A NOVEL FUNCTION FOR NEUREXIN AND NEUROLIGIN FAMILY MEMBERS IN PROMOTING NEURITE OUTGROWTH

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ABSTRACT

Neurexins and neuroligins are heterophilic cell adhesion molecules which interact with the cytoskeleton and play a role in synaptogenesis. However, they are also expressed prior to synapse formation, suggestive of additional functions. Other neuronally expressed cell adhesion molecules play a role in promoting neurite outgrowth in addition to their adhesive functions. Here, we demonstrate a novel role for neuroligin 1 in promoting neurite outgrowth in N1E-115 neuroblastoma cells. Further, we identify both the acetylcholinesterase-like domain and PDZ motif as essential requirements for this process. We demonstrate that neuroligin 2 and neurexin 1β can produce the cytoskeletal rearrangements required for neurite outgrowth in N1E-115 cells. Finally, we show expression of nlgn1, nlgn2, nrxn1 and nrxn2 in the developing murine spinal cord. Insight into the outgrowth-promoting effects of the neurexin-neuroligin complex is especially pertinent as defects in neurexin and neuroligin family members have been linked to the autism spectrum of neurodevelopmental disorders.

RESUMÉ

Les neurexines et les neuroliguines sont des molécules d'adhésion cellulaire hétérophiliques. Elles peuvent interagir avec le cytosquelette et jouent un rôle dans la synaptogénèse. De plus, ces protéines sont aussi exprimées avant la formation des synapses ce qui suggère qu'elles pourraient avoir des fonctions additionnelles. Il a déjà été démontré que des molécules d'adhésion cellulaire exprimées dans les neurones peuvent promouvoir l'extension neuritique. Nous démontrons ici que neuroliguine 1 engendre l'extension de neurites dans les cellules de neuroblastomes N1E-115. De plus, nous avons démontré que son domaine acetylcholinesterase et le motif PDZ sont essentiels à ce processus. Nous montrons que neuroliguine 2 et neurexine 1β engendrent des réarrangements du cytoskelette favorisant l'extension de neurites dans les cellules N1E-115. Finalement, nous montrons que nlgn1, nlgn2, nrxn1 et nrxn2 sont exprimés dans la colonne vertébrale de souris en développement. Puisque les neurexines et les neuroliguines ont été associées aux troubles du spectre autistique, une meilleure compréhension des effets du complexe neurexine-neuroliguine sur la croissance neuronale est essentielle afin d'étudier ces maladies neurologiques.

ABBREVIATIONS

AChE, acetylcholinesterase; CAMs, cell adhesion molecules; C-terminal, carboxy terminal; EGF, epidermal growth factor; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, haemagglutinin; LNS, Laminin/Neurexin/Sex hormone-binding globulin; N-cadherin, neural cadherin; NCAM, neural cell adhesion molecule; NLGN, neuroligin; NRXN, neurexin; N-terminal, amino terminal; RT-PCR, reverse transcription-PCR; TFR, transferrin receptor.

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CHAPTER 1: INTRODUCTION

A number of neuronally expressed cell adhesion molecules (CAMs) have been shown to promote the outgrowth of neurites in cultured cells [1], a process thought to be similar to the extension of dendritic or axonal processes by neurons in the developing nervous system [2]. Members of the neurexin (NRXN) and neuroligin (NLGN) families of CAMs are expressed prior to synapse formation in the developing rodent nervous system [3-6] and can be localized to growth cones [7]. Furthermore, these proteins participate in intracellular interactions which could mediate the cytoskeletal rearrangements required for neurite outgrowth [8-14] . Here, we sought to demonstrate a novel role for the heterophilic NRXN-NLGN cell adhesion complex in promoting neurite outgrowth.

CAMs in neurite outgrowth

Several neuronally-expressed families of CAMs play well-established roles in promoting neurite outgrowth during development via a contact-mediated outgrowth mechanism. The interaction of these CAMs with their ligands, which may be molecules expressed on the surface of other cells or components of the extracellular matrix, produces both a mechanical adhesive component and a signaling component by which intracellular pathways are activated which ultimately lead to neurite outgrowth [15]. CAMs which have been shown to play this role include L1, neural (N)-cadherin and the neural cell adhesion molecule (NCAM). These molecules mediate interactions with their extracellular binding partners and interact intracellularly with components of the actin cytoskeleton and several signaling pathways [15]. Intriguingly, all three proteins interact

in *cis* with the fibroblast growth factor receptor (FGFR), and the tyrosine kinase activity of FGFR is required for the neurite outgrowth promoted by these CAMs [1]. Additional signaling occurs through the intracellular regions of all three CAMs [15]. The well-characterized role of these CAMs in neurite outgrowth suggests that two other families of neuronally expressed heterophilic CAMs, the NRXNs and NLGNs, may play a similar role during development.

Structure of NRXNs

The NRXN protein family was first discovered when NRXN1α was identified as a receptor for α-latrotoxin, a component of black widow spider venom causing massive presynaptic neurotransmitter release [16]. The NRXNs were found to be highly polymorphic single-transmembrane domain proteins with extracellular repeats bearing resemblance to other proteins involved in axon guidance and synaptic development [17]. The three NRXN genes are each transcribed from two promoters, creating a long α form and a shorter β form consisting of an N-terminally truncated version of the protein with a short β-specific sequence at the N terminus (Fig. 1A) [18]. The extracellular domain of the α-NRXNs consists of six LNS domains and three EGF-like regions, grouped into three repeats consisting of an LNS A and B domain flanking a central EGF-like domain (Fig. 1A) [18]. These LNS domains are named for the proteins that contain them, namely laminin, NRXNs, and sex hormone-binding globulin [19]. Each of these domains is independently folded, and individual LNS domains interact with distinct binding partners [19]. Of these structural units, β-NRXNs retain only the sixth LNS domain. Alpha- and β-NRXNs share an extracellular O-linked glycosylation domain (Fig. 1A). The short

intracellular C-termini of the α and β forms of each NRXN protein are identical [18]. All three NRXN family members are subject to extensive alternative splicing, with five splice sites in the α-NRXNs and two in the β-NRXNs (Fig. 1A). These splice sites are independently regulated, allowing for the production of a staggering multitude of distinct NRXN isoforms [20]. This diversity has been proposed as a possible underlying cause of cell-cell recognition during neurological development [21]. This alternative splicing also regulates the ability of NRXNs to interact with their binding partners; primarily, an insert at splice site 4, located in LNS 6, regulates binding of NRXNs to their heterophilic binding partners, the NLGNs [22].

Structure of NLGNs

NLGN1 was identified in a search for potential binding partners for NRXNs [22]. Binding was found to be stoichiometric and Ca^{2+}-dependent. The NLGN1 protein was found to migrate at about 116 kDa and consist of a single chain of 843 amino acids, corresponding to a molecular weight of about 95 kDa. The difference between the predicted size of NLGN1 and its observed size was accounted for by extensive glycosylation; the extracellular domain of NLGN1 contains five N-glycosylation consensus sites and a Ser-Thr-rich stalk region that is highly O-glycosylated. NLGN1 is also alternatively spliced at two sites, denoted “A” and “B”, indicating a diversity of NLGN isoforms more limited than that of NRXNs, but nonetheless significant. NLGN1 was found to be a type I transmembrane protein, with a large extracellular N-terminus of 651 amino acids and a relatively short intracellular C-terminus of 126 amino acids (Fig.

1B). The bulk of the extracellular domain is composed of a catalytically inactive domain bearing significant homology to acetylcholinesterase and other esterase domain-containing proteins (Fig. 1B). This acetylcholinesterase-like (AChE-like) domain is catalytically inactive due to the absence of the active site serine residue required for function [22]. Further, the active site of the AChE-like domain is not accessible to substrate [23].

Shortly after the discovery of NLGN1, two other NLGN family members were cloned and named NLGN2 and NLGN3 [24]. The NLGN family shares a high degree of sequence homology, with 52% of residues shared among all three NLGN forms. The transmembrane and extracellular regions, particularly the AChE-like domain, share the highest degree of homology. All three NLGNs share the same sites of alternative splicing (Fig. 1B), although the alternatively spliced sequences are distinct in each family member. As in NLGN1, the active site serine is replaced with a glycine residue in NLGN2 and NLGN3 [24].

A fourth NLGN family member was later identified in humans in a database search and screen of RNA transcripts from brain; however, it was found to be expressed primarily in non-neuronal tissues, and its expression level in brain was surprisingly low [25]. NLGN4 transcripts produced a mature protein which was found to be closest in sequence to human NLGN3 and likewise lacks the serine required for catalytic activity [25]. This database search also identified a possible fifth NLGN only present in humans [25]. This NLGN was later positively identified and named NLGN4Y, as it is located on the Y chromosome and differs from human NLGN4 by only 19 amino acids [26].

More recently, a highly divergent mouse NLGN4 was identified [27]. While this NLGN molecule shares many characteristics with the other NLGNs, including NRXN binding and localization to dendritic spines, it is highly divergent from human NLGN4, suggesting that NLGN4 may have been under less stringent evolutionary restraints than the other NLGN family members [28]. NLGN4 is not detectable in the brains of newborn mice, and contributes only 3% of the NLGN protein in adult murine brain, suggesting that it plays a lesser role than other NLGN family members in the murine brain [27].

NRXN–NLGN binding

NRXNs and NLGNs as CAMs

NRXN–NLGN binding produces functional heterophilic cell adhesion complexes [29]. The cell adhesion properties of NRXNs and NLGNs were determined by overexpression of NLGN1 or NRXN1β in *Drosophila* S2 cells; these normally nonadherent cells formed aggregates when mixed, but not in the absence of Ca^{2+} or when soluble NRXN1β was added to block binding [29]. The kinetics of this aggregation were similar to that of aggregation mediated by other CAMs, such as the cadherins, suggesting that the function of NRXNs and NLGNs as CAMs is physiologically relevant [29]. As other neuronally-expressed CAMs have been shown to play a role in neurite outgrowth, the fact that NRXNs and NLGNs function as CAMs presents the possibility that they too may promote neurite outgrowth during development.

Ca^{2+}-dependence of the NRXN-NLGN complex

As half-maximal NRXN-NLGN binding requires a concentration of approximately 2 μM free Ca^{2+}, the binding sites have a high affinity for Ca^{2+} and are likely saturated in vivo, making Ca^{2+} ions a structural element of the NRXN-NLGN interaction [29]. Multiple experiments suggested that NLGN, not NRXN, was responsible for Ca^{2+} binding [29]. However, the EF hand motif initially thought to mediate this binding in NLGN1 does not bind Ca^{2+} [23]. Two sites within the NRXN LNS4 domain suspected to be involved in Ca^{2+} binding based on homology with the laminin LNS domain are localized to the NRXN-NLGN binding interface (Graf et al., 2006). Mutation of these sites disrupts the ability of NRXN to bind NLGNs, confirming that NRXNs are responsible for Ca^{2+} binding (Graf et al., 2006).

Modulation of binding by alternative splicing

NLGN binding was originally believed to be limited to β-NRXNs lacking an insert at splice site 4 [22,24]. It is now understood that the NLGNs can bind to both α- and β-NRXNs, and that the presence or absence of an insert at splice site 4 of NRXNs and splice site B of NLGNs modifies the affinity of this interaction [30]. Crystal structures of the NRXN1β-NLGN1 complex localized the splice sites of both proteins to close to the binding interface, confirming the effects of alternative splicing on this interaction [23]. The binding properties of different splice variants of NLGN1 and NRXN1β have been thoroughly mapped by surface plasmon resonance and affinity chromatography [31].

While NLGNs were originally believed to bind only to β-NRXNs, binding of α-NRXNs to all three NLGNs was later demonstrated; the presence of an eight-residue

insert at splice site B in NLGN1 prevented this binding in earlier trials [32]. The lack of binding of insert-positive NLGNs to α-NRXNs resulted from steric hindrance due to the N-glycosylation of Asn-303 in the insert at splice site B [32,33]. NLGNs bound to α-NRXNs through LNS 6, as is the case for β-NRXNs; the differential binding abilities appear to result from the different N-terminal sequences of α- and β-NRXNs [32]. Thus, the presence of an insert at splice site B of NLGN prevents the binding of α-NRXNs, while the β-NRXNs are able to bind regardless of the status of splice site B [32]. Splice site B appears to be used only in NLGN1 [34].

Further study of the NRXN1β LNS domain responsible for mediating binding to NLGNs found that the presence of an insert at splice site 4 is not an absolute regulator of NLGN binding. Rather, the presence of this insert abolishes binding of NRXN1β to NLGN1 and NLGN4, but has a more modest effect on binding to NLGN2 and NLGN3 [35]. In an assay of the ability of insert-positive NRXN1β to induce clustering of NLGNs and associated proteins, the presence of the insert permitted clustering of NLGN2 but prevented clustering of NLGN3, suggesting that the presence or absence of this insert has a subtle and complex effect on the NRXN-NLGN interaction [35]. Consistent with these findings, x-ray and neutron scattering analysis found that splice site 4 is not directly located to the NRXN-NLGN binding interface, but rather to its edge [34]. Splice site 4 of NRXNs and splice site 2 of NLGNs are located at roughly opposite sides of the binding interface, suggesting that their presence or absence could produce subtle, additive effects on binding [23].

