV – synaptic vesicle

VGAT – Vesicular GABA transporter

VGluT-1 – Vesicular glutamate transporter 1

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CHAPTER I: INTRODUCTION

THE SYNAPSE

The human brain contains an estimated 50 to 100 billion neurons and collectively these neurons form 100 to 500 trillion synapses. Each synapse can be described as a point of connection between a neuron and a postsynaptic cell that allows intercellular communication via the transmission of signals also known as neurotransmitters (C. Dean et al., 2003). This web of neurons, connected to one another via synapses, gives rise to a highly complex and dynamic neural circuit that allows for perception, learning and memory. Although years of research have been dedicated to the understanding of how this neural networking forms, many of the precise mechanisms and key players that underlie synapse development have yet to be elucidated.

SYNAPSE STRUCTURE

Synapses are composed of three main parts (Fig 1): the presynaptic terminal, the synaptic cleft and the postsynaptic terminal (N. E. Ziv and C. C. Garner, 2004). The presynaptic terminal typically originates from an axon and contains anywhere from hundreds to thousands of neurotransmitter filled vesicles also known as synaptic vesicles (SVs). In response to stimulation, these SVs dock, fuse and release their contents at the terminal into the synaptic cleft from a specialized region of the presynaptic plasma membrane called the active zone. The synaptic cleft is a narrow intercellular gap approximately 20 nm in width where neurotransmitters are released and diffuse across to the postsynaptic terminal (N. E. Ziv and C. C. Garner, 2004). Released neurotransmitters then bind to receptors that have been recruited to postsynaptic sites of cell-cell contact by structural and scaffold proteins. This assembly of

Figure 1

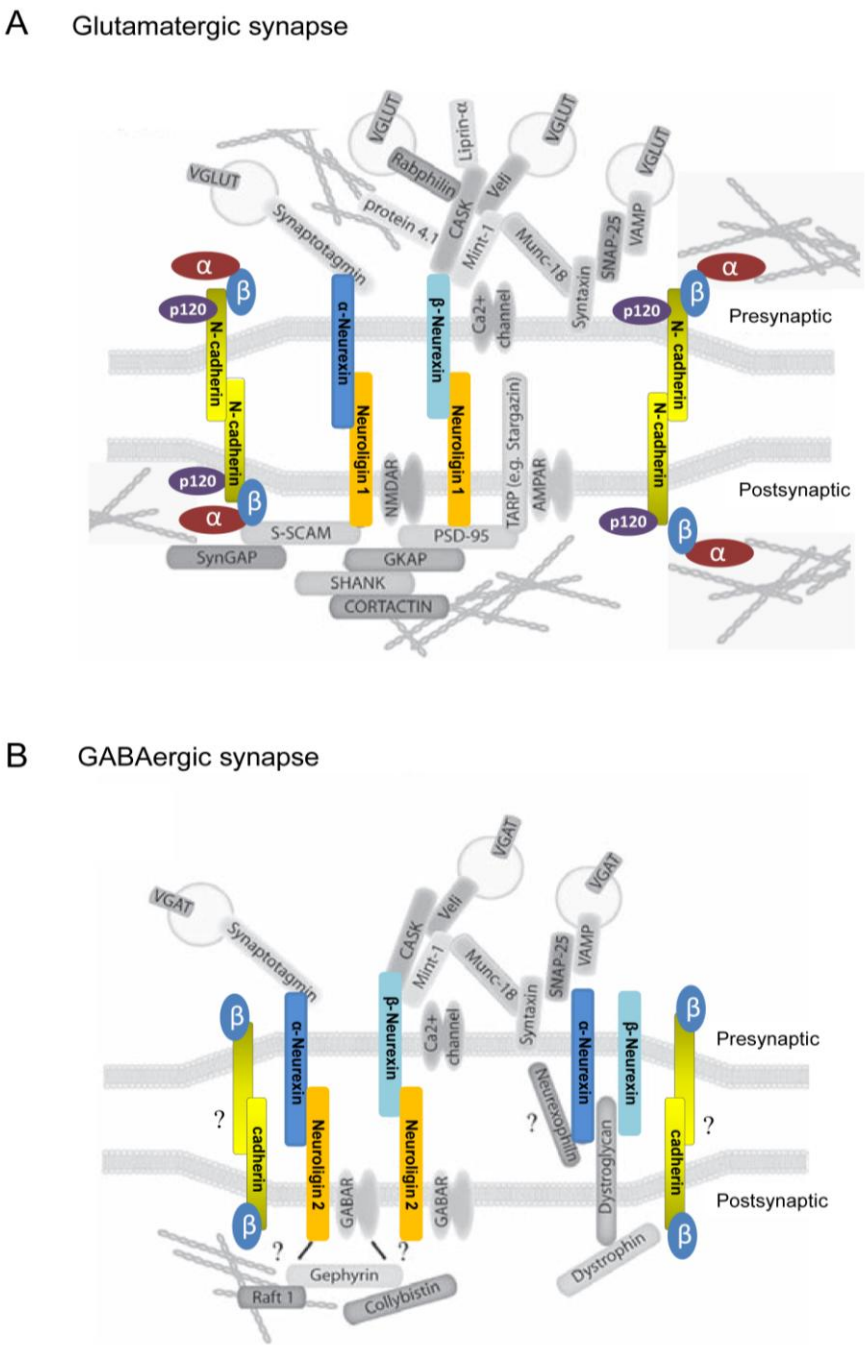

Figure 1. Schematic representation of neuroligins and cadherins at GABAergic and glutmatergic synapses. **(A)** N-cadherin makes transsynaptic homophilic interactions while NL1 makes heterophilic interactions with α and β neurexin isoforms. **(B)** NL2 is present at GABAergic synapses but no specific cadherins have been identified. Figure adapted from Lise et al., 2006.

proteins in the postsynaptic membrane make up an electron-dense structure referred to as the postsynaptic density (PSD). The pre- and postsynaptic membranes are held together by a matrix consisting of a wide variety of cell adhesion molecule (CAM) families as well as extracellular matrix proteins (ECMs) that span the synaptic cleft (Garner et al., 2006).

Several classes of synapses exist in the central nervous system (CNS), two of which are important in mediating the rate of action potential firing. Excitatory synapses are formed between axons and dendritic spines and generate excitatory postsynaptic potentials that increase the probability of the post synaptic neuron to reach action potential threshold (J. N. Levinson and A. El-Husseini, 2005). In contrast, inhibitory synapses are formed on the shaft of dendrites and generate inhibitory postsynaptic potentials that decrease the probability of the postsynaptic neuron to reach action potential threshold (J. N. Levinson and A. El-Husseini, 2005). It is therefore the collective input of both inhibitory and excitatory signals in the postsynaptic neuron that determine whether or not the postsynaptic cell reaches the threshold to fire an action potential (Sanes et al, 2000).

Signal transmission by inhibitory and excitatory synapses is mediated by different classes of neurotransmitters. GABA and glycine are neurotransmitters that are released at inhibitory synapses and glutamate is released at the majority of excitatory synapses. Other neurotransmitters that are commonly released at synapses are acetylcholine, dopamine and serotonin. Although more than one type of excitatory and inhibitory synapse exist, the current study will focus specifically on glutamatergic and GABAergic synapses.

GLUTAMATERGIC SYNAPSES

Excitatory synaptic transmission at glutamatergic synapses is mediated by the release of the neurotransmitter glutamate, which is transported into synaptic vesicles via the vesicular glutamate transporter (VGluT) (Fig 1A). Released neurotransmitters are received by glutamate receptors such as NMDA receptors, AMPA receptors and mGLURs that are situated on the postsynaptic membrane (A. K. McAllister, 2007). These receptors are anchored to the postsynaptic membrane by excitatory synapse specific scaffold molecules such as members of the PSD-95 family and ProSAP/Shank family.

GABAERGIC SYNAPSES

At GABAergic presynaptic terminals, the neurotransmitter GABA is packaged into SVs by the vesicular GABA transporter (VGAT) (Fig 1B)(F. A. Chaudhry et al., 1998). GABA is then released into the synaptic cleft and received at the postsynaptic membrane by GABA receptors that are anchored by inhibitory specific scaffold molecules such as gephyrin. It has been proposed that collibystin, another inhibitory synapse specific scaffold molecule, mediates the synaptic localization of gephyrin (K. Harvey et al., 2004) and that gephyrin in turn clusters $GABA_A$ and glycine receptors at synapses (F. Fischer et al., 2000; S. Levi et al., 2004). However, in comparison to excitatory synapses, much less is known about the molecular make up of the pre- and postsynaptic compartments of inhibitory synapses (Fig 1B).

THEORY OF SYNAPTOGENESIS

Early stages of synapse formation or synaptogenesis, involve the establishment of cell-cell contacts by filopodia that originate from axons and dendrites. Initial axon-dendrite contact is often transient (Arikkath, 2008). However once target recognition is established, complex molecular assembly of the pre- and postsynaptic machinery occurs. This process includes the

recruitment and assembly of various receptors, signalling molecules and scaffolding proteins to the synaptic site (Arikkath, 2008). Many of these synaptic proteins are present in neurons before synapses are formed (Fletcher et al, 1991) and are shuttled throughout axons and dendrites in heterogenous clusters of proteins, known as transport packets before they are brought to developing synaptic sites. In presynaptic assembly, molecular components of the presynaptic compartment are delivered to nascent synapses in multi-molecular complexes. These complexes are thought to exist in at least two forms: Piccolo transport vesicles (PTVs) and synaptic vesicle protein transport vesicles (STVs). PTVs carry active zone proteins such as piccolo, bassoon, N-cadherin, and RIM1, as well as proteins that mediate SV exocytosis including, SNAREs, Syntaxin, SNAP25, Munc13, and Munc18 (A. K. McAllister, 2007). STVs transport synaptic vesicle (SV) proteins as well as other proteins necessary for exo- and endocytosis such as VAMP2/synaptobrevin II, synapsin, synaptotagmin and calcium channels (R. G. Zhai et al., 2001).

Cell-cell contact is necessary for synaptogenesis to occur and has been speculated to be the initiating step of synapse formation. However, some have argued that axon-dendrite contact is not always required to begin the formation of functional synaptic compartments. In 2003, Krueger et al. demonstrated that functional SV release sites also known as “orphan boutons” can form in the absence of postsynaptic specializations, suggesting that the formation of functionally mature presynaptic release sites does not require contact with a postsynaptic membrane (S. R. Krueger et al., 2003). This contradicted the common theory that recruitment of presynaptic components and assembly of presynaptic machinery comes after the establishment of cell-cell contact. They further proposed that these orphan clusters can translocate along axons and be recruited to axodendritic contact sites and initiate the formation of novel synapses (S. R. Krueger et al., 2003). These findings therefore propose that synapse formation can occur through more than one mechanism.

The mechanism by which postsynaptic proteins are delivered to postsynaptic sites is less clear than the assembly of the presynaptic molecular complex. Some studies have shown postsynaptic proteins such as NMDAR and SAP-102 to be transported in transport packets similar to STVs (Sans et al, 2003). However, others such as Bresler et al. (2004) have reported that NMDAR localize to synapses from a diffuse pool (Bresler et al. 2004). Therefore, whether postsynaptic proteins aggregate at synaptic sites from diffuse cytoplasmic pools or are delivered in discrete packets is still unclear.

The timeline of molecular events that underlie synapse formation remains vague and studies examining the order of pre- versus postsynaptic assembly give contradicting results. Washbourne et al. (2002) reported that postsynaptically localized NMDA receptors cluster earlier than synaptic vesicle proteins (P. Washbourne et al., 2002), thus suggesting that postsynaptic assembly precedes presynaptic assembly. Also at excitatory glutamatergic synapses, postsynaptic proteins such as ionotropic glutamate receptors are present in dendrites before synapses are formed (Craig, 1993). In contrast, others have observed postsynaptic proteins such as PSD-95 and NMDA receptors gradually accumulate at sites apposed to clusters of the presynaptic protein Bassoon, thus suggesting that presynaptic assembly precedes the recruitment of postsynaptic elements (H. V. Friedman et al., 2000; C. L. Waites et al., 2005).

CELL ADHESION MOLECULES (CAMS) AT THE SYNAPSE

At the synapse, physical contact between the pre- and postsynaptic membrane is largely mediated through CAMs. Many CAMs can function to mediate cell-cell adhesion and/or as signalling molecules within (A. M. Hinsby et al., 2004) and outside of the nervous system (R. O. Hynes, 2002). Since cell-cell contact often precedes synapse formation as mentioned above,

CAMs have been considered to be attractive candidates to trigger synaptogenesis and to potentially coordinate synapse differentiation bidirectionally. There are a number of CAM families that are found both pre- and postsynaptically including members of the cadherin (U. Tepass et al., 2000), Eph, Ephrin, immunoglobulin (G. Rougon and O. Hobert, 2003), and neurexin/neuroligin family (M. B. Dalva et al., 2007). In addition to providing mechanical cell-cell adhesion, certain CAMs can induce synapse formation in HEK cell/neuron coculture assays including neuroligins (NLs) (P. Scheiffele et al., 2000), EphB receptors (M. S. Kayser et al., 2006), SynCAM (T. Biederer et al., 2002), Netrin-G ligand (NGL) (S. Kim et al., 2006), and signal regulatory proteins (SIRPs). Most recently, an entirely new family of transmembrane proteins called Leucine rich-repeat Transmembrane proteins (LRRTMs) have been identified from an expression screen for synaptogenic proteins and have also been shown to induce presynaptic differentiation in coculture assays (M. W. Linhoff et al., 2009). These findings therefore strongly suggest that CAMs not only function in providing mechanical adhesion between neurons but also trigger the formation of synapses. However, the mechanisms by which they mediate their synapse promoting actions are not well understood.

In summary, growing evidence suggests that synaptic CAMs have two major functions at the synapse: 1) to mediate physical contact and stabilize the pre-and postsynaptic terminals, 2) to act as signal transduction molecules for example to trigger synapse formation (Hortscht 2009).

CADHERINS

History

Cadherins are one of the most extensively studied families of synaptic adhesion proteins. They were originally identified as glycoproteins, responsible for calcium dependent homophilic adhesion during morula compaction in the preimplantation mouse embryo (Sanes et al, 2000) (Peyrieras, 1983). Today, the cadherin superfamily includes over 100 members and is further classified into subfamilies such as classic cadherins, protocadherins and desmosomal cadherins (Obst-Pernberg, Redies, 1999). A common characteristic shared among cadherins that distinguish this family from other CAM families is the presence of repetitive subdomains or cadherin repeats in the extracellular domain. These cadherin repeats contain calcium binding sites that help stiffen the extracellular domain in the presence of calcium that in turn allows for cell-cell adhesion (Horstcht, 2009) (Nager et al, 1996). Since their initial discovery, the function of cadherins has expanded from mediating mechanical cell-cell adhesion (Sanes et al, 2000), to regulating a variety of other biological processes including, cell sorting in tissues, organization in development, coordinating cell movement and synapse assembly and maintenance (Colman et al, 2007).

Classic cadherins

Classic cadherins were the first cadherins to be identified and to date are the most extensively studied members of the cadherin superfamily. In vertebrates, classic cadherins have five cadherin (EC1-EC5) repeats in the extracellular domain, each comprised of approximately 110 amino acid residues (Fig 2) (Takeichi, 1995). In type I cadherins, the most distal cadherin repeat (EC1) is highly conserved and is important for homophilic adhesion and trans-cadherin interactions (Patel, 2006). The extracellular domain of classical cadherins is linked to a single transmembrane domain that is connected to a cytoplasmic tail.

Figure 2

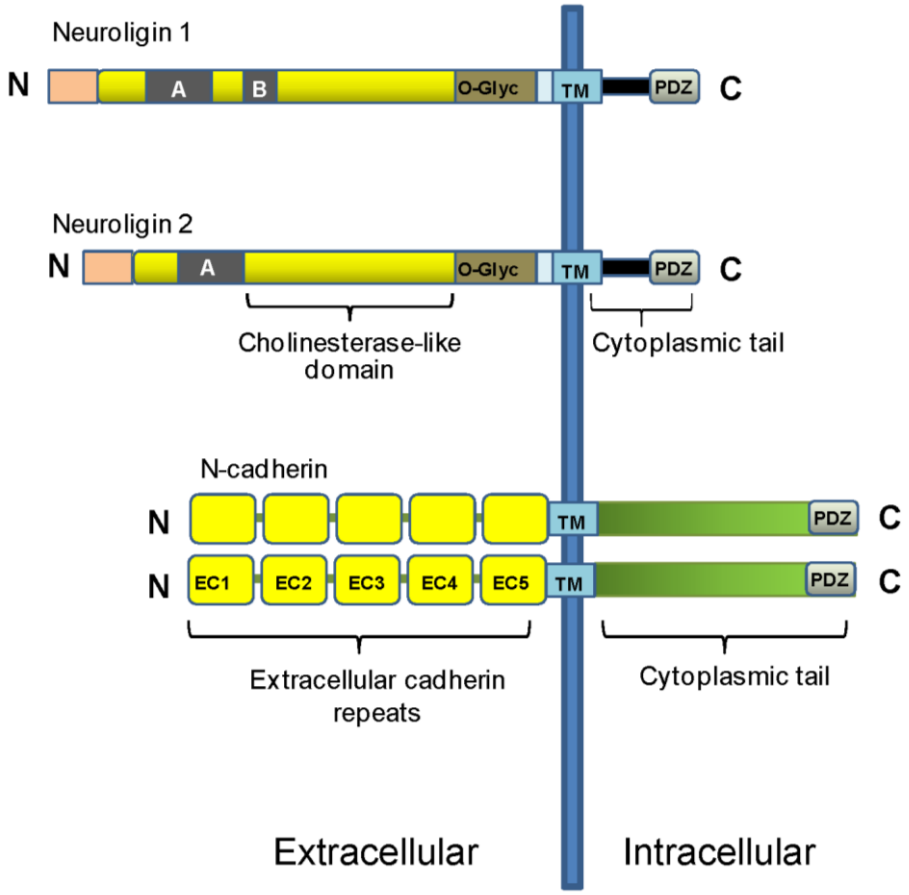

Figure 2. Schematic representation of neuroligins and N-cadherin. Neuroligins interact with neurexins via a cholinesterase-like domain found on the extracellular domain. The cholinesterase-like domain contains two sites (A and B) of alternative splicing. N-glycosylation sites are thought to mediate neuroligin-neurexin affinity. A single transmembrane domain connects the extracellular domain to a cytoplasmic tail containing a PDZ binding motif. N-cadherin has five cadherin repeats in the extracellular domain with EC1 being important for trans-cadherin interactions. The extracellular domain is linked to a single transmembrane domain that is connected to a cytoplasmic tail. O-Glyc=N-glycosylation sites;TM=transmembrane domain; PDZ= PDZ binding domain; EC=Extracellular domain

This cytoplasmic tail is highly homologous among cadherin isoforms and interacts with many cytoplasmic proteins such as catenins that will be described more in detail below (Fig 1A)(Redies, 1999, 2000). The cadherin cytoplasmic domain also includes a highly conserved PDZ binding site that is thought to interact with a variety of PDZ-domain-containing proteins (F. Demontis et al., 2006).

