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Current Opinion in Neurobiology

Control of neural development and function by glial neuroligins

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Neuroligins are a family of cell adhesion molecules, which are best known for their functions as postsynaptic components of the trans-synaptic neurexin–neuroligin complexes. Neuroligins are highly conserved across evolution with important roles in the formation, maturation and function of synaptic structures. Mutations in the genes that encode for neuroligins have been linked to a number of neurodevelopmental disorders such as autism and schizophrenia, which stem from synaptic pathologies. Owing to their essential functions in regulating synaptic connectivity and their link to synaptic dysfunction in disease, previous studies on neuroligins have focused on neurons. Yet a recent work reveals that neuroligins are also expressed in the central nervous system by glial cells, such as astrocytes and oligodendrocytes, and perform important roles in controlling synaptic connectivity in a non-cell autonomous manner. In this review, we will highlight these recent findings demonstrating the important roles of glial neuroligins in regulating the development and connectivity of healthy and diseased brains.

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Current Opinion in Neurobiology 2019, **57**:163–170

This review comes from a themed issue on **Molecular neuroscience**

Edited by **Yishi Jin** and **Tim Ryan**

https://doi.org/10.1016/j.conb.2019.03.007

Introduction

Classically, synapses in the brain are defined as apposition of two neuronal elements; a pre-synaptic bouton from an axon and a post-synaptic density that is localized to a dendrite. Neurotransmitter release-mediated communication (i.e. synaptic transmission) at these close neuron-neuron contacts form the basic functional unit of the nervous system. Formation of these synaptic structures is thought to be controlled by homophilic and heterophilic transcellular interactions between a number of cell adhesion proteins (reviewed in Refs. [1–3]) that adhere specific axons to their proper postsynaptic partners. These trans-synaptic cell adhesion molecule (CAM) complexes ultimately recruit neurotransmitter receptors necessary for synaptic communication.

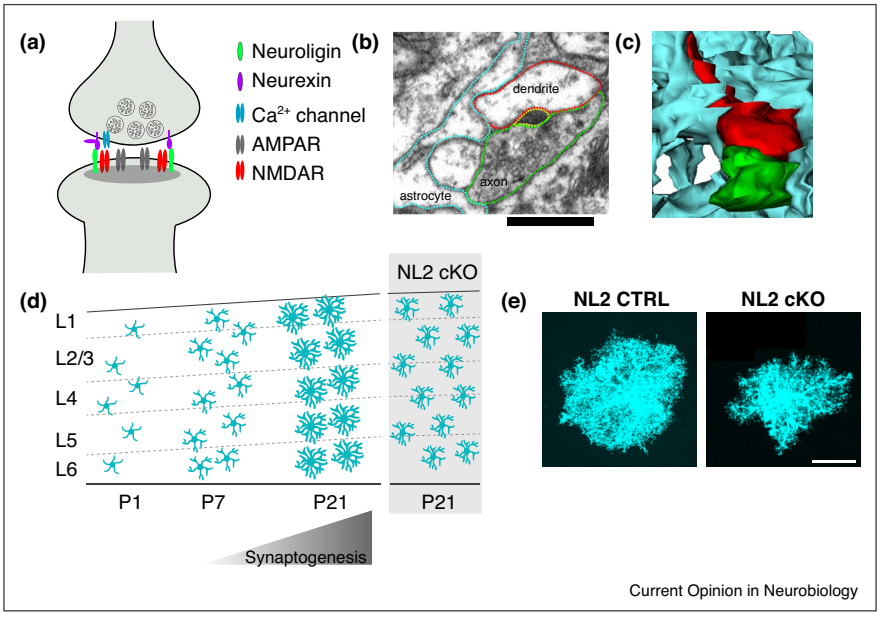
The neuroligin (NL) family of CAMs are composed of five members in humans, four homologs of which are present in mice [4]. Over the past three decades, NLs have been extensively studied for their roles in regulating synaptogenesis and synaptic function. Thus far, an overwhelming majority of these studies regarded NLs primarily as neuronal post-synaptic proteins [5,6]. The functions of postsynaptic NLs are accomplished by the trans-synaptic interactions with presynaptic neurexins (NRXs) [7] (Figure 1a). A complex recognition code between different neuroligin and neurexin isoforms and splice variants exists, which is thought to provide synapse subtype specific recognition between axons and dendrites [8–10]. While loss of a single NL is not lethal in mice, global ablation of NLs1–3 results in perinatal death [6], underscoring the importance of these proteins in development. The emphasis of NLs in CNS development and function is further signified as mutations in both NL3 and NL4 are associated with Autism Spectrum Disorders (ASD) [11,12], in which synaptic dysfunction is thought to underlie the complex social and cognitive pathologies.