Structure of the NRXN-NLGN complex

The soluble extracellular domain of NLGN1 constitutively forms dimers with two associated NRXN1β molecules [33]. Analytical sedimentation confirmed that NLGN1 proteins sediment as dimers, while NRXN1β molecules exist as monomers in the absence of NLGN1 [31]. No higher order NLGN1 oligomers were detected by sedimentation equilibrium analysis [33]. The presence or absence of the O-glycosylated stalk region had no effect on dimerization [33].

X-ray and neutron scattering analysis of the AChE-like domains of NLGNs 1-4 indicated that the four NLGNs exist as dimers with highly similar structural configurations and confirmed the interaction of each dimer with two NRXN molecules located on opposite sides of the dimer [34,36]. Intriguingly, a recent study revealed the presence of NLGN1-NLGN3 and NLGN2-NLGN3 binding in brain extracts, while co-immunoprecipitation of NLGN1 and NLGN2 was not observed [6]. It remains to be determined whether these interactions represent heterodimers or higher-order associations of homodimers [6].

When NRXNs were first visualized by immunoelectron microscopy, a subset of NRXN molecules were localized to the postsynaptic membrane, long believed to be the exclusive domain of NLGNs [37]. This raised the possibility that NRXNs and NLGNs may interact in *cis* within the plane of the membrane. While this interaction has not been visualized or characterized directly, *cis*-expression of NRXN inhibited *trans*-interaction of NRXNs and NLGNs, suggesting *cis*-binding occurs under physiological conditions.

Intriguingly, *trans*-binding of NRXNs to NLGN1 was equally disrupted by postsynaptically expressed NRXN1β possessing or lacking an insert at splice site 4 [37].

Expression patterns of NRXNs and NLGNs

Distribution of NRXN isoforms in the CNS

Initial experiments on the expression pattern of NRXN family members found that all three nrxn genes are expressed at significant levels only in brain [20]. The different NRXN isoforms were found to be expressed in distinct classes of neurons, but to have a somewhat overlapping pattern of expression [20]. In situ hybridization experiments revealed a sharper delineation in the expression of NRXN isoforms containing or lacking the insert at splice site 4 [22]. The cortex and cerebellum of adult rat brains showed more mRNA for insert-negative transcripts of NRXN1 and NRXN2 than their insert-positive counterparts, while the pattern was reversed for several deep structures. Insert-negative NRXN3 transcripts were present at high levels in the hippocampus, which lacked the insert-positive transcript altogether [22]. The significance of this distribution has not yet been determined.

NRXNs were initially suspected to be localized to synapses on the basis of their interaction with components of the exocytic machinery [16]. An antibody to NRXNs initially found that they were located to synaptic sites [17]. A second antibody directed towards the intracellular C-terminus and capable of detecting all six major forms of NRXN proteins demonstrated that NRXN family members were, indeed, localized to

synapses in culture and *in vivo*, and that NRXNs were localized to growth cones of developing axons originating from pontine explants [7]. NRXNs are thus ideally positioned to mediate neurite outgrowth during development. When NRXNs were definitively localized by immunogold electron microscopy, it was discovered that a pool of NRXNs is present in the postsynaptic membrane, in addition to the long-presumed presynaptic population of NRXN molecules [37]. This was consistent with earlier results which implicated NRXNs in postsynaptic NMDA receptor function in a cell-autonomous manner [38].

Distribution of NLGN isoforms in the CNS

NLGNs 1, 2 and 3 are expressed primarily in neurons in rodents [6,22,24]. However, NLGN3 is significantly expressed in glia during development and into adulthood [39]. Unlike the NRXNs, the pattern of NLGN expression in the brain of newborn and adult mice was revealed by *in situ* hybridization to be almost entirely overlapping, with very few areas of differential expression [27]. NLGN1 was found by immunohistochemistry to be present in neurons in all areas of the brain [4]. Expression patterns of NLGN1 and NLGN2 in rat cerebellum at postnatal day 2 (P2) were analyzed by *in situ* hybridization and found to overlap to a high degree [40]. At P8, around the time of synaptogenesis, NLGN1 and NLGN2 expression was found to overlap in both Purkinje and cerebellar granule cells [40]. As with the NRXNs, the significance of this overlapping expression has not yet been determined.

Subcellular fractionation of adult rat brain revealed enrichment of NLGN1 in synaptic plasma membranes, but was unable to differentiate between pre- and

postsynaptic localization [22]. NLGN3 was also localized to synapses by subcellular fractionation [6]. Another investigation of NLGN1 localization reported that soluble NRXN fragments bound to the entire surface of hippocampal neurons, but that this signal was concentrated in neurites [22]. Immunocytochemistry in adult rat brains demonstrated colocalization of NLGN1 with PSD-95 and a subunit of the NMDA receptor [4]. NLGN1 was then definitively localized to the postsynaptic membrane by immunogold electron microscopy [4]. This localization is consistent with the identification of a dendritic targeting motif in the short intracellular C-terminus of NLGNs [41,42].

Developmental expression of NRXNs and NLGNs in the CNS

Consistent with their well-characterized role in synapse development, NRXNs and NLGNs are expressed at high levels during the period of synaptogenesis, which proceeds during the first few weeks of postnatal life [4]. However, they have also been shown to be expressed in the nervous system prior to synaptic development, raising the possibility that NRXNs and NLGNs may play a role in neurite outgrowth.

A low level of NLGN expression is evident at E12 and E16, although it takes off after birth, when synaptic proteins tend to be expressed [4]. NLGN3 expression in rat also reaches its peak around the time of synaptogenesis, but expression takes off very early in development; NLGN3 protein was detectable at E12, with strong expression as early as embryonic day 16 [6]. A similar pattern was detected for NLGNs 1,2, and 3 [27]. NLGNs 1-3 are detectable by quantitative western blotting in the brains of newborn mice, while NLGN4 is only detectable in adults. Over the course of development, levels of all NLGN proteins increase 2- to 3-fold, but NLGN4 contributes only 3% of the total NLGN protein

present in adult mouse brain. Analysis of mRNA transcripts over development revealed a similar pattern of expression, with NLGNs 1, 2, and 3 detectable at low levels in newborn mice [27]. This indicates that NLGNs have a wide pattern of expression in the brain at developmental stages prior to synapse formation.

Expression of NRXNs and NLGNs in other tissues

While NRXN and NLGN isoforms are primarily expressed in neuronal tissues in rodents, some isoforms are expressed at low levels in other tissues. Expression of both NRXNs and NLGNs was observed in the rodent pancreatic beta-cell secretory apparatus, which bears developmental and structural similarities to the neuronal exocytic machinery [43]. Indeed, NRXN-NLGN interactions have been shown to play a role in insulin secretion in this system [43].

While NLGN3 is expressed primarily in the brain in rodents, northern blot analysis demonstrated expression of human NLGN3 in a number of non-neuronal tissues, including heart, pancreas and skeletal muscle [44], indicating that NLGN3 has a broader pattern of expression in humans than in rodents. The identification of NLGN4 in humans was surprising, as this transcript was found primarily in non-neuronal tissues, including skeletal muscle, heart, liver, and pancreas, but at very low levels in brain [25]. It has been suggested that NLGN expression outside the nervous system may play a role in other adhesive functions, although NRXN expression in non-neuronal tissues besides the pancreas has not been demonstrated [17,25] and NLGNs do not produce homophilic interactions [29]. This raises the intriguing possibility that NLGNs may have additional binding partners which are expressed outside the CNS.

Intracellular interactions of NRXNs and NLGNs

The C-termini of NRXNs and NLGNs participate in a number of intracellular interactions which link these adhesion molecules to the cytoskeleton and to components of intracellular signaling pathways. NRXNs 1-3 participate in PDZ-mediated intracellular interactions with CASK, a scaffolding molecule primarily expressed in brain and localized to the pre- and postsynaptic densities in mature neurons [8]. A second region in the C-terminus of NRXNs, adjacent to the membrane, also plays a role in binding to CASK [8]. CASK forms a tripartite complex with Mints and Veli, and Mint1 and Mint2 can also bind directly to the NRXN C-terminus in a PDZ-dependent manner, providing an alternate mechanism of recruiting the complex to NRXNs [9]. As CASK can act as a structural molecule and also as a CaM kinase, the interaction of NRXNs with CASK may play important signaling functions [8]. Through CASK, NRXNs are able to interact with protein 4.1 and nucleate the assembly of actin filaments, providing a mechanism for linking NRXN function to the cytoskeletal rearrangements required for neurite outgrowth [10].

The C-terminal PDZ motif of NLGNs 1-3 mediates a direct physiological interaction with the third PDZ domain of PSD-95 [11]. NLGNs 1-3 were also found to bind to the related scaffolding proteins PSD-93 and SAP-102 [11]. The recently discovered NLGN4 also interacts with PSD-95 [25]. In addition to binding to these scaffolding proteins, NLGNs interact with the brain-specific synaptic scaffolding molecule (S-SCAM) through their PDZ domains [12]. S-SCAM also binds to NLGNs via a WW domain [13]. S-

SCAM, in turn, binds beta-catenin, providing a link between NLGNs and the cytoskeleton [14].

Studies of the structure of the NRXN-NLGN complex have consistently pointed to a 2:2 molecular ratio, consisting of NLGN dimers tightly associated to two individual NRXN molecules [31,33,34]. However, studies of the synaptogenic function of NRXN indicated that at least four NRXN monomers had to be aggregated in order for NRXN to promote synaptogenesis [7]. This discrepancy has led some to propose that higher-order complexes may be formed through the interactions of associated intracellular proteins [34,36]. Evidence that NRXNs and NLGNs participate in a multitude of interactions with diverse structural and signaling molecules supports our hypothesis that these proteins may signal to produce the cytoskeletal reorganization required for neurite outgrowth.

Current understanding of NRXN and NLGN function

Role in promoting synaptogenesis

Strikingly, presentation of NRXN or NLGN family members alone to the surface of neuronal cells can trigger the development of synaptic specializations, suggesting that the NRXN-NLGN complex may function as a bidirectional cue for synapse development. Expression of NLGN family members in noneuronal cells can trigger the development of presynaptic specializations in contacting axons [40], and NRXNs can also produce the aggregation of components of both excitatory and inhibitory postsynaptic densities [45]. While the deletion of the O-glycosylated linker region of NLGN1 slightly reduced the

ability of NLGN1 to form presynaptic specializations, removal of the AChE-like domain abolished this effect entirely [40]. The addition of a soluble form of NRXN1β also abolished this effect, indicating that the synaptogenic activity of NLGN1 is dependent upon its interaction with presynaptic NRXNs [40].

A later study demonstrated that the AChE-like region of NLGN alone, coated onto beads, was sufficient to produce the development of presynaptic specializations when presented to the surface of hippocampal neurons [7], suggesting that NLGN produces this effect simply by clustering NRXNs. Mutations of NLGN1 which reduced its synaptogenic activity in a similar assay were mapped to regions of the AChE-like domain responsible for oligomerization in AChE itself. These mutations were shown to abolish interactions between individual NLGN molecules, indicating that oligomerization of NLGN1 is required for its function [7].

Much work has focused on the localization of specific NRXNs and NLGNs to excitatory or inhibitory synapses specifically. The interest in this area has stemmed from the role of NRXNs and NLGNs in synaptic development, and the belief that specific combinations of NRXN and NLGN isoforms could act to specify synaptic identity during synaptogenesis [46]. NLGN2 appears to be localized specifically to inhibitory synapses [5], and mice lacking NLGN2 show a decrease in the number of inhibitory synapses without a corresponding decrease in overall synaptic number [47]. However, NLGN2 can also be detected at excitatory synapses under conditions of heightened PSD-95 expression [45], suggesting that regulation of PSD-95 levels may be a mechanism to determine the localization of NLGN2 *in vivo*. While the localization of NLGN1 to excitatory synapses

specifically appears to be more stringent [4,45], NLGN3 has been definitively localized to both excitatory and inhibitory synapses [6]. The involvement of the myriad splice variants of NRXNs and NLGNs has introduced a tremendous level of complexity to this question [48].

Potential role as cell-surface receptors

Several characteristics of the NRXNs and NLGNs support a function as cell-surface receptors. For example, the O-glycosylation domain of NLGN1 creates a carbohydrate-rich region near the membrane, a feature found in many other cell-surface receptors [22]. The extracellular structure of NRXNs resembles that of a cell-surface receptor [17,18], and several extracellular binding partners for NRXNs have been identified aside from the NLGNs. Specifically, the neurexophilins, a family of small proteins of unknown function with a structural resemblance to neuropeptides, have been shown to bind to the extracellular domain of α-NRXNs, but not β-NRXNs [8].

Phenotype of animals lacking NRXN or NLGN family members

Studies of animals deficient for one or multiple NRXNs or NLGNs have provided some insights into the function of these proteins *in vivo*. The similarity among members of each family and their largely overlapping patterns of expression suggests that there may be the ability for compensation within the NRXN family and the NLGN family. Indeed, mice deficient in NLGN1 are viable and have no reproductive defects [4]. Mice lacking NLGN2 display marked behavioral phenotypes and show a loss of inhibitory function, but have no decrease in overall synaptic number [47]. However, triple knockout mice lacking NLGNs 1, 2, and 3 have decreased body mass and die within 24 hours after

birth due to respiratory anomalies in the brainstem [27]. NLGN4 is not upregulated in the brains of these knockout mice to compensate for these defects. The loss of NLGNs 1-3 does not grossly affect the cytoarchitecture of the brain or alter synaptic density, although synaptic function is disrupted [27].

