Region-Specific Phosphorylation Determines Neuroligin-3 Localization to Excitatory Versus Inhibitory Synapses

Brain-Region-Specific Phosphorylation Targets Neuroligin-3 to Distinct Synapse Types

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Keywords

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SUMMARY

Neuroligin-family postsynaptic adhesion proteins control the development and function of neuronal synapses. The four mammalian paralogs operate at distinct subsets of synapses, where they interact with presynaptic adhesion and postsynaptic scaffolding proteins to coordinate transmitter receptor recruitment. Rodent Neuroligin-1 functions specifically at excitatory synapses, while Neuroligin-2 and Neuroligin-4 function specifically at inhibitory synapses. The synapse-type specificity of Neuroligin-3, mutations of which cause autism-spectrum disorders in humans, is less apparent and still poorly characterized. We demonstrate that Neuroligin-3 specifically localizes to subsets of excitatory synapses in cortical regions, and to inhibitory synapses in subcortical regions. We further show that region-specific phosphorylation of Neuroligin-3 regulates its association with the Gephyrin scaffold at inhibitory postsynapses. Our data reveal a mechanism that controls the synapse-type association of Neuroligin-3 across the brain and likely contributes to the very diverse synapse pathologies that are caused by Neuroligin-3 perturbation in mouse models of autism-spectrum disorders.

INTRODUCTION

Autism-spectrum disorders (ASDs) comprise a set of early-onset neuropsychiatric conditions with strong genetic underpinnings that indicate synaptic etiology (Bourgeron, 2015). Mutations in the Neuroligin (NL) family of postsynaptic adhesion molecules were among the first identified monogenetic causes of non-syndromic autism in humans (Jamain et al., 2003), and have since provided key molecular insights into synaptic pathologies that may underlie ASDs (Südhof, 2008; Krueger et al., 2012; Südhof, 2017). Among the four NL genes, mutations of *Nlgn3* have provided some of the most extensively studied genetic models of autism. These *Nlgn3* mutations cause a range of synaptic endophenotypes that vary depending on the brain region examined. They include effects on fast glutamatergic and GABAergic transmission, and on metabotropic glutamatergic and endocannabinoid signaling (Baudouin et al., 2012; Rothwell et al., 2014; Földy et al., 2013; Tabuchi et al., 2007; Radyushkin et al., 2009).

The mouse synapse types affected by *Nlgn3* mutations are more diverse than those observed with other NLs, which are more consistently synapse-type selective. NL1 localizes to excitatory synapses, and its deletion causes defects at glutamatergic synapses (Song et al., 1999; Chubykin et al., 2007; Chanda et al., 2017). NL2 displays a synapse-type specificity that is complementary to that of NL1 as it localizes to and functions at inhibitory synapses and at subtypes of cholinergic and dopaminergic synapses (Graf et al., 2004; Varoqueaux et al., 2004; Poulopoulos et al., 2009; Takács et al., 2013; Uchigashima et al., 2016; Chanda et al., 2017). NL4 function in mice appears to be restricted to subsets of inhibitory synapses (Hoon et al., 2011; Hammer et al., 2015; Zhang et al., 2018), most prominently glycinergic. The synaptic specificity of NL3 has been examined in cultured neurons (Budreck and Scheiffele, 2007) and in the cerebellum (Baudouin et al., 2012), where a

mixed localization to excitatory and inhibitory synapses was detected. In contrast, NL3 in retina was found only at inhibitory synapses (Südhof, 2017).

The absence of an obvious synapse-type specificity for Nlgn3, as well as the broad range of synaptic defects in *Nlgn3* mutants, prompted us to more systematically examine NL3 localization across brain regions and to search for potential mechanisms that may regulate distinct synapse specificities of NL3 in different parts of the brain. Based on localization studies with knockout-validated antibodies, we show that NL3 selectively associates with either excitatory or inhibitory synapses in a highly regulated manner, with strikingly distinct patterns in cortical versus subcortical brain areas. We further identify a post-translational regulatory mechanism that controls the synapse-type specificity of NL3 in a brain-area-dependent manner, where serine phosphorylation of NL3 at its Gephyrin binding-site prevents an association with the scaffold at inhibitory postsynapses, while retaining its affinity for scaffolds at excitatory synapses.

RESULTS

NL3 Overexpression in Cultured Neurons Causes Non-Specific Dendritogenic and Synaptogenic Effects

As seen in previous studies involving exogenous expression of NLs (Levinson et al., 2005; Poulopoulos et al., 2009; Schnell et al., 2014), we found that overexpression of HA-NL3 in cultured hippocampal neurons causes notable changes in neuron morphology, including increases in dendritic arborization and in the number of dendritic filopodia. These effects were elicited via the NL3 cytoplasmic domain, as overexpression of a truncated NL3 variant lacking the intracellular domain (HA-NL3$^{\Delta ICD}$) did not produce these effects (Figure 1).

In addition, NL3 overexpression in cultured neurons caused pronounced effects on the density of presynaptic contact sites (Figure 1A-1C), as previously demonstrated (Chubykin et al., 2007; Levinson et al., 2005; Chih et al., 2005; Chen et al., 2010; Schnell et al., 2012). Overexpression of HA-NL3 increased the density of both excitatory and inhibitory presynaptic contacts as seen by immunolabeling for the corresponding presynaptic marker proteins VGluT1 and VIAAT (Figure 1B and 1C). These effects of NL3 overexpression on the recruitment of presynaptic terminals appear to be mediated by the NL3 extracellular domain, since full-length and ΔICD variants of NL3 were equally effective (Figure 1B and 1C).