Numerous recent studies, investigating gene expression in different CNS cell types, demonstrated that NLs are expressed in multiple glial cells, particularly astrocytes and oligodendrocytes, with cell-intrinsic and extrinsic functions. In this review, we will focus on three NLs (NLs1–3), which have been well studied in rodents and humans and we will highlight studies demonstrating: 1) the sufficiency of glial NLs to mediate neuronal contacts via NRXs, 2) the functional roles of glial neuroligins in glial development and synaptic connectivity, and 3) how glial NLs participate in the pathophysiology of diseases such as glioma and neurodevelopmental disorders.

Neuroligin family proteins are expressed in glial cells

Heterologous connections can be formed between neurons and non-neuronal cells that express NLs [7,13,14]. Exogenous expression of NLs in fibroblast-like cells, such as HEK293T or COS7, is sufficient to attract axonal

Figure 1



Astrocytic NL2 controls astrocyte morphogenesis. **(a)** Schematic of neuroligin–neurexin interactions at the synapse. Pre-synaptic α-neurexins and β-neurexins interact with postsynaptic neuroligins to recruit receptors to the post-synaptic density and calcium channels and synaptic vesicles to the active zone. **(b)** Electron micrograph depicting an astrocyte (cyan) contacting both a dendrite (red) and an axon (green) at the synapse. **(c)** 3D reconstruction of b. **(d)** Schematic of postnatal mouse astrocyte development. From P7 to P21 astrocyte territories rapidly increase in size and infiltrate the neuropil, coincident with synaptogenesis. With loss of astrocytic NL2 (cKO) astrocytes are smaller and less complex, with decreased ability to infiltrate the neuropil. **(e)** Max projections of confocal z-stacks of medial prefrontal cortical astrocytes at P21. $NL2^{loxP/+}$ or $^{loxP/loxP}$, both carrying a floxed Rosa-TdTomato allele, were postnatally injected with Cre plasmid to produce sparse CTRL or cKO astrocytes, respectively. Scale bar = 25 μm. Images from 1b–c are courtesy of Dr W. Chris Risher, Marshall University.

contacts and cluster presynaptic proteins at the heterologous connection via transcellular interactions with axonal NRXs [7,14]. However, until recently, the endogenous expression of NLs in non-neuronal cells was largely ignored. Moreover, astrocytic NL expression may be lost during long-term culturing in serum-containing media, which is well-known to cause astrocytes to lose their *in vivo* gene expression profile [15]. There were several studies, however, that investigated NL expression in the CNS and have revealed the presence of NLs in cultured Schwann cells *in vitro* and *in vivo*, in astrocytes in the spinal cord and retina, as well as in oligodendrocytes and NG2+ oligodendrocyte progenitor cells [16–18].

In the last decade, decreasing sequencing costs and improved cell purification methods have enabled large efforts to characterize cell-type specific transcriptomes in the CNS. These datasets are comprised of FACS sorting or cell-specific ribosome purification [19–21], and single cell analyses [22,23] of brain cells, as well as acutely purified human astrocytes [24]. Mining of these datasets reveals that NLs1–3 are expressed in astrocytes and oligodendrocytes to an equivalent or higher level compared to neurons [20,24]. In astrocytes specifically, NL transcripts maintain constant expression throughout the lifetime of the animals [25•,26] suggesting long-term involvement in maintaining astrocyte-neuron interactions and astrocytic function. Recently, these data were corroborated in astrocytes via *in situ* hybridization of NL mRNA in the mouse cortex, and by RT-PCR and western blotting for NL mRNA and protein, respectively, in isolated astrocytes from rat brains [27••]. Together, these findings provide strong evidence for NL expression in CNS glia.