Mice lacking even one of the three α-NRXNs have impaired survival, double knockouts do not survive past the first week, and triple knockout mice perish on the first day of life [49]. This decreased survival was due to respiratory impairment at the level of the brainstem. The mice showed a slight decrease in synaptic density, but did not show any gross abnormalities in brain mass or axonal pathfinding. The most striking defect was a reduction in synaptic function resulting from a loss of coupling between calcium channels and the presynaptic release machinery [49]. This effect could be rescued by NRXN1α but not NRXN1β, implicating the large extracellular sequences present only in α-NRXNs in regulating Ca^{2+} channels [50]. The same mice were shown to have some defects in postsynaptic NMDA receptor function [38].

While the defects in axonal pathfinding observed in even the NRXN or NLGN triple knockout mice were not severe, showing more defects at the level of the synapse, this does not negate the hypothesis that NRXNs and NLGNs are involved in promoting neurite outgrowth. In fact, the defects observed in NRXN or NLGN knockout animals are consistent with those seen in mice deficient in NCAM or N-cadherin, two CAMs whose roles in promoting neurite outgrowth are well documented [15].

Implication in autism spectrum disorders

NRXN-NLGN interactions have recently been implicated in autism spectrum disorders in humans. Mutations in NLGN3 and NLGN4 were first identified in sibling pairs diagnosed with autism-spectrum disorders [26], leading to a number of other discoveries of genetic links to NRXNs and NLGNs [36]. One mutation in human NLGN3 producing familial autism has been shown to interfere with the processing of NLGN3, leading to intracellular retention [51]. Studies of other families with autism-spectrum disorders have revealed chromosomal mutations corresponding to the positions of NLGN1 and NLGN2 [52,53]. A screen of a large number of autism patients revealed structural variants in the signal peptide of NRXN1β, though no variants in NRXN2 and NRXN3 were uncovered in this population [54]. Intriguingly, polymorphisms in NRXN3 have been implicated in alcohol dependence [55], and mice lacking NLGN2 display a marked behavioral phenotype characterized by anxiety-like behaviors [47]. Taken together, these data provide evidence that defects in NRXNs and NLGNs are associated with a range of social and behavioral phenotypes. Given that the NRXN-NLGN cell adhesion complex has been implicated in certain autism spectrum disorders, studies on the effects of these protein families on neurite outgrowth may yield insight into the development of certain neurological systems.

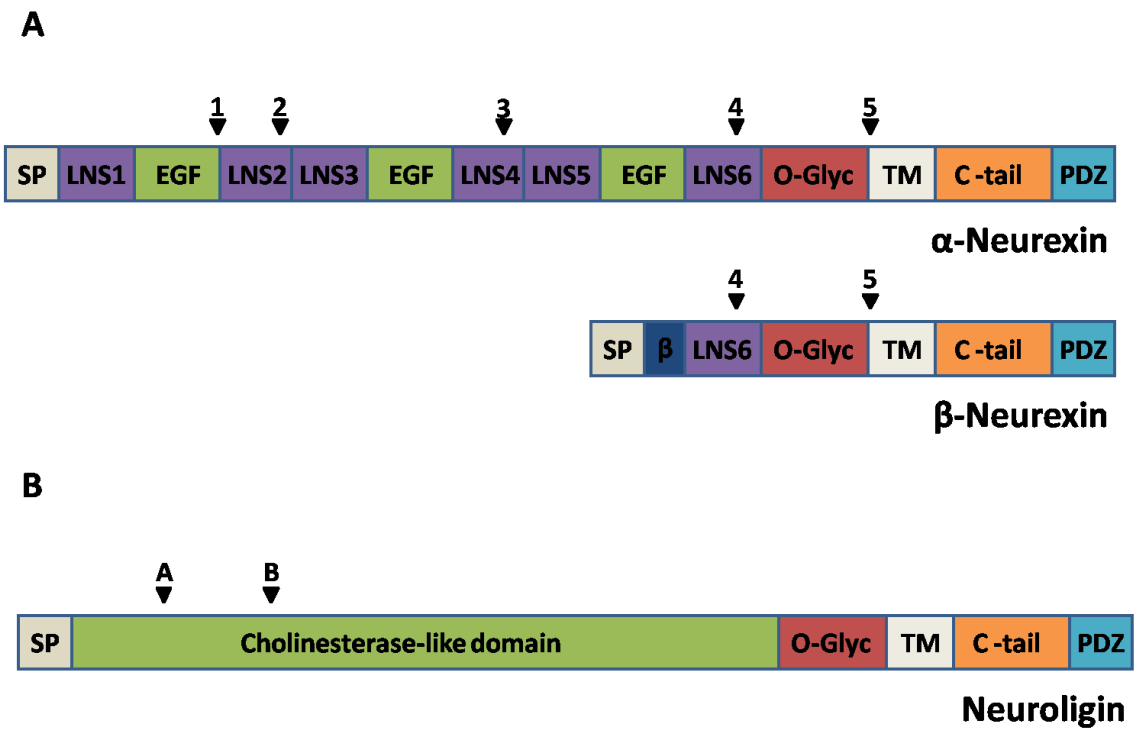

Fig. 1. Schematic representation of the generalized domain structure of (A) neurexins and (B) neuroligins. SP, signal peptide; LNS, laminin / neurexin / sex hormone-binding globulin domain; EGF, epidermal growth factor-like domain; O-Glyc, O-glycosylation domain; TM, transmembrane domain; PDZ, PDZ motif; β, β-specific sequence. Arrowheads indicate the positions of splice sites.

CHAPTER II: A NOVEL FUNCTION FOR NEUROLIGIN 1 IN PROMOTING NEURITE OUTGROWTH IN N1E-115 NEUROBLASTOMA CELLS

A Novel Function for Neuroligin 1 in Promoting Neurite Outgrowth in N1E-115 Neuroblastoma Cells

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Abbreviations: AChE, acetylcholinesterase; CAMs, cell adhesion molecules; C-terminal, carboxy terminal; FGFR, fibroblast growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HA, haemagglutinin; LNS, Laminin/Neurexin/Sex hormone-binding globulin; N-cadherin, neural cadherin; NCAM, neural cell adhesion molecule; NLGN, neuroligin; NRXN, neurexin; N-terminal, amino terminal; RT-PCR, reverse transcription-PCR; TFR, transferrin receptor.

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CONTRIBUTION OF AUTHORS

The writing of the manuscript was a collaborative effort between myself and Dr. Bedford with contributions from Dr. Lamarche-Vane. Mathura Thevarajah assisted with production of constructs. I performed the experiments in all of the figures and carried out all quantification and statistical analysis.

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ABSTRACT

Neurexins and neuroligins are heterophilic cell-adhesion molecules which interact with the cytoskeleton and play a role in synaptogenesis. However, they are also expressed prior to synapse formation, suggestive of additional functions. Other neuronally-expressed cell-adhesion molecules also play a role in promoting neurite outgrowth. Here, we demonstrate a novel role for neuroligin 1 in promoting neurite outgrowth in N1E-115 neuroblastoma cells. Furthermore, we identify both the acetylcholinesterase-like domain and PDZ motif as essential requirements for this process. Identification of this novel role for neuroligin 1 is particularly pertinent as neuroligin family members are linked to the autism spectrum of neurodevelopmental disorders.

1. Introduction

The neurexins (NRXNs) and neuroligins (NLGNs) are two families of neuronally-expressed cell adhesion molecules (CAMs) [1-4] that assemble to form a heterophilic adhesion complex [5], which has previously been implicated in the process of synaptogenesis [6,7]. NRXNs and NLGNs are however also expressed prior to synaptogenesis, as early as embryonic day 12 [4,8], suggestive of additional roles in the nervous system. Three NRXN (NRXN 1-3) and four NLGN (NLGN 1-4) genes exist in rodents, each encoding a single-transmembrane domain protein subject to extensive alternative splicing [1-4]. Each NRXN protein can be expressed as a long α or an amino (N)-terminal truncated β form [1,2]. Both forms bind NLGNs through a conserved extracellular LNS (Laminin/Neurexin/Sex hormone-binding globulin) domain [1-4] and nucleate the assembly of actin filaments through an intracellular interaction with a CASK / protein 4.1 complex [9]. NLGNs, in turn, interact with NRXNs through a catalytically inactive extracellular acetylcholinesterase (AChE)-like domain [3,4,10] and with a number of actin-associated scaffolding molecules, including PSD-95, predominantly through an intracellular carboxy (C)-terminal PDZ motif [11].

Other neuronally-expressed CAMs such as L1, the neural cell adhesion molecule (NCAM) and neural (N)-cadherin have been shown to mediate multiple functions in addition to their roles at the synapse. Amongst these is the induction of neurite outgrowth [12], which is the extension of processes by neuronally-derived cells and involves the rearrangement of the cytoskeleton [13]. We therefore set out to examine whether the NRXN-NLGN complex is able to induce neurite outgrowth. Here we demonstrate that one component of this complex, NLGN1, can promote this activity in N1E-115

neuroblastoma cells and that interactions through both the AChE-like domain and PDZ motif are essential for this process.

2. Materials and Methods

2.1. Plasmids and constructs

Murine haemagglutinin (HA)-tagged NLGN1 (A+B site negative) was kindly provided by Peter Scheiffele (Columbia University, New York, NY, USA). HA-tagged transferrin receptor (HA-TfR, Belouzard and Rouille, 2006) was kindly provided by Yves Rouille (Institut Pasteur de Lille, Lille Cedex, France). To generate NLGN1-ΔN, a XhoI / KpnI digested PCR product produced using primers ΔN-5' and ΔN-3' was swapped with the wild-type XhoI / Kpn I fragment, deleting the AChE-like domain. To generate NLGN1-ΔPDZ, a KpnI / SphI digested PCR product produced using primers ΔPDZ-5' and ΔPDZ-3' was swapped with the wild-type KpnI / SphI fragment, deleting the PDZ motif. To generate NLGN1-ΔΔ, ΔN and ΔPDZ were KpnI / SphI digested and ligated. For primers see supplementary material online.

2.2 Reverse-transcription (RT)-PCR

Total RNA was isolated from N1E-115 cells and mouse hindbrain tissue using TRIZOL reagent (Invitrogen). First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen), and PCR performed as described [14]. For primers see supplementary material online.

2.3 Cell culture and transfection

N1E-115 mouse neuroblastoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics at 5% CO2. Cells were plated at a concentration of 5.6x103 cells cm-2 onto coverslips coated with 25 μg ml-1 laminin (VWR Canlab) in Hank's Balanced Salt Solution (Invitrogen) and transfected using Lipofectamine 2000 (Invitrogen).

2.4 Immunofluorescence

N1E-115 cells were processed for immunofluorescence 16 hours post-transfection as described [14], using mouse anti-HA (HA.11, Covance) and an alexa-488-conjugated goat anti-mouse secondary (Invitrogen). Cells were counterstained with rhodamine-conjugated phalloidin and DAPI. Images were acquired on a Zeiss Axiovert 135 microscope using Zeiss oil immersion 63x objective.

2.5 Quantification and statistical analysis

A neurite was defined as a process equal to or exceeding one cell body length. Phalloidin staining was used to assess the health of the cells. At least 150 healthy transfected cells were counted for each experiment and condition. All data are averages of 3 or more independent experiments ±SEM. Statistical significance was determined by paired two-way Student's t-test. Asterisks indicate statistically significant differences; *$p<0.05$, **$p<0.01$, ***$p<0.001$, NS = not significantly different.

3. Results

3.1 Expression of nrxns and nlgns in N1E-115 cells

N1E-115 neuroblastoma cells are a commonly used system for characterizing the role of molecules in inducing neurite outgrowth [15]. Therefore, we used these cells to investigate a role for the NRXN-NLGN complex in inducing neurite outgrowth. We first examined the endogenous expression of nrxn and nlgn genes in N1E-115 cells by RT-PCR. PCR primers were designed to recognize all isoforms of each nrxn or nlgn tested, and adult mouse hindbrain was used as a positive control for these primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a loading control. All primers produced a PCR product of the appropriate size in mouse hindbrain but not in water control. PCR products were obtained for nrxn2, nlgn1, and nlgn2 in N1E-115 cells (Fig. 1). Therefore, nrxn2, nlgn1, and nlgn2 but not nrxn1 are endogenously expressed in N1E-115 cells.

3.2 NLGN1 promotes neurite outgrowth in N1E-115 cells

To characterize the role of NLGN1 in promoting neurite outgrowth, we transiently transfected N1E-115 cells with HA-NLGN1, processed them for immunofluorescence and quantified the proportion of HA-NLGN1 transfected cells with neurites as compared to a HA-tagged transferrin receptor (HA-TFR) control. HA-TFR does not induce neurite outgrowth above background levels (Supplementary Fig. S1). HA-TFR transfected cells showed a predominantly rounded phenotype (Fig. 2A, left), while HA-NLGN1 transfected cells were characterized by long, thin neurites (Fig. 2A, right). Further we

found 36.6±3.4% of HA-NLGN1 and 19.2±2.6% of HA-TFR transfected cells bore neurites, representing a statistically significant 2.20±0.20-fold increase for HA-NLGN1 in neurite outgrowth over HA-TFR control (Fig. 2B).