Overall, these data show that NL3 overexpression in cultured neurons elicits the same potent synaptogenic and dendritogenic effects that are commonly caused by overexpression of NL family members (Chubykin et al., 2007; Levinson et al., 2005; Chih et al., 2005), without specificity for either excitatory or inhibitory synapses.

Endogenous NL3 Localizes to Subsets of Excitatory Synapses in Cortical and of Inhibitory Synapses in Subcortical Areas

To systematically examine the differential localization of endogenous NL3 to excitatory versus inhibitory synapse types across the rodent brain, we used

an antibody we had previously generated against NL3 (Varoqueaux et al., 2006). To reliably determine NL3 localization, we first assessed the specificity of the antibody for endogenous NL3 by comparing immunoreactivity in brains from wild type (WT) versus NL3 knockout (KO) mice (Figure 2A). We determined that our anti-NL3 antibody displayed minimal immunoreactivity on brains lacking NL3, thus confirming that the punctate immunofluorescence signal in WT brains corresponds to endogenous NL3 clusters.

With this validated antibody, we examined the patterns of NL3 distribution across regions of the adult mouse brain. We detected punctate patterns of NL3 immunoreactivity in most parts of the brain. Interestingly, NL3 puncta appeared in two distinct sizes depending on the brain region examined. Small NL3 puncta appear in cerebral cortex, hippocampus, and the molecular layer and deep nuclei of cerebellum. Large NL3 puncta are prevalent in olfactory bulb, basal ganglia, thalamus, brain stem, and the granule-cell layer of cerebellum (Table 1).

We next investigated whether these puncta correspond to synaptic clusters of NL3, and whether they display excitatory or inhibitory synapse-type specificity. By colabeling endogenous NL3 together with excitatory and inhibitory postsynaptic markers, PSD-95 and Gephyrin, respectively, we found that indeed the majority of NL3 puncta overlap with synaptic markers, indicating a predominantly synaptic localization of NL3 clusters. Furthermore, NL3 showed a strong excitatory vs. inhibitory postsynapse preference that varied between regions. Puncta size largely corresponded to distinct synapse-type specificities, such that in areas with small puncta, NL3 colocalized with PSD-95 at excitatory postsynapses (Figure 2B), with no appreciable overlap with Gephyrin at inhibitory postsynapses (e.g. cortex in Figure 2D). In areas with large puncta, NL3 specifically colocalized with Gephyrin (Figure 2D) with no appreciable overlap with PSD-95 (e.g. brainstem in Figure 2B). In cerebellum, NL3 displays a more refined layer-specific selectivity. NL3 localizes to excitatory synapses in the molecular layer and deep nuclei, to inhibitory synapses in the granule cell layer, and to subsets of both excitatory and inhibitory synapses in cerebellar glomeruli (Table 1; Figure 2, Supplementary Figure 1), consistent with independent observations previously made using a distinct anti-NL3 antibody (Baudouin et al., 2012). Together, these data show that endogenous NL3 displays strong synapse-type selectivity that is differentially regulated across brain areas.

Quantification of the fraction of total excitatory and inhibitory postsynaptic puncta that contain NL3 indicates that in all cases NL3 has further synapse subtype specificities in all areas examined. Of the total PSD-95 puncta, overlap with NL3 reached 30% in cortex, 60-70% in hippocampus, and 45% in the molecular layer of the cerebellum. The degree of overlap in these areas was significantly above random overlap as assessed by inverting the channel overlays (Figure 2C). This indicates that excitatory synapse localization is specific in these areas, while the partial coverage indicates that only certain subtypes of excitatory synapses contain NL3. Correspondingly, in areas like olfactory bulb, striatum, thalamus, and brainstem, all NL3 puncta localize together with Gephyrin, whereas only about half of the total Gephyrin puncta contain NL3 (Figure 2E).

We further examined whether the NL3-positive fraction of inhibitory synapses segregates with glycinergic or GABAergic receptors, and found no

such preference. Rather, NL3 was detected at $GABA_AR$-positive, GlyR-positive, and mixed GABAergic-glycinergic postsynapses in brainstem (Supplementary Figure 1). These data reveal a general pattern of binary and mutually exclusive synapse-type specificity for NL3, and further selectivities – yet to be determined– for subsets of excitatory synapses in cortical areas and subsets of inhibitory synapses in subcortical areas. This robust and regulated synapse-type selectivity of NL3, which varies depending on brain region and neuron type, is outstanding among other NL family members, which show more consistent preferences for synapse types throughout the brain.

Region-Specific Serine Phosphorylation of NL3 Regulates Synapse-Type Selectivity

The region-specific synapse-type selectivity of NL3 indicates the existence of a regulatory mechanism that determines whether NL3 will associate with excitatory or inhibitory postsynaptic specializations. The cytoplasmic domain of NL3 contains binding sites for both PSD-95, the key scaffold protein of excitatory glutamatergic synapses, and for Gephyrin, the key scaffold protein of inhibitory GABAergic and glycinergic synapses (Song et al., 1999; Meyer et al., 2004; Poulopoulos et al., 2009) (Figures 3A and 4). In view of this, we hypothesized that these binding sites may be regulated by phosphorylation in a region-specific manner. To test this hypothesis, we applied a targeted phospho-proteomic approach to address the phosphorylation status of the Gephyrin-binding site of endogenous NL3 immunopurified from rat brain (Figure 3B). For this purpose, we chose to digest with endoproteinase Asp-N (cleaving N-terminally of Asp residues) and subsequently enrich for phosphopeptides. Analysis by mass spectrometry (MS) detected a phosphopeptide that unequivocally represented a new NL3 phosphorylation site at serine 799 (S799), which is part of the NL3 consensus motif that mediates binding to Gephyrin (Figure 3C). Mass spectrometric sequencing of this monophosphorylated peptide species yielded no evidence of phosphorylation on proximal residues, including the tyrosine 792 (Y792) that corresponds to a known phosphorylation site in NL1 (Giannone et al., 2013).

