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Posttranslational modifications of neuroligins regulate neuronal and glial signaling

Jaehoon Jeong^{1,*}, Jeremiah D. Paskus^{1,2,*}, and Katherine W. Roche¹

¹Receptor Biology Section, National Institute of Neurological Disorders and Stroke (NINDS), National Institutes of Health, Bethesda, MD 20892, USA

²Interdisciplinary Program in Neuroscience, Georgetown University Medical Center, Washington, DC 20057, USA

Summary

This review covers the dynamic regulation of neuroligin isoforms, focusing on posttranslational events including phosphorylation, glycosylation and activity-dependent cleavage. There is a growing literature on how phosphorylation confers an isoform-specific level of modulation affecting a variety of protein interactions. In addition, recent studies describe activity-dependent proteolytic cleavage of neuroligins, revealing a broader role for neuroligins than just synaptic ‘glue’. Interesting new research implicates the cleaved extracellular fragments of neuroligins in promoting glioma. These reports on cell signaling mediated by the cleavage products of neuroligins suggest novel and important roles for neuroligins in neuro-glial signaling.

Introduction

Cell adhesion molecules play a critical role in the assembly of synaptic components to form and promote maturation of functional synapses [1]. Neuroligins (NLGNs) are a class of postsynaptic cell adhesion molecules in neurons [2,3] that are incredibly synaptogenic in many assays including neuron-HEK cell co-cultures and cultured primary neurons, although NLGNs are not required for synapse formation *in vivo* [4,5]. A variety of mutations in NLGN genes such as copy number variants, protein truncations, and amino acid substitutions have been identified in patients with autism spectrum disorders (ASDs) and alter social behavior and memory formation in mouse models [6,7]. Collectively, this evidence supports a critical role for NLGNs in proper neuronal function. Four NLGN genes (NLGN1 to NLGN4) have been identified in mice and five NLGN genes (NLGN1, 2, 3, 4X, and 4Y) are found in human [2,8]. The different NLGN isoforms display distinct subcellular localization patterns. NLGN1 is localized to glutamatergic excitatory synapses, whereas NLGN2 is mainly localized to GABAergic inhibitory synapses [9–11]. NLGN3 is localized to both excitatory and inhibitory synapses [12], and NLGN4 is known to be localized to

*These authors contributed equally to the work

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GABAergic synapses in the central nervous system and glycinergic synapses in the retina in mice [13,14].

Each NLGN isoform shows great similarity in structure and conservation of amino acid sequence (Fig. 1). A large extracellular acetylcholinesterase-like domain is involved in the specificity of trans-synaptic interactions with the presynaptic neurexin (NRXN) family [9]. NLGN homo- and hetero-dimerization are also mediated by the extracellular domain [12,15,16]. A short cytoplasmic tail (c-tail) is separated from the extracellular region by a single transmembrane domain and is exposed to a variety of intracellular regulatory signals [2,17]. Multiple intracellular protein-protein interactions with the c-tails of NLGNs have been characterized, elucidating novel mechanisms of isoform-specific subcellular localization and synapse specialization. However, defined protein-interaction domains are generally conserved in all NLGN isoforms (Fig. 1). For example, postsynaptic density protein 95 (PSD-95), the prototypical scaffolding protein at glutamatergic synapses, is a known binding partner of NLGN1; and, gephyrin, an inhibitory scaffolding molecule, interacts with NLGN2 [11,18]. However, the PSD-95 binding site and gephyrin-binding domain are conserved in all NLGN isoforms. Therefore, binding partner specificity cannot be attributed entirely to divergence in amino acid sequence. Recent studies have demonstrated the importance of posttranslational modifications as a critical regulator of NLGN isoform-specific functional differences in trafficking and protein-protein interactions.

Phosphorylation

Recently, several studies have demonstrated that NLGNs, like many other synaptic proteins, are regulated by phosphorylation. NLGN phosphorylation can dynamically control trafficking, protein interactions and synaptogenic efficacy. For example, T739 was identified as a CaMKII phosphorylation site on NLGN1 and experiments revealed that T739 phosphorylation is increased upon synaptic activation in cultured neurons and following sensory experience *in vivo* [19]. Expression of a non-phosphorylatable mutant, NLGN1 T739A, displayed reduced surface levels of NLGN1, and a deficit in NLGN1 synaptogenic activity [19]. Thus dynamic phosphorylation of NLGN1 in response to synaptic activity is essential for efficient protein trafficking to the cell surface and activity-dependent synaptogenesis.