Classic cadherins are further subdivided into type I and type II classes, both of which are structurally very similar but differ in their adhesive properties (Patel, 2006). Type I classic cadherins typically form homophilic interactions. In contrast, type II classic cadherins frequently make both homophilic and heterophilic interactions (Y. Shimoyama et al., 2000). However, it should be noted that some type I classic cadherin isoforms such as N-cadherin and R-cadherin have been reported to make very weak heterophilic interactions as well (H. Inuzuka et al., 1991). Classic cadherins exist in two forms; a monomeric form and a cis-dimeric form, the latter being strongly adhesive (Tamura, 1998). It has been proposed that at target adhesion sites, cadherin monomers aggregate to form cis-dimers that then interact with cadherin cis-dimers of the opposing membrane to form a stable adhesive intercellular connection (Colman, 1997; Shapiro, 1995) . Thus it is thought that cadherins regulate adhesion strength by controlling the accumulation of cadherin cis-dimers at cell-cell contact sites (Shan, 2000).

Cytoplasmic partners of Classic cadherins

The cytoplasmic half of the cadherin adhesion complex includes a family of cytoplasmic partners known as catenins that directly or indirectly interact with the cytoplasmic C-terminus tail of cadherins. Members of the catenin family are thought to help mediate cadherin based adhesion by anchoring cadherins to the actin cytoskeleton. The catenin superfamily is further classified into three main families: the α-catenin family, β-catenin family, and the p120catenin family (Fig IA). β- catenin binds directly to the cytoplasmic tail of cadherin via 12 armadillo

repeats of its central domain. The C-terminal domain of β-catenin includes a PDZ motif that can bind to a variety of PDZ proteins such as the scaffold protein S-SCAM (W. Nishimura et al., 2002). Catenins can also interact with other catenin molecules. β-catenin has been shown to recruit α-catenin to adherens junctions and the N-terminal domain of β-catenin has been shown to bind α-catenin (D. B. Ivanov et al., 2001). α-catenin in turn has been shown to bind to actin filaments, both *in vitro* and *in vivo* systems (Rimm, 1995). Therefore α-catenin is thought to link the cadherin adhesion complex to the actin cytoskeleton (D. B. Ivanov et al., 2001). Interestingly, evidence from Dreese et al. suggest that α-catenin does not anchor the cadherin/β-catenin complex to the actin cytoskeleton by binding to them simultaneously but instead mediates this connection by mutually exclusively switching back and forth between interacting with β-catenin and actin filaments (F. Drees et al., 2005; S. Yamada and W. J. Nelson, 2007). Like β-catenin, p120 catenin also interacts with the cytoplasmic domain of cadherins via its armadillo repeats (R. C. Ireton et al., 2002) and requires cadherin for proper localization to junctions (M. A. Thoreson et al., 2000). In addition to binding to cadherins, p120 catenin also interacts with Rho GTPases (regulators of cytoskeleton dynamics) (Anastasiadis, 2000) suggesting that like α-catenin, p120-catenin may also control the interplay between cadherins and the actin cytoskeleton. Several studies have indicated the importance of p120 in cadherin-mediated adhesion including a study that demonstrated that the degradation of the cadherin adhesion complex is dependent on p120 (R. C. Ireton et al., 2002; K. Xiao et al., 2003). In young neurons, p120 catenins initially bind to cadherins but are replaced by presenilin at later stages of neuronal development (M. E. Rubio et al., 2005). Cadherins also bind to δ-catenin, a neuron specific catenin member (J. Zhou et al., 1997). δ-catenin associates with a number of PDZ proteins including erbin, AMPA receptor binding protein (ABP), GluR-interaction protein (GRIP) and densin-180 (J. Arikkath, 2009). The p120 catenin family also includes two lesser known catenins, Armadillo repeat gene deleted in velocardiofacial syndrome (ARVCF) and p0071 (B. Walter et al.). ARVCF and p0071 have PDZ-binding motifs that have been

shown to interact with synaptic PDZ proteins (R. P. Laura et al., 2002; H. Ohno et al., 2002) and are both detected in the nervous system, however, their distribution or function in the nervous system is unclear (H. Sirotkin et al., 1997; B. Stahl et al., 1999). In addition to acting as binding partners of cadherin, catenins can also bind to a number of other proteins, including PDZ domain containing proteins, protein kinases, protein phosphatases as well as effectors of the actin cytoskeleton (M. Deguchi et al., 2000; F. Drees et al., 2005; S. Boguslavsky et al., 2007; T. Ichii and M. Takeichi, 2007). This therefore gives the ability for the cadherin adhesion complex to interact with a diverse range of binding partners that likely allows the complex to modulate several signalling pathways.

Cadherin Function

In addition to mediating cell-cell adhesion, cadherins have been to shown to play roles in cell sorting, cell motility and synapse assembly. Selective cell sorting is thought to be mediated in part by the expression of specific cadherins (Townes, Holtfretter 1955). Cadherins make homophilic interactions and therefore highly selective adhesion can occur between cells expressing cadherins of the same type to not only mediate cell sorting but also tissue organization. Although a single type of tissue can express many forms of cadherins, several isoforms have been shown to be preferentially expressed in particular tissues, for example, E-cadherin is expressed in all epithelial tissue, N-cadherin is expressed in neural tissue and muscle, R-cadherin is expressed in the forebrain and bone, and cadherin-6 in the kidney (J. M. Halbleib and W. J. Nelson, 2006).

Cadherins have also been reported to act as signalling molecules. N-cadherin has been shown to cooperate with fibroblast growth factor (FGF) receptors to activate signalling pathways correlated with signalling pathways found in carcinomas (J. Hulit et al., 2007). N-cadherin and E-cadherin can also activate MAPK-dependent signalling pathways by interacting with EGF

receptors (P. Doherty et al., 2000; S. Pece and J. S. Gutkind, 2000). In neurons, proper cadherin expression is also necessary for normal activation and regulation of neurite outgrowth. In the developing frog retina, expression of a functionally inactive dominant-negative form of N-cadherin prevents proper neurite outgrowth (Reihl, Johnson 1996). Axons have also been shown to adhere to substrates with recombinant N-cadherin and further project to areas with higher concentrations of N-cadherin (P. Doherty et al., 1991; P. Doherty et al., 2000). In contrast, T-cadherin has been shown to inhibit axon outgrowth (Fredette, ranscht 1994). Thus different cadherins may have the ability to inhibit or promote neurite outgrowth.

Localization in nervous system

Several members of the cadherin superfamily, in particular classical cadherins, have been shown to be expressed in the CNS. In the mouse brain, type II classical cadherins, cadherin-6, cadherin-8 and cadherin-11 are expressed in a region specific manner such as in the medial geniculate body (cad6), the ventral posterior thalamic nucleus (cad6/cad11) and the anteroventral thalamic nucleus (cad6/cad8). Cadherins aggregate at sites of cell-cell contact and electron microscopy (EM) images of hippocampal neurons have shown that cadherins cluster at synaptic contact sites, localizing to regions flanking the active zone (N. Uchida et al., 1996). Several cadherins have been identified at the synapse such as N-cadherin, R-cadherin, cadherin-7, cadherin-6, and cadherin-6B (K. Obst-Pernberg and C. Redies, 1999). The region specific expression pattern and synaptic localization of cadherin isoforms suggest that they may play a role in forming the neural network via cadherin –mediated cell-cell adhesion. It has been proposed that cadherins may mediate synapse specificity by localizing to specific synapse subclasses. In the hippocampus, immunolabelling for N-cadherin and E-cadherin revealed that their synaptic distribution is mutually exclusive. Furthermore, electron microscopy (EM) analysis identified other synaptic junctions that were not labelled for N- or E-cadherin (A. M.

Fannon and D. R. Colman, 1996). Other studies have also provided evidence that cadherins mediate synapse specificity. Benson and Tanaka (1998) demonstrated that the synaptic distribution of N-cadherin changes over the course of synaptic development. N-cadherin is initially present at both glutamatergic and GABAergic synapses (7 DIV) and becomes restricted to glutamatergic synapses as synapses mature (17 DIV) in hippocampal cultures (D. L. Benson and H. Tanaka, 1998). Moreover, immunolabeling for β-catenin demonstrated that β-catenin remains present at both glutamatergic and GABAergic synapses thus suggesting that other forms of cadherins are present at synapses absent of N-cadherin (D. L. Benson and H. Tanaka, 1998). However, to date no cadherins have been identified at mature GABAergic synapses. Recent studies have suggested that the cadherin isoforms, cadherin-11 and cadherin-13 may potentially localize to GABAergic synapses because they have been shown to promote the formation of GABAergic synapses in cultured hippocampal neurons (S. Paradis et al., 2007). These findings all provide supportive evidence that different cadherins localize to different synapse subclasses. This differential synaptic localization may reflect how cadherins can mediate synapse specificity.

N-cadherin and synapse formation

Neuronal cadherin (N-cadherin) is the most widely distributed cadherin in the CNS and has been well characterized in neurons (Arikkath, 2008). It is a type I classical cadherin and is found at both pre- and postsynaptic terminals of synapses (Fannon, 1996). Findings from several studies have provided suggestive evidence that cadherins function as regulators of synapse formation. N-cadherin is present in vesicle-like clusters prior to synaptogenesis and some of these clusters have been shown to correspond to dense-core vesicles that are known to transport essential components to nascent synapses (M. Shapira et al., 2003). Therefore, cadherins are thought to be one of the first synaptic proteins that are delivered to nascent synapses. In support of this theory, both *in vitro* and *in vivo* studies have demonstrated that N-

cadherin is present early at nascent synapses (O. Bozdagi et al., 2004; J. D. Jontes et al., 2004). *In vitro* time-lapse studies, have shown that N-cadherin is initially present at all immature synapses at both the pre-and postsynaptic membranes (D. L. Benson and H. Tanaka, 1998). *In vivo,* N-cadherin has been shown to be transported with active zone components to sites of nascent synaptic contact in Rohan-Beard neurons (J. D. Jontes et al., 2004).

Several studies involving alterations of cadherin expression have also provided suggestive evidence that cadherins can regulate synapse formation and morphology. When hippocampal neurons express a dominant negative cadherin that disrupts cadherin-cadherin intercellular interactions, a decrease in synapse number is observed, as well as impairment in synapse function (Togashi, 2002). Furthermore, dendritic spines become filopodia-like processes or bifurcated spine heads instead of mushroom shaped-spines that are characteristic of wildtype neurons (H. Togashi et al., 2002). Knock down of N-cadherin expression levels using RNA interference (RNAi) technology has also been shown to reduce the number of spines by approximately 50% (Saglietti, 2007). A loss in spine number and a reduction in mEPSC frequency is also observed when AHACD, a peptide that disrupts the function of N-cadherin is applied to hippocampal neurons (Mysore, 2008). N-cadherin may also be necessary for synaptic assembly. Interference of cadherin 6B function with antibodies results in the disruption of normal PSD-95 distribution in retinal neurons (Y. Honjo et al., 2000). Depletion of catenins that are members of the cadherin-catenin complex also exhibit changes in synapse phenotype and density. Loss of β-catenin expression results in a mislocalization of the synaptic vesicles along the axon and a decrease in the number of synaptic reserve pool vesicles (S. X. Bamji et al., 2003). Also, p120-catenin and δ-catenin loss of function studies result in a decrease in synapse density (J. Piedra et al., 2003; P. Cerruti Mainardi, 2006). These findings taken together therefore suggest the importance of the cadherin adhesion complex in synapse formation. Interestingly, although several studies have shown how a disruption of N-cadherin

function can alter synapse formation, N-cadherin overexpression *in vitro* does not appear sufficient to induce synapse formation (Sara, 2005) nor is it sufficient to recruit postsynaptic elements such as PSD-95 (Nam and Chen 2005). N-cadherin's inability to induce synapse formation might be isoform specific, as other recent reports have suggested other cadherin forms to promote synapse formation (S. Paradis et al., 2007).

Other studies have proposed that N-cadherin is more important for the initial adhesion and stabilization of young synapses and are dispensable in mature synapses. Although Togashi et al (2002) observed a significant reduction in synapse number following disruption of cadherin-cadherin interactions, they also observed a less dramatic loss of presynaptic terminals in older hippocampal neurons (21 DIV) in comparison to younger neurons (8 DIV) (H. Togashi et al., 2002). A separate study further investigated this phenomenon, by also perturbing intercellular cadherin-cadherin interactions of hippocampal neurons at early stages of synapse formation (0-5 DIV) and examining the neurons at an age when synapses are more mature (10-20 DIV)(O. Bozdagi et al., 2004). When synapse density was measured, they found that the synapse density of mutant neurons to be comparable to wildtype neurons (O. Bozdagi et al., 2004). Therefore, it is possible that the importance of N-cadherin in synaptogenesis peaks at the initial adhesion stage and decreases as synapses mature where other alternative mechanisms can come into play to maintain mature synapses.

Cadherins and plasticity

Several studies have suggested that classical cadherins regulate synaptic plasticity by controlling pre- and postsynaptic function and by synaptic adhesion. Chimeric cultures with N-cadherin knockout neurons from embryonic stem cells acting as postsynaptic cells and wildtype neocortical neurons as presynaptic cells have demonstrated that the absence of N-cadherin

results in an impairment in presynaptic vesicle exocytosis and recycling (Jungling, Gottman 2006). Postsynaptically, neurons from hippocampal cultures that expressed an extracellular domain lacking dominant-negative form of N-cadherin recorded reduced mEPSC amplitudes (T. Okuda et al., 2007).

The calcium concentration at the mammalian synapse allows for both cis- and trans-cadherin interactions to occur, thus it is hypothesized that Ca^{2+} is dynamically regulated in the synaptic cleft that in turn modulate cadherin-cadherin based adhesion and signalling (Heupel, Baumgartner, Golenhofen 2008). Studies involving calcium ion treatment, have demonstrated that high concentrations of calcium rigidify and dimerize cadherin molecules. This dimerization in turn generates a strong adhesive state that is resistant to proteolytic degradation (Tanaka, Shan, Colman 2000). Furthermore, cadherin mediated adhesion has been shown to respond to changes in synaptic activity. Tanaka et al. reported that synaptic activity promotes the dimerization of N-cadherin, which results in strengthened adhesion across the synapse (Tanaka 2000). Also, induction of L-LTP in hippocampal slices increases the number of synapses that contain N-cadherin suggesting that N-cadherin is necessary to hold synaptic contacts and establish L-LTP (O. Bozdagi et al., 2000). Studies involving the impairment of cadherin function also support the correlation between cadherin and synaptic plasticity. When a dominant-negative cadherin construct that disrupts intercellular cadherin interactions is expressed in neurons, a decrease in synapse size, synaptic vesicle recycling and synaptic transmission is observed (O. Bozdagi et al., 2004). Similarly, interference of N- or E-cadherin function by antibodies directed against the extracellular domain of cadherin inhibits LTP in hippocampal slice cultures (Tang 1998). *In vivo,* LTP was also altered in hippocampal neurons of cadherin-11-knockout mice (Manabe 2000). Also, long-term memory was impaired when N-cadherin function disrupting peptides were introduced into the dorsohippocampal area of mice (C. Schrick et al., 2007).

Cadherins have also been suggested to mediate synaptic plasticity by interacting with other synaptic membrane proteins such as AMPA receptors (AMPARs). AMPARs are glutamate receptors that mediate fast excitatory synaptic transmission in the CNS and the overexpression of the AMPAR subunit GluR2 has been shown to promote spine growth (M. Passafaro et al., 2003; C. Y. Tai et al., 2008). Biochemically, both the N-terminal domain of AMPA receptors and AMPA receptor subunit GluR2 can interact with N-cadherin. Furthermore, N-cadherin was shown to recruit GluR2 in hippocampal neurons suggesting that N-cadherin and AMPARs may work together to regulate synapse development (L. Saglietti et al., 2007). Cadherin-catenin interactions may also regulate synaptic plasticity. Depolarization of neurons results in an enrichment of β-catenin in dendritic spines (S. Murase et al., 2002). Also a loss of αN-catenin results in long term memory defects and neurons from δ-catenin knockout mice exhibit deficits in CA1 LTP (C. Park et al., 2002; I. Israely et al., 2004).