Next, we sought to identify kinases with substrate specificity for NL3-S799 using *in vitro* assays with purified kinases and substrate peptide (NL3 785-805; RLTALPDYTLTLRRSPDDIPL). We applied this assay both in a candidate approach (Supplementary Figure 2A) and a larger-scale screening approach (Supplementary Figure 2B), and detected phosphate transfer onto the NL3 substrate peptide with various kinases. However, we did not identify any kinases that displayed substrate-specificity for the S799 residue as compared to other phosphorylatable residues of the peptide. As such, the kinase responsible for NL3-S799 phosphorylation remains elusive.

We next investigated the endogenous distribution of phospho-S799-NL3 and whether it follows the region-specific patterns of NL3 synapse specificities. To do so, we raised phospho-specific antibodies in rabbits immunized against the corresponding NL3 phospho-S799 peptide (RRpSPDDIP). From the resulting antibodies, even the best performing antibody (6808) displayed only moderate preference for the phosphorylated over the non-phosphorylated peptide. To increase its phospho-specificity, we mixed antibody 6808 with an excess of non-phosphorylated peptide to compete with binding to non-phosphorylated NL3 epitopes. This increased the

binding specificity of antibody 6808 for phosphopeptide vs. non-phosphopeptide from approximately 3-fold with antibody alone to 20-fold with the antibody-peptide mix (Figure 3D). This provided us a critical reagent for the detection of endogenous phospho-S799-NL3.

To investigate the region-specificity of endogenous phospho-S799-NL3, we immunoblotted samples from different brain regions using the phospho-specific 6808 antibody-peptide mix. To validate the detection specificity of our immunoblots, and to determine whether homologous phosphorylation occurs in NLs other than NL3, brain region samples were obtained from WT as well as NL3 KO mice to serve as controls. Homogenates from both WT and KO genotypes were prepared from five brain regions, i.e. neocortex, hippocampus, striatum, thalamus, and cerebellum. Western blots juxtaposing these samples were carried out on the same membranes. These samples were immunoblotted for actin and NL3. Similar levels of actin across all lanes indicated equal total protein load. NL3 immunoreactivity appeared as a specific band with electrophoretic mobility corresponding to 100-110 kDa, which was absent in NL3 KO samples. Similar levels of total NL3 immunoreactivity were detected across WT lanes from all brain areas examined (Figure 3E).

Having validated the preparation, we proceeded to probe for phospho-S799-NL3 immunoreactivity using the 6808 antibody-peptide mix. We identified specific phospho-S799-NL3 immunoreactive bands in the anticipated 100-110 kDa range, displaying robust intensity in WT hippocampus, and moderate intensity in WT cortex and cerebellum. Equivalent bands were absent from the corresponding NL3 KO samples, confirming that the phospho-specific signal comes from NL3. This finding is particularly important, given that the Gephyrin-binding epitope containing the S799 phosphorylation site is highly conserved among NLs, and immunoreactivity could also come from NL1 or NL4. The complete loss of the phospho-specific band in NL3 KOs shows that NL1 and NL4 are not phosphorylated at the homologous site, indicating that this phosphorylation mechanism is specific to NL3.

Importantly, while NL3 was robustly expressed in all areas tested, phospho-specific NL3 bands seen in hippocampus, neocortex, and cerebellum were not seen in striatum or thalamus (Figure 3E), the latter areas being those in which all NL3 puncta colocalize with Gephyrin at inhibitory synapses (Figures 2 and 3E). Indeed, the region specificity of the phospho-S799-NL3 band corresponds to the pattern of NL3 localization at excitatory synapses, with NL3 being present at the majority of excitatory synapses in hippocampus, and in more limited subsets of excitatory synapses in neocortex and cerebellum. Given these results, we conclude that the phospho-S799-NL3 band indeed identifies a novel type of phosphorylation of native NL3 in a region-specific manner that correlates with its differential selectivity for excitatory synapses *in vivo*.

Phosphomimetic Mutation of S799 in the Gephyrin-Binding Site of NL3 Inhibits its Interaction with Gephyrin

The S799 phosphorylation site is located within the R797-T812 epitope identified as the site on NL3 that directly interacts with Gephyrin (Poulopoulos et al., 2009), indicating that this phosphorylation may regulate the NL3-

Gephyrin interaction. Given that we detected phospho-S799-NL3 specifically in areas where NL3 does not colocalize with Gephyrin, and that NL3 remains unphosphorylated in subcortical areas where NL3 and Gephyrin do colocalize, we hypothesized that S799 phosphorylation of NL3 may inhibit the binding and association of NL3 with Gephyrin. To test this, we produced phosphomimetic S799D ($NL3^{S799D}$) and phospho-null S799A ($NL3^{S799A}$) mutants to genetically mimic the phosphorylated and unphosphorylated forms of NL3. We used these variants in a series of experiments that were designed to assess their association with Gephyrin in yeast, cell lines, cultured neurons, and *in vivo*.

We first used yeast-two-hybrid assays to assess the effect of $NL3^{S799D}$ and $NL3^{S799A}$ mutations on the interactions of the NL3 cytoplasmic domain with Gephyrin and with the PDZ domains of S-SCAM, a scaffold protein of excitatory postsynapses. Consistent with our hypothesis, Gephyrin binding of NL3 was abolished by the phosphomimetic $NL3^{S799D}$ mutation, while the PDZ-mediated interaction was left intact (Figure 4A). The phospho-null $NL3^{S799A}$ mutation left Gephyrin and PDZ interactions unaffected (Figure 4A). The same effects were seen with homologous mutants of all other NL cytoplasmic domains (Figure 4B, Supplementary Figure 3). These data indicate that serine phosphorylation of the Gephyrin-binding site of NLs prevents the interaction with Gephyrin.