Glial neuroligins control neural development via transcellular interactions with neurexins

Neuronal NLs are well studied for their functions in controlling formation of synapses [5,7] and synaptic strength [6,28]. These functions of NLs are dependent on their ability to transcellularly interact with NRXs (Figure 1a). In contrast, through interactions mediated by their intracellular C-terminal domain, NLs recruit actin remodeling proteins to control dendritic arborizations and stabilize postsynaptic structures [5,29,30]. In rodent neurons, NL1 directly interacts with Rho-GTPase activating proteins (GAPs) to ultimately stimulate assembly of F-actin and activation of this signaling pathway results in increased spine density, synaptogenesis, and enhancement of long-term potentiation in the

hippocampus [31]. Moreover, NLs are sufficient to recruit Wave Regulatory Complex (WRC) proteins to the cell membrane to stimulate F-actin assembly, synaptic bouton growth and synaptic transmission [32,33•].

CNS glia are highly polarized cells, with many branches extending to contact neurons, synapses, and blood vessels. These branches are maintained primarily by the actin cytoskeleton and disrupting this branching impairs glial cell function [34–36]. Although limited in number, studies that investigated the functions of glial NLs revealed that these CAMs also control glial structure and function via mediating cell–cell interactions that are critical for neural development. Here, we will highlight some of these findings.

The *Drosophila* protein gliotactin is a homolog of neuroligin-3 that is required for blood–brain barrier formation

Gliotactin is a transmembrane glycoprotein in *Drosophila melanogaster*, with a homolog also present in *C. elegans* [37,38]. *Drosophila* gliotactin was originally characterized in peripheral glia [37]. Gliotactin fly mutants exhibit grossly normal proliferation and localization of the glia, yet the mutants display a partial paralysis due to incomplete formation of the blood–brain barrier [37]. Similar to vertebrate NLs, gliotactin has an extracellular cholinesterase-like domain, which is rendered inactive due to a missing serine residue [37]. It was proposed that NL3 is the vertebrate homolog of gliotactin, based on NL3 expression in ensheathing glia of the olfactory epithelium, and in astrocytes of the retina and spinal cord [16]. However, the amino acid homology between gliotactin and any of NLs1–3 is similar (~30%). In the future, it will be interesting to investigate how loss of NLs disrupts the development and function of glia in the olfactory epithelium, spinal cord and retina in mammals.

Gliotactin protein is also found in the septate junction, a structure that separates cellular compartments made up by epithelia and glia of *Drosophila* [39]. The authors of this study found that early in embryogenesis gliotactin co-localizes with neurexin, and there is a reciprocal dependency for proper localization of each protein. Furthermore, gliotactin-null mutants failed to hatch into larvae. Intriguingly, while gliotactin re-expression sufficiently rescued this phenotype, despite 40% sequence homology, overexpression of *Drosophila* NL (*Dnl*, NL2 homolog) did not [39]. This suggests that specific functions of gliotactin/NL3 are necessary for maintaining these cellular junctions. Similarly, in the mouse brain overexpressing different NLs or NL chimeras in place of another fail to completely rescue deficits indicating that each NL have non-redundant functions in different aspects of neural development [40].

Neuroligin–Neurexin adhesions control myelin-axon contacts

Oligodendrocytes are highly complex glial cells that myelinate axons in the CNS. Multiple oligodendrocyte processes contact nearby neuronal axons, where they tightly wrap newly synthesized myelin around. Proctor *et al.*, using cultured oligodendrocytes and neurons, found that knockdown of NL3 results in severely stunted branching of oligodendrocyte processes [18]. This finding begged the question of whether myelination was disrupted as well. To investigate this, Proctor *et al.* performed a myelin wrapping assay both *in vitro* and *ex vivo* with Purkinje neurons of the cerebellum by quantifying the number of Myelin Basic Protein (MBP)+ oligodendrocyte-wrapped axons. In this system, the authors incubated the cultures with control media or with media containing NL1-ECD (extracellular domain), a more competitive binder of NRXs than endogenous NLs [18,41]. NL1-ECD incubation of cultured cells or slices resulted in a sharp decrease in the percentage of myelinated axons, suggesting that endogenous NL–NRX interactions play a role in myelination. However, knockdown of NL3 was carried out in a co-culture system of oligodendrocytes and neurons and thus effects of neuronal NL3 in oligodendrocyte maturation cannot be ruled out in this context [18]. In the future, it will be important to disentangle the contributions of oligodendrocytic NLs by conducting cell-type specific manipulations to conclusively show a role for glial NLs in myelination.