Some neurite outgrowth-promoting factors can also induce multiple neurites or neurite branching [16,17]. To address these possibilities, we assessed the percent of cells with multiple neurites or branched neurites among HA-NLGN1 or HA-TFR expressing neurite-positive cells. We found 26.4±2.9% and 14.9±3.3% of HA-NLGN1 and 26.9±1.8% and 13.2±2.6% of HA-TFR expressing neurite-positive cells with multiple neurites or branched neurites, respectively (Fig. 2C and D), representing no significant difference in either measure. Taken together, these results show NLGN1 promotes neurite outgrowth, but not the induction of multiple neurites per cell or neurite branching, in N1E-115 cells.

3.3 Deletion of either the AChE-like domain or the PDZ motif partially disrupts NLGN1-mediated neurite outgrowth

To identify which domain(s) of NLGN1 mediates the neurite outgrowth phenotype, we generated two NLGN1 mutants and tested them in the N1E-115 assay. These were HA-ΔN, lacking the AChE-like domain and thus the ability to dimerize and interact with endogenous NRXNs [10], and HA-ΔPDZ, lacking the C-terminal PDZ motif, abolishing interactions with a number of scaffolding molecules [11] (Fig. 3A). We found 27.2±1.7% of HA-ΔN and 25.0±2.2% of HA-ΔPDZ transfected cells bore long, thin neurites (Fig. 3B-D) and were morphologically similar to HA-NLGN1 transfected cells. These values were significantly below the proportion of HA-NLGN1 transfected cells with neurites

(41.7±2.6%) (Fig. 3D), indicating that deletion of either the AChE-like domain or the PDZ motif partially disrupts NLGN1-mediated neurite outgrowth. However, these values represented statistically significant 1.56±0.08 and 1.45±0.16-fold increases over HA-TFR control respectively (Fig. 3D), indicating that both mutants retain partial function. Together, these results show both the AChE-like domain and PDZ motif are required for the full function of NLGN1 in promoting neurite outgrowth.

3.4 Deletion of both the AChE-like domain and PDZ motif abolishes NLGN1-mediated neurite outgrowth.

To exclude the possibility that additional domains of NLGN1 contribute to promoting neurite outgrowth, we generated a double mutant, HA-ΔΔ, lacking both the AChE-like domain and PDZ motif (Fig. 4A), and tested it in our N1E-115 assay system. We found HA-ΔΔ transfected cells displayed predominantly rounded phenotypes (Fig. 4B) and showed no significant difference in neurite outgrowth over control, with 21.2±0.9% of HA-ΔΔ and 19.2±1.0% of HA-TFR transfected cells bearing neurites (Fig. 4C). Taken together, our results demonstrate that only the AChE-like domain and PDZ motif of NLGN1 are required, strongly suggesting that protein-protein interactions with these regions mediate NLGN1-dependent neurite outgrowth.

4. Discussion

Here we identify a novel function for NLGN1 in promoting neurite outgrowth in NIE-115 neuroblastoma cells. We show that (i) certain NRXN and NLGN family

members are expressed in N1E-115 cells, (ii) NLGN1 transfected cells are characterized by long thin neurites, (iii) NLGN1 promotes neurite outgrowth but not neurite initiation or branching, (iv) NLGN1 deletion mutants lacking either the AChE-like domain or PDZ motif retain partial function but that (v) a NLGN1 deletion mutant lacking both the AChE-like domain and PDZ motif fails to promote neurite outgrowth. Together, these results indicate NLGN1 mediates neurite outgrowth by a mechanism that involves both interactions through its extracellular AChE-like domain and intracellular PDZ motif.

The ability of NLGN1 to produce neurite outgrowth is consistent with the role of other neuronally-expressed CAMs [12]. Intriguingly, overexpression of NLGN1 did not produce an increase in the proportion of positive cells with either branched neurites or multiple neurites, suggesting that NLGN1 promotes the outgrowth of existing neurites, but is not involved in neuritogenesis.

Our results show HA-ΔN, which cannot bind NRXNs, possesses only partial neurite outgrowth activity, suggesting that interactions between HA-NLGN1 and endogenous NRXN2 (Fig. 1) are important for the effects of NLGN1. This could result from interactions between HA-NLGN1 on a transfected cell and NRXN2 on contacting cells or interactions on the surface of a transfected cell [18]. Additionally our results show HA-ΔPDZ also possesses only partial neurite outgrowth activity, suggesting interactions with scaffolding molecules and thus the actin cytoskeleton are equally important. The complete loss of the neurite outgrowth phenotype with the HA-ΔΔ mutant indicates that additional intracellular interactions are not involved. The maintenance of a partial phenotype with the single mutants also raises the possibility that NLGN1 may function at

least partly through a coreceptor. The outgrowth-promoting CAMs N-cadherin, L1, and NCAM all require binding to the fibroblast growth factor receptor (FGFR) for signal transduction [12]. It will therefore be interesting to examine whether a coreceptor can modulate NLGN1 neurite outgrowth functions.

NRXNs have been localized to the growth cones of developing axons [10]. The presence of NLGNs on growth cones however is unlikely, due to a dendritic targeting motif [19]. It is more likely that NLGN1 plays a role in the extension of dendritic processes to meet incoming axons during development and during adult plasticity. Such a role is consistent with reported findings showing the density of dendritic protrusions in cultured rat hippocampal neurons is dependent on NLGN1 levels and the ability of NLGN1 to bind NRXNs [20].

Defects in members of the NRXN and NLGN families have been associated with cases of familial autism-spectrum disorders in humans [21,22]. While the range of symptoms and underlying causes of these disorders are complex, autism-spectrum disorders are associated with aberrations in dendrites and dendritic spine morphologies [23]. Our finding that NLGN1 can promote neurite outgrowth indicates one functional mechanism of the NRXN-NLGN complex that may be compromised in autism spectrum disorders.

5. Acknowledgements

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6. References

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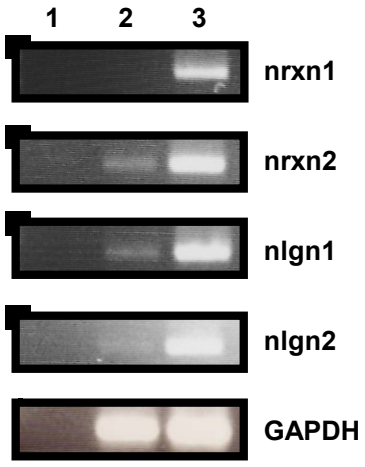

Fig. 1. Expression of nrxns and nlgns in N1E-115 cells. RT-PCR of (1) water control or RNA isolated from (2) N1E-115 cells or (3) adult mouse hindbrain for nrxn1, nrxn2, nlgn1, nlgn2 and GAPDH control.

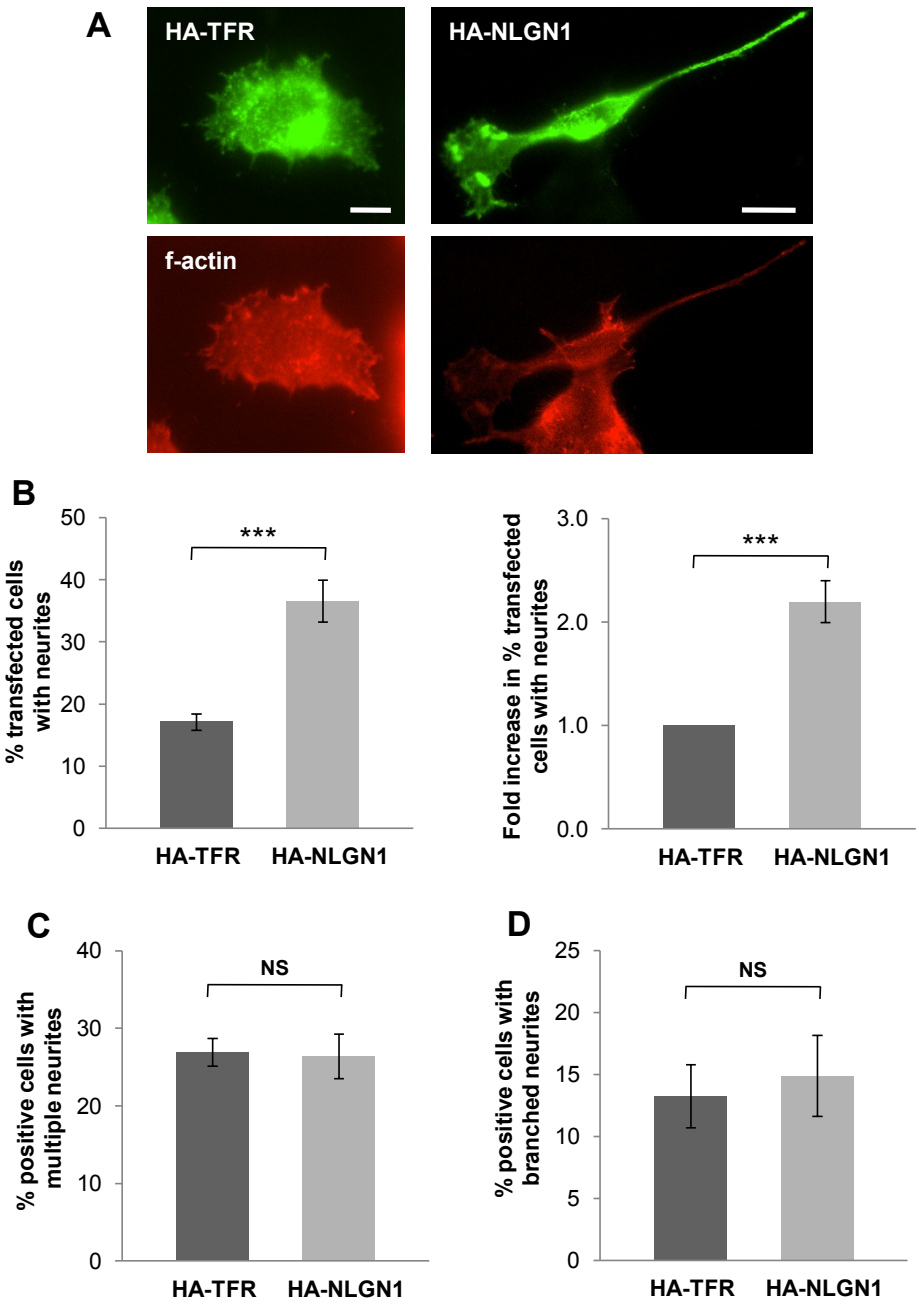

Fig. 2. NLGN1 promotes neurite outgrowth in N1E-115 cells. (A) Typical N1E-115 cells transfected with HA-TFR (left) or HA-NLGN1 (right) processed for immunofluorescence (top panels) and stained with phalloidin (bottom panels). Scale bars, 10μm. (B) Percent

(%) of HA-TFR or HA-NLGN1 transfected cells with neurites, absolute values (left) and fold increase in percent with neurites normalized to HA-TFR (right) (n=8). For statistical details see Materials and Methods. (C) Percent of neurite-positive HA-TFR or HA-NLGN1 transfected cells with multiple neurites, absolute values (n=8). (D) Percent of neurite-positive HA-TFR or HA-NLGN1 transfected cells with branched neurites, absolute values (n=8).