We next examined the association of full-length NL3 and its $NL3^{S799D}$ and $NL3^{S799A}$ mutants with Gephyrin membrane clusters in heterologous mammalian cells. Such cell-based clustering assays have been used extensively in Gephyrin biology to examine its association with membrane proteins, including glycine receptors (Meyer et al., 1995), $GABA_A$ receptors, and NLs (Poulopoulos et al., 2009). HA-tagged NL3 constructs were expressed in COS7 cells together with constructs expressing GFP-Gephyrin and myc-tagged Collybistin lacking its autoinhibitory SH3 domain (myc-$CB2^{SH3-}$), a constitutively active form of Collybistin that induces Gephyrin clusters on the plasma membrane in heterologous cells (Kins et al., 2000; Poulopoulos et al., 2009; Soykan et al., 2014). GFP-Gephyrin membrane microaggregates induced pronounced co-clustering of HA-NL3 (Figure 5A-B), which we quantified using cross-correlation analysis as done previously (Poulopoulos et al., 2009). The phosphomimetic HA-$NL3^{S799D}$ mutant displayed a significant reduction in co-clustering with underlying Gephyrin microaggregates by approximately one-third compared to WT HA-NL3 or HA-$NL3^{S799A}$ (Figure 5B), consistent with a reduction in Gephyrin scaffold binding upon phosphomimetic mutation of S799 in the Gephyrin-binding site of NL3.

We went on to test the effects of HA-$NL3^{S799D}$ and HA-$NL3^{S799A}$ mutants in cultured neurons. Overexpression of WT HA-NL3, as well as HA-$NL3^{S799A}$ phospho-null mutant, caused an increase in the number of Gephyrin clusters over the corresponding phosphomimetic HA-$NL3^{S799D}$ mutant (Figure 5C-D). This abating effect of the phosphomimetic mutant on Gephyrin clustering is consistent with a loss of the ability to interact with Gephyrin at the neuronal plasma membrane. Together, our results obtained with cultured cells support the notion that phosphorylation of S799 in the Gephyrin-binding site of NL3 perturbs its interaction with Gephyrin and the protein scaffold at inhibitory postsynapses.

To assess the function of NL3 phosphorylation in a system that –unlike cultured neurons– retains native regional specificities and regulation, we used *in utero* electroporation to investigate the effects of NL3 phospho-mutants on Gephyrin clustering *in vivo*. We expressed GFP together with NL3 constructs in layer 2/3 neurons of the cerebral cortex (Figure 6A), where endogenous NL3 is exclusively localized to excitatory postsynapses (Figure 2B) and is phosphorylated at S799 (Figure 3E). Three weeks after birth, we compared the effects of WT HA-NL3, phosphomimetic HA-NL3^{S799D}, or phospho-null HA-NL3^{S799A} on the numbers of perisomatic Gephyrin clusters formed in electroporated neurons (Figure 6B). Consistent with the hypothesis that cortical NL3 is phosphorylated to prevent Gephyrin association, neurons overexpressing phospho-null HA-NL3^{S799A} displayed a significant increase in the numbers of perisomatic Gephyrin clusters as compared to neurons overexpressing the phosphomimetic HA-NL3^{S799D} mutant or WT HA-NL3 (Figure 6C). The fact that WT HA-NL3 behaves like the phosphomimetic mutant in cortex indicates that WT HA-NL3 is phosphorylated at S799 by endogenous mechanisms in cortex, which results in a loss of effect on Gephyrin scaffolds. Taken together, these data support the notion of an *in vivo* function of S799 phosphorylation of the NL3 Gephyrin-binding site to confer excitatory synapse specificity in cortical areas of the brain.

DISCUSSION

The four members of the NL family of postsynaptic cell adhesion proteins play key roles in synapse assembly, maturation, and function (Krueger et al., 2012; Baudouin and Scheiffele, 2010; Südhof, 2017; Südhof, 2008). NL3 is the most abundant NL in the rodent brain (Varoqueaux et al., 2006) and currently the focus of intense research, owing to the identification of NL3 mutations that cause autism in humans (Jamain et al., 2003; Sanders et al., 2011; Bourgeron, 2015) and autism-like behaviors in mice (Tabuchi et al., 2007; Radyushkin et al., 2009). NLs display distinct synapse-type specificities, which have been documented for NL1, NL2, and NL4 in rodents (Song et al., 1999; Varoqueaux et al., 2006; Graf et al., 2004; Varoqueaux et al., 2004; Hoon et al., 2011; Hammer et al., 2015). In the case of NL3, however, a clear pattern of synapse-type specificity has not emerged.

We investigated the localization of NL3 across various brain regions employing KO mice as controls in order to validate signal specificity with maximal stringency. Our data demonstrate a clear pattern of distinct regional synapse-type specificities: NL3 associates with subsets of excitatory glutamatergic synapses in the cerebral cortex, hippocampus, and certain areas of the cerebellum; conversely, NL3 associates with inhibitory synapses in the olfactory bulb, basal ganglia, thalamus, brain stem, and the granule cell layer of cerebellum. These findings illustrate the versatile and highly regulated role of NL3 at different synapse subtypes, which will help to interpret the diverse synaptic defects observed in *Nlgn3* mutant mice.