NEUROLIGINS

History and complex

In addition to cadherins, the neuroligin-neurexin adhesion complex has also been characterized as an important regulator in synaptogenesis. Neuroligins were discovered as binding partners of neurexins by affinity chromatography on immobilized β-neurexin (K. Ichtchenko et al., 1995). Since their discovery, various isoforms of neuroligins have been identified in chicken, rodents (NL1-NL3), and in humans (NL1-NL5) (Dean 2006). In neurons, neuroligins are postsynaptic transmembrane proteins that make trans-synaptic heterophilic interactions with their presynaptic partner neurexins via an esterase-like domain located on the extracellular domain (Dalva 2007). This esterase-like domain is homologous to acetylcholinesterase (AchE) but lacks cholinesterase activity (Kang and Craig, 2007) and contains one to two sites of alternative splicing (domain A and B). These alternative splicing sites can generate various neuroligin isoforms that bind either α- or β-neurexins (Fig 2). In addition to binding neurexins, this esterase-like domain has been shown to mediate neuroligin oligomerization that in turn is thought to control neuroligin-neurexin binding (C. Dean et al., 2003). Proximal to the transmembrane domain lie five potential N-glycosylation sites and a Ser-Thr-rich domain that allows O-linked glycosylation (K. Ichtchenko et al., 1995). This glycosylation has been shown by several studies to be important in modulating the affinity of neuroligin – β-neurexin interactions (Y. A. Ushkaryov et al., 1994; K. Ichtchenko et al., 1995). Neuroligin- neurexin binding is also dependent upon other factors such as the presence of calcium ions, alternative splicing of the extracellular domain of both proteins, and the dimerization of neuroligins. A single-pass transmembrane region connects the extracellular domain to a C-terminal cytoplasmic tail (K. Ichtchenko et al., 1995).

Cytoplasmic partners of neuroligins

The cytoplasmic tail of neuroligins is highly conserved among neuroligin members and can bind several PDZ proteins via a PDZ binding motif (M. Irie et al., 1997; G. Meyer et al., 2004). Neuroligins are present at both glutamatergic and GABAergic synapses (J. N. Levinson and A. El-Husseini, 2005). At glutmatergic synapses in neurons, neuroligins bind to PSD-95, a postsynaptic scaffold molecule that has been shown to assemble postsynaptic machinery by clustering protein complexes including ion channels, receptors (i.e. NMDAR) and other scaffold proteins such as GKAP (Fig 1A) (M. F. Lise and A. El-Husseini, 2006). At GABAergic synapses, the neuroligin isoform NL2 binds to the E-domain of gephyrin via a gephyrin binding (GB) domain on the cytoplasmic tail and can recruit gephyrin to synapses (Fig 1B) (E. R. Graf et al., 2004). A recent study has demonstrated that NL2 also activates collibystin, a scaffold molecule that is tightly associated with gephyrin and also suggested to cluster $GABA_A$ receptors to synapses (A. Poulopoulos et al., 2009).

Localization in the nervous system

Neuroligins are predominantly expressed in the nervous system. Furthermore, *in situ* hybridization analysis in rat tissue has shown that all three neuroligin isoforms NL1-3 are expressed in the brain (J. Y. Kwon et al., 2004). NL1 is expressed exclusively in CNS neurons and predominantly localizes to glutamatergic synapses (J. Y. Song et al., 1999). NL2 is also expressed in the CNS, however in contrast to NL1, it has been shown to be present primarily at GABAergic synapses (F. Varoqueaux et al., 2004). NL2 is also present outside the CNS such as in the pancreas, lung, endothelia, uterus and colon (A. T. Suckow et al., 2008). However the functional role of NL2 at these areas is unclear (A. T. Suckow et al., 2008). In contrast to NL1 and NL2, NL3 is present at both glutamatergic and GABAergic synapses (E. C. Budreck and P. Scheiffele, 2007). Non-neuronal cells also express neuroligins. Glial cells, such as immature astrocytes, Schwann cells, and olfactory ensheathing glia express NL3 in developing mice and

rats (M. Gilbert et al., 2001). In humans, three different NL3 isoforms are also expressed in the heart, skeletal muscle, placenta and pancreas (R. A. Philibert et al., 2000). Finally, NL4 is expressed in similar tissues as the NL3 isoforms as well as in the brain, and liver (M. F. Bolliger et al., 2001).

Neuroligin function

Like cadherins, neuroligins function both as cell adhesion molecules and as signalling molecules in synapse formation. The adhesive properties of neuroligins have been demonstrated by cell aggregation studies that have shown that cells expressing β-neurexins only aggregate in the presence of cells that express NL 1 (T. Nguyen and T. C. Sudhof, 1997). In neurons, this neuroligin-neurexin interaction is also thought to mediate synapse specificity via alternative splicing of the extracellular esterase-like domain of neuroligins (Dalva 2007). Neuroligins are thought to also mediate synapse specificity by localizing to synapse subclasses. As mentioned previously, NL1 has been shown to primarily localize to glutamatergic synapses (Song 1999) and NL2 has been shown to predominantly localize to GABAergic synapses (Varoqueax 2004). Neuroligins in association with scaffold proteins have also been proposed to modulate the balance between glutamatergic and GABAergic synapses. When PSD-95 is overexpressed, the distribution of NL2 is shifted from GABAergic to glutamatergic synapses (J. N. Levinson et al., 2005). Corresponding with this observation, electrophysiological studies report an increase in the ratio of excitatory to inhibitory synaptic currents in PSD-95 overexpressing cells (J. N. Levinson et al., 2005). Similarly, when PSD-95 is knocked down using RNAi, there is an increase in VGAT clusters that contain NL1 that may indicate a shift of NL1 distribution from glutmatergic to GABAergic synapses (K. Gerrow et al., 2006). Therefore, neuroligins are not only important in determining synapse specificity but may also maintain inhibitory/excitatory balance that is essential for proper neural networking. Mutations in the

genes encoding NL3 and NL4 of the X chromosome and NL4Y of the Y chromosome have been linked to cases of autism, a neurological disorder that is correlated to an imbalance of the GABAergic/glutamatergic synapse ratio (J. L. Rubenstein and M. M. Merzenich, 2003; J. B. Vincent et al., 2004).

Neuroligins and synapse formation

The strong correlation between neuroligins and synapse formation were initially sparked by a study in 2000, where Scheiffele and his colleagues demonstrated that ectopic neuroligin expression from non-neuronal cells could induce the formation of presynaptic structures in contacting axons (P. Scheiffele et al., 2000). This finding has since led to many studies examining neuroligins' role as synaptogenic molecules. Similar to coculture studies, overexpression of three neuroligin isoforms (NL1, NL2, NL3) in primary hippocampal cultures have been shown to enhance synapse formation (Chih, 2005) of both glutamatergic and GABAergic synapses. Interestingly, NL2 overexpression enhanced GABAergic synapse formation more strongly than NL1 or NL3 overexpression which may reflect the preferential localization of NL2 to GABAergic synapses (B. Chih et al., 2005). Furthermore, RNAi-mediated reduction of NL1, NL2, or NL3 expression results in an overall decrease in synapse number (Chih 2005). Although these neuroligin expression studies have shown that neuroligin overexpression dramatically enhances synapse formation, the mechanisms that explain the enhanced synapse number phenotype are unclear. Some have proposed that transsynaptic signalling of neuroligins with neurexins is important (B. Chih et al., 2006). Others have shown that neuroligins and the postsynaptic scaffold molecule PSD-95 can recruit each other to synapses, thus suggesting that cross-talk between neuroligins and other postsynaptic proteins regulate synapse formation (C. Dean and T. Dresbach, 2006). Despite not having yet

uncovered a signalling mechanism, both overexpression and depletion of neuroligin studies *in vitro* demonstrate that neuroligin expression influences synapse formation.

Interestingly, results from *in vivo* studies of neuroligin knockout mice do not agree with *in vitro* study observations. Although neuroligin knockdown experiments demonstrate a reduction in synapse number *in vitro*, surprisingly, NL1-3 triple knockout mice do not exhibit any in change synapse density in the hippocampus (Varoqueaux 2006). The absence of a phenotype *in vivo* has been suggested to reflect the ability of other synaptic proteins to functionally compensate for the loss of neuroligins *in vivo* that may not be observed when neuroligin expression is acutely reduced in RNAi experiments done *in vitro*.

Neuroligins are also thought to control synapse specificity because different neuroligin isoforms are present at different synapse subclasses. However, the complete mechanisms by which neuroligins are targeted to synapses have not been elucidated. In 2006, Chih et al demonstrated that alternative splicing of the extracellular domain regions A and B of neuroligins controls the localization of neuroligins to glutamatergic and GABAergic synapses (B. Chih et al., 2006). This finding suggests that alternative splicing of the extracellular domain of neuroligins and neuroligin-neurexin interactions mediate proper neuroligin localization and synapse specificity. It has also been suggested that cross-talk between neuroligins and post synaptic scaffold proteins control synaptic localization of neuroligins. For example, PSD-95 overexpression restricts NL1 to glutamatergic synapses and can also recruit NL2 to glutamatergic synapses (J. N. Levinson and A. El-Husseini, 2005). Therefore, there is evidence that support both hypotheses that the extracellular domain and that the intracellular domain is important for proper localization of neuroligins to synapses. However, whether one is more important than the other for synaptic localization remains unclear.

Neuroligins and plasticity

Electrophysiological studies conducted in both *in vitro* and *in vivo* systems have suggested that neuroligins can mediate synapse plasticity. *In vivo,* NL1-3 triple knockout mice exhibit impairment in synaptic function (F. Varoqueaux et al., 2006). In these animals, a significant reduction in GABA-mediated and glutamatergic synaptic transmission is observed and further analysis of levels of synaptic proteins showed that these mice also had lower levels of synaptic vesicle proteins, thus suggesting that the absence of neuroligins causes presynaptic dysfunction (F. Varoqueaux et al., 2006). In other studies, silencing NL1 expression using virus-mediated RNAi in the amygdala resulted in impaired LTP and also demonstrated that NL1 is necessary for the storage of associative fear memory (J. Kim et al., 2008). Results from neuroligin overexpression studies have also provided supportive evidence that neuroligins are important in synaptic plasticity. Chubykin (2007) reported that NL1 overexpression leads to an increase in excitatory synaptic transmission and that NL1 and NL2 overexpression specifically enhanced excitatory and inhibitory transmission respectively. Furthermore, mEPSC amplitude has also been reported to increase at synapses overexpessing NL-1 (O. Prange et al., 2004). However, this was only observed when cells were coexpressing PSD-95, suggesting that neuroligins may control synapse plasticity by working with other postsynaptic molecules such as PSD-95 (O. Prange et al., 2004). Behavioural studies of NL1 overexpressing mice showed deficits in memory acquisition, a shift in synaptic activity towards increased excitation and an impairment in LTP (R. Dahlhaus et al., 2009). Thus both loss and gain of function studies *in vitro* and *in vivo,* indicate that neuroligins act as mediators of synaptic plasticity.

RATIONALE

Cadherins and neuroligins have both been shown to play critical roles in the early stages of synapse establishment and have been suggested to promote presynaptic assembly and to localize synaptic vesicles (P. Scheiffele et al., 2000; S. X. Bamji et al., 2003; O. Bozdagi et al., 2004). Although cadherins and neuroligins have been very well studied independently, to date no one has investigated their spatial or functional relationship to one another. It is still unknown whether some synapses express only cadherins and others only neuroligins or if synapses express both. Their functional relationship to one another has also yet to be investigated. Are cadherins and neuroligins acting in concert in a common pathway or are they acting independently in parallel pathways?

Past studies have suggested a possible physical interaction between cadherins and neuroligins. In 2002, Nishimura et al. demonstrated that Synaptic Scaffolding Molecule (S-SCAM), is recruited to synapses via interactions with cadherin's cytoplasmic binding partner, β-catenin (Nishimura, 2002). Following this finding, Iida et al. presented that the cytoplasmic domain of neuroligin binds to the PDZ and WW domain of S-SCAM, and that S-SCAM can recruit NL-1 (J. Iida et al., 2004) and NL-2 (K. Sumita et al., 2007) to synapses. The findings of these two studies taken together suggest a possible S-SCAM mediated link between cadherins and neuroligins.

OVERALL AIM

This thesis will attempt to investigate the spatial and functional relationship between cadherins and neuroligins. The spatial relationship will be investigated by examining distribution of neuroligin and cadherin isoforms at GABAergic and/or glutamatergic synapses. The functional relationship will be studied by knockdown and overexpression studies of neuroligin and cadherin isoforms. Results of the present study will help us understand whether different CAM families work together to regulate proper synapse development.

CHAPTER II: MATERIALS AND METHODS

siRNA AND OVEREXPRESSION CONSTRUCTS

N-cadherin siRNA (Dharmacon Inc., cat# J-091851-09-0019), and a previously used NL1 siRNA (B. Chih et al., 2005) were transfected into rat hippocampal neurons to suppress expression of endogenous N-cadherin and NL1, respectively. The siRNA-resistant N-cadherin-CFP construct was made by using site-directed mutagenesis (Stratagene) to introduce five silent point mutations into the N-cadherin coding sequence. The following primer was used: gctggtctggaccgagagaaaGTCCAGCAATACACCTTAAtaattcaagccactgacatg. The siRNA-resistant HA-NL1 construct was made in a similar way, using the primer, ccatggcggctcttacatGGAGGGAACAGGTAATCTGTatgatgggagtgtc.
ON-TARGET *plus* non-targeting siRNA that is designed not to target any known gene in the cell was used as a control (Dharmacon Inc., cat# J-091851-09-0019).

CELL CULTURES

Primary hippocampal cultures were prepared by removing whole hippocampi from E18 Sprague Dawley rats. Hippocampi were rinsed 3x in Hank's Ca^{2+}- Mg^{2+}-free, phenol-red-containing BSS (HBSS) (Invitrogen Canada Inc., Burlington, ON), incubated for 20 minutes at 37°C in 0.25% trypsin (Worthington, Lakewood, NJ), and then rinsed another 3 times in HBSS. Tissue was dissociated by repeated trituration with a p200 pippetteman. Dissociated cells were plated onto 18mm borsilicate glass coverslips of a 12 well dish at a density of 75,000 cells per well. Glass coverslips were pre-treated by soaking them in concentrated nitric acid overnight, followed by sterilization with dry heat (>225°C) overnight, exposing them to UV light for 30 minutes, and finally by coating them in 600ul of 0.5mg/ml borate butter in poly-L-lysine overnight.

Dissociated cells were first plated into 1 ml of plating media [10% heat inactivated horse serum (Gibco),86.55% MEM supplemented with BSS (Invitrogen Canada Inc., Burlington, ON), 0.45% glucose, 1% sodium pyruvate (Invitrogen Canada Inc., Burlington, ON), 1% GlutaMax (Invitrogen), 1% streptomycin/penicillin (Invitrogen Canada Inc., Burlington, ON)]. 4 hours after plating, the plating media was aspirated off and replaced with 2 ml per well of maintenance media [2% B27 supplement (Invitrogen Canada Inc., Burlington, ON), 1% GlutaMax (Invitrogen Canada Inc., Burlington, ON), 1% streptomycin/penicillin (Invitrogen Canada Inc., Burlington, ON) and 96% Neurobasal medium (Gibco)]. Cell cultures were maintained at 37°C in 5% CO_2 in a Hepa Class 100 incubator. Maintenance media was changed twice a week by replacing half the old media for fresh maintenance media per well.

IMMUNOCYTOCHEMISTRY

Synapse Density

14 DIV primary rat hippocampal cultures were fixed for 2 minutes with 4%sucrose/4% paraformaldehyde. The fixative was aspirated off and then replaced with ice cold 100% methanol and kept at -20°C for 7 minutes. Methanol was aspirated off and cultures were permeabilized for 10 minutes in 0.1% Triton-X in PBS, blocked for 1 hour in 10% goat serum to reduce nonspecific staining. Following blocking, coverslips were incubated with primary antibodies at 4°C overnight in humidification chambers to prevent evaporation. Primary antibodies were diluted in 1% goat serum. The presynaptic glutamatergic marker VGluT-1 was detected using a guinea pig anti-VGluT-1 (1:1000 dilution) (Synaptic Systems, Goettingen, Germany) and the postsynaptic glutamatergic marker PSD-95 was detected using a mouse anti-post-synaptic-density-95 (PSD-95) (1:500) (Affinity BioReagents, Golden, CO). Cells were washed with three exchanges of PBS at 10 minute intervals and incubated for 1 hour with secondary antibodies at room temperature. Synaptic markers were visualized using donkey

anti guinea pig Cy5 (1:300 dilution) (Jackson Immuno Research, West Grove, PA) and goat anti mouse Texas Red-X (1:250 dilution) (Molecular Probes, Eugene, OR) secondary antibodies diluted in 1% goat serum. Cells were subsequently washed with PBS 3 times at 10 minute intervals, and were mounted by applying Prolong mounting reagent (Invitrogen Canada Inc., Burlington, ON) onto glass slides and inverting the coverslips onto the slides. Slides were sealed using generic nail polish. All slides were stored at 4°C in dark boxes when not in use to prevent fading of fluorescence.