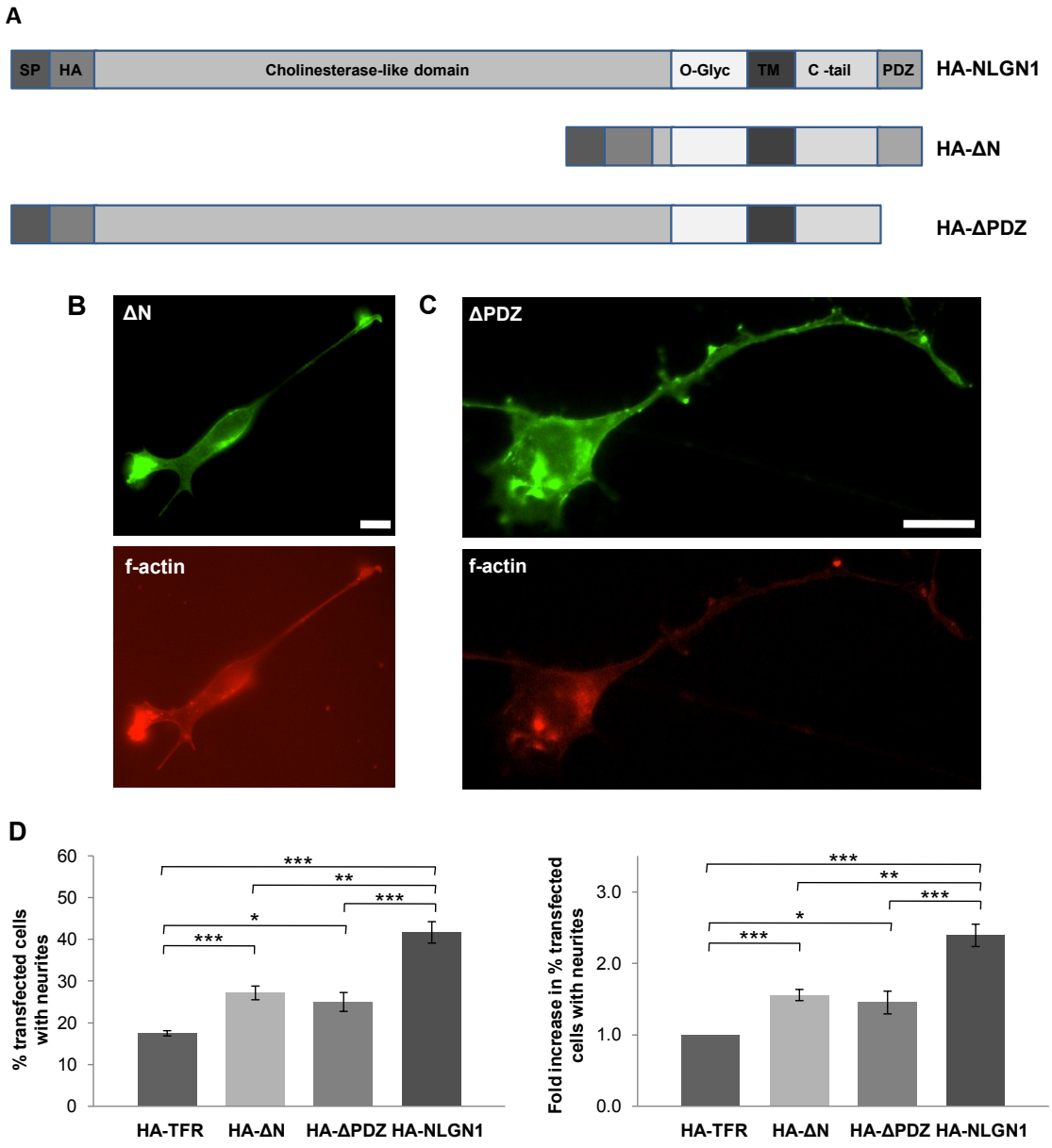

Fig. 3. Deletion of either the AChE-like domain or PDZ motif partially disrupts NLGN1-mediated neurite outgrowth. (A) Schematic representation of the domain structure of HA-NLGN1, HA-ΔN and HA-ΔPDZ. SP, signal peptide; O-Glyc, O-glycosylation domain;

TM, transmembrane domain and PDZ, PDZ motif. Typical HA-ΔN (B) and HA-PDZ (C) transfected N1E-115 cells showing long thin processes. Top, immunofluorescence; bottom, phalloidin. Scale bars, 10μm. (D) Percent of HA-TFR, HA-ΔN, HA-ΔPDZ or HA-NLGN1 transfected cells with neurites, absolute values (left) and fold increase in percent with neurites normalized to HA-TFR (right) (n=7).

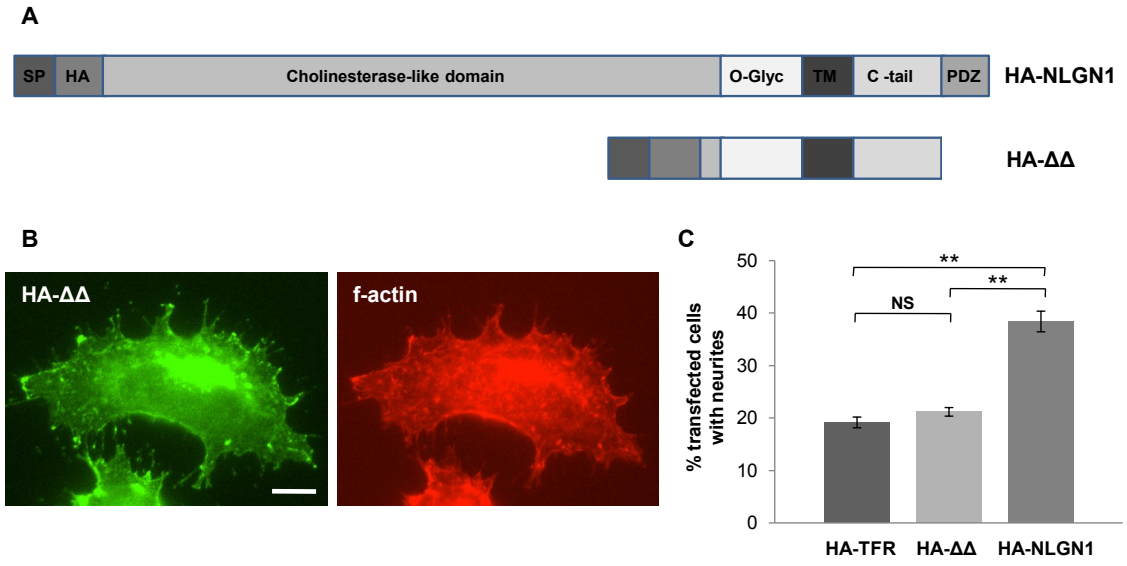

Fig. 4. Deletion of both the AChE-like domain and PDZ motif abolishes NLGN1-mediated neurite outgrowth. (A) Schematic representation of the domain structure of HA-NLGN1 and HA-ΔΔ. For domain details see Fig. 3. (B) Typical HA-ΔΔ transfected N1E-115 cell. Left, immunofluorescence; right, phalloidin. Scale bar, 10µm. (C) Percentage of HA-TFR, HA-ΔΔ or HA-NLGN1 transfected cells with neurites, absolute values (n=4).

Supplementary Table S1.

PCR primers used to generate deletion constructs and for RT-PCR

Deletion primers

ΔN-5’	TAGCGCTACCGGACTCAGATC
ΔN-3’	GTTGGTACCAATGCGTAGTCCGGAACGTCG
ΔPDZ-5’	CCAATAAGGTAAATCTCTGGCTGGAGCTGG
ΔPDZ-3’	CTATACGCATGCCTATGAATGTGAATGGGGGTGTGG

RT-PCR primers

Nrxn1-5’	CAACCCCACCAGAGTAGGTG
Nrxn1-3’	CCCTCCTGATCGCATTCCCTG
Nrxn2-5’	CAGGTGCAGTGGAGGTGATCC
Nrxn2-3’	CCTTGCTTGGCGTCTTGGGG
Nlgn1-5’	ACAGGATGATCCCAAGCAAC
Nlgn1-3’	CGTGATCCAAGTCAGTGTGC
Nlgn2-5’	CCAAGGGCCTATGACCGCTTC
Nlgn2-3’	GCTGCAGCGATACTAGCTCC

The oligonucleotides are written 5’ to 3’ and restriction sites are underlined.

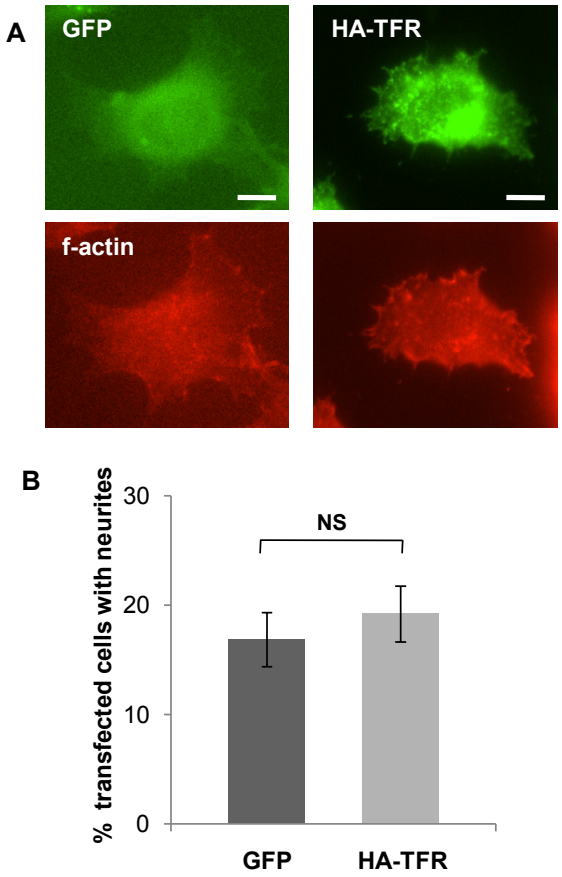

Fig. S1. HA-TFR does not induce neurite outgrowth in N1E-115 cells. (A) Typical N1E-115 cells transfected with pEGFP-C1, Clontech (left) or HA-TFR (right) processed for immunofluorescence (top panels) and stained with phalloidin (bottom panels). GFP-transfected cells were stained with phalloidin only. Scale bars, 10μm. GFP is commonly used as a control for morphological analyses and did not induce morphological changes in transfected cells (left). HA-TFR (right) did not induce morphological changes in transfected cells and had the added advantage of being a single-transmembrane domain

protein which clearly delineates the membrane and can be detected with the same antibody as NLGN1. (B) Percentage of GFP or HA-TFR transfected cells with neurites, absolute values (n=3). HA-TFR did not induce an increase in the percent of cells with neurites above the levels seen in GFP-transfected cells, which is consistent with the background levels of cells that spontaneously extend neurites in culture.

CHAPTER III: FURTHER CHARACTERIZATION OF THE ROLE OF NRXNS AND NLGNS IN NEURITE OUTGROWTH

Abstract

NRXNs and NLGNs are two families of neuronally expressed CAMs which have been implicated in synaptogenesis and are expressed in the murine brain prior to synaptic development. We have previously demonstrated that one member of this family, NLGN1, induces neurite outgrowth when expressed in N1E-115 neuroblastoma cells, a process which is thought to be mechanistically similar to the extension of axonal or dendritic processes in the developing nervous system. Here, we further investigate the effects of NLGN1 on neurite outgrowth in N1E-115 cells and characterize the effects of NLGN2 and NRXN1β on N1E-115 cell morphology. Further, we examine the expression pattern of NRXN and NLGN family members in the murine spinal cord at a developmental timepoint prior to synaptogenesis. We find that the effect of NLGN1 on neurite outgrowth in N1E-115 is not dose-dependent and that expression of NLGN1, NLGN2 or NRXN1β has distinct effects on N1E-115 cell morphology. Furthermore, we identify the spinal cord as a site of early expression of NRXN and NLGN family members, suggesting that these molecules may play a role in spinal cord development prior to synaptogenesis.

Introduction

Neurite outgrowth refers to the extension of processes by neuronally-derived cells in culture. Mechanisms used by cells in culture to extend processes are thought to be similar to those underlying the extension of dendritic or axonal processes by neurons in the developing nervous system [2]. A number of soluble and contact-mediated protein cues act to promote the outgrowth of neurites and other processes [56]. Several lines of evidence led us to investigate a potential role for the NRXN and NLGN families of heterophilic CAMs in promoting neurite outgrowth. Other neuronally-expressed CAMs possess well-characterized outgrowth-promoting activity [1]. Like many well-established outgrowth and guidance molecules [56], NRXNs have been localized to growth cones of developing axons, where they might detect and respond to environmental cues [7]. Both the NRXNs and NLGNs interact intracellularly with components of the scaffolding machinery [8-13], providing a mechanism for linking these proteins to the cytoskeletal rearrangements which underlie neurite outgrowth [57]. Finally, while NRXNs and NLGNs are involved in synaptic development [40,45], which proceeds over the course of the first few weeks of postnatal life [4], they can be detected in the mammalian brain as early as embryonic day 12 [3-6,28], which strongly suggests additional developmental functions. We therefore set out to demonstrate and characterize a role for the NRXN-NLGN complex in promoting neurite outgrowth. We previously identified a novel role for one component of this complex, NLGN1, in promoting neurite outgrowth in N1E-115 neuroblastoma cells (Chapter 2, Fig. 2). Further, we showed that both the extracellular domain responsible for binding to NRXNs [7,22] and the intracellular PDZ motif mediating interactions with a number of scaffolding molecules [11,12] are necessary and

sufficient for this activity (Chapter 2, Fig. 3 and 4). Based on our identification of the outgrowth-promoting function of NLGN1, the strict dendritic localization of NLGN1 in mature neurons [41,42], and the effects of NLGN1 on spine density in cultured neurons [58], we proposed that NLGN1 may play a role in the extension of dendritic filopodia during neuronal development (Chapter 2).

After establishing a novel role for NLGN1 in promoting neurite outgrowth, we next chose to investigate the possibility that other NRXN and NLGN family members may possess this activity. The rodent NLGNs 1-3 share a high degree of sequence homology, suggesting that other members of this family could also be involved in promoting neurite outgrowth [24]. NRXN binding to NLGN1 appears to be involved in NLGN1-mediated outgrowth, since abolishing the domain responsible for NRXN binding diminishes the ability of NLGN1 to produce this effect (Chapter 2, Figure 3). As NRXN-NLGN interaction in *cis* have been observed [37], and NRXNs also possess the characteristics suggestive of a role in neurite outgrowth [3,7-10], signaling to the cytoskeleton could occur partially downstream of endogenous NRXNs on a NLGN1-transfected cell. We chose to examine the effects of one NRXN and one additional NLGN on cell morphology in N1E-115 neuroblastoma cells. We selected NLGN2 for expression in N1E-115 cells due to its high degree of sequence homology with NLGN1 [24] and its strong endogenous expression in the developing and adult nervous system [5]. NRXN1β was selected for expression in N1E-115 cells as it is the best studied of the NRXN family members[18,33,37] and is expressed in the developing and adult nervous system [3].