Mechanisms that Determine Neuroligin Specificities for Postsynaptic Scaffolds

All NLs contain binding sites for the key scaffold protein of inhibitory postsynapses, Gephyrin (Poulopoulos et al., 2009) (Figure 3), and for PDZ-containing scaffolds of excitatory postsynapses, such as PSD-95 and S-SCAMs (Song et al., 1999; Meyer et al., 2004) (Supplementary Figure 3). *In vitro* and often in culture, NLs associate with and affect synapse types non-selectively (e.g. Figures 1 and 5). *In vivo*, however, NLs display highly selective synapse-type specificities, indicating that endogenous regulatory mechanisms determine the preference of a given NL to associate with either excitatory or inhibitory postsynaptic scaffolds. Interestingly, the emerging regulatory mechanisms that determine synapse-type specificity of NLs are distinct for different NL family members.

The mechanism by which NL2 and NL4 consistently associate with Gephyrin involves their ability to bind and activate the Gephyrin-binding protein Collybistin, which NL1 and NL3 cannot (Poulopoulos et al., 2009; Soykan et al., 2014). The specificity of NL1 for excitatory synapses appears to stem from constitutive phosphorylation of a tyrosine residue within its Gephyrin-binding motif that blocks interaction with Gephyrin (Giannone et al., 2013). Here, we identify a new phosphorylation-based mechanism that determines the synapse-type specificity for NL3, which unlike mechanisms for the other NLs, does so in a striking region-specific manner.

Using mass spectrometry, we detected a previously unknown serine phosphorylation site within the Gephyrin-binding motif of NL3. Using phosphomimetic mutants, we determined that this phosphoserine is predicted to block the interaction of NL3 with Gephyrin, similar to the aforementioned phosphotyrosine in the Gephyrin-binding motif of NL1 (Giannone et al., 2013). We generated and validated a phospho-specific antibody against this epitope, and confirmed that phosphorylation only occurs in the areas where NL3 loses its association with Gephyrin and localizes to excitatory synapses. This phosphorylation manifests in a region-specific and possibly cell-type-specific manner, making the corresponding kinase(s), phosphatase(s), and upstream regulators of particular future interest, as these remain to be identified despite our efforts (Supplementary Figure 2).

The Gephyrin-binding motif is highly conserved across all NLs, and the phosphorylation-targeted serine residue is present in NL1, 3, and 4. Interestingly, NL2 has alanine in the homologous position (Figure 3A), effectively making it a phospho-null variant that is hence always able to bind Gephyrin, in agreement with the consistent association of NL2 with inhibitory synapses *in vivo*. Despite this highly conserved context, and the fact that our phospho-specific antibody would equivalently recognize phospho-serine in the homologous position of any of the three NLs that harbor it, the phosphorylation event only appears in NL3, since no phospho-specific bands were detected in NL3 KO lysates (Figure 3E). Additionally, the phosphotyrosine found in the Gephyrin-binding site of NL1 was not detected in the homologous site of NL3 in our mass spectra, even though our samples were enriched for phosphopeptides. Indeed, we did detect MS/MS spectra showing this tyrosine to be unphosphorylated in NL3 (Figure 3B). Together, these data indicate that distinct mechanisms have evolved to regulate the synapse-type specificities of the four NL family members, and that in the case of NL3 this regulation is region-specific.

An interesting intersection when considering the distinct synapse type specificities of NL family members is the fact that NLs appear on the neuron surface as dimers and higher-order oligomers (Dean et al., 2003; Comoletti et al., 2003; Comoletti et al., 2007; Budreck and Scheiffele, 2007; Koehnke et al., 2008; Poulopoulos et al., 2012; Shipman and Nicoll, 2012), which include NL heterodimers. Indeed, NL3 in particular appears to interact with other NLs *in cis*, as abundant NL1-3 heterodimers were previously isolated as crosslinked adducts from hippocampal, striatal, and cerebellar neuron cultures (Poulopoulos et al., 2012). Furthermore, NL2-NL3 complexes were co-immunoprecipitated from whole brain lysates (Budreck and Scheiffele, 2007). Considering these findings together with the findings presented here, in particular regarding the localization of NL3 to inhibitory synapses in the striatum and the NL1-3 heterodimers isolated from striatal cultures, the possibility arises that a fraction of NL1 may localize to subtypes of inhibitory synapses as NL1-3 heterodimers in a subset of striatal synapses. The possibility of such distinct and neuron-specific variations of NL dimer combinations enriches the diversity of NL specificities at distinct synapse subtypes. Correspondingly, future circuit-level analyses of NL types at distinct synapses would benefit from the perspective of the operational NL unit at the synapse being the dimer.

NL3 Synapse Specificities vis-à-vis NL3 Synaptic Phenotypes

The function of NL3 at various synapses has been a focus of intense research, particularly in light of the close link between *Nlgn3* mutations and ASDs. A substantial number of mouse genetic studies identified strikingly diverse synaptic phenotypes in different brain regions and neuron types of NL3 mutant mice, indicating diverse synapse specificities for NL3 across the brain. We looked at broad specificities for inhibitory vs. excitatory synapses and did not address more refined subtypes of those broad categories. At this level, the previously reported synaptic phenotypes of *Nlgn3* mutants are largely consistent with our localization data. In the CA1 region of the hippocampus, where we find NL3 to be heavily phosphorylated and to localize to excitatory postsynapses (Figures 2B, 2C and 3E), *Nlgn3* KO causes a decrease in the frequency of miniature excitatory postsynaptic currents (mEPSCs) (Etherton et al., 2011a, 2011b). In the basal ganglia, where we find unphosphorylated NL3 to extensively localize to inhibitory postsynapses (Figures 2D and 3E), recordings from NL3 KO D1 medium spiny neurons of the ventral striatum show a specific loss of miniature inhibitory postsynaptic currents (mIPSCs) (Rothwell et al., 2014). Additional synaptic defects were identified in metabotropic glutamate receptor signaling in the cerebellum (Baudouin et al., 2012) and in presynaptic cannabinoid signaling at CCK synapses in hippocampus (Földy et al., 2013). While it is possible that a minor NL3 fraction below our detection threshold does localize to inhibitory CCK synapses in hippocampus, our findings, as well as the presynaptic nature of this phenotype, may indicate that the effect of NL3 perturbation on inhibitory CCK transmission in hippocampus reflects a network-level phenotype, rather than a direct effect of NL3 dysfunction at CCK-pyramidal cell synapses. On aggregate, the remarks above illustrate how the identification of region-specific patterns of synapse-type specificities of NL3 presented here may

contribute to refine mechanistic interpretations of mouse genetic functional data.