VGluT-1 and NL2 localization upon coexpression of N-cadherin-CFP and NL1 siRNA

10 DIV cells were cotransfected with GFP and control siRNA, N-cadherin-CFP, NL1 siRNA or N-cadherin-CFP plus NL1 siRNA. At 14 DIV, cultures were fixed with ice cold 100% methanol at -20°C for 10 minutes, permeabilized for 10 minutes in 0.01% Triton-X in PBS, and blocked for 1 hour in 10% goat serum. Following blocking, coverslips were incubated with primary antibodies at 4°C overnight in humidification chambers to prevent evaporation. Primary antibodies were diluted in 1% goat serum. The presynaptic glutamatergic marker VGluT-1 was detected using a guinea pig anti-VGluT-1 (1:1000 dilution) (Synaptic Systems, Goettingen, Germany) and NL2 was detected using a rabbit anti-NL2 (1:500) (generous gift from Dr. Ann Marie Craig, UBC). Cells were visualized using goat anti-rabbit Texas-red (1:250 dilution) (Molecular Probes, Eugene, OR) and donkey anti-guinea pig Cy5 (1:300 dilution) (Jackson Immuno Research, West Grove, PA). Cells were mounted and stored as described above.

N-cadherin and NL1 localization

14 DIV primary rat hippocampal cultures were fixed for 2 minutes with 4%sucrose/4% paraformaldehyde. The fixative was aspirated off and then replaced with ice cold 100% methanol and kept at -20°C for 7 minutes. Methanol was aspirated off and cultures were

permeabilized for 10 minutes in 0.01% Triton-X in PBS, blocked for 1 hour in 10% goat serum to reduce nonspecific staining. All antibodies were diluted in 1% goat serum. The presynaptic glutamatergic marker VGluT-1 was detected using a guinea pig anti-VGluT-1 (1:1000 dilution) (Synaptic Systems, Goettingen, Germany) and N-cadherin was detected using a rabbit anti-N-cadherin (1:300 dilution) (generous gift from Dr. David Coleman, McGill University). Cells were visualized using goat anti-rabbit Texas-red (1:250 dilution) (Molecular Probes, Eugene, OR) and donkey anti-guinea pig Cy5 (1:300 dilution) (Jackson Immuno Research, West Grove, PA). Cells were mounted and stored as described above.

N-cadherin and NL2 localization

At 14 DIV, cultures were fixed with ice cold 100% methanol at -20°C for 10 minutes, permeabilized for 10 minutes in 0.01% Triton-X in PBS, and blocked for 1 hour in 10% goat serum. All antibodies were diluted in 1% goat serum. The presynaptic glutamatergic marker VGluT-1 was detected using a guinea pig anti-VGluT-1 (1:1000 dilution) and the presynaptic GABAergic marker VGAT was detected using a guinea pig anti-VGAT (1:2000 dilution) (Synaptic Systems, Goettingen, Germany). N-cadherin was detected using a mouse anti-N-cadherin (1:300) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and NL2 was detected using a rabbit anti-NL2 (1:500) (generous gift from Dr. Ann Marie Craig, UBC). Cells were visualized using goat anti-rabbit Alexa 488 (1:1000 dilution), anti-mouse Texas-red (1:250 dilution) (Molecular Probes, Eugene, OR) and donkey anti-guinea pig Cy5 (1:300 dilution) (Jackson Immuno Research, West Grove, PA). Cells were mounted and stored as described above.

TRANSFECTION

Transfection solutions were prepared by incubating 0.7 ug of GFP, N-cad-CFP or HA-NL1 DNA plus 1 ug of the oligo siRNA with 0.8 ul of Lipofectamine 2000 (Invitrogen Canada Inc., Burlington, ON) in 50 ul of Opti-MEM transfection media (Invitrogen Canada Inc.,

Burlington, ON) for 20 minutes to allow DNA-containing liposomes to form. After the incubation period, the transfection solution was added to 10 DIV cultures in a drop wise motion and then gently shaken to evenly distribute the transfection media within the maintenance media.

For N-cadherin/NL1 localization, 0.35 ug of GFP-NL1 was transfected into 10 DIV cultures with the transfection method described above.

WESTERN ANALYSIS

Human embryonic Kidney (HEK) 293 cell lines were maintained with Dulbecco's modified Eagle's medium with 10% FBS and 1% streptomycin and penicillin. 24 hours after passage or at ~60% confluence, cells were transfected using Lipofectamine 2000 (Invitrogen Canada Inc., Burlington, ON). Cells were then harvested and lysed in lysis buffer containing 0.5% NP40, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10% glycerol, 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 1 Complete protease inhibitor mixture tablet per 7 ml (Roche Applied Science) 24 hours after transfection. Proteins were separated and visualized as previously described (Levinson et al., 2005). Primary antibodies: mouse anti-HA (1:1000, Babco), rabbit anti-GFP (1:1000, Synaptic Systems). HRP-conjugated goat anti-mouse or rabbit secondary antibodies (1:3000, Biorad) were used. Blots were visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

CONFOCAL IMAGING AND IMAGE ANALYSIS

Transfected hippocampal neurons were imaged using an Olympus Fluoview 1000 confocal microscope (10X/0.30 UPlan FL N; 20X/0.75 UPlan SApo; 60X/1.4 Oil Plan-Apochromat). All images in a given experiment were captured with a single Z-slice and analyzed with the same exposure time and conditions.

IMAGE ANALYSIS AND QUANTIFICATION

***Neuronal masks**:* To examine the density of synapses along a single, transfected neuron, a 'mask' was made of the GFP fluorescence by ImageJ image analysis program. The GFP cells were thresholded until the all neurites were highlighted in their entirety. Using the 'selection' application, an outline of the highlighted neurites was generated. Cell bodies and any background noise were eliminated by deselecting those areas until a representative mask that only outlined the neurites of the transfected cell was achieved.

***Co-localization analyses*:** Images were analyzed using ImageJ with co-localization plugins downloaded from the program's website (http://grove.ufl.edu/~ksamn2/plugins.html#COLOC, http://rsb.info.nih.gov/ij/plugins/colocalization.html). Thresholded puncta were obtained by subtracting background immunofluorescence signal of each analyzed image. Background-subtracted immunofluorescence clusters for all imaging channels were correlated for overlapping signal. Co-localization of immunopositive puncta was done by measuring the frequency of overlapping puncta in two channels. Points of co-localization were defined as regions greater than 3 pixels in size where the intensity ratio of the two channels was greater than 50. All the puncta were examined in a field.

CHAPTER III: RESULTS

FUNCTIONAL RELATIONSHIP OF N-CADHERIN AND NL1

To investigate the functional relationship between N-cadherin and NL1, overexpression or knockdown studies or a combination of both were done to examine their effects on synapse density. Primary hippocampal neurons were transfected at 10 DIV with a previously described NL1 RNAi (B. Chih et al., 2005), an N-cadherin RNAi, or constructs expressing epitope-tagged N-cadherin or NL1. At 14 DIV, transfected neurons were fixed and immunolabeled for the glutamatergic presynaptic marker, VGluT-1, and the glutamatergic postsynaptic marker, PSD95. A synapse was defined as point of colocalization between a VGluT-1 and PSD95 positive cluster. A mask of the transfected cell was made by making an outline of the transfected neuron. Synapse density of the transfected cell was measured by counting the number of points of colocalization between VGluT-1 and PSD95 positive clusters along the length of the neurites within the mask. Control neurons were cotransfected with GFP and a pool of nonspecific control siRNA. All measurement values were normalized to control siRNA expressing cells. Oligo siRNAs were used to knockdown the expression of N-cadherin and NL1 and were validated by western blot as described below.

RNAi and construct validation

To validate N-cadherin and NL1 specific siRNAs, HEK cells were cotransfected with N-cadherin-CFP plus N-cadherin siRNA or HA-NL1 plus NL1 siRNA. Western analysis showed that N-cadherin siRNA knocked-down N-cadherin-CFP expression but could not knockdown E-cadherin-CFP expression, thus demonstrating that the siRNA is specifically targeted to N-cadherin and cannot knockdown the expression of E-cadherin isoform (Fig 3A). Western

Figure 3

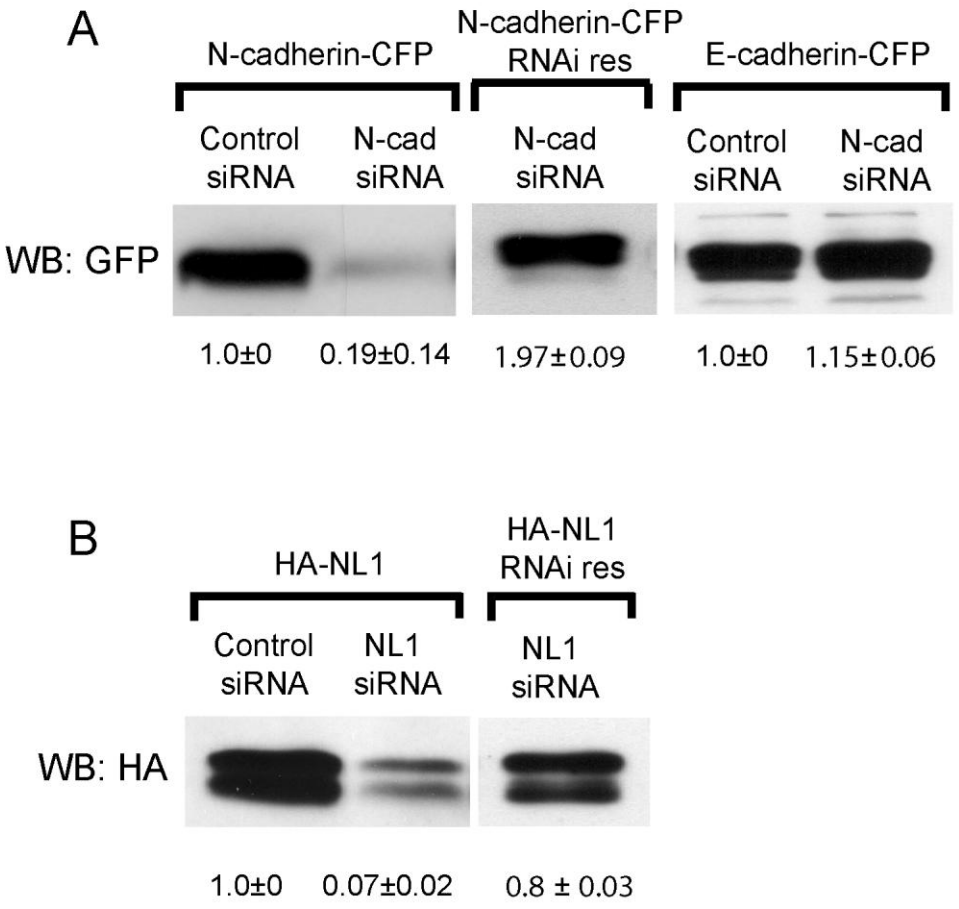

Figure 3. Knockdown of N-cadherin and NL1 using siRNA. **(A)** Anti-GFP antibody was used to detect the levels of N-cad-CFP expression (~150 kDa). HEK293 cells transfected with N-cadherin siRNA plus wildtype N-cadherin-CFP display a significant decrease in N-cadherin-CFP levels compared to cells transfected with control, nonspecific siRNA plus wildtype N-cadherin-CFP. **(A)** Conversely, N-cadherin siRNA did not attenuate levels of siRNA-resistant N-cadherin-CFP. **(B)** Anti-HA antibody was used to detect the levels of HA-NL1 expression (~116 kDa). HEK293 cells transfected with NL1 siRNA plus wildtype HA-NL1 exhibit a significant decrease in HA-NL1 levels compared to cells transfected with control nonspecific siRNA plus wildtype HA-NL1. **(B)** Conversely, NL1 siRNA did not attenuate levels of siRNA-resistant HA-NL1. N=3 cultures and 3 blots.

analysis of HEK 293 cotransfected with NL1 siRNA and HA-NL1 NL1 showed that NL1 siRNA knocked-down HA-NL1 expression (Fig 3B).

Off-target effects are a common caveat that should be considered when using siRNAs for knockdown studies. Off-target effects arise when the siRNA pairs with and thus silences the expression of multiple genes at a time (C. J. Echeverri et al., 2006). As a result, it is possible that the observed phenotype is not due to the silencing of the target gene. To avoid this potential complication, rescue experiments were done to verify that the knockdown phenotypes observed in the present study were in fact caused by silencing N-cadherin or NL1 expression. To rescue the N-cadherin knockdown phenotype, cells were cotransfected with N-cadherin siRNA plus a siRNA insensitive N-cadherin expressing construct. The siRNA insensitive N-cadherin should be able to compensate for the loss of endogenous N-cadherin and thus reverse the siRNA knockdown effect. siRNA insensitive N-cadherin and NL1 overexpressing constructs were designed (by Joshua N Levinson, PhD candidate, UBC) and were validated by western analysis. HEK 293 cells expressing both N-cadherin siRNA and N-cad-CFP displayed a robust expression of N-cadherin, indicating that N-cad-CFP was indeed insensitive to the RNAi (Fig 3A). Similarly, cells expressing both NL1 siRNA and HA-NL1 strongly expressed NL1, thus indicating that HA-NL1 was also insensitive to the RNAi (Fig 3B).

Knockdown of N-cadherin or NL1 decreases synapse density

To examine the effects of N-cadherin and NL1 depletion on synapse density, neurons were transfected with N-cadherin siRNA, NL1 siRNA or both. N-cadherin siRNA expression significantly reduced the density of synapses by 50±5%, and NL1 siRNA expression reduced the density of synapses by 66±3% (Fig 4A, D). This decrease in synapse density is comparable to the approximate 50% decrease observed in previous experiments utlilizing an N-cad siRNA (L. Saglietti et al., 2007) and the approximate 60% decrease observed in previous

Figure 4

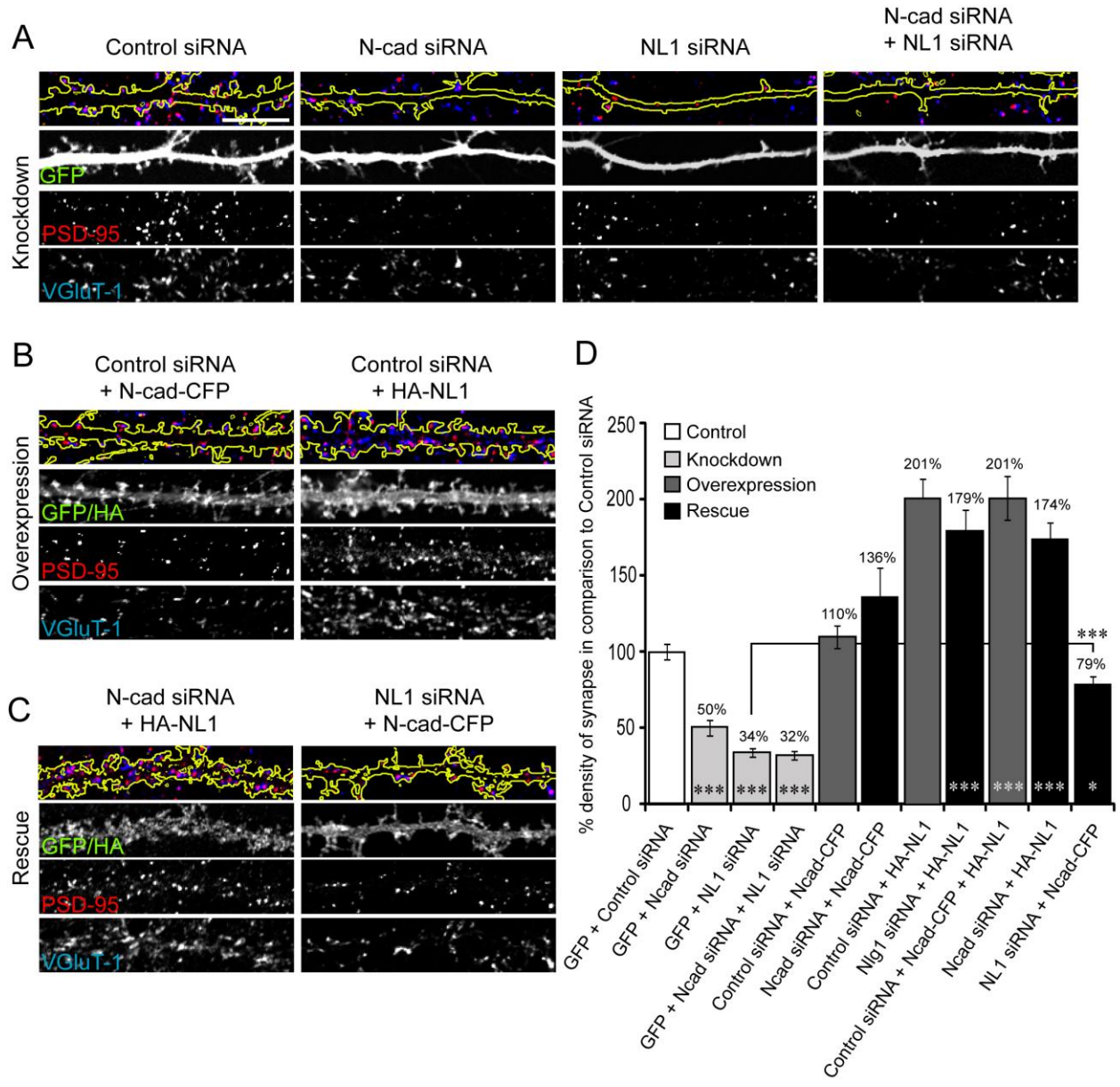

Figure 4. N-cadherin and NL1 functionally interact to regulate synapse formation. **(A,B,C)** Confocal images of 14 DIV neurons transfected at 10 DIV with the indicated constructs and immunolabeled with VGluT-1 and PSD-95. Top panels illustrate merged images of masks of GFP/HA transfected neurites (yellow outline) merged with VgluT-1 (blue) and PSD-95 (red) immunolabelling. **(A)** Confocal images of GFP control, N-cadherin siRNA and NL1 siRNA expressing cells. **(B)** Confocal images of N-cadherin and NL1 overexpressing cells. **(C)** Confocal images of N-cadherin and NL1 rescue cells. **(D)** Quantification of the density of synapses in transfected neurons normalized to cells transfected with control siRNA. Scale bar = 10µm; N = 640 µm of neurite per cell from 21-50 cells from 4 separate cultures; asterisk on bottom of each bar indicates significance with respect to control; *p<0.01,**p<0.005 ***p<0.001

experiments utilizing NL1 siRNA (B. Chih et al., 2005) thus validating our method of analysis. Interestingly, expression of both NL1 and N-cadherin siRNAs resulted in a similar deficit in synapse density compared to cells expressing NL1 siRNA alone (32±3% of control) (Fig 4A, D). It is possible that NL1 and N-cadherin promote the formation of synapses through a similar pathway. Indeed, if these two proteins mediated synapse formation using parallel pathways, or if different populations of synapses were being affected, one would expect an additive effect of the double knockdown. Thus the presented data suggest that the two adhesion molecules are acting in a similar pathway to regulate synapse formation.