Finally, we sought to investigate additional regions in the developing nervous system where the NRXN-NLGN complex could act to promote neurite outgrowth. One of the best-characterized processes which result from neurite outgrowth is the process of axon guidance, by which axons extend over long distances, responding to a variety of guidance cues, to connect with their targets. The spinal cord is a site of extensive axon outgrowth and guidance, as sensory and motor axons extend to make exquisitely wired connections with target cells [56]. If NRXNs and NLGNs are implicated in neurite outgrowth in the developing spinal cord, we would expect to see their expression early in embryonic life. However, studies of the developmental expression of NRXNs and NLGNs have focused on the embryonic brain. We therefore analyzed the endogenous expression of NRXN and NLGN family members in the murine spinal cord at a developmental time point concurrent with the earliest expression of these genes in the murine brain. We chose to analyze the expression of nrxn1, nrxn2, nlgn1 and nlgn2 as these genes are all expressed at high levels in the developing murine brain and are not expressed outside the nervous system [3-5].

Taken together, this work further characterizes the effects of NRXN and NLGN family members on cell morphology and demonstrates an additional site where the NRXN-NLGN complex may function during development.

Materials and Methods

Plasmids and constructs

Murine haemagglutinin (HA)-tagged NLGN1 (A+B site negative), HA-NLGN2, and HA-NRXN1β (site 4 negative) were kindly provided by Peter Scheiffele (Columbia University, New York, NY, USA). pEGFP-C1 was purchased from Clontech.

Reverse-transcription (RT)-PCR

Total RNA was isolated from N1E-115 cells and mouse hindbrain tissue using TRIZOL reagent (Invitrogen). First strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen), and PCR performed as described [59]. Primers used were:

Nrxn1-5’	CAACCCCACCAGAGTAGGTG
Nrxn1-3’	CCCTCCTGATCGCATTCCCTG
Nrxn2-5’	CAGGTGCAGTGGAGGTGATCC
Nrxn2-3	CCTTGCTTGGCGTCTTGGGG
Nlgn1-5’	ACAGGATGATCCCAAGCAAC
Nlgn1-3’	CGTGATCCAAGTCAGTGTGC
Nlgn2-5’	CCAAGGGCCTATGACCGCTTC
Nlgn2-3’	GCTGCAGCGATACTAGCTCC

Cell culture and transfection

N1E-115 mouse neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and antibiotics at 5% CO_2. Cells were

plated at a concentration of $5.6x10^3$ cells cm^{-2} onto coverslips coated with 25 μg ml^{-1} laminin (VWR Canlab) in Hank's Balanced Salt Solution (Invitrogen) and transfected using Lipofectamine 2000 (Invitrogen).

Immunofluorescence

N1E-115 cells were processed for immunofluorescence 16 hours post-transfection as described [59], using mouse monoclonal anti-HA (HA.11, Covance) and a fluorescein (FITC)-conjugated donkey anti-mouse secondary (Jackson Labs). Cells were counterstained with rhodamine-conjugated phalloidin and DAPI. GFP-transfected cells were counterstained only. Images were acquired on a Zeiss Axiovert 135 microscope using Zeiss oil immersion 63x objective.

Quantification and statistical analysis

A neurite was defined as a process of length equal to or exceeding one cell body length. Phalloidin staining was used to assess the health of the cells. At least 150 healthy transfected cells were counted for each experiment and condition. All data are expressed as averages of 3 or more independent experiments ±SEM. Statistical significance was determined by paired two-way Student's t-test. Asterisks indicate statistically significant differences; *$p<0.05$, **$p<0.01$, ***$p<0.001$, NS = not significantly different.

Results

NLGN1-dependent neurite outgrowth is not dose-dependent

We previously controlled for the possibility that expression of a membrane protein alone could induce neurite outgrowth in N1E-115 cells by expressing a transmembrane protein, the transferrin receptor (HA-TFR), which is unlikely to be associated with the neurite outgrowth machinery. HA-TFR did not produce an increase in neurite outgrowth over a more commonly used GFP control (Chapter 2, Fig. S1). We next sought to control for the possibility that the level of NLGN1 expression alone could explain the observed neurite outgrowth phenotype. To address this, we transiently transfected N1E-115 cells with HA-NLGN1 at low (one-half the amount of plasmid recommended by the manufacturer's protocol), mid (according to manufacturer's protocol), or high (twice the amount of plasmid recommended by the manufacturer's protocol) doses. We then processed the transfected cells for immunofluorescence and quantified the proportion of HA-NLGN1 transfected cells with neurites as compared to GFP control. We found no significant difference in neurite outgrowth between any of the three HA-NLGN1 conditions, with 28.4±3.0% of HA-NLGN1 low, 28.8±2.3% of HA-NLGN1 mid, and 27.8±2.3% of HA-NLGN1 high transfected cells bearing neurites. However, these conditions represented statistically significant 2.37±0.56-fold, 2.28±0.27-fold, and 2.23±0.36-fold increases respectively over the 13.8±2.5% of GFP transfected cells bearing neurites (Fig. 1). Thus, expression of NLGN1 induces neurite outgrowth in N1E-115 cells in a dose-independent manner.

NRXNs and NLGNs induce cytoskeletal rearrangements and alterations in cell morphology

Phenotypic analysis of N1E-115 cells transfected with NRXN or NLGN family members produced distinct phenotypes consistent with rearrangements of the cytoskeleton (Fig. 2). N1E-115 cells transfected with GFP demonstrated a phenotype indistinguishable from that of untransfected cells, characterized by adherence to the substrate and minimal membrane specializations (Fig. 2, A-B). NLGN1 expression produced an increase in the incidence of cells possessing a single long neurite with filopodia along its length (Fig. 2, C-D). Expression of NLGN2 produced cells with a number of short, broad neurites (Fig. 2, E-F). Expression of NRXN1β produced cells with many filopodia and lamellipodia, suggestive of activation of the cytoskeleton (Fig. 2, G-H). Thus, expression of both NRXN and NLGN family members in N1E-115 cells induces cytoskeletal rearrangements and alterations in cell morphology.

Expression of NRXNs and NLGNs in embryonic mouse spinal cord

While expression of NRXNs and NLGNs has been demonstrated as early as embryonic day 12 in murine brain [3-6,28], the expression pattern of NRXNs and NLGNs in the developing spinal cord has not been addressed. As the spinal cord is a major site of axon outgrowth during development, we sought to analyze the endogenous expression of nrxn and nlgn genes in spinal cord at embryonic day 13.5 by RT-PCR. PCR primers were designed to recognize all isoforms of each nrxn or nlgn tested, and adult mouse hindbrain was used as a positive control for these primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression was used as a loading control. All primers produced a PCR product of the appropriate size in mouse hindbrain but not in water control. PCR products were obtained for all sets of primers tested (Fig. 3). Thus,

nrxn1, nrxn2, nlgn1 and nlgn2 are endogenously expressed in the murine spinal cord at embryonic day 13.5.

Discussion

We have previously demonstrated a novel role for NLGN1 in promoting neurite outgrowth in N1E-115 neuroblastoma cells (Chapter 2). Here, we further characterize the roles of NRXNs and NLGNs in promoting neurite outgrowth in N1E-115 cells and analyze the expression of these genes in the developing murine spinal cord. We show that (i) the effect of NLGN1 on neurite outgrowth in N1E-115 cells is not dose-dependent, (ii) expression of other NRXN and NLGN family members produces distinct morphological changes in N1E-115 cells, and (iii) that NRXN and NLGN family members are expressed in the murine spinal cord as early as embryonic day 13.5. Taken together, these results show that NLGN1-dependent neurite outgrowth is not dependent on expression level and that other members of the NRXN and NLGN families can produce the cytoskeletal rearrangements required for this neurite outgrowth. Further, our results suggest that the outgrowth-promoting effects of the NRXN-NLGN complex may play a role in neural development in the spinal cord.

Dose-independence of neurite outgrowth induced by NLGN1expression in N1E-115 cells

In order to control for the possibility that the neurite outgrowth observed upon NLGN1 expression resulted from the levels of the protein instead of its activity, we quantified the neurite outgrowth phenotype of N1E-115 cells transfected with low,

medium, or high amounts of HA-NLGN1. Our results demonstrate that NLGN1 promotes neurite outgrowth in N1E-115 cells in a dose-independent manner. This dose-independence could result from the saturation of endogenous NRXN2 molecules or downstream signaling molecules in N1E-115 cells. Coexpression of NLGN1 and NRXN family members would help to clarify this question, and might produce even more dramatic neurite outgrowth phenotypes. Taken together with our finding that an unrelated membrane protein, HA-TFR, does not promote neurite outgrowth, the dose-independence of the neurite outgrowth phenotype observed upon NLGN1 expression suggests that NLGN1 specifically produces neurite outgrowth in N1E-115 cells through interactions with cytoskeletal proteins and signaling molecules.

Phenotypic effects of NRXN and NLGN expression in N1E-115 cells

Our morphological studies suggest that, in addition to NLGN1, other NRXN and NLGN family members can induce cytoskeletal rearrangements and alterations in cell morphology in N1E-115 cells. NLGN1-transfected cells most often displayed long, thin neurites, often with filopodia along their length (Fig. 2, C-D). This is consistent with the long neurites observed in our previous qualitative and quantitative experiments (Chapter 2, Fig. 2). Expression of NLGN2 in N1E-115 cells produced cells with shorter, broader processes, which also bore filopodia and were indicative of cytoskeletal rearrangements (Fig. 2, E-F). The different observed effects of NLGN1 and NLGN2 on neurite outgrowth are surprising considering the close relationship between these two molecules. NLGN1 and NLGN2 share 59% sequence homology, identical sites of alternative splicing, and similar binding properties [24]. However, the greatest regions of divergence between the

two molecules occur in the region of the intracellular C-termini [24], which is likely to mediate the intracellular interactions required to produce cytoskeletal rearrangements. Indeed, the NLGN2 sequence includes several short stretches of amino acids for which there are no counterparts in NLGN1 or NLGN3. This intracellular sequence divergence may explain the difference in effect of NLGN2 as compared to NLGN1 in neurite outgrowth. To investigate this possibility, it would be interesting to assess the morphological effects of mutant forms of NLGN1 and NLGN2 in which the intracellular domains had been swapped. As NLGN1 and NLGN2 are exclusively localized *in vivo* to the postsynaptic compartment of excitatory and inhibitory synapses, respectively [4,5,45], the differential effects of these proteins on neurite outgrowth may underlie the different dendritic morphologies associated with these synapse types. Further quantification and characterization of the differences between outgrowth mediated by NLGN1 and NLGN2 could help to clarify the mechanism of NLGN1-mediated neurite outgrowth.

Expression of NRXN1β in N1E-115 cells produced complex cytoskeletal rearrangements characterized by large numbers of lamellipodial and filopodial structures per transfected cell (Fig. 2, G-H), as opposed to the single long process most frequently observed in NLGN1 transfected cells. This raises the possibility that NRXN1β may signal more strongly to induce cytoskeletal rearrangements than NLGN1, and could therefore be a more potent outgrowth-promoting molecule. If so, this could reflect distinct roles for NRXNs and NLGNs during neuronal development. Unlike NLGNs, which are postsynaptic molecules and for which we have proposed a role in dendritic outgrowth, NRXNs have been localized to the growth cones of developing axons in culture [7] and are primarily presynaptic molecules in the adult nervous system [17,37].

As such, their hypothetical role in neurite outgrowth would be much closer to the roles played by well-characterized growth cone receptors such as DCC [60]. If NRXNs indeed promote outgrowth more potently than NLGNs, this could reflect the far longer distances which must be covered by developing axons, as opposed to developing dendrites, during neuronal development.

An alternate explanation could be that overexpression of NRXN1β produces extravagant cytoskeletal rearrangements in N1E-115 cells through *cis* interactions with endogenous NLGNs within the plane of the membrane. In such a scenario, the cytoskeletal rearrangements would result from signaling downstream of endogenous NLGNs rather than downstream of the overexpressed NRXN1β proteins. It is possible that the extent of cytoskeletal rearrangements and neurite outgrowth observed upon overexpression of NLGN1 (Chapter 2) was limited by the number of endogenous NRXN molecules available to stimulate outgrowth. Such a scenario would be consistent with the comparable levels of neurite outgrowth we observed with three different transfection levels of NLGN1 (Fig. 1). It is possible that under our assay conditions (Chapter 2), the number of endogenous NRXNs available was not sufficient to stimulate all NLGN1 molecules. Thus, the effects observed upon transfection of NRXN1β may result from a more complete stimulation of endogenous NLGN1 or stimulation of endogenous NLGN2. One way to test this would be to assess the neurite outgrowth phenotype of untransfected N1E-115 cells cultured in either typical medium or medium supplemented with the purified soluble extracellular domain of NRXN1β [40]. This would permit us to assess the effects of NRXN1β binding to endogenous NLGNs while ensuring that only NLGNs could mediate signaling to the cytoskeleton.