Another phosphorylation site on NLGN1, S752, was identified in a characterization of enriched phospho-peptides from mouse synaptic membranes [20]. Notably, S752 is located in a conserved region of the c-tail that has been identified as regulating NLGN1 and 3 function at excitatory synapses [21]. For NLGN1, mutation of a nearby residue (E747N) decreased AMPAR and NMDAR currents compared to WT when expressed on a null NLGN knockdown background. In contrast, the analogous mutation in NLGN3 (E740N) resulted in a defect in AMPAR current enhancement only. Interestingly, the phosphorylation site S752 is five amino acids away from E747 and the analogous serine residue is not conserved in NLGN3 (A745). Investigation of any potential effect of the E747N mutation on S752 phosphorylation would be informative, and might illuminate functional differences between NLGN1 and 3 at excitatory synapses.

In another study, the tyrosine phosphorylation of Y782 within the gephyrin binding motif of NLGN1 was reported. Using biochemical assays, researchers identified tyrosine phosphorylation of ectopic and endogenous NLGN1 [22]. Importantly, phosphorylation at Y782 attenuated NLGN1 binding to gephyrin and application of a broad tyrosine kinase inhibitor reduced PSD-95-GFP recruitment with co-transfected NLGN1 in neurons. The NLGN1/NRXN1 β interaction has been suggested to stimulate Y782 phosphorylation, implicating a master regulatory role of the Y782 site for NLGN1 selective binding with PSD-95 [22].

As with NLGN1, there is also evidence that NLGN2 is regulated by phosphorylation. Most notably, S713 in human NLGN2 (S714 mouse and rat NLGN2) has been reported by several groups to be phosphorylated and important for regulating protein interactions and NLGN activity [20,23–29]. Although phosphorylation of neighboring S718 is observed in the post-mortem human brain tissue of frontotemporal lobar degeneration (FTLD) patients [26] and S721 phosphorylation is also identified in mouse brain samples using a phosphoproteomic approach [27], thus far functional studies have been made primarily on the S714 phosphorylation site. NLGN2 S714 phosphorylation negatively modulates its binding to gephyrin [24]. Furthermore, phosphorylation on NLGN2 S714 is believed to provide a binding motif for Pin1 (peptidyl-prolyl isomerase), that subsequently affects protein folding or promotes phosphorylation of nearby Y770 [24]. These events may attenuate NLGN2/gephyrin binding. Consistent with this model, NLGN2/gephyrin interactions at GABAergic post-synaptic sites are increased and a concomitant enhancement of synaptic recruitment of $\gamma 2$ subunit-containing GABA_ARs are observed in Pin1 knockout animals. Furthermore, a serine analogous to NLGN2 S714 in NLGN3 can be phosphorylated in human, mouse, and rat [25,27,30]. Serine phosphorylation at the consensus S-P motif in both NLGN2 and 3 is consistent with proline directed kinases playing an important regulatory role for multiple NLGN isoforms. Further investigation of this distinct molecular regulation is important.

NLGN2 interacts with two key inhibitory postsynaptic scaffolding proteins, gephyrin and collybistin [11,31]. The binding of NLGN2 with gephyrin recruits cytoplasmic gephyrin and collybistin complexes to the plasma membrane. The gephyrin-collybistin complexes are tethered to the plasma membrane and help recruit inhibitory receptors, such as GABA_A receptors and glycine receptors. As discussed above, tyrosine phosphorylation within the gephyrin binding domain of NLGN1 can affect gephyrin binding. Likewise, NLGN2 has the analogous tyrosine (770 in NLGN2), which is conserved in all NLGN isoforms, and it is critical for NLGN2 binding with gephyrin. Mutation of Y770 to alanine significantly reduces NLGN2 binding with gephyrin and collybistin activation [31]. However, whether the NLGN-gephyrin interaction is necessary for NLGN2 function at inhibitory synapses has not been clearly answered. Recently, a detailed study proposed that NLGN2 functions at inhibitory synapses by gephyrin-dependent and gephyrin-independent mechanisms [23]. Like the autism-associated mutation (R705C), or the mutation that blocks gephyrin binding (Y770A), phosphorylation of S714 regulates NLGN2 function in inhibitory synapse responses [23]. Further studies are required to fully understand the complicated regulatory mechanism of NLGN2 at inhibitory synapses.