Astrocytic neuroligins control astrocyte and synapse development

In the rodent cortex, astrocytes are born from the radial glial stem cells perinatally and over time develop a complex, bush-like morphology to infiltrate the neuropil and to tightly interact with synaptic structures (Figure 1b–d). Through their structural and functional association, astrocytes play many critical roles in the formation and maturation of CNS synapses [42]. During development, astrocytes secrete soluble synaptogenic factors including thrombospondins [43], hevin [44,45], glypicans, [46]and chordin-like 1 [47], which strongly stimulate formation of excitatory synapses and recruitment of NMDA and AMPA receptors to postsynaptic sites (reviewed in Ref. [48]). Furthermore, in the mouse cortex the major period of synapse formation (P7-P14) corresponds to the time period in which astrocytes become morphologically complex by innervating the neuropil and associating with synapses (Figure 1d).

To determine the molecular mechanisms that mediate astrocyte morphogenesis in the mammalian CNS, Stogsdill *et al.*, utilized an astrocyte-neuron co-culture system. Astrocytes grown alone in culture exhibit a simple morphology; however, in the presence of neurons, astrocytes rapidly develop a stellate morphology [49], which is mirrored *in vivo* [50]. In this system, short hairpin RNA (shRNA) to knockdown astrocytic

NLs (NL1–3) resulted in a drastic reduction in astrocyte branching after co-culturing with neurons. Reintroduction of shRNA-resistant NLs completely rescued this phenotype. Reduction of neuronal NRXs in this system similarly perturbed astrocyte morphology [27••], demonstrating that transcellular interactions of astrocytic NLs and neuronal NRXs control astrocyte morphological development, *in vitro*. These findings were confirmed *in vivo*. Silencing or overexpression of astrocytic NLs1–2 caused a reduction or increase, respectively, in astrocyte neuropil infiltration and territory volume [27••]. Specifically, loss of astrocytic NL2 resulted in diminished neuropil infiltration of astrocyte processes both at P7 and P21, while loss of astrocytic NL1 reduced complexity only at P7 which was corrected by P21. Conversely, loss of astrocytic NL3 only reduced astrocyte complexity later in development [27••]. These findings suggest that there are non-redundant developmental roles for astrocytic NLs. In summary, this study revealed a previously unknown role of NLs in controlling astrocytic morphological development by mediating the adhesions between astrocyte processes and neuronal NRXs. It will be important for future studies to elucidate the signaling mechanisms downstream of NLs in astrocytes that mediate the morphological elaboration of these glial cells.

Because astrocytes play important roles in synapse development and loss of astrocytic NL2 significantly decreased neuropil infiltration of perisynaptic astrocyte processes [27••], Stogsdill *et al.* also tested if synapse formation within the territories of mutant astrocytes was perturbed. Using the Cre-LoxP system in conjunction with *in vivo* electroporation, the authors deleted NL2 from a sparse population of astrocytes in the visual cortex and found that loss of NL2 in an astrocyte leads to significantly fewer excitatory synapses formed within the domain of that astrocyte. No significant changes to inhibitory synapse formation was observed in the same assay [27••]. These findings were unexpected as neuronal NL2 is proposed to be exclusively localized to inhibitory synapses [51] and specifically control inhibitory synapse formation and function [52,40].

Astrocytes are also important regulators of synaptic transmission, controlling the uptake of extracellular glutamate [53] and releasing neuroactive molecules [54]. To determine the role of astrocytic NL2 on synaptic function, Stogsdill *et al.*, conditionally deleted NL2 from astrocytes and performed electrophysiological recordings of layer V cortical pyramidal neurons. They found that loss of astrocytic NL2 leads to a significant decrease in both frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs). Interestingly, a significant increase in miniature inhibitory postsynaptic current (mIPSCs) frequency was also observed [27••]. Taken together, these findings revealed a novel role for astrocytic NL2 in regulating synapse numbers and excitation/inhibition balance in the mouse cortex.

The mechanisms governing the effects of astrocytic NL2 on the local control of excitatory synapse numbers and global control of excitation/inhibition balance remain unknown. It is likely that astrocytic NL2 functions through distinct mechanisms from neuronal NL2. One possibility is astrocytic NL2, by stabilizing astrocyte-synapse contacts, ensures the delivery of pro-synaptogenic factors to nascent excitatory synapses to promote synaptogenesis. Another possibility is NL–NRX interactions between astrocytes and the neuronal elements of the synapse forms a diffusion barrier for neuronal counter parts thus trapping them within the synaptic cleft and enhancing their stability (Figure 2a). Alternatively, astrocytic NL2 could compete with neuronal NL2 for presynaptic NRXs thus negatively affecting the function or number of synapses (Figure 2b). These two models are not mutually exclusive and may be occurring at distinct synapse types.