Endogenous expression of NRXNs and NLGNs in embryonic spinal cord

Our finding that NRXNs and NLGNs are expressed in the developing murine spinal cord (Fig. 3) raises the possibility that the NRXN-NLGN complex plays a role in the spinal cord prior to synaptogenesis. The ability of NLGN1 to promote neurite outgrowth (Chapter 2) and the effects of other NRXN and NLGN family members on cell morphology (Fig. 2) suggest that the NRXNs and NLGNs may function to promote axonal and dendritic outgrowth in the spinal cord during development. While synaptic density and gross structure of the spinal cord has not been addressed in NRXN or NLGN knockout animals, there is some evidence to suggest that loss of components of the NRXN-NLGN complex leads to spinal cord defects. Mice lacking NLGN2 display defects in motor coordination [47], suggestive of spinal cord defects. NLGN2 knockout mice also display a decrease in pain sensitivity [47], which could be related to defects in afferent spinal cord projections. Taken together, these results further characterize the effects of NRXNs and NLGNs on neurite outgrowth and cell morphology and point to a region where outgrowth promoted by the NRXN-NLGN complex could play a role during development.

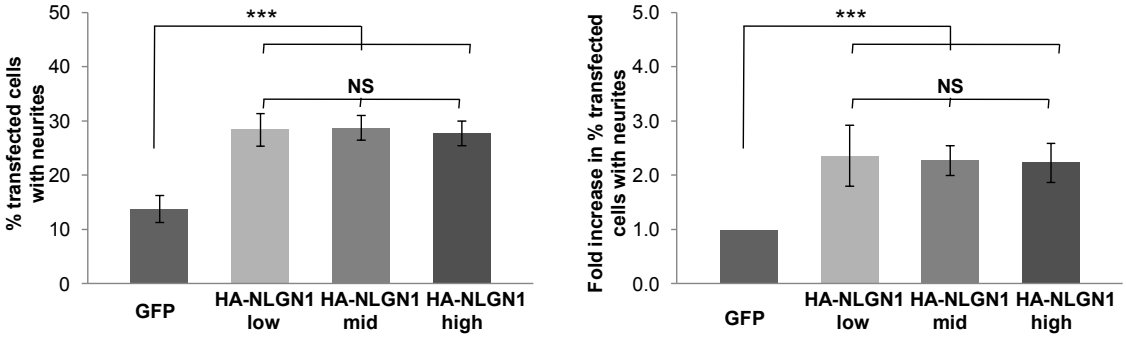

Fig. 1. NLGN1-mediated neurite outgrowth is not dose-dependent. Percent (%) of GFP, HA-NLGN1 low, HA-NLGN1 mid, or HA-NLGN1 high transfected cells with neurites, absolute values (left) and fold increase in percent with neurites normalized to GFP (right) (n=3). For statistical details see Materials and Methods.

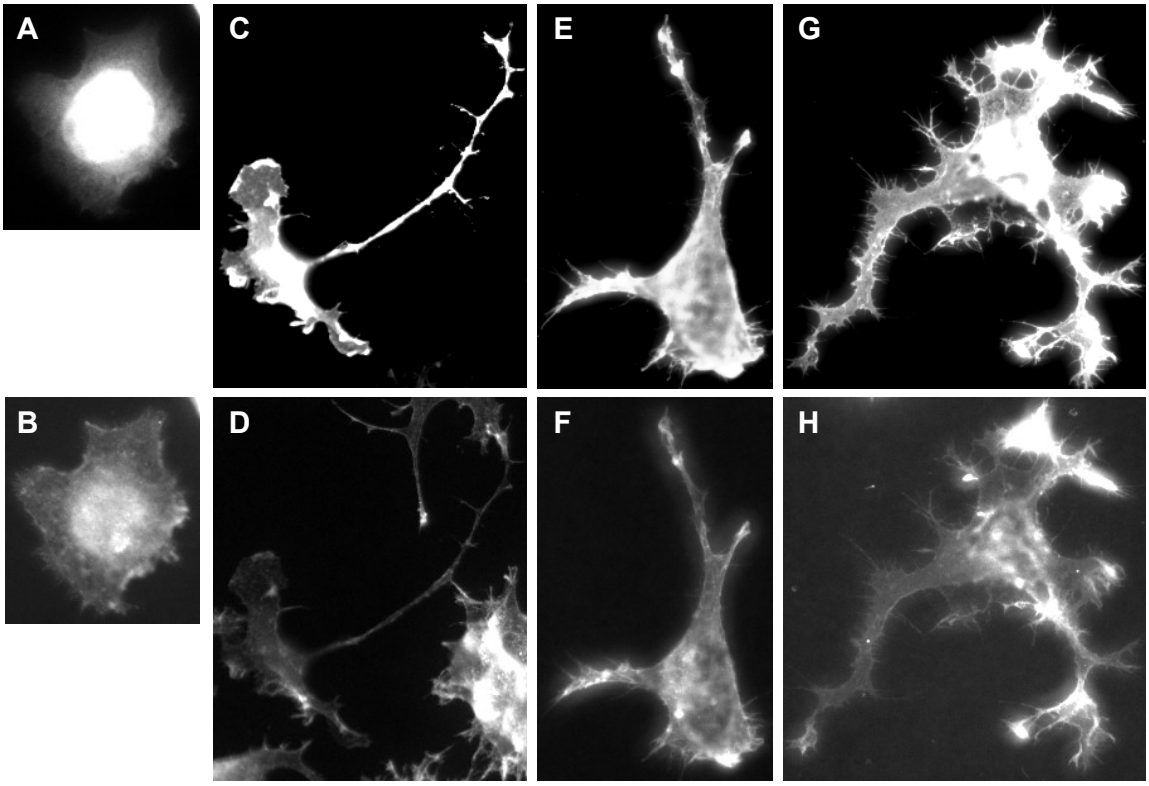

Fig. 2. NRXN and NLGN family members induce cytoskeletal rearrangements in N1E-115 cells. Typical N1E-115 cells transfected with (A-B) GFP, (C-D) HA-NLGN1, (E-F) HA-NLGN2 or (G-H) HA- NRXN1β processed for immunofluorescence (A,C,E,G) and stained with phalloidin (B,D,F,H).

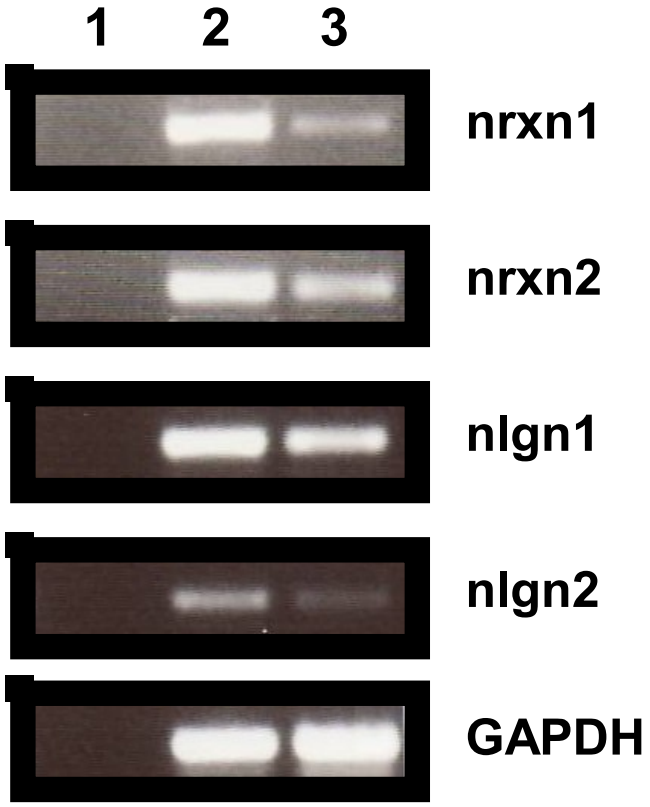

Fig. 3. Expression of nrxns and nlgns in embryonic mouse CNS. RT-PCR of (1) water control or RNA isolated from (2) E13.5 murine spinal cords or (3) adult mouse hindbrain for nrxn1, nrxn2, nlgn1, nlgn2 and GAPDH control.

CHAPTER IV: GENERAL DISCUSSION

The NRXNs and NLGNs are two families of neuronally expressed CAMs which assemble to form a heterophilic cell adhesion complex [17,18,22,24,28,29]. In addition to its adhesive function, the NRXN-NLGN complex has been implicated in the process of synaptogenesis during development [40,45]. However, NRXNs and NLGNs are expressed prior to synaptogenesis in the developing rodent brain [3-6], suggesting that they may play additional roles during development. Other neuronally expressed CAMs have been shown to promote neurite outgrowth in cultured neuroblastoma cells [1], a process thought to be analogous to the outgrowth of neuronal processes in the developing nervous system [2]. NRXNs and NLGNs participate in intracellular interactions which could mediate the cytoskeletal rearrangements required for neurite outgrowth [8-14]. Based on these lines of evidence, we set out to investigate the possibility that the NRXN-NLGN complex could induce neurite outgrowth.

We have demonstrated a novel role for NLGN1 in promoting neurite outgrowth in the N1E-115 neuroblastoma cell line (Chapter 2). This effect is specific and not dose-dependent, as the overexpression of an unrelated transmembrane protein, the transferrin receptor, does not induce neurite outgrowth in this system (Chapter 2, Fig. S1), and the neurite outgrowth induced by NLGN1 is unchanged under different expression conditions (Chapter 3, Fig. 1). NLGN1 promotes neurite outgrowth, but not the induction of multiple neurites or branched neurites (Chapter 2, Fig. 2), suggesting that NLGN1 functions to promote the elongation of existing processes. Further, the AChE-like domain responsible for mediating NRXN binding and the PDZ motif responsible for interactions with a number of intracellular scaffolding molecules are required for the outgrowth-promoting activity of NLGN1 (Chapter 2, Fig. 3 and 4), suggesting that NLGN1

functions by interacting with NRXNs and clustering intracellular proteins which ultimately produce cytoskeletal rearrangements leading to neurite outgrowth. Finally, other members of the NRXN and NLGN families produce distinct morphological phenotypes characterized by certain cytoskeletal rearrangements when expressed in N1E-115 cells (Chapter 3, Fig. 2). Taken together, our results demonstrate that the NRXN-NLGN complex functions to promote the outgrowth of neurites. Since NRXNs and NLGNs are expressed in the brain [3-6,28] and spinal cord (Chapter 3, Fig. 3) early in embryonic development, the outgrowth-promoting effects of these molecules could act to produce the outgrowth of neuronal processes prior to synaptogenesis. The predominantly presynaptic localization of NRXNs [7,17,37] and the postsynaptic localization of NLGNs [4,41,42] suggest that these protein families may function to promote the outgrowth of axons and dendrites, respectively. Further studies will clarify the importance of NRXN-NLGN interactions for the early development of the nervous system.

Mechanism of action

Structure of the NRXN-NLGN complex in neurite outgrowth

We have demonstrated that NLGN1 can cause neurite outgrowth in the N1E-115 neuroblastoma cell line, and that other NRXN and NLGN family members can produce cytoskeletal rearrangements and changes in cell morphology. Our studies present some interesting insights into the possible mechanism of neurite outgrowth mediated by the NRXN-NLGN complex. While NLGN1 produces neurite outgrowth in N1E-115 cells, it

does not induce an increase in the proportion of neurite-positive cells with either multiple neurites or branched neurites. This finding contrasts the actions of NLGN1 with a number of molecules implicated in neurite outgrowth which have also been shown to play a role in promoting the incidence of multiple neurites or branched neurites [61,62]. This suggests that while NLGN1 promotes the outgrowth of existing processes, it does not promote the induction of novel processes. In order to confirm that the action of NLGN1 is specifically related to outgrowth, neurite length per process and per cell could be measured and compared in untransfected cells and cells expressing NLGN1.

Our findings also raise the question of the structure of the NRXN-NLGN complex in neurite outgrowth. One explanation for our results is that overexpressed NLGN1 molecules produced outgrowth by interacting with endogenous NRXNs on the surface of a transfected cell. Interactions in *cis* between NLGNs and a pool of postsynaptically localized NRXNs have been observed in mature neurons [37]. However, our analysis revealed occasional pairs of cells with neurites which appeared to fasciculate with one another (data not shown), raising the possibility that at least some of the NLGN1-mediated neurite outgrowth may have occurred as a result of interactions in *trans*. As NLGNs are specifically localized to dendrites in mature neurons [41,42], it is unlikely that NRXN-NLGN interactions in *cis* on the surface of growth cones contribute to our proposed NRXN-mediated axon outgrowth during development. We find it more likely that NRXN molecules on the growth cones of developing axons could interact with NLGNs on the surface of contacting or fasciculating neurons to produce axon outgrowth. Intriguingly, NLGN-mediated dendritic outgrowth could proceed as a result of interactions either in *cis* or in *trans*, as a result of the dendritic localization of both

NRXNs and NLGNs [37,41,42]. Additional studies will be required to determine which configuration of the NRXN-NLGN complex is responsible for neurite outgrowth and changes in cell morphology produced by NRXN and NLGN family members.

If both NRXNs and NLGNs can indeed signal to the cytoskeleton in *cis* or in *trans*, this signaling could add an increased level of complexity to outgrowth mediated by the NRXN-NLGN complex. Interactions in *trans* could produce effects analogous to those of the ephrin and Eph families of outgrowth cues, which interact in *trans* to produce bidirectional signaling [63]. Interactions in *cis* might produce increased complexity in the outgrowth response due to additive or conflicting signals downstream of the NRXN and NLGN components of this complex.