It remains to be determined whether some autism-associated *Nlgn3* point mutants, such as the R451C mutation that leads to gain-of-function effects (Tabuchi et al., 2007), alter the endogenous localization or synapse specificities of NL3. These avenues of future research, along with the determination of the particular synapse subtypes that NL3 associates with in cortical and subcortical regions, with the identification of cell-type-specific kinase and phosphatase pathways that regulate S799 phosphorylation, and with the assessment of the effect of mutations on these endogenous properties, will help understand how synaptic dysfunction along select yet diverse nodes of circuitry in cortical and subcortical regions converge onto the manifestations of ASD.

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AUTHOR CONTRIBUTIONS

Conceptualization, N.B. and Al.P.; Methodology, L.P.T., K.D., H.U., O.J., N.B., Al.P. Investigation, L.P.T., B.A., An.P., K.D., T.S., M.C.A., O.Y., D.K, M.H., H.H., P.R.L., L.D., M.S.-P., J.J.E.C., Al.P.; Formal Analysis L.P.T., B.A., An.P., K.D., T.S., H.H.H., Al.P.; Writing – Original Draft, Al.P.; Writing – Review & Editing, L.P.T., An.P., K.D., M.S.-P., O.J., N.B., Al.P.; Visualization, L.P.T., B.A., An.P., K.D., A.P.; Supervision L.P.T., M.S.-P., H.U., O.J., N.B., Al.P.; Project Administration N.B. and A.P.; Funding Acquisition M.S.-P., N.B, and Al.P.

FIGURE TITLES AND LEGENDS

Figure 1. NL3 overexpression in cultured neurons induces non-specific dendritogenic and synaptogenic effects
(A) Neurons at DIV7 expressing control GFP or HA-NL3 (green), immunolabeled for the excitatory presynaptic marker VGluT1 (magenta); scale bar, 20 μm.
(B-C) Quantification of excitatory VGluT1 (B) and inhibitory VIAAT (C) punctum density onto neurons transfected with control GFP, full-length HA-NL3, and HA-NL3/ΔICD. Sample images of neurons expressing HA-NL3 (bottom inset) and control GFP (top inset); scale bar, 20 μm; ANOVA $p < 0.001$, $n \geq 36$ cells per condition from 3 independent experiments.
(D) Representative tracing of neurite arborization of transfected neurons.
(E-F) Quantification of dendritic features. Number of dendrites intersecting concentric circles (Sholl analysis) and (F) density of filopidia per 10 μm of dendrite length from neurons transfected with control GFP, full-length HA-NL3, or cytoplasmic truncation HA-NL3/ΔICD; $n \geq 60$ cells per condition from 4 independent experiments. Exogenous NL3 overexpression promotes dendritic complexity via its cytoplasmic domain, and promotes both excitatory and inhibitory synaptogenesis via its extracellular domain.

Figure 2. Endogenous NL3 localizes to excitatory synapses in cortical, and to inhibitory synapses in subcortical regions
(A) Validation of NL3 antibody immunolabeling in WT versus *Nlgn3* KO tissue shows NL3-specific punctate signal. Cortex displays small puncta and thalamus displays large puncta (scale bar, 45 μm).
(B) NL3 colocalizes with subsets of PSD-95 puncta in cerebral cortex, hippocampus CA1 stratum oriens, and cerebellum molecular layer (scale bar, 3 μm) but not in brainstem (scale bar, 14 μm).
(C) Quantification of NL3 percentage overlapping with PSD-95 puncta versus randomized overlap in cerebral cortex (Cx), hippocampus CA1 stratum radiatum (CA1 SR), stratum oriens (CA1 SO), and cerebellum molecular layer (CB ML).
(D) NL3 significantly colocalizes with Gephyrin in brainstem, thalamus centrolateral nucleus, and globus pallidus, but not in cerebral cortex (scale bar, 7 μm).
(E) Quantification of the percentage of NL3 that overlaps with Gephyrin, and the percentage of Gephyrin that overlaps with NL3 in thalamus and brainstem. NL3 is fully localized to a subset of inhibitory postsynapses in thalamus and brainstem.

Table 1. Abundance, size, and synapse-type localization of NL3 puncta across brain regions.
The left column lists brain regions (bold) and subregions included in the analysis. Column two tabulates the relative abundance and size of NL3 puncta. Abundance is indicated by 1 (low) to 3 (high) marks, * for small puncta and ◊ for large puncta. Lack of punctate immunoreactivity in a region is indicated by /. The third and fourth columns note the presence (+) or absence (-) of NL3 puncta colocalizing with Gephyrin or PSD-95, respectively. Areas not examined are marked as n.a.

Figure 3. Identification of endogenous serine phosphorylation within the Gephyrin-binding site of NL3

(A) Alignment of the intracellular domains of rat NL1-NL3 and human NL4. Conserved and conservative residues are in red. PDZ- and Gephyrin-binding sites are indicated in blue. The serine within the Gephyrin-binding site corresponding to NL3 phospho-S799 is highlighted in green.