NLGN3 and 4X are both located on the X chromosome and are implicated in ASDs as multiple mutations have been identified in autistic patients. Although most mutations are in extracellular domains, a point mutation in the cytoplasmic region of NLGN4X, R704C, was identified in an autistic patient [32]. Interestingly, an isoform-specific PKC phosphorylation site (T707) has been characterized nearby, and the autism mutant (NLGN4X R704C) eliminates T707 phosphorylation [33]. Furthermore, expression of a phosphomimetic mutation of NLGN4X T707 (T707D) increased spine number and augmented both AMPAR- and NMDAR-mediated currents dramatically compared to WT, thus demonstrating that NLGN4X can be transformed from a modest potentiator of excitatory synaptic transmission to a robust one – with a single modification.

Although phosphorylation sites are most often at intracellular sites, which are accessible to intracellular kinases, several extracellular residues of NLGNs have been identified recently as phosphorylation sites. In a study of the phosphoproteome of a human melanoma cell line [34] phosphorylation of S102 and S105 in the extracellular region of NLGN1 were identified. Similarly, a single phosphorylation site, T163, has been identified in the extracellular region of NLGN2 by phosphoproteomic analysis of melanoma tissue from mutant mice [35]. As with many other phosphorylation sites found in the extracellular regions of membrane proteins, a detailed physiological role of these phosphorylation sites in NLGNs is unknown. It will be important to validate these extracellular residues as being utilized and identify the extracellular kinases to better understand a possible role in the neurodevelopmental process.

Glycosylation and other modifications

NLGNs are heavily glycosylated [36–38] and multiple putative glycosylation sites are located in the extracellular region adjacent to the transmembrane domain. Glycosylation plays an important role in NLGN surface expression and protein folding [39] as recently reviewed [3]. Interestingly, N-linked glycosylation at N303 in the alternatively spliced B insertion of NLGN1 modulates its binding to NRXN α/β and regulates presynaptic differentiation [15,40]. Aberrant glycosylation is also implicated in the pathology resulting from variants identified in NLGNs in patients with ASD such as NLGN3 R451C and NLGN4X R87W [39]. Interestingly, the effect of the R87W mutation on the protein processing and export of NLGN4X resembles that of the R451C mutation in NLGN3 [38], although there were differences in the extent to which the mutation affects surface expression and synaptogenic efficacy. The NLGN3 mutant still retains some intact trafficking, NRXN1 β binding and synaptogenic activity.

Only recently have posttranslational modifications such as ubiquitination and methylation been reported for NLGNs. Specifically ubiquitination at K749 and methylation at R750 in the cytoplasmic region of NLGN2 were identified in proteomic studies using mouse tissue or human cell lines, respectively [41,42]. Although detailed functional analyses have not been conducted, these residues are located within the critical region of NLGN1 and 3 [21], suggesting ubiquitination or methylation could render isoform-specific features to NLGN2 for inhibitory synaptic function. More studies will shed light on the role these modifications

might play in synaptogenic properties of NLGN2 and if similar modifications exist in other isoforms.

Cleavage

Recent studies have revealed activity-dependent cleavage as a potent posttranslational modification regulating NLGNs. In Peixoto et al., cultured cortical neurons were stimulated with KCl to increase synaptic activity resulting in a dramatic loss of NLGN1 at synapses [43]. The authors demonstrated that NLGN1 cleavage is dependent on NMDAR activation and CaMKII signaling, and concluded that MMP9 was the most likely enzyme for the cleavage. To determine the precise cleavage site, the authors generated NLGN1 with sequential deletions and amino acid substitutions in the extracellular domain (ECD) and these experiments implicated the juxtamembrane stalk (amino acids 672–695) as the potential site of cleavage. Using a thrombin inducible cleavage mutant of NLGN1, the authors show that cleavage of NLGN1 destabilizes NRXN1 β , which results in decreases in synaptic strength by reducing the presynaptic release probability at excitatory synapses. To test this *in vivo*, the authors use pharmacological activation and a dark rearing behavior paradigm to increase neuronal activity and observed an increase in the soluble NLGN1-derived amino terminal fragments (NTFs), but no increase of NLGN1-NTFs in the MMP9 KO mouse. All these data reveal robust activity-dependent cleavage of NLGN1 *in vivo*.