Overexpression of NL1, but not N-cadherin induces the formation of supernumerary synapses

In addition to the N-cadherin and NL1 knockdown studies, the effects of N-cadherin or NL1 overexpression on synapse density was also examined. To study the effect of N-cadherin overexpression on synapse density, neurons were transfected with a CFP-tagged N-cadherin expressing construct. Overexpression of N-cadherin did not enhance synapse density compared to control neurons ($p= 0.28$ Student's T-test) (Fig 4B, D). This result supports findings of previous work that have shown that N-cadherin overexpression alone is insufficient to induce synapse formation (Y. Sara et al., 2005). To study the effect of NL1 overexpression on synapse density, neurons were transfected with an HA-tagged NL1 expressing construct. In contrast to N-cadherin overexpression, NL1 overexpression enhanced synapse density to 200±13% of control. This phenotype is in agreement with previous studies that have also shown that NL1 overexpression triggers the formation of synapses (B. Chih et al., 2005). Overexpression of both N-cadherin and NL1 resulted in a synapse density similar to that seen with NL1 alone, further demonstrating that overexpression of N-cadherin is insufficient to

promote the formation of supernumerary synapses (Fig. 4D). We define supernumerary synapses as those that are in excess of the number of synapses observed in control cells.

Rescue of knockdown in neurons

As a further control to confirm that the phenotype observed in cells expressing N-cadherin and NL1 siRNAs were specifically due to knockdown of these two targeted proteins, hippocampal cultures were cotransfected with N-cadherin/NL1 siRNA and RNAi insensitive N-cadherin/NL1 overexpressing constructs described above. Overexpression of N-cadherin in N-cadherin knockdown cells completely rescued synapse loss. Indeed, synapse density in cells expressing N-cadherin siRNA + N-cadherin-CFP were statistically similar to control cells ($p=0.7$; Student's T-Test) and to cells expressing N-cadherin alone ($p=0.2$; Student's T-test) (Fig 4D). Similarly, overexpression of HA-NL1 in NL1 knockdown cells rescued the deficit in the density of synapses and as a result, synapse density of NL1 siRNA + HA-NL1 expressing cells were statistically similar to cells expressing NL1 alone ($p=0.086$; Student's T-test). These data therefore demonstrate that the phenotypes of N-cad or NL1 siRNA expressing cells are not due to off-target effects.

NL1 rescues the synaptic phenotype of N-cadherin knockdown

Although knockdown of neuroligin expression *in vitro* reportedly results in a significant decrease in synapse density, NL 1-3 triple knockout mice surprisingly do not exhibit any change in synapse number (Varoqueaux, 2006). This difference of *in vitro* versus *in vivo* phenotypes has been hypothesized to be a result of functional compensation by other unknown synaptic molecules. However, a search for candidate molecules that may functionally compensate for neuroligin loss has never been investigated. Since double knockdown of N-cadherin and NL1 in

the present study indicated that these proteins are utilizing a similar pathway to promote synapse formation, we wanted to test the functional interaction between N-cadherin and NL1. Could N-cadherin and NL1 functionally compensate for one another to regulate synapse formation when the expression of one is reduced? To determine whether NL1 could rescue synapse loss following N-cadherin knockdown, cells were cotransfected with N-cadherin siRNA and HA-NL1. Overexpression of NL1 in an N-cadherin knockdown background significantly increased synapse density compared to cells expressing N-cadherin siRNA alone (Fig. 4A-D). This observation is most likely due to the ability of NL1 to induce supernumerary synapse formation. Furthermore, N-cadherin siRNA did not significantly reduce the synapse density observed following NL1 overexpression in comparison to NL1 expression alone ($p=0.13$ Student's T-test). These results thus further support the conclusion that N-cadherin and NL1 act in a common pathway to promote synapse formation, and suggest that NL1 can functionally compensate for N-cadherin.

N-cadherin partially rescues the synaptic phenotype of NL1 knockdown

To examine whether N-cadherin could rescue the NL1 knockdown phenotype, cells were cotransfected with NL1 siRNA and N-cadherin-CFP. Overexpression of N-cadherin in an NL1-knockdown background resulted in a significant increase in synapse density (79±5%) compared to NL1 knockdown cells (34±3%; $p=1.3x10^{-17}$ Student's T-test). However, N-cadherin overexpression in an NL1-knockdown background could not fully rescue the NL1 knockdown phenotype (Fig 4C,D). This finding therefore suggests that the overexpression N-cadherin can partially rescue the synaptic deficits observed in NL1 knockdown cells. These results taken together suggest that although N-cadherin overexpression is not sufficient to induce supernumerary synapse formation, it is able to at least partially rescue synapse loss observed following knockdown of NL1.

NL2 LOCALIZATION UPON NL1 KNOCKDOWN

In 2002, Nishimura et al. demonstrated that Synaptic Scaffolding Molecule (S-SCAM), can be recruited to synapses via N-cadherin's cytoplasmic partner, β-catenin (W. Nishimura et al., 2002). Following this study, Iida et al. reported that S-SCAM can recruit NL1 to synapses (J. Iida et al., 2004). The results of these studies taken together therefore suggested a physical interaction between N-cadherin and NL1 via S-SCAM. Interestingly, S-SCAM has also been shown to recruit NL2 to synapses suggesting that S-SCAM can localize different NL isoforms to synapses (K. Sumita et al., 2007). Although NL2 is often thought to be a GABAergic synapse-specific molecule, however, overexpression of the glutmatergic postsynaptic molecule PSD95 has been shown to shift NL2 localization from GABAergic to glutamatergic synapses (J. N. Levinson et al., 2005). This study therefore demonstrated that the distribution of NL2 is not necessarily fixed to GABAergic synapses. In the present study, a partial rescue was observed following N-cadherin overexpression in a NL1 knockdown background. It is possible that when N-cadherin is overexpressed, N-cadherin may also recruit NL2 to synapses to promote synapse formation.

To investigate whether N-cadherin overexpression enhances the localization of NL2 at glutamatergic synapses, 10 DIV primary rat hippocampal cultures were cotransfected with N-cadherin, NL1 siRNA, or both. Cells were fixed at 14 DIV and then immunolabeled using an anti-VGluT-1 to mark glutamatergic synapses and anti-NL2 (Fig 5A). The proportion of NL2-positive clusters that colocalized with VGluT-1 was similar in control and N-cadherin overexpressing cells suggesting that N-cadherin overexpression is not sufficient to recruit NL2 (Fig 5A). Despite this, overexpression of N-cadherin significantly enhanced the proportion of NL2-positive clusters that colocalized with VGluT-1 clusters in a NL1 knockdown background (Fig 5B). Taken together, these results suggest that N-cadherin can recruit NL2 in the absence of NL1 but not under wildtype conditions.

Figure 5

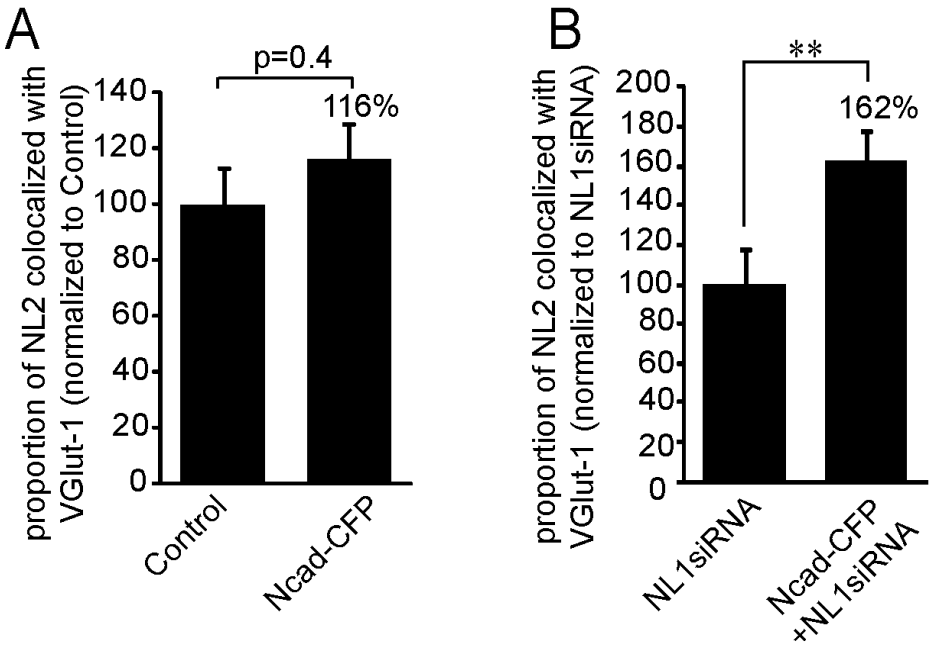

Figure 5. The proportion of NL2 at glutamatergic synapses is increased in NL1 knockdown cells overexpressing N-cadherin. (A) The proportion of NL2 associated with VGluT-1 is similar between controls cells and those overexpressing N-cadherin. **(B)** In NL1 knockdown cells, overexpression of N-cadherin significantly enhances the proportion of NL2 associated with VGluT-1. N = 840 µm of neurite per cell from 9-15 cells from 2 separate cultures.

SPATIAL LOCALIZATION OF N-CADHERIN, NL1 AND NL2

Spatial relationship of N-cadherin and NL1

N-cadherin and NL1 have been shown to preferentially localize to glutamatergic synapses (D. L. Benson and H. Tanaka, 1998; J. Y. Song et al., 1999). Although the synaptic distributions of N-cadherin and NL1 have previously been analyzed independently, their spatial distribution in relation to one another is still unclear. To examine the spatial distribution of N-cadherin and NL1 at glutamatergic synapses, 10 DIV primary rat hippocampal cultures were transfected with a GFP-tagged NL1 construct (as antibodies specific to NL1 were unavailable). The transfected cultures were then fixed at 14 DIV and the distribution of N-cadherin was examined by immunolabelling the cultures with an anti-N-cadherin antibody (Fig 6A). To study the distribution of NL1 and N-cadherin at glutmatergic synapses specifically, the fixed cultures were also immunolabeled for the glutmatergic synaptic marker VGluT-1.

Figure 6

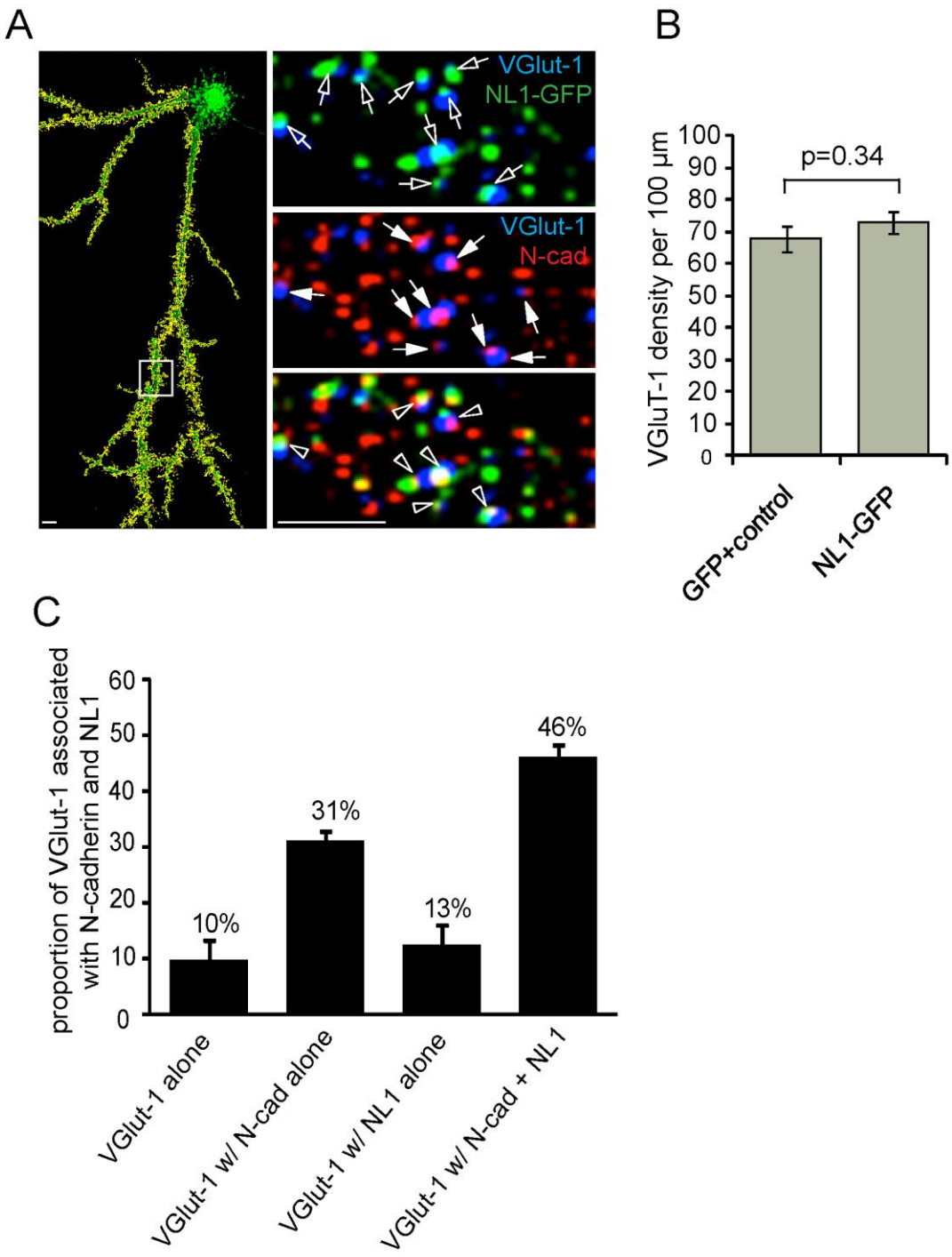
A
VGlut-1
NL1-GFP
VGlut-1
N-cad
B
VGluT-1 density per 100 µm
100
90
80
70
60
50
40
30
20
10
0
p=0.34
GFP+control
NL1-GFP
C
proportion of VGlut-1 associated with N-cadherin and NL1
60
50
40
30
20
10
0
10%
31%
13%
46%
VGlut-1 alone
VGlut-1 w/ N-cad alone
VGlut-1 w/ NL1 alone
VGlut-1 w/ N-cad + NL1

Figure 6. Spatial distribution of NL1 and N-cadherin at glutamatergic synapses.
(A) Confocal images of 14 DIV neurons transfected at 10 DIV with GFP-NL1 and immunolabeled with VGluT-1 and N-cadherin. Low magnification image (left) illustrates a neuron transfected with GFP-NL1 with a mask outlining the area of the cell analyzed (the cell body was manually removed from the mask). A higher magnification of the region of interest demonstrates the mask of the area (top left), GFP-NL1 following thresholding plus VGluT-1 (top right), VGluT-1 and N-cadherin (middle right) and merged image (bottom right). Points of GFP-NL1 colocalization with VGluT-1 are indicated by open arrows. Points of N-cadherin colocalization with VGluT-1 are indicated by closed arrows. Points of triple colocalization are indicated by arrowheads. **(B)** Quantification of VGluT-1 puncta density per 100 µm of neurite. **(C)** Quantification of the proportion of VGluT-1 puncta within the mask that is associated with GFP-NL1, N-cadherin, or both. N = 857.6 µm of neurite per cell from at least 27 cells from 3 separate cultures. Scale bar = 10 µm.

One caveat of using neuroligin expressing constructs is that the overexpression of neuroligins has been reported to enhance synapse formation (Chih, 2005). To avoid this confounding problem, cultures were transfected with minimal amounts of DNA of GFP-NL1, a neuroligin expressing construct which we find to have less of an effect on synapse formation. To further ensure that the acquired data was not biased by cells that exhibited enhanced synapse formation due to neuroligin overexpression, data analysis focused only on cells expressing low levels of GFP-NL1. Indeed, when cells expressing low levels of GFP-NL1 were analyzed, the density of VGluT-1 clusters was comparable to GFP controls (68±19 puncta/100 µm for GFP and 73±17 puncta/100 µm for GFP-NL1, p=0.34 Student's T-test). We therefore concluded that this was an appropriate method to examine the distribution of NL1 at glutmatergic synapses (Fig 6B).