(B) Coomassie stained SDS-PAGE of anti-NL3 immunopurified eluate from rat brain extract. The extracted NL3 band as identified by mass spectrometry is boxed. Light (IgG_{25}) and heavy (IgG_{50}) immunoglobulin chains from the antibody are indicated.

(C) Fragment ion mass spectrum of the doubly charged precursor of the monophosphorylated peptide NL3(791-800). The inset shows the mass spectrum of the parent phosphopeptide (M_{obs} = 1300.6354, M_{calc} = 1300.6176, relative mass error = 13.7 ppm). Although a contaminating parent peptide ($[M+2H^+]^{2+}$ = 650.294) was co-isolated for fragmentation with the target parent peptide ($[M+2H^+]^{2+}$ = 651.325), conclusive N-terminal b-ion (b_2, b_3, b_7 in their non-phosphorylated forms) and C-terminal y-ion (y_5-y_8 in their phosphorylated forms) series, together with the neutral loss of the phospho moiety from y_3, clearly indicates phosphorylation on S799 while excluding the other potential phosphorylation sites of the peptide DYTLTLRRSP.

(D) Spot blots assessing the phosphospecificity of the 6808 antibody-peptide mix, targeting the epitope containing phosphorylated S799 on NL3. Phosphorylated and corresponding unphosphorylated peptides spotted on nitrocellulose at increasing amounts (50 ng to 2 µg). Immunoreactivity was measured by infrared fluorescence of dye-conjugated secondary antibody.

(E) Western blots of lysates from cerebral cortex (Cx), hippocampus (Hp), striatum (St), thalamus (Th), and cerebellum (Cb), probed for actin (loading control, bottom), NL3 (middle), and phosphorylated at S799 NL3 (NL3-pS, top). Antibody 6808 recognizes a band at the size of NL3 that is absent in *Nlgn3* KO lysates, indicating specific recognition of native phosphorylated NL3. While NL3 was detected in all regions examined, bands corresponding to NL3-pS were specifically detected as a major band in hippocampus, and minor bands in cortex and cerebellum.

Figure 4. Phosphomimetic mutations of serine at the Gephyrin-binding site of NLs disrupt interaction with Gephyrin.

(A) Yeast-two-hybrid assays using NL3 intracellular domains as bait, with WT S799 ($NL3^{ICD}$), phospho-null (~S799A), or phosphomimetic (~S799D) variants used against prey constructs of empty vector control (∅), Gephyrin (Geph), or S-SCAM fragment encompassing PDZ domains 1-3 (PDZ). Left: base plate showing comparable growth of transformed yeast cultures across all plate segments. Right: colorimetric β-galactosidase reaction on colony-lift replicate membrane. Positive interactions between NL3 mutants and postsynaptic scaffolds is indicated by a blue/green color reaction.

(B) Yeast-two-hybrid assays with bait intracellular domains from each of the four NLs in WT (ICD) and their corresponding Gephyrin-binding site phospho-null (~S->A) and phosphomimetic (~S->D) mutations versus full-length Gephyrin prey constructs. Phosphomimetic mutation at the Gephyrin-binding serine residue disrupts interaction with Gephyrin.

Figure 5. Phosphomimetic mutation of serine at the Gephyrin-binding site of NL3 disrupts membrane co-clustering with Gephyrin.
(A) Membrane co-clustering assays in COS7 cells transfected with GFP-Gephyrin (green) and myc-CB2^{SH3-} to induce Gephyrin membrane microaggregates. HA-NL3 (magenta) or its corresponding phospho-null (HA-NL3^{S799A}) or phosphomimetic (HA-NL3^{S799D}) mutants were transfected to assess co-clustering with Gephyrin membrane microaggregates (scale bar, 10 µm for main panel, 3.6 µm for inset).
(B) Pearson's correlation coefficients between GFP-Gephyrin microaggregates and surface-stained HA-NL3, HA-NL3^{S799A}, or HA-NL3^{S799D}; $p < 0.01$, one-way ANOVA with post-hoc Bonferroni from 3 independent experiments.
(C) Expression of HA-NL3, phospho-null (HA-NL3^{S799A}), and phosphomimetic (HA-NL3^{S799D}) mutants in DIV14 cultured neurons, immunolabeled for endogenous inhibitory postsynaptic marker Gephyrin (green) and inhibitory presynaptic marker VIAAT (magenta), merged in bottom row (scale bar, 10 µm).
(D) Quantification of dendritic ($p < 0.001$, one-way ANOVA with post-hoc Bonferroni, $n \geq 60$ cells per condition from 5 independent experiments) and perisomatic ($p < 0.01$, one-way ANOVA with post-hoc Bonferroni, $n \geq 35$ cells per condition from 3 independent experiments) Gephyrin clusters in neurons transfected with HA-NL3 or corresponding mutants. Phosphomimetic mutation at the Gephyrin-binding serine specifically abolishes NL3 interaction with Gephyrin in yeast, hinders NL3-Gephyrin co-clustering in cell lines, and NL3-mediated Gephyrin recruitment in cultured neurons.

Figure 6. Expression of NL3 mutants *in vivo*.
(A) HA-NL3 constructs expressed together with GFP in the cerebral cortex after *in utero* electroporation. Scale bar, 100 µm.
(B) Representative cell bodies of electroporated neurons expressing GFP (grey) together with NL3 WT (HA-NL3), phospho-null (HA-NL3^{S799A}), and phosphomimetic (HA-NL3^{S799D}) mutants. Dashed lines enclose fields used to classify clusters as perisomatic. Endogenous Gephyrin (green) and VIAAT (magenta) immunolabeling is shown separately and merged (bottom). Scale bar, 5 µm.
(C) Quantification of perisomatic Gephyrin clusters around electroporated cells; $p < 0.01$, $n \geq 85$ cells per condition from 7 independent experiments. Overexpressed WT NL3 in cortex behaves like the phosphomimetic mutant; it is deficient in recruiting postsynaptic Gephyrin, even though it recruits VIAAT terminals, indicating HA-NL3 is being natively phosphorylated at the Gephyrin-binding site.