Another study also reported the activity-dependent cleavage of NLGN1 and showed that ADAM10 and γ -secretase activity triggered the accumulation of the NTF and C-terminal fragment (CTF) of NLGN in cultured neurons and mouse brain tissue [44]. The studies also implicated the juxtamembrane domain as the likely site of extracellular cleavage and showed synaptic activity and NMDA receptor activation can trigger NLGN1 cleavage *in vivo*. Although treatment with the MMP inhibitor GM6001 inhibited the extracellular cleavage in both studies, Suzuki et al. found that treatment with MMP2, 3, 9, 12 or 15 inhibitors did not block the generation of NLGN1-NTFs, whereas treating neuronal cultures with INCB3619, an ADAM10/17 inhibitor, abolished the secretion of NLGN1-NTFs. Knockdown and knockout of ADAM10 in neuronal cultures resulted in the reduction in the accumulation of NLGN1-NTFs. Finally, overexpression of NLGN1 in ADAM10 KO fibroblasts eliminated the formation of NLGN1-NTF. These experimental findings implicate ADAM10 as the critical protease.

How different proteases target distinct NLGN isoforms, and how they might work in concert, is an intriguing question. Proteases generally have liberal, poorly defined, and/or no consensus sequence, complicating investigation. It is important to note, however, that the presumptive cleavage location of NLGN1 at the juxtamembrane stalk is within a highly divergent region across all NLGN isoforms could therefore lead to isoform-specific regulation. Taken together, differential cleavage events are likely mediated by a combination of appropriate sequence, isoform interactions, in conjunction with NLGN subcellular localization and perhaps additional factors like dimerization or glycosylation. Given that activity appears to regulate both cleavage and phosphorylation, it is intriguing to consider how these events may interrelate. More studies in this area are needed to elucidate potential influences of post-translational modifications on NLGN proteolysis.

N-terminal proteolysis of NLGN1 generates a corresponding intracellular CTF, which can be detected with antibodies against the C-terminal domain of NLGN1 as a band at ~20 kDa, which is consistent with its predicted mass and is absent in the NLGN1 KO brain [43]. Moreover, it has been reported that NLGN1-CTFs are subject to intramembrane cleavage by γ -secretase, and that inhibition of γ -secretase blocks the generation of smaller NLGN1-CTFs [43]. A recent study reported that the overexpression of TAT-tagged NLGN1-CTF in cortical neurons was sufficient to augment spine and synapse number, which is consistent with a prior report demonstrating that the overexpression of NLGN1-CTF (membrane bound), but not the free C-terminal domain of NLGN1, increases spine density [43,45]. This CTF effect was mediated by the activation of the LIM-domain protein kinase/cofilin pathway. However, in the same study, TAT-tagged NLGN1-CTF was reported to enable long-term potentiation (LTP) in CA1 in conflict with a report demonstrating that NLGN1-mediated LTP is dependent on its extracellular domain [46]. The basis of these seeming inconsistencies requires more investigation.

Although growing evidence supports independent functions of NLGN-CTFs, it remains to be seen what role free NLGN-NTFs play in synaptic transmission. Interestingly, a novel role for NLGN3-NTFs as a mitogen has recently been described [47], based on findings that optogenetic stimulation of synaptic activity drives the growth of xenografted human gliomas in mice. The authors performed *in vitro* assays to define the molecular basis of the mitogenic activity and demonstrated that it was a secreted factor. Using mass spectrometry, they determined that the cleaved NTF fragment of NLGN3 was responsible for the accelerated glioma growth. Indeed, application of conditioned media containing the ectodomain of NLGN3, or recombinant NLGN3 induced the proliferation of various lines of high-grade glioma cells. Remarkably, secreted NLGN3 regulated glioma proliferation could be blocked through the concurrent addition of recombinant NRXN. Furthermore, it was found that secreted NLGN3 promoted glioma growth through activation of the PI3K-mTOR pathway resulting in the feedforward expression of NLGN3 in glioma cells. How NLGN3 might activate the PI3K-mTOR pathway, or how it results in the feedforward expression of NLGN3 in glioma cell lines, is not clear. Most importantly, the glial receptor for cleaved NLGN3 NTF is not known. Therefore, many questions remain to be answered including what function NLGN isoforms have in non-neuronal cell types and if there is a non-pathogenic role for NLGN3 NTFs.

NLGNs and glia

NLGN3 is thought to be a homologue of the *Drosophila Melanogaster* protein gliotactin, which is expressed in glial cells during development and is essential in the formation of the blood-brain barrier [48]. NLGN3, as a potential vertebrate gliotactin, was found via PCR to be expressed developmentally in various glial cell types, particularly in astrocytes of the retina and spinal cord, olfactory ensheathing glia, and in Schwann cells. Indeed, sequencing of the entire transcriptomes of astrocyte and oligodendrocyte lineage cells, potential origin cells of gliomas, has revealed that NLGN1-3 transcripts are highly enriched in all of these glial cell types [49]. Some of these *in silico* data have been verified. It has been shown using RT-PCR that NLGN1, 2 and 3 transcripts are expressed in juvenile and senescent NG2 cells (oligodendrocyte precursors) and that the expression of NLGN3 decreases significantly with