N-cadherin and NL1 colocalize at a subset of glutamatergic synapses

To examine the distribution of NL1 and N-cadherin with VGluT-1 on the GFP-NL1 expressing cell, a mask outlining the transfected neuron was made (Fig 6A) and all NL1, N-cadherin and VGluT-1 positive clusters within the mask were analyzed. The distribution of N-cadherin and NL1 at glutamatergic synapses was determined by measuring the proportion of VGluT-1 clusters within the mask that colocalized with N-cadherin and GFP-NL1. The data analysis identified separate populations of VGluT-1 positive clusters that were apposed to N-cadherin, NL1, and N-cadherin plus NL1. Of the VGluT-1 positive clusters, 10±3% of the clusters did not colocalize with either N-cadherin or NL1 (Fig 6C). 31±2% of VGluT-1 clusters colocalized only with N-cadherin and only 13±3% colocalized with only NL1. Finally, 46±2% of VGluT-1 clusters colocalized with both N-cadherin and NL1 (Fig 6C). These results demonstrate that of the VGluT-1 clusters that were associated with N-cadherin and/or NL1, the majority of VGluT-1 positive clusters contained both N-cadherin and NL1, and therefore suggests that approximately half of glutamatergic synapses contain both N-cadherin and NL1 at

the same synapse. Furthermore, very few VGluT-1 positive clusters were unassociated with either CAM thus emphasizing the importance of the presence of N-cadherin and NL1 at glutamatergic synapses.

Spatial distribution of N-cadherin and NL2

Time lapse studies have shown that although N-cadherin is initially present at both GABAergic and glutamatergic synapses, its distribution becomes restricted to glutamatergic synapses as neurons mature (D. L. Benson and H. Tanaka, 1998). Therefore, N-cadherin is commonly thought as a glutamatergic synapse specific cadherin. However, findings from the same studies have also suggested that N-cadherin is not exclusively found at glutmatergic synapses. At 14 DIV, although N-cadherin is mainly found at glutamatergic synapses, N-cadherin has also been shown to be present at approximately 10% of GABAergic synapses (D. L. Benson and H. Tanaka, 1998). Furthermore, β-catenin is present at both glutamatergic and GABAergic synapses (D. L. Benson and H. Tanaka, 1998) indicating that other cadherins are present at GABAergic synapses. In spite of this suggestive evidence, cadherins have yet to be identified at mature GABAergic synapses. The present study identified subsets of glutamatergic synapse that contain NL1, N-cadherin or both. It is possible that like glutamatergic synapses, subsets of GABAergic synapses exist. Furthermore, we also found that approximately 60% of glutamatergic synapse contained NL1. This raised the question of whether other neuroligin isoforms are at glutamatergic synapses as well. Neuroligin isoform, NL2, is one of few proteins that have been shown to mainly localize to GABAergic synapses (P. Scheiffele et al., 2000; F. Varoqueaux et al., 2004; B. Chih et al., 2006). Like NL1 and N-cadherin, the spatial distribution of N-cadherin and NL2 at glutamatergic or GABAergic synapses is unknown to date.

N-cadherin and NL2 colocalize in a subset of glutamatergic synapses

To study the spatial distribution of NL2 and N-cadherin at glutamatergic synapses, cultures were immunolabeled with antibodies against NL2, N-cadherin as well as VGluT-1 to mark glutamatergic synapses (Fig 7A). Although it is thought that NL2 preferentially localizes to GABAergic synapses, studies have shown NL2 to be weakly present at glutamatergic synapses in 14 DIV neurons (J. N. Levinson et al., 2005). Results of our colocalization analysis showed that 39±4% of the NL2 clusters associated with VGluT-1. More specifically, 18±2% of NL2 clusters colocalized with both VGluT-1 and N-cadherin, and 21±2% localized with VGluT-1-positive clusters alone (i.e. in the absence of N-cadherin) (Figure 8A). 41±4% of NL2 clusters colocalized with N-cadherin only. This was surprising because NL2 and N-cadherin are thought to localize to GABAergic and glutamatergic synapses respectively, and therefore one would predict that there would be minimal overlap in their synaptic distribution.

Interestingly, our analysis demonstrated that over half of the N-cadherin positive clusters (57±4%) did not localize with VGluT-1 or NL2 (Figure 8B). To see such a large fraction of N-cadherin unassociated with VGluT-1 was very surprising because N-cadherin is thought to preferentially localize to glutamatergic synapses (D. L. Benson and H. Tanaka, 1998). 31±3% of N-cadherin clusters localized with VGluT-1. More specifically, 26±3% colocalized with VGluT-1 alone and 5±1% of N-cadherin clusters colocalized with both VGluT-1 and NL2. The low colocalization rate of all three proteins demonstrates that N-cadherin is not commonly localized together with NL2 at glutamatergic synapses.

Of the total VGluT-1 positive clusters, 28±4% did not colocalize with either N-cadherin or NL2. 60±4% colocalized with N-cadherin and of this 45±3% of VGluT-1 clusters colocalized with only N-cadherin (Figure 8C). In contrast to N-cadherin, only 26±4% of VGluT-1 clusters colocalized with NL2 and of this, 14±2% colocalized with both N-cadherin and NL2, and 12±2% of VGluT-1 clusters colocalized with NL2 alone (in the absence of N-cadherin).

Figure 7

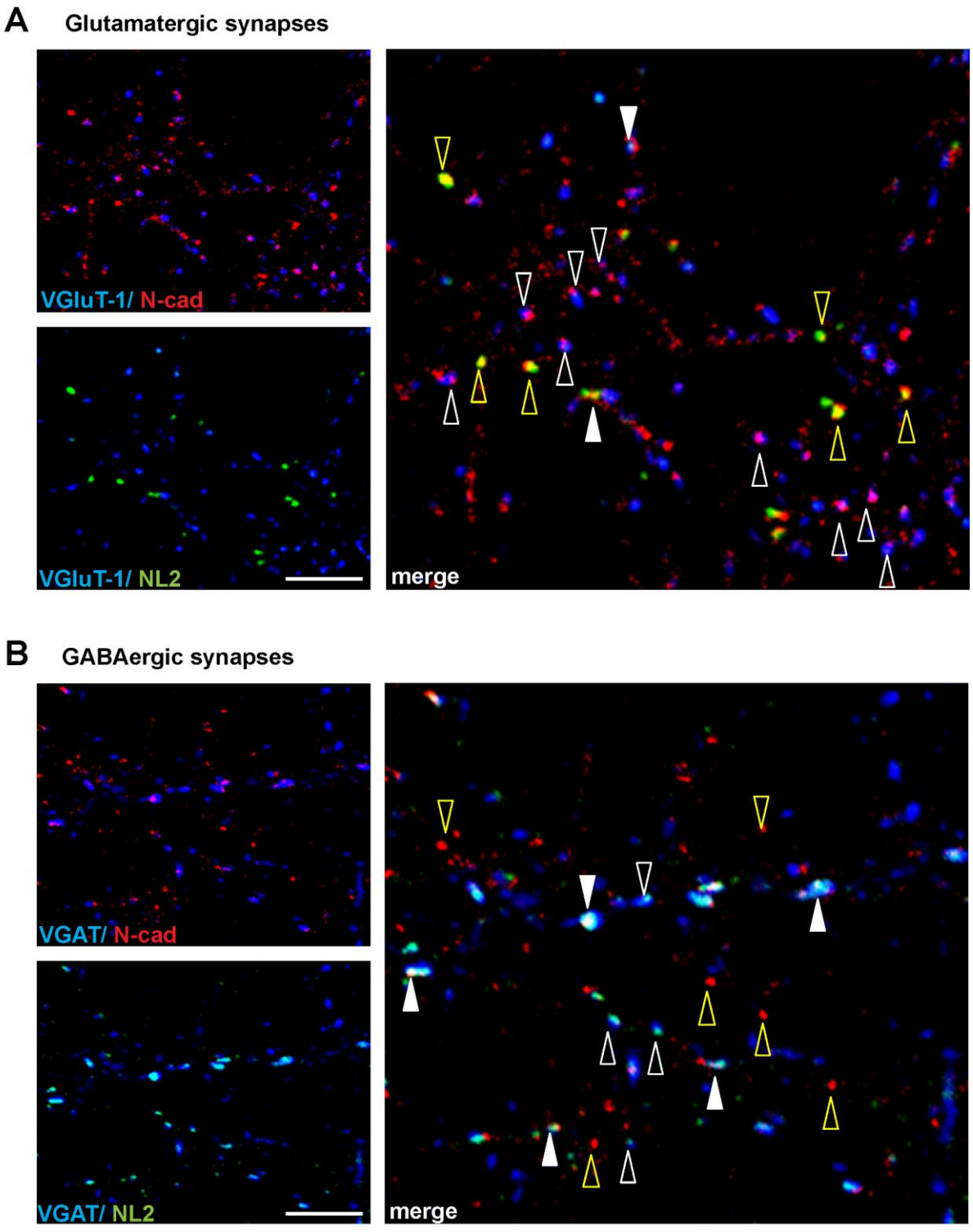
A
Glutamatergic synapses
VGluT-1/ N-cad
VGluT-1/ NL2
merge
B
GABAergic synapses
VGAT/ N-cad
VGAT/ NL2
merge

Figure 7. Spatial distribution of NL2 and N-cadherin at glutamatergic and GABAergic synapses. **(A)** Confocal images of 14 DIV neurons immunolabeled with N-cadherin, NL2 and VGluT-1. N=32 images from 3 separate cultures. **(B)** Confocal images of 14 DIV neurons immunolabeled with N-cadherin, NL2 and VGAT. GABAergic synapses are marked by the GABAergic presynaptic marker VGAT. N=42 images from 4 separate cultures. Points of colocalization between N-cadherin and VGluT-1/VGAT are shown as hollow arrowheads. Points of colocalization between N-cadherin and NL2 are shown as yellow arrowheads. Points of triple colocalization between N-cadherin, NL2 and VGluT-1/VGAT are shown as solid arrowheads. Scale bar = 10 µm.

Figure 8

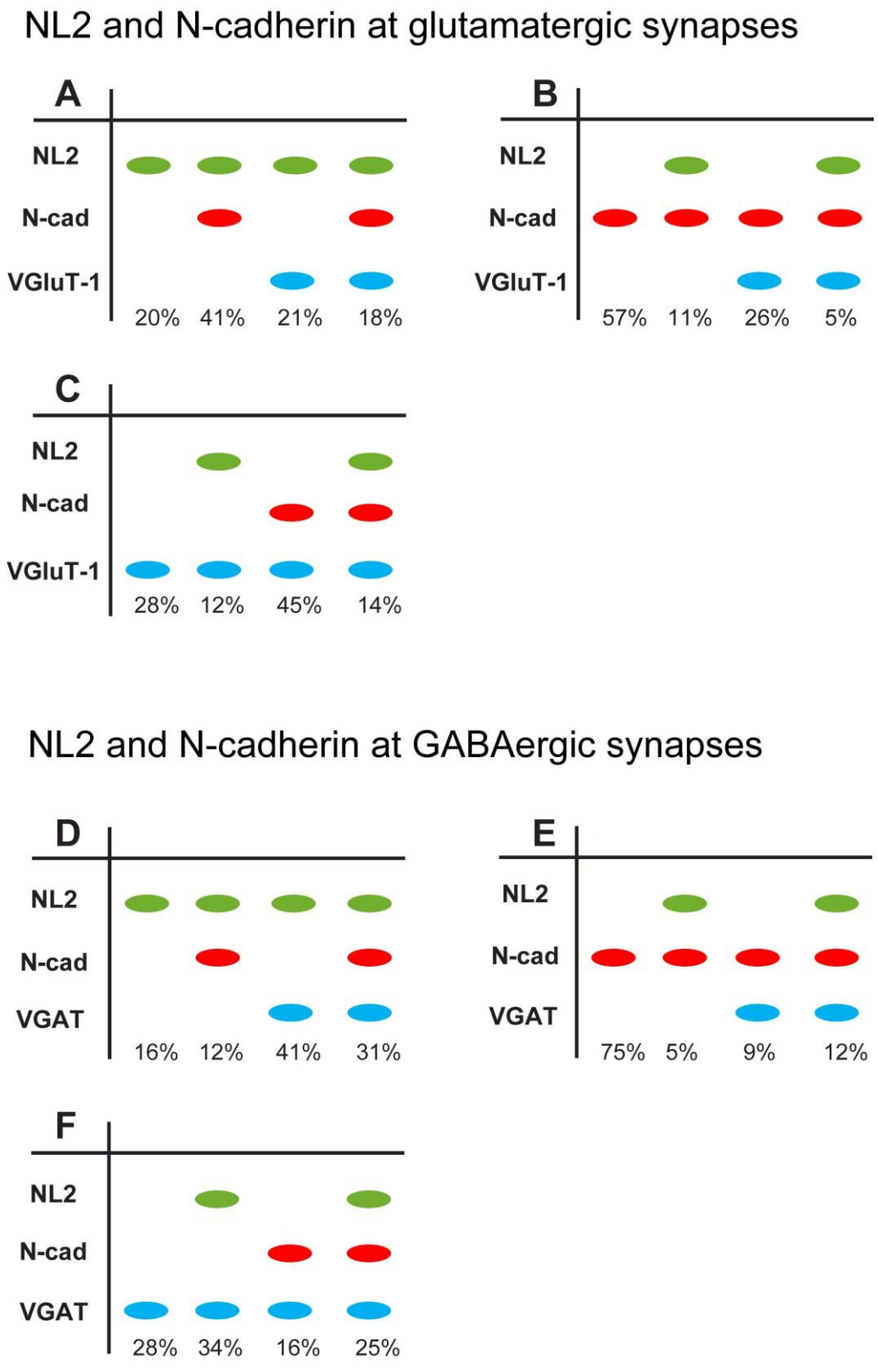
NL2 and N-cadherin at glutamatergic synapses
A
NL2
N-cad
VGluT-1
20%
41%
21%
18%
B
NL2
N-cad
VGluT-1
57%
11%
26%
5%
C
NL2
N-cad
VGluT-1
28%
12%
45%
14%
NL2 and N-cadherin at GABAergic synapses
D
NL2
N-cad
VGAT
16%
12%
41%
31%
E
NL2
N-cad
VGAT
75%
5%
9%
12%
F
NL2
N-cad
VGAT
28%
34%
16%
25%

Figure 8. Spatial distribution of NL2 and N-cadherin at glutmatergic and GABAergic synapses. (A,B,C) Spatial distribution of NL2 and N-cadherin at glutmatergic synapses (VGluT-1). **(A)** Proportion of NL2 clusters (green) that colocalized with N-cadherin (red) and/or VGluT-1 (blue) . **(B)** Proportion of N-cadherin clusters that colocalized with NL2 and/or VGluT-1. **(C)** Proportion of VGluT-1 clusters that colocalized with NL2 and/or N-cadherin. N = 3, n = 32. **(D,E,F)** Spatial distribution of NL2 and N-cadherin at GABAergic synapses (VGAT). **(D)** Proportion of NL2 clusters that colocalized with N-cadherin and/or VGAT. **(E)** Proportion of N-cadherin clusters that colocalized with NL2 and/or VGAT. **(F)** Proportion of VGAT clusters that colocalized with NL2 and/or N-cadherin. N = 4; n = 42

This demonstrates that NL2 does not preferentially localize to glutamatergic synapses. Furthermore, the higher rate of colocalization between VGluT-1 with N-cadherin than with NL2 demonstrates that N-cadherin preferentially localizes to glutamatergic synapses more than NL2.

In summary, these findings support prior findings that suggest that N-cadherin preferentially localizes to glutamatergic synapses and that NL2 does not. However, studying the localization of N-cadherin and NL2 side by side also demonstrated that the synaptic expression of N-cadherin and NL2 is not mutually exclusive.

NL2 and N-cadherin colocalize in a subset of GABAergic synapses

To study the spatial distribution of NL2 and N-cadherin at GABAergic synapses, cultures were immunolabeled with antibodies against NL2, N-cadherin as well as VGAT to mark GABAergic synapses (Fig 7B). Previous studies have shown that NL2 preferentially localizes to GABAergic synapses (F. Varoqueaux et al., 2004). Indeed, our data has also shown that NL2 predominantly localizes to GABAergic synapses (72±3%) (Figure 8D). More specifically, 31±3% of NL2 positive clusters colocalized with both N-cadherin and VGAT, whereas 41±3% of NL2 positive clusters colocalized with VGAT only. 43±4% of NL2 associated with N-cadherin positive clusters. Of this, 31±3% colocalized with N-cadherin and VGAT, and only 12±2% of NL2 colocalized with N-cadherin only, demonstrating that NL2 is more commonly associated with N-cadherin when N-cadherin is present at GABAergic synapses. These results thus far demonstrate that NL2 is strongly correlated with GABAergic synapses.

In contrast to NL2, 75±2% of N-cadherin clusters were found alone (in the absence of N-cadherin and VGAT) thus showing that N-cadherin is not commonly associated with GABAergic synapses (Figure 8E). 20±1% of N-cadherin colocalized with VGAT only. 17±1% of N-cadherin colocalized with NL2 positive clusters. Of this, only 5±1% of N-cadherin colocalized with NL2

alone and 12±1% colocalized with both N-cadherin and VGAT. The low rate of colocalization between N-cadherin and NL2 or VGAT demonstrated that N-cadherin is not commonly associated with NL2 or GABAergic synapses.