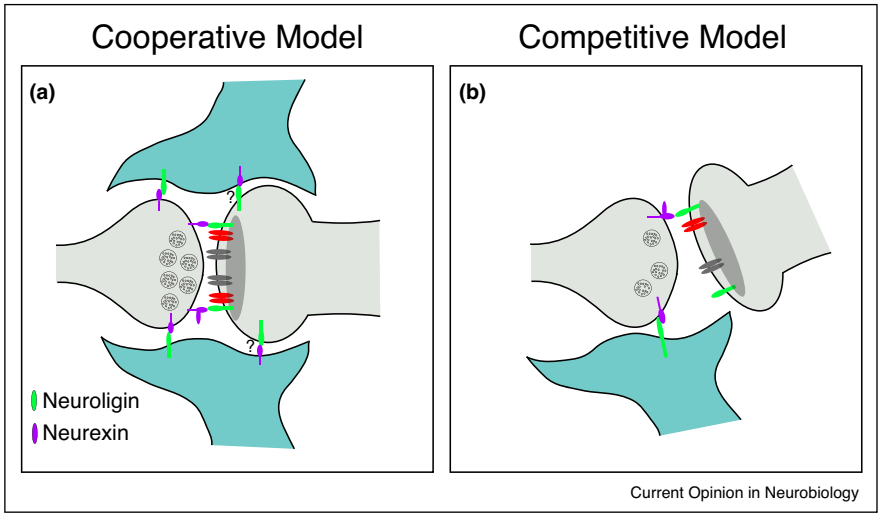
Roles of glial neuroligins in disease

NL mutations are linked to several neurological disorders including ASD and schizophrenia. Moreover, expression and function of NL family proteins are known to be affected in neurodevelopmental disorders [12]. The studies we summarized above strongly implicate glial NL loss-of-function in disease pathogenesis. Furthermore, behavioral deficits in single NL knockout (KO) mice include anxiety and locomotor phenotypes, among others [55–57]; however, for example in the case of NL2, conditional loss only in neurons does not phenocopy the constitutive KO [58]. Thus, it is crucial to understand how glial NLs control neural development and function to fully understand the complex biology of these important proteins in diseases of the nervous system. A number of recent studies started to elucidate the importance of glial NLs in disease pathogenesis, which we will review next.

Secreted neuroligin-3 affects glioma proliferation and growth

High grade glioma (HGG) is a devastating brain tumor that arises from neural and oligodendrocyte precursors [59]. A recent study found that neural activity promotes proliferation of HGG cells in a xenograft mouse model of human HGG [60]. The authors demonstrated that neural activity stimulated the release of soluble factors into the tumor environment and subsequently found a secreted form of NL3 to be necessary and sufficient to promote HGG proliferation in an activity-dependent manner [60]. Interestingly, HGG cells normally do not express NL3 protein, but when exposed *in vitro* to recombinant human NL3 protein, HGG cells induce NL3 transcription and translation via feedforward tumor-microenvironment crosstalk [60]. A genetic dissection of cell-types contributing to secreted NL3 revealed that both neurons and oligodendrocyte precursors secrete NL3, as well as the

Figure 2



Proposed models for astrocytic NL function at synapses. **(a)** Schematic demonstrating a cooperative model of astrocytic (blue) NLs interacting with neuronal (grey) NRXs (or vice versa, question mark). These transcellular interactions may stabilize synapses by restricting the diffusion of neuronal NRXs and NLs from the synaptic cleft. Conversely, in a competitive model **(b)**, binding of astrocytic NLs to neuronal NRXs diminishes synaptic strength by replacing neuronal NLs.

HGG cells [61••]. The latter is of clinical significance as NL3 abundance in tumors is inversely correlated with survival [60]. Lastly, Venkatesh *et al.* delineated the mechanism of NL3 secretion by identifying the protease that cleaves NL3, thus yielding an exciting potential therapeutic target for HGG. However, currently we do not know whether NL3 is also secreted from healthy cells to mediate normal neural function, a critical insight that is necessary for the development of proper therapeutic strategies for HGG.