age [50]. Intriguingly, it has also been suggested that axo-glial communication through neuronally expressed NRXN and oligodendrocyte expressed NLGN3 may mediate myelination and oligodendrocyte differentiation [51]. It has been shown that oligodendrocytes make contact with heterologous cells expressing NRXN isoforms, and that saturating amounts of NLGN-ECDs block this interaction *in vitro* and *in vivo*, stalling oligodendrocyte differentiation and thus myelination [51]. Moreover, knockdown of NLGN3 in oligodendrocytes using siRNAs further impedes their differentiation and morphological complexity.

Other evidence for glial roles includes a study that the astrocyte-secreted protein hevin (high endothelial venule protein/SPARC-like protein 1), a factor known to modulate excitatory synaptic transmission, is critical in the assembly of thalamocortical synapses by acting as a bridge between the interaction of NRXN1 α and NLGN1 β [52]. This report demonstrates a novel role of glial secreted factors in modulating NRXN-NLGN adhesive signaling by the bridging of two isoforms that have been reported not to interact directly. Additionally, it has also been shown that astrocyte-derived transforming growth factor beta 1 induces the assembly of inhibitory synapses and clustering of NLGN2 in a CaMKII dependent manner [53]. Taken together, these data demonstrate novel mechanisms by which glial-derived soluble factors regulate both excitatory and inhibitory synaptic transmission through NRXN-NLGN interactions and NLGN trafficking.

Conclusions

The research on how NLGNs are modified in response to neuronal activity has greatly expanded our understanding of the molecular mechanisms underlying their physiological roles. However, the physiological relevance of the NLGN modifications are not fully understood, and many critical questions are still unanswered. Nevertheless, novel findings concerning the function of neuroligins in cancer and glial cells has opened up exciting new avenues for exploring their function in both basic and translational biology. Continued work in this field will help clarify the complex, and crucial, role neuroligins play in synapse development, maintenance, and plasticity.

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Highlights

- NLGNs are regulated by multiple posttranslational modifications
- Posttranslational modifications allow isoform-specific NLGN functions
- Activity-dependent cleavage of NLGNs yields functional NLGN fragments
- Neurons and glia communicate to modulate NLGN-NLXN signaling

Figure 1. Posttranslational modification sites in the juxtamembrane stalk and cytoplasmic regions of neuroligin isoforms

A cartoon of the structure of a neuroligin is depicted on the left and an alignment of the juxtamembrane and cytoplasmic regions of mouse neuroligin isoforms are aligned on the right. The residues that are phosphorylated are indicated with a red box. The residue identified in an autism patient is shaded in black. A reported ubiquitination site (light green) and methylation site (orange) are indicated. Residues that were identified in human neuroligins are marked at the analogous residues within the mouse neuroligin sequence. The divergent juxtamembrane stalk region is highlighted in green, with critical cleavage residues highlighted in yellow. An asterisk (*) indicates conserved amino acid residues between neuroligin isoforms; a colon (:) indicates conservation between amino acids with strongly similar properties; and, a period (.) indicates conservation between weakly similar amino acids.

Figure 2. Regulation of neuroligins by posttranslational modifications

Neuroligins are single transmembrane proteins that form homo- and hetero- dimers. A schematic drawing of neuroligins is shown as a monomer for convenience. The transmembrane regions are depicted as a dark blue rectangle. The B splicing site (light blue), phosphorylation sites (black circles), glycosylation sites (green diamonds), autism mutation sites (red), ubiquitination sites (purple) and methylation sites (yellow) are indicated with a legend shown in the box. Regulatory roles of each posttranslational modification on neuroligins are described in the text boxes if functional studies have been reported for the relevant site. The amino acid residues are numbered as in the reference paper.

Figure 3. Activity-dependent cleavage of neuroligins

(A) Activity triggers the cleavage of the NLGN1-NTF, mediated by MMP9 and/or ADAM10. The resultant free NLGN1-CTF displays autonomous functional roles similar to full-length NLGN1, increasing spine number and potentiating excitatory neurotransmission. NLGN1-CTF is subsequently degraded by γ -secretase. (B) Activity triggers cleavage of NLGN3, releasing the soluble NLGN3-NTF, which mediates activity-dependent glioma growth. (C) Expression of neuroligin isoforms in glial cells. Astrocyte-derived hevin acts as a linker protein, bridging the interaction of NLGN1B and NRXN1 α .