Finally, results from colocalization analysis of VGAT association with N-cadherin and NL2, showed that the majority (59±2%) of VGAT clusters colocalized with NL2 positive clusters (Figure 8F). Of this pool of VGAT clusters, 25±2% of VGAT colocalized with both N-cadherin and NL2, and 34±2% of VGAT colocalized with NL2 alone. Interestingly 41±4% of VGAT clusters colocalized with N-cadherin clusters and of this 16±2% of VGAT colocalized to N-cadherin alone and 25±2% of VGAT colocalized with both N-cadherin and NL2. This was surprising because N-cadherin has been shown to be excluded from most GABAergic synapses during development (D. L. Benson and H. Tanaka, 1998). Finally, 28±3% of VGAT clusters did not associate with either N-cadherin or NL2. These results taken together, demonstrate that NL2 is tightly associated with GABAergic synapses but also suggests that a small subpopulation of VGAT clusters also associates with N-cadherin.

In summary, NL2 is more strongly associated to GABAergic synapses than N-cadherin. However, similar to the distribution of N-cadherin and NL2 at glutamatergic synapses, the distribution of these CAMs does not appear to be exclusive to GABAergic synapses. Furthermore, we have identified subpopulations of GABAergic synapses that contain no NL2 or both NL2 and N-cadherin.

CHAPTER IV: DISCUSSION

FUNCTIONAL RELATIONSHIP OF CADHERINS AND NEUROLIGINS AT SYNAPSES

Previous studies have suggested that cadherins and neuroligins share some functional commonality at synapses. They are both necessary for establishing synaptic connections *in vitro,* and depletion or disruption of their function often results in impairment in synapse function and loss (D. L. Benson and H. Tanaka, 1998; H. Togashi et al., 2002; B. Chih et al., 2005). Indeed, when N-cadherin or NL1 expression was knocked down in the present study, a similar loss in synapse density was observed. Also, findings by Nishimura et al. (2002) and Iida et al. (2004) have implied that cadherins and neuroligins may physically interact via the scaffolding molecule S-SCAM. Nishimura et al. had found that the cadherin cytoplasmic partner β-catenin, can recruit S-SCAM to synapses (W. Nishimura et al., 2002), while in a separate study, S-SCAM was reported to be able to recruit and localize neuroligins to synapses (J. Iida et al., 2004) thus implying a possible link between cadherins and neuroligins. However, the potential interaction between the two CAMs was never investigated and whether β-catenin can recruit neuroligins to synapses via S-SCAM remains unclear. Furthermore, it remains to be elucidated whether cadherins and neuroligins are acting in concert via a common pathway or independently of one another via separate parallel pathways to mediate synapse formation.

NEUROLIGIN AND N-CADHERIN KNOCKDOWN AND SYNAPSE DENSITY

Disruption of neuroligin or cadherin function both lead to a decrease in synapse number as well as impairment in synapse function. Both current and previous studies have demonstrated that knockdown of neuroligin leads to a more robust loss of synapse density in

comparison to knockdown of N-cadherin expression. However, whether the combined knockdown of N-cadherin and neuroligins such as NL1, would result in an even more robust synapse loss phenotype remained unknown. An increase in synapse loss upon combined knockdown may suggest an additive effect and therefore indicate that N-cadherin and NL1 are acting independently in parallel pathways. However, synapse loss in N-cadherin plus NL1 double knockdown cells did not exceed loss in synapse density observed when NL1 was knocked-down alone. This result therefore suggests that N-cadherin and NL1 are acting in a common pathway to mediate synapse formation.

Although the effects of the double knockdown of N-cadherin and NL1 expression strongly suggest that N-cadherin and NL1 are acting in a common pathway, the possibility that they are acting in parallel pathways also cannot be completely dismissed. It is possible that an additive effect was not observed if a maximum limit in synapse loss was reached. If indeed the minimum synapse density necessary for cell survival was reached with NL1 knockdown alone, then any additional synapse loss by N-cadherin knockdown could have been masked when both N-cadherin and NL1 expression were knocked down together.

NEUROLIGIN AND N-CADHERIN OVEREXPRESSION AND SYNAPSE DENSITY

Since the publication of Scheiffele's co-culture study that demonstrated that neuroligin expression induces the formation of presynaptic terminals (P. Scheiffele et al., 2000), neuroligins have been extensively studied as potential synaptogenic molecules. *In vitro,* overexpression of neuroligins in neurons has also been shown to induce the formation of supernumerary synapses (B. Chih et al., 2005). In contrast, N-cadherin overexpression does not appear to be sufficient to induce synapse formation *in vitro* (Y. Sara et al., 2005). Indeed in the present study, no change in synapse density was observed when N-cadherin alone was

overexpressed and enhancement in supernumerary synapse formation was observed only when NL1 was expressed. Furthermore, when N-cadherin and NL1 were co-expressed, synapse density did not exceed that of NL1 expression alone further demonstrating that N-cadherin expression is not sufficient to enhance synapse number. To date it is unclear why NL1 but not N-cadherin overexpression can enhance supernumerary synapse formation. If N-cadherin and NL1 are acting in a common pathway, it is possible that N-cadherin overexpression cannot enhance supernumerary synapse formation because it is limited by the amount of NL1 present. If this is so, one would predict supernumerary synapse formation to occur when the limiting factor, NL1, is made limitless. Indeed when NL1 was expressed alone or co-expressed with N-cadherin, an enhancement in the formation of supernumerary synapses was observed. Therefore, if N-cadherin and NL1 are acting in a common pathway, we propose that NL1 is a limiting factor in this pathway that regulates synapse formation.

FUNCTIONAL COMPENSATION BETWEEN CADHERINS AND NEUROLIGINS

In vitro, the loss of NL expression results in a dramatic decrease in synapse number. Interestingly in *in vivo* studies, no change in synapse density is reported in NL1-3 triple knockout mice (F. Varoqueaux et al., 2006). The absence of a synapse density phenotype in neuroligin knockout animals may indicate that other synaptic CAMs may be able to functionally compensate for the loss of neuroligins. To date, no study has investigated whether different synaptic CAM families can functionally compensate for one another. Cadherins and neuroligins are attractive candidates to test for functional compensation because they are synaptic CAMs that share commonality in their roles in synapse formation and function, and also because of their possible physical interaction via S-SCAM.

The results of the present study demonstrated that NL1 overexpression can fully rescue the N-cadherin knockdown phenotype thus suggesting that NL1 overexpression is able to compensate for the functional loss of N-cadherin. Furthermore, NL1 overexpression not only rescued but also restored synapse density to levels comparable to when NL1 is overexpressed alone. Synaptic localization of NL1 also remained unaffected when NL1 was overexpressed in an N-cadherin knockdown background suggesting that NL1 can still localize appropriately to synapses even when N-cadherin is depleted. The present data thus strongly suggests that NL1 is able to functionally compensate for N-cadherin in synapse formation.

If we are proposing that N-cadherin and NL1 are acting in a common pathway to regulate synapse formation, which is upstream of the other? In the present study, we propose that N-cadherin overexpression is insufficient to enhance supernumerary synapse formation because the amount of NL1 is limiting. In contrast, NL1 expression was able to enhance supernumerary synapse formation even in the absence of N-cadherin suggesting that NL1 is not limited by N-cadherin. Therefore, if N-cadherin and NL1 are acting in a common pathway, we propose that N-cadherin is limited by NL1 because it functions upstream of NL1 (Illustration 1).

Interestingly, when N-cadherin was overexpressed in NL1 knockdown cells, we observed a partial rescue of the NL1 knockdown phenotype. These results were unexpected since numerous studies have reported N-cadherin expression to be insufficient to induce synapse formation *in vitro (Y. Sara et al., 2005)* and since in the present study, N-cadherin overexpression could not induce supernumerary synapse formation. It is possible that a partial rescue was observed because N-cadherin functions in an additional signalling pathway that cannot induce supernumerary formation but can induce synapse formation when there is a loss in synapse number.

Taking all of the results of the present study together, we propose the following model whereby, N-cadherin regulates synapse formation via two separate pathways (Illustration 1). In Pathway A, N-cadherin and NL1 are regulating synapse formation via a common pathway whereby N-cadherin lies upstream of NL1 and N-cadherin is dependent on the presence of NL1 to regulate synapse formation. It is possible that the overexpression of N-cadherin cannot induce synapse formation because its ability to induce synapse formation is limited by the presence of NL1. In contrast to Pathway A, we propose that in a second pathway, Pathway B, N-cadherin is not dependent on the presence of NL1 and although this pathway cannot induce supernumerary synapse formation, this pathway can detect and partially compensate when there is a loss in synapse number. Previous studies have shown that S-SCAM can interact and recruit NL2 to synapses (K. Sumita et al., 2007). Furthermore, overexpression and knockdown of NL2 has been shown to enhance and decrease glutamatergic synapse number respectively (B. Chih et al., 2005). The present study has shows that NL2 becomes more associated with VGluT-1 clusters when N-cadherin is overexpressed in NL1 knockdown cells. However, NL2 localization does not change upon N-cadherin expression alone. This finding suggests that N-cadherin recruits NL2 in the absence of NL1 expression. We therefore propose that Pathway B involves N-cadherin's recruitment of NL2 that can the rescue synapse loss that is observed following loss of NL1 expression.

CADHERINS AND NEUROLIGINS AT GABAERGIC AND GLUTAMATERGIC SYNAPSES

As CAMs, cadherins and neuroligins are thought to mechanically hold pre- and post-synaptic terminals in apposition by binding to one another or to another transynaptic partner. Both cadherins and neuroligins have been shown to aggregate at synaptic contact sites, and specific isoforms have been shown to preferentially distribute themselves to glutamatergic and GABAergic synapses. However, the spatial relationship between neuroligins and cadherins with respect to one another is unknown at glutamatergic and GABAergic synapses.

Illustration 1

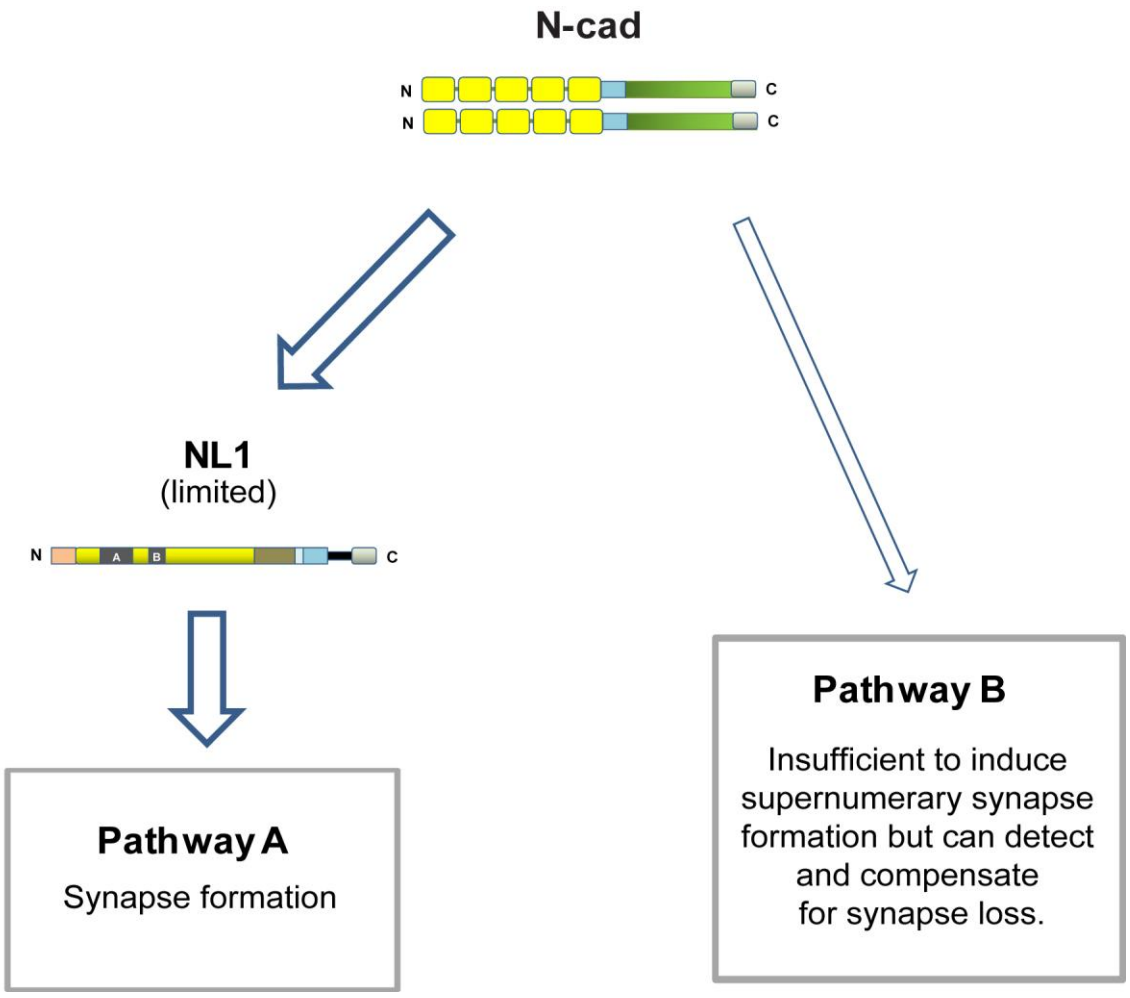

Illustration 1. Model of the functional relationship between NL1 and N-cadherin. Pathway A: N-cadherin and NL1 are in a common pathway where N-cadherin functions upstream of NL1. N-cadherin`s ability to regulate synapse formation is limited by NL1. Pathway B: A second pathway that cannot induce supernumerary synapse formation but N-cadherin expression can detect and compensate for synapse loss. Rescue of synapse loss in this pathway may be due to the recruitment of NL2 by N-cadherin.

N-cadherin and NL1 at glutamatergic synapses

Although the distributions of N-cadherin and NL1 have been analyzed separately at synaptic compartments, their spatial distribution in relation to one another is unknown. Do these CAMs occupy the same glutamatergic synapses or are they expressed at separate glutamatergic synapses? Analysis of the spatial distribution of NL1 and N-cadherin in cultured neurons confirmed that NL1 and N-cadherin mainly localize together at glutamatergic synapses. Interestingly, although approximately half of glutmatergic synapses contained both N-cadherin and NL1, several other subpopulations of glutmatergic synapses also emerged from our spatial distribution analysis. These subpopulations included glutamatergic synapses that contained only N-cadherin, only NL1, and neither N-cadherin nor NL1. It is unclear what these other subpopulations of glutamatergic synapses represent. However, we propose that these different subpopulations may represent distinct populations of glutmatergic synapses, or perhaps represent snapshots of different developmental stages during synapse formation.

Previous studies have demonstrated that N-cadherin is present in neurons prior to synaptogenesis and is detected in dense-core vesicles that are delivered to nascent synapses (M. Shapira et al., 2003). N-cadherin can be detected at synapses as early as 3 DIV (D. L. Benson and H. Tanaka, 1998) and is initially present at both glutamatergic and GABAergic synapses before its distribution becomes restricted to only glutamatergic synapses (7 DIV) (D. L. Benson and H. Tanaka, 1998). Such studies have thus implied that N-cadherin is one of the earliest CAMs present at synapses during development. In contrast, although N-cadherin is reported to be clustered at glutamatergic synapses by 7 DIV, NL1 is only weakly clustered at this time point and becomes significantly more clustered in mature 14 DIV neurons (J. N. Levinson et al., 2005). Furthermore, NL1 has been reported to be present at only a few glutamatergic synapses at 7 DIV and becomes better associated with glutamatergic synapses by 14 DIV neurons (K. Gerrow et al., 2006). It is possible that the clustering and synaptic

localization of NL1 follows after the synaptic localization of N-cadherin. Past studies by Nishimura et al. and Iida et al. that indicated a physical interaction between cadherins and neuroligins via β-catenin and S-SCAM, may explain this temporal lag between the localization of N-cadherin and NL1 to synapses (W. Nishimura et al., 2002; J. Iida et al., 2004). These two studies taken together imply that cadherins can recruit neuroligins to synapses. It is therefore possible that cadherins are observed at synapses earlier than neuroligins because they may mediate proper synaptic localization of neuroligins.

In the present study, we identified distinct populations of glutamatergic synapses that contained neither N-cadherin nor NL1, either N-cadherin or NL1, or both. These synapses may represent different subsets of glutamatergic synapses or may represent glutamatergic synapses caught at different stages of synapse development. If N-cadherin is necessary to recruit neuroligins, it is possible that those glutamatergic synapses that only contained N-cadherin represent very young synapses where N-cadherin has just arrived at the synapse but has not yet recruited NL1. Synapses that contain both N-cadherin and NL1 may represent synapses at a more mature stage when the complete assembly of synaptic components has been established. N-cadherin/NL1 colocalization data analysis also identified synapses that contained only NL1. Sharing and recycling of synaptic vesicles between synapse may suggest that synaptic components are also recycled and shared between pre-existing and newly forming synapses (S. E. Ahmari et al., 2000). Because cadherins are among the first CAMs to be present at newly formed synapse, it is possible that when synapses are eliminated, N-cadherin is shipped away prior to neuroligins to form new synapses elsewhere. Therefore, it is possible that synapses containing only NL1 may represent fully mature or disassembling synapses where N-cadherin has already been transported away and NL1 is left behind.