Neuroligins and autism spectrum disorder

Genetic analyses of rare autism spectrum disorder (ASD) variants reveal a potential association with NL3 and NL4 mutations in humans [11,62]. Studies in a NL3 knock-in mouse mimicking a mutation found in ASD patients (R451C) revealed social behavior deficits [63], increased repetitive behavior [64] and disruptions in the excitation/inhibition balance in the cortex [65], indicating conserved mechanisms of NL3 function in circuits governing ASD phenotypes. The contributions to these phenotypes from glial NL3 have not been explored. However, astrocytic NL involvement in ASD pathogenesis has not been completely ignored. Valproic acid (VPA) is a common anti-epileptic drug associated with increased occurrence of ASD when administered to pregnant women [66]. VPA mechanisms of action are thought to be limited to neurons by shifting the excitatory/inhibitory balance of CNS synapses [67]. Astrocytes also respond to VPA treatment, at least *in vitro*, and robustly upregulate transcription of NL1 in response [68]. This finding suggests that changes in transcellular astrocyte-neuronal contacts mediated by NL–NRX interactions may occur after VPA treatment. Whether synaptogenesis and/or synaptic function is impaired by VPA-treated astrocytes remains an interesting avenue of exploration. Lastly, recent genome-wide analysis of ASD implicates MDGAs (MAM domain-containing glycosylphosphatidylinositol anchors) in ASD susceptibility [69]. Interestingly MDGAs interact with NL2 in *cis*, blocks the NL2-NRX interaction and subsequently reduces the density of inhibitory synapses, *in vitro* [70,71]. These findings suggest a homeostatic mechanism of NL2 interactions in excitatory/inhibitory synapse numbers, a hallmark of ASD pathology. Yet, whether MDGAs interact with glial NLs to control similar functions is an open question.

Glial neuroligins and schizophrenia

Previous studies point toward disrupted synaptic connectivity as a major driver of pathology in the neurodevelopmental disorder schizophrenia (SZ) [72]. Interestingly, one study identified multiple damaging genetic variants in the NL2 gene among SZ patients [73]. In particular, the authors showed that one variant, R215H, exhibited membrane trafficking defects and diminished transcellular interactions with NRX1. A knock-in NL2-R215H mouse exhibited decreased inhibitory synapse proteins, corresponding deficits in inhibitory synaptic transmission and SZ-like behaviors including altered anxiety and stress responses [74,75]. While these studies do not specifically implicate glial NL2 in SZ pathogenesis, another study performed transcriptomic analysis on glial progenitors cells (GPCs) derived from SZ patient and control fibroblasts and revealed downregulation of NLs1–3 in these

progenitor cells [76•]. Strikingly, transplantation studies showed that chimeric mice containing GPCs from SZ patients exhibited reduced astrocyte complexity, and behavioral abnormalities reminiscent of SZ [76•]. While a causal link for NLs in regulating astrocyte morphogenesis and astrocyte-synapse interactions in SZ has not been made, it is a hypothesis worth pursuing given the important roles of NLs in astrocytes [27••].

Conclusion and future direction

In summary, NLs have essential functions in glia, which mediate critical roles in the development and function of the CNS. The findings summarized here point toward numerous avenues of exploration for future studies, which should include addressing the following questions:

1 What pathways are downstream of astrocyte and oligodendrocyte NLs to mediate morphological development of these cells?
2 Do oligodendrocytic NLs control myelination?
3 How do astrocytic NLs control synaptic connectivity?
4 Do astrocytic NLs direct astrocyte processes to particular synapses?
5 Do glial NLs contribute to neurodevelopmental disease, including ASD and SZ?

Future studies regarding NLs should be carefully designed to assess cell-type specific functions of these genes. Furthermore, identification and characterization of unique NL splicing and post-translational modifications in glia would constitute an informative avenue to investigate. *In vivo* cell type specific tagging of endogenous NLs using CRISPR/Cas9 mediated techniques, in place of antibodies for protein visualization, will be a valuable tool to identify subcellular localization of NLs in glia and determine where glial NLs are located with respect to synapses. Taken together, future investigation of NL function in glia will begin to elucidate the complete mechanisms of actions for these important, disease-linked molecules in the CNS.

Conflict of interest statement

Nothing declared.

Acknowledgement

The relevant work in Eroglu laboratory is supported by N.I.H.R01-NS102237 to C.E.

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