Signaling downstream of the NRXN-NLGN complex

We are also interested in determining which downstream effectors could mediate the increase in neurite outgrowth resulting from NLGN1 overexpression, as well as the changes in cell morphology observed upon overexpression of NLGN2 and NRXN1β. There have been no reports of activation of the intracellular region of NLGN1, for example, by phosphorylation, in the transduction of synaptogenic signals. Instead, this activation is thought to result from the aggregation of the intracellular partners of NLGN1 [45]. Indeed, the effect of NRXNs binding to NLGNs in an assay of synaptogenic activity was thought to be related to the ability of NRXNs to cluster NLGNs and bring their associated intracellular partners into closer proximity [45]. This is consistent with the apparent role of soluble NLGNs in the development of presynaptic specializations via the clustering of NRXNs [7]. We therefore sought to eliminate the ability of NLGN1 to

interact with its intracellular partners to determine if this was sufficient to eliminate its outgrowth-promoting activity. Since a near-complete C-terminal deletion was not stably expressed at the surface of N1E-115 cells, we produced a more conservative deletion mutant, lacking the C-terminal PDZ motif (HA-ΔPDZ). Deletion of the C-terminal PDZ motif of NLGN1 was not sufficient to completely abolish the outgrowth-promoting effects of this molecule (Chapter 2, Fig. 3). This suggested to us that a secondary region within the C-terminus could be playing a role in signal transduction to produce alterations of cytoskeletal morphology and dynamics. One possibility was that the interaction of the NLGN1 C-terminus with S-SCAM could mediate an interaction with the cytoskeleton through β-catenin [13,14]. However, our double mutant, lacking both the PDZ motif and the bulk of the acetylcholinesterase-like domain (HA-ΔΔ), was unable to produce any level of neurite extension above background (Chapter 2, Fig. 4), suggesting that the remaining intracellular C-terminus is not involved in the observed neurite outgrowth phenotype. The neurite outgrowth produced by NLGN1 is therefore dependent on its ability to both bind to NRXNs and to interact with intracellular partners through the PDZ motif, suggesting that the accumulation of actin as a result of these interactions produces the neurite outgrowth phenotype. Indeed, in N1E-115 cells expressing HA-NLGN1 or HA-ΔN, we observed accumulations of filamentous actin which colocalized with the α-HA signal (Chapter 2, Fig. 2A and 3B). This colocalization appeared to be markedly decreased in N1E-115 cells expressing HA-ΔPDZ or HA-ΔΔ constructs (Chapter 2, Fig. 3C and 4B), suggesting that the accumulation of actin resulted from interactions with the PDZ motif. Quantitative studies analyzing the frequency and intensity of this colocalization, as well as colocalization in N1E-115 cells expressing NLGN2 and

NRXN1β, could shed light on the mechanism of neurite outgrowth mediated by the NRXN-NLGN complex.

Possible involvement of RhoGTPases in downstream signaling

We previously demonstrated that expression of NLGN1 induced neurite outgrowth in N1E-115 cells, and that this effect was mediated by the AChE-like domain responsible for NRXN binding and the PDZ motif responsible for binding to intracellular scaffolding molecules (Chapter 2). While expression of NLGN2 and NRXN1β did not produce a traditional neurite outgrowth phenotype in N1E-115 cells, both proteins induced significant changes in cell morphology indicative of cytoskeletal rearrangements (Chapter 3, Fig. 2). As cytoskeletal rearrangements underlie neurite outgrowth processes [57], it is likely that the effects of NLGN2 and NRXN1β are mechanistically similar to those of NLGN1. These cytoskeletal effects are likely to be based on the activation of one or more RhoGTPases. The RhoGTPases are a family of small molecules which act as molecular switches, cycling between an active GTP-bound state and an inactive state in which GDP is bound [64]. Activated RhoGTPases relay signals to produce intracellular changes, of which the most important is rearrangement of the cytoskeleton. The three best characterized RhoGTPases, RhoA, Rac1, and Cdc42, produce distinct effects on the cytoskeleton and cell morphology upon activation [64]. Activation of one or a combination of these molecules can lead to the production of the cytoskeletal and membrane structures associated with neurite outgrowth or alternately can lead to neurite retraction [57].

Filopodia, which are fine protrusions of the actin cytoskeleton often found on growth cones and implicated in the outgrowth of axons [65], are promoted by activation of the small GTPase Cdc42 [64]. Lamellipodia are broader membrane structures defined by a network of actin filaments of varying orientations [66] which are promoted by Rac activation [67]. Conversely, activation of Rho leads to neurite retraction in N1E-115 cells [68] and growth cone collapse in developing axons [57].

The phenotype of NLGN1 transfected cells is suggestive of the activation of Rac1 and Cdc42. NLGN1-transfected cells frequently display a single long neurite with filopodia along the length, as well as lamellipodial structures which can often be observed on the side of the cell body opposing the neurite (Chapter 3, Fig. 2 C-D). Neurite outgrowth induced by DCC (deleted in colorectal cancer) in N1E-115 cells requires the activity of Cdc42 and Rac1, but not RhoA [60]. The phenotypic similarity of NLGN1 and DCC transfected cells [60] suggests that Rac1 and Cdc42 may also be involved in neurite outgrowth downstream of NLGN1. Further, neurite outgrowth in response to serum starvation in N1E-115 cells is mediated by Rac and Cdc42 activity [69], suggesting that neurite outgrowth mediated by NLGN1 may proceed via a similar mechanism.

NLGN2 transfected N1E-115 cells, in contrast, present short, broad processes more similar to lamellipodia, with some distal filopodial structures (Chapter 3, Fig. 2 E-F). This suggests that the balance of small RhoGTPase activation may be biased towards higher Rac1 activation and lower Cdc42 activation in these cells. It is also possible that Cdc42 is not activated at all in these cells, as the phenotype of N1E-115 cells expressing

HA-NLGN2 is similar to the lamellipodia bearing short microspikes observed in N1E-115 cells expressing both DCC and a dominant-negative Cdc42 [60].

N1E-115 cells transfected with NRXN1β showed a robust induction of filopodial formation (Chapter 3, Fig. 2 G-H), indicative of rearrangement of the cytoskeleton and suggestive of Cdc42 activation. However, NRXN1β expression did not induce the outgrowth of the long, thin processes observed upon NLGN1 expression, despite the filopodial structures common to both. Cdc42 activity is linked to the production of long neurites as well as filopodia [57], raising the question of what signals to the cytoskeleton differentiate the two phenotypes.

Implications for neural development

Possible involvement of NRXNs and NLGNs in axonal and dendritic outgrowth

We have shown that NRXN and NLGN family members can promote neurite outgrowth and changes in cell morphology in the N1E-115 neuroblastoma cell line. What role might outgrowth mediated by the NRXN-NLGN complex play in the development or plasticity of the nervous system? During development, axons are required to grow long distances to make connections with their targets, setting up the exquisitely wired adult nervous system. The molecules that mediate this outgrowth are localized to the membrane of the growth cone, the highly motile structure at the leading edge of the axon responsible for sampling the environment and responding to soluble or contact-mediated guidance cues [56]. Consistent with their predominantly presynaptic localization in

mature neurons, NRXNs have been localized to the growth cones of developing axons originating from pontine explants [7,37]. This localization, coupled with our observation that NRXN1β can induce cytoskeletal rearrangements and changes in cell morphology (Chapter 3, Fig. 2 G-H), led us to propose that NRXNs may play a role in promoting the outgrowth of axons during development. Further quantitative analysis of the ability of NRXNs to promote neurite outgrowth in N1E-115 cells will provide insight into this question. Unlike NRXNs, evidence for the presence of NLGNs on the surface of growth cones is lacking. Indeed, this localization is unlikely, due to the intracellular dendritic targeting motif of NLGNs [41,42] and their postsynaptic localization in mature neurons [4]. It is more likely that the NLGNs play a role in the extension of dendritic processes to meet incoming axons during development and during adult plasticity. Filopodial extensions initiate the elaboration of dendritic arbors and appear to represent initial stages in the development of functional postsynaptic structures [57]. Together with the dendritic localization of NLGN1 [41,42], our finding that NLGN1 promotes neurite outgrowth in N1E-115 neuroblastoma cells (Chapter 2) suggests that NLGN1 may function in the nervous system to promote dendritic development. Like NLGN1, NLGN2 possesses a dendritic targeting sequence; however, it is localized specifically to inhibitory synapses [5,24]. Further qualitative and quantitative analysis of the differences between the NLGN1 and NLGN2 phenotypes in N1E-115 cells will provide us with insight into the possible role of NLGN1 and NLGN2 in the developing nervous system.

If NRXNs and NLGNs are involved in the outgrowth of axons and dendrites, respectively, we would expect loss of these proteins to lead to a decrease in synaptic number. Indeed, changes in the level of NRXNs and NLGNs have a profound effect on

synaptic number and identity in cultured neurons [7,40,45,58,70-72]. However, knockout animals lacking NRXN and NLGN family members display relatively mild defects. Deletion of NLGN2 leads to a decrease in the number of inhibitory synapses which does not appear to correspond to an overall decrease in synaptic number [47]. Triple NLGN knockout mice similarly did not present a decrease in overall synapse number [27]. Deletion of all three α-NRXNs reduced the density of inhibitory synapses in mouse brainstem without a corresponding increase in the density of excitatory synapses, suggesting that inhibitory synaptic development is inhibited in these animals [49]. However, this may not reflect a lack of importance of NLGN1 in dendritic outgrowth and synapse formation. Many knockout animals deficient in other, well-characterized neurite outgrowth-promoting molecules display surprisingly modest defects, almost certainly as a result of a high degree of redundancy among outgrowth cues [56]. Indeed, it is possible that other outgrowth and cell adhesion systems are able to compensate for the loss of NRXNs and NLGNs in these animals. Further, these studies frequently assess synaptic density in one specific region of the brain, such as the hippocampus [27,47]. The motor deficits observed in some NRXN and NLGN knockout animals [47], as well as our finding that NRXNS and NLGNs are expressed in the spinal cord during embryonic development (Chapter 3, Fig. 3), suggests that decreases in synapse number might be observed in the spinal cord of knockout animals. Finally, consistent with the phenotype of NRXN and NLGN knockout animals, mice deficient in NCAM or N-cadherin, two CAMs whose roles in neurite outgrowth have been well characterized, do not display gross defects in axonal outgrowth or pathfinding [15].

Role of RhoGTPase activity in axonal and dendritic outgrowth

There is some debate over which RhoGTPases regulate the development and plasticity of dendritic filopodia, spines, and arbors [57]. Of these molecules, Rac has been the most strongly implicated in promoting spine formation and maintenance, while RhoA appears to be a negative regulator of dendritic spines [73]. However, Rac activity generally promotes a more lamellipodial mode of outgrowth than Cdc42, which is generally associated with the long, thin processes characteristic of the phenotype of NLGN1-transfected N1E-115 cells [57]. The role of Cdc42 in dendritic spine morphology is less well-characterized. Knockout animals deficient in Cdc42 have severe defects in axon outgrowth, but do not show strikingly altered dendritic morphology [74]. However, other studies have shown a positive effect of Cdc42 on spine morphogenesis [75,76]. Further experiments are required to link NRXN and NLGN function to the activities of specific RhoGTPases in N1E-115 cells and potentially in the nervous system.

Possible role of NRXN/NLGN-mediated outgrowth in adults

While the role of NRXNs and NLGNs has been primarily investigated during synaptogenesis, and our work has focused on the period prior to synaptic development, expression of NRXN and NLGN family members continues to increase into adulthood [3-6]. Levels of NLGN1 protein continue to increase at least until P60 in mice, long after the peak of synaptogenesis [4], suggesting that NLGN1 may play an important role later in adulthood as well as during development. In addition to the role that NRXNs and NLGNs play in synaptic adhesion in the adult nervous system, the high level of expression of these proteins in later postnatal rodents could be indicative of a role in

promoting extension of axon collaterals and dendritic filopodia in response to learning or other events requiring neuronal plasticity.

Involvement of NRXNs and NLGNs in autism spectrum disorders

Autism spectrum disorders are complex disorders characterized by a continuum of social, linguistic, cognitive and motor deficits [77]. These disorders affect roughly 6 out of 1000 children and have a high concordance rate. The underlying causes of autism-spectrum disorders appear to be complex [77]. However, in recent years, a number of studies have revealed disruptions in NRXNs [54,78-80] and NLGNs [26,52,53,81] in individuals or families with autism-spectrum disorders. Moreover, these genes have been linked to a variety of other behavioral disorders not related to autism [82,83] as well as to a predisposition to addiction [55,84].

Knockout mice lacking NLGN3 or NLGN4 demonstrate social and behavioral deficits, making them ideal model systems for studying heritable forms of autism [85,86]. Deletion of NLGN2 produces mice with an altered behavioral phenotypes characterized by increased anxiety [47], and some studies report similar defects in knockin mice with a NLGN3 mutation associated with autism-spectrum disorders in humans [87,88]. While at least one line of animals deficient in a component of the NRXN-NLGN complex displays a decreased brain volume [85], it remains to be seen whether these animals display defects in dendritic structure or axonal pathfinding, either in the brain or spinal cord. Further studies into this question may clarify the involvement of NRXN and NLGN

deficiencies in autism-spectrum disorders and shed light on the possible function of the NRXN-NLGN complex in promoting the outgrowth of neuronal processes in the developing nervous system.

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