Although it is known that cadherins and neuroligins are present at synapses early in development, the temporal aspect of synaptic localization of N-cadherin and NL1 has not been

examined side by side to date. Live imaging of neurons cotransfected with fluorophore-tagged N-cadherin and NL1 constructs would be an informative method to further understand the dynamics of these CAMs in neurons and may reveal the temporal order of N-cadherin and NL1 recruitment to synapses.

The results of the present study have demonstrated that N-cadherin and NL1 are commonly associated with glutamatergic synapses and are often found together at the glutamatergic synapses. The results of the present study also suggest that neither of these CAMS are present at all glutamatergic synapses nor are they always associated together at the same synapses. Although NL1 is thought to be exclusively localized to glutamatergic synapses, it would be of interest to also investigate whether the distribution of NL1 at GABAergic synapses to confirm whether NL1 is exclusively localized to glutamatergic synapses.

N-cadherin and NL2 at glutamatergic and GABAergic synapses

In comparison to glutamatergic synapses, much less is known about the molecular make up of GABAergic synapses. Furthermore, no cadherin isoforms have been identified at GABAergic synapses to date. However, they are thought to be present at GABAergic synapses because β- catenin has been detected at GABAergic synapses (D. L. Benson and H. Tanaka, 1998). In contrast to cadherins, the neuroligin isoform, NL2, has been shown to preferentially localize to GABAergic synapses and its overexpression has been shown to enhance GABAergic synapse formation more strongly in comparison to overexpression of other neuroligin isoforms (F. Varoqueaux et al., 2004; B. Chih et al., 2005). Indeed, our analysis of NL2's spatial distribution also showed that NL2 preferentially distributes itself to GABAergic synapses.

Although the presented data showed that N-cadherin preferentially localizes to glutamatergic synapses and NL2 to GABAergic, the data also revealed that this distribution was

not mutually exclusive. In contrast to previous reports, N-cadherin was also detected at group of GABAergic synapses and similarly, NL2 at a group of glutamatergic synapses. Upon further analysis, several subpopulations of GABAergic/glutamatergic synapses that contained mixtures of N-cadherin and NL2 or only N-cadherin were identified.

Overlapping N-cadherin and NL2 distribution

Like the subpopulations of glutamatergic synapses identified from our NL1/N-cadherin spatial distribution analysis, it is unclear what these subpopulations of synapses represent. However, we propose that these subpopulations represent subtypes of GABAergic/glutamatergic synapses or synapses at different developmental stages.

Although, N-cadherin, NL1 and NL2 are often described as glutamatergic or GABAergic synapse specific CAMs, their synaptic distributions may not be exclusive to glutamatergic or GABAergic synapses. Although NL1 has been shown to become strongly enriched at glutamatergic synapses, the same study has also shown NL1 to be weakly present at GABAergic synapses in mature cells (14 DIV) (J. N. Levinson et al., 2005). Similarly, NL2 has also been shown to predominantly localize to GABAergic synapses but remain weakly present at glutamatergic synapses (J. N. Levinson et al., 2005). The percentage of N-cadherin present at glutamatergic synapses has not been quantified to date. However, approximately 10% of GAD65 labelled clusters (GABAergic synapse) have been shown to associate with N-cadherin positive clusters at 14 DIV (D. L. Benson and H. Tanaka, 1998) which may account for the population of N-cadherin containing GABAergic synapses that were identified in our analysis.

TEMPORAL CHANGES IN N-CADHERIN AND NL2 DISTRIBUTION

The distribution of CAMs at synapses has been shown to change over the course of synaptic development. In hippocampal cultures, N-cadherin is present at both glutamatergic

and GABAergic synapses in young neurons before its localization is restricted to glutamatergic synapses at 7 DIV (D. L. Benson and H. Tanaka, 1998). Like NL1, NL2 is only weakly clustered in young neurons (7 DIV) and gradually becomes more enriched at GABAergic synapses as neurons mature (14 DIV). Furthermore, the majority of NL2 clusters do not face presynaptic specializations in young neurons (4 DIV) in comparison to older neurons (14 DIV) (F. Varoqueaux et al., 2004). It is therefore likely that the synaptic localization of N-cadherin precedes the localization of NL2. A physical interaction is also thought to occur between cadherin and NL2, since NL2 has also been shown to be recruited to synapses via interactions with S-SCAM (K. Sumita et al., 2007). Therefore, it is possible that N-cadherin may help orchestrate synapse formation by recruiting other CAMs such as NL2 to GABAergic synapses. The presence of N-cadherin at GABAergic synapses in young neurons and the absence of N-cadherin at mature GABAergic synapses also suggest that N-cadherin may play a dominant role in the initial stages of synapse formation for both glutamatergic and GABAergic synapses. Analysis of the spatial distribution of N-cadherin and NL2 at GABAergic synapses identified a population of GABAergic synapses that only contained N-cadherin. If the subsets of GABAergic synapse identified in the present study represent specific stages of synapse development, GABAergic synapses that only contained N-cadherin may represent very young GABAergic synapses where N-cadherin has not yet recruited NL2. Furthermore, it is possible that GABAergic synapses that associated with both N-cadherin and NL2 may represent young synapses where N-cadherin is in the process of recruiting NL2. Finally, because N-cadherin is only present at GABAergic synapses in young neurons, GABAergic synapses with only NL2 may represent fully mature GABAergic synapses.

Although N-cadherin has been reported to be present at both glutmatergic and GABAergic synapses early in synapse development, it is unknown how N-cadherin's distribution becomes restricted to glutamatergic synapses. It is possible that N-cadherin does not

preferentially localize to glutamatergic over GABAergic but may appear so because of an initial high glutmatergic to GABAergic synapse ratio. If glutamatergic and GABAergic synapses are formed at a similar rate early in development but glutamatergic synapse formation dominates later, this would result in a high glutamatergic to GABAergic synapse ratio, and the distribution of N-cadherin may only appear to favour glutamatergic synapses. Regardless of whether the GABAergic synapses that associated N-cadherin represent synapses of a specific developmental stage or an unknown GABAergic synapse subclass, the present study can conclude that the distribution of N-cadherin is not exclusive to glutamatergic synapses. Furthermore, the present study demonstrates that although N-cadherin and NL2 predominantly localize to glutamatergic and GABAergic synapses respectively, their spatial distributions are not mutually exclusive. Finally, subpopulations of glutamatergic and GABAergic synapses that contained both CAMs were identified and may represent new subclasses of synapses or may represent different stages of synapse development.

In spite of suggestive evidence that indicates the presence of other cadherins at GABAergic synapses, no cadherins have been identified at mature GABAergic synapses to date (D. L. Benson and H. Tanaka, 1998). Although N-cadherin has been the cadherin of focus in synapse formation, it would be of interest to identify other cadherin isoforms that are potentially present at GABAergic synapses and to investigate whether cadherins and neuroligins regulate synapse formation via a common pathway at GABAergic synapses as well. Cadherin-11 and cadherin-13 may be promising candidates since knockdown of their expression has been shown to decrease GABAergic synapse number *in vitro* (S. Paradis et al., 2007). Also, E-cadherin and N-cadherin have been reported to have mutually exclusive spatial distributions in the hippocampus and therefore E-cadherin may also be present at GABAergic synapses.

FINAL REMARKS

The present study has provided the first suggestive evidence that cadherins and neuroligins may be acting together to mediate synapse formation. We propose that N-cadherin is acting in a common pathway with NL1 where N-cadherin is acting upstream of NL1 and NL1 is limiting. The present study also demonstrated that N-cadherin and NL1 are commonly present together at glutamatergic synapses by immunofluorescence. Furthermore, our data demonstrated that although N-cadherin is thought to be predominantly localized to glutamatergic synapses and NL2 to GABAergic synapses, their spatial distributions are not mutually exclusive.

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APPENDIX A

During the period when my thesis was being read by my advisory committee, some additional results were generated to address some questions that were raised from my thesis. The following appendix includes new data that was acquired.

Figure A1

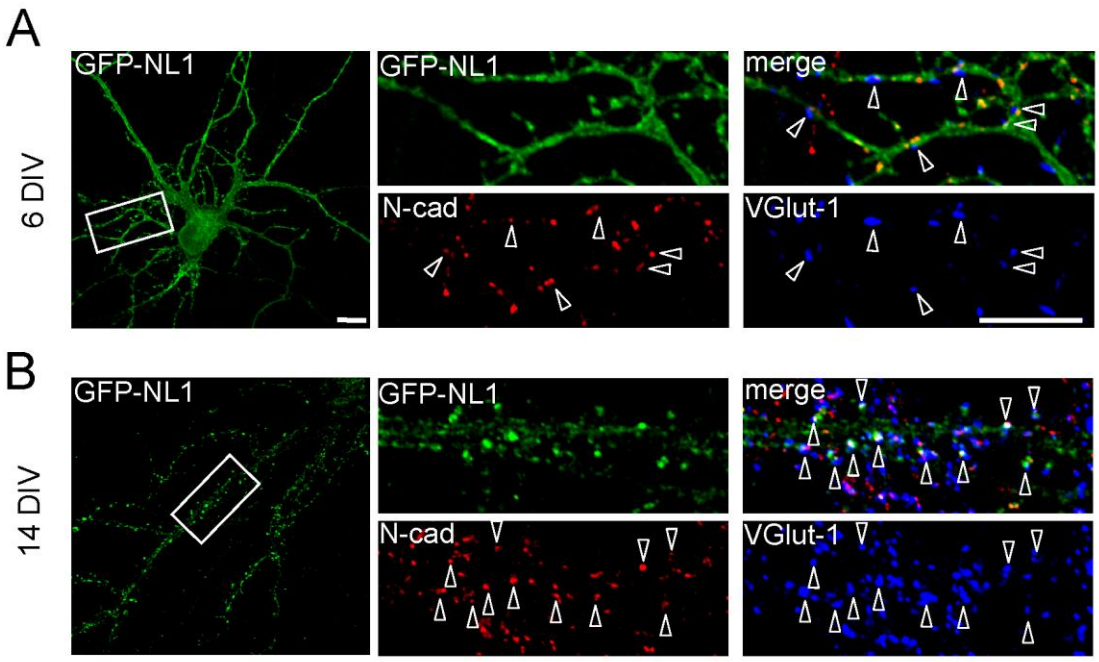

Figure A1. To investigate the temporal localization of N-cadherin and NL1, the distribution of both cell adhesion molecules was examined at 6 DIV and 14 DIV in primary rat hippocampal cultures. Confocal images of neurons were transfected at 2 or 10 DIV with GFP-NL1, and immunolabelled with VGluT-1 (blue) and N-cadherin (red). GFP-NL1 is diffusely distributed along neurites in 6 DIV cells **(A)** but is clustered at 14 DIV **(B)**. N-cadherin is clustered at both 6 and 14 DIV. VGluT-1 clusters colocalize with N-cadherin at 6 DIV and colocalize with both N-cadherin and NL1 clusters at 14 DIV. N=3, n=23-27 cells. Scale bars = 10µm.

Figure A2

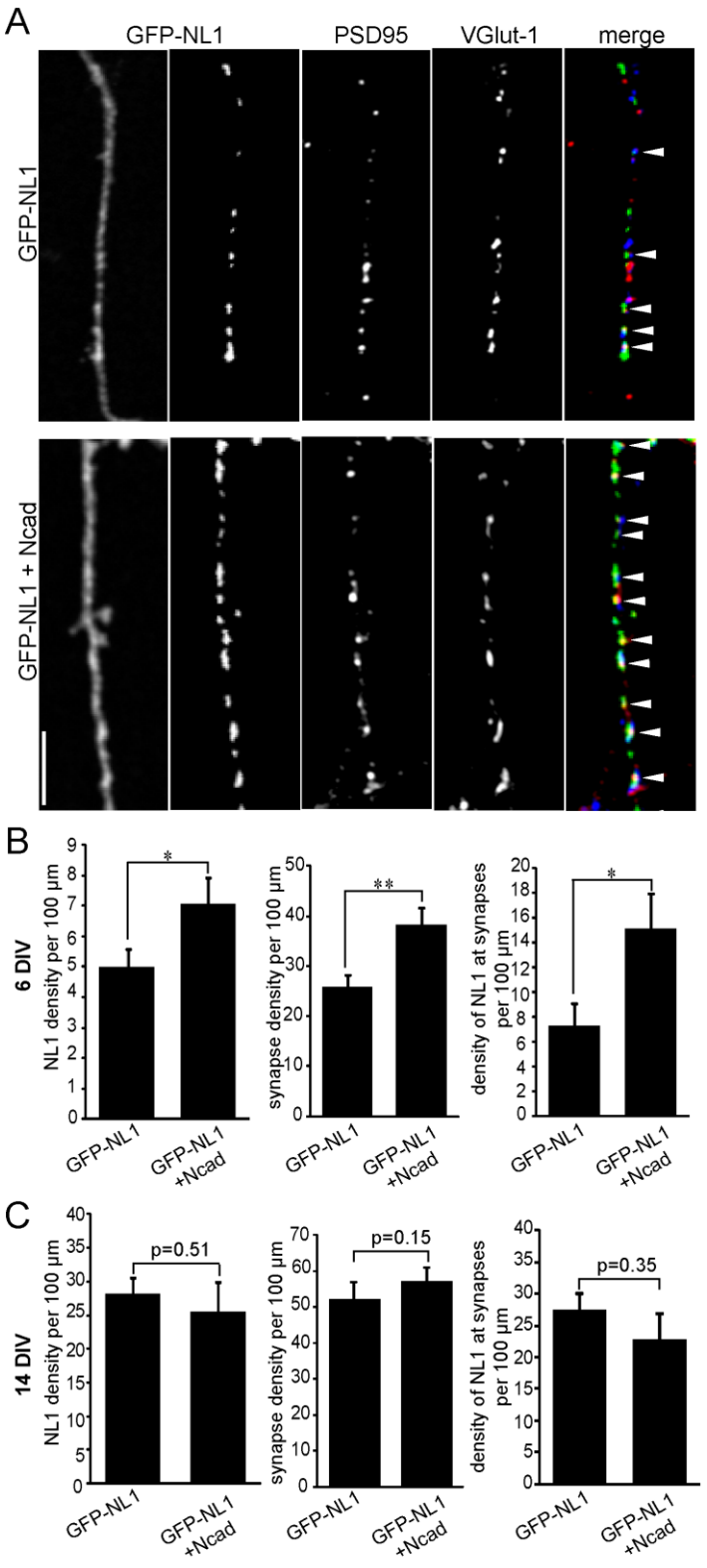
A
GFP-NL1
PSD95
VGlut-1
merge
GFP-NL1
GFP-NL1 + Ncad
B
6 DIV
NL1 density per 100 µm
synapse density per 100 µm
density of NL1 at synapses per 100 µm
*
**
*
GFP-NL1
GFP-NL1 +Ncad
C
14 DIV
NL1 density per 100 µm
synapse density per 100 µm
density of NL1 at synapses per 100 µm
p=0.51
p=0.15
p=0.35
GFP-NL1
GFP-NL1 +Ncad

Figure A2. To investigate whether N-cadherin overexpression can enhance clustering of NL1, hippocampal cultures were transfected at 2 or 10 DIV with GFP-NL1 or N-cadherin-CFP+GFP-NL1 and fixed 4 days later. (A) Confocal images of 6 DIV neurons transfected with GFP-NL1 or N-cadherin-CFP+GFP-NL1, and immunolabelled with VGluT-1 and PSD-95. Raw images of GFP-NL1 are shown on far left, and thresholded images shown in second column. **(B)** Quantification of 6 DIV neurons demonstrates an increase in NL1 density, synapse density (defined as a PSD-95/VGluT-1 co-cluster), and density of synaptically-localized Nl1 in N-cadherin overexpressing cells. **(C)** Quantification of 14 DIV neurons demonstrates no change in either Nl1 densith, synapse density of density of synaptically-localized Nl1 in N-cadherin overexpressing cells. N=3, n=20. *p<0.05, **p<0.01; Student's t-test. Scale bar = 10µm.

Figure A3

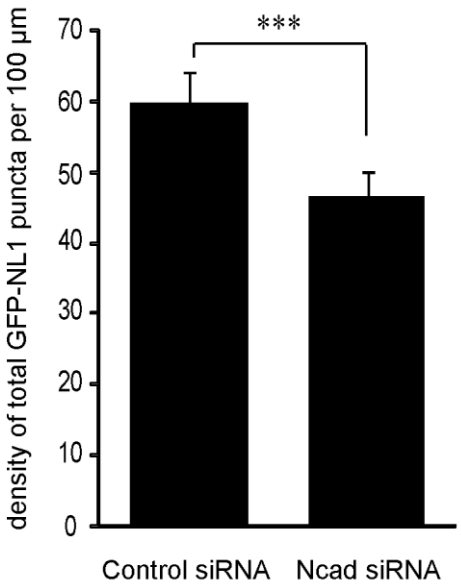

Figure A3. The density of NL1 clusters is decreased in N-cadherin siRNA expressing cells. The density of NL1 clusters in 14 DIV cells transfected at 10 DIV with either control or N-cadherin siRNA were assayed. There was a significant decrease in NL1 cluster density in Ncadherin siRNA expressing cells (Ncadherin siRNA=46±3 puncta per 100µm in comparison to control cells (Control siRNA=60±4 puncta per 100µm). 60N=3, n=25-26.***p<0.01; Student's t-test.