STAR METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and resources from this study are available upon request and availability. Requests should be directed to and will be fulfilled by the Lead Contacts, Nils Brose (brose@em.mpg.de) and Alexandros Poulopoulos (apoulopoulos@som.umaryland.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All animal experiments were designed and carried out in compliance with animal welfare guidelines of the European Community Council Directive 86/609/EEC and approved by the Bioethical Committee of Turin University issued and by the state of Lower Saxony, Germany according to the corresponding permits 33.9-42502-04-13/1359, and 33.19-42502-04-13/1052. For information on mouse and rat experimental strains see Key Resources Table below.

METHOD DETAILS

Plasmids

NL3 construct variants were produced from an HA-tagged expression construct of NL3 previously cloned from rat cDNA into pCMV (Poulopoulos et al., 2009). Site-directed mutagenesis using the Quikchange method (Stratagene) was used to produce S799A and S799D mutants. Full length HA-NL3 and respective mutants were subcloned into vector pRaichu for expression in cell lines or neurons. Cytosolic domains of NL3 and corresponding mutants were subcloned into vector pLexN for yeast-two-hybrid assays. pRaichu (generously provided by Jun-ichi Miyazaki, Osaka) is based on the pCAGGS vector backbone (Mochizuki et al., 2001). pLexN is prey construct modification of pBTM116 (Vojtek et al., 1993). pEGFPN1, pRaichu-NL3 and corresponding S799A and S799D variants, as well as pRaichu-NL3-ECD (NL3 extracellular domain with stop codon replacing position K735) were used for transfections. Previously published myc-CB2^{SH3-} (Harvey et al., 2004) and GFP-Gephyrin (Fuhrmann et al., 2002) were used for COS7 cell transfections. For *in utero* electroporation, NL3 and corresponding mutants were subcloned into F(SYN)UGW-RBN (Weston et al., 2011), generously provided by Christian Rosenmund and Ralf B. Nehring, Berlin, and co-injected with GFP expression plasmid pFUGW (Addgene Plasmid #14883 from David Baltimore's lab).

For yeast-two-hybrid assays, the intracellular domains of rat NL1-NL3 and mouse NL4 were subcloned into pLexN to serve as bait constructs, as previously described (Poulopoulos et al., 2009). Corresponding mutant bait constructs of NL3 S799A and S799D as well as the homologous mutant variants of NL1, NL2, and NL4 were produced with site-directed mutagenesis. Plasmid vector pVP16-3 (Vojtek et al., 1993) was used for prey constructs of

full-length Gephyrin (Poulopoulos et al., 2009) and the sequence comprising the three tandem PDZ domains of S-SCAM (PDZ1-3, residues 422–497 of P_446073) (Meyer et al., 2004). Coding regions of all plasmids were verified by Sanger sequencing.

Cell culture and transfection

COS7 cells were plated directly onto glass coverslips, cultured in DMEM supplemented with 10% fetal bovine serum, and transfected with FuGENE6 (Roche) according to standard protocols.

Primary neuron cultures were prepared from cortex of wild-type (WT) C57BJ embryos at embryonic day (E) 16. Cultures were prepared as described previously (Tuffy et al., 2010), with papain substituted for trypsin to obtain better cell recovery following tissue digestion and trituration. Cells were plated on poly-L-lysine-coated glass coverslips at a density of 25,000-75,000 cells/cm^2. Neurons were transfected at day in vitro (DIV) 3 with Lipofectamine 2000 using standard protocols. Analyses were carried out at DIV 7-14.

Antibodies

The following antibodies were used for immunocytochemistry on cell lines and cultured neurons (ICC), immunohistochemistry on brain sections (IHC), immunohistochemistry on brain sections after *in utero* electroporation (IUE), Western blotting (WB), and immunoprecipitation (IP): Mouse-anti Actin (AC40, Sigma, 1:5000 for WB), Mouse-anti-myc (9E10, 1:1,000, Sigma-Aldrich for ICC), Mouse anti-gephyrin (3B11; Synaptic Sytems; 1:500 dilution for ICC, IUE); mouse anti-gephyrin (mAb7a; Synaptic System; 1:1000 dilution for IHC); mouse anti-PSD-95 (6G6-1C9; Abcam; 1:500 dilution for ICC); mouse anti-PSD-95 (clone K28/43; NeuroMab; 1:1000 dilution for IHC); rabbit anti-GFP (598 MBL; 1:1000 dilution IUE); rabbit anti-GFP (132003; Synaptic Systems; 1:1000 dilution for IC); guinea pig anti-$GABA_AR\gamma2$ (1:2000; provided by Dr. Jean-Marc Fritschy, Zurich); mouse anti-GlyRα1 (Mab4a; 1:100; provided by Dr. Heinrich Betz, Frankfurt); guinea pig anti-VIAAT (131004; Synaptic Systems; 1:1000 dilution for IC, IUE); guinea pig anti-VGluT1 (135304; Synaptic Systems; 1:1000 dilution for IC, IUE); rabbit anti-HA (715500; Invitrogen; 1:1000 dilution for IC, 1:4000 dilution for WB).

Custom-made NL3 antibody 804 (1:1000 dilution for WB, 5-10 µl antiserum for IP) was previously described and validated in NL3 KO samples (Varoqueaux 2006), and by mass spectrometric identification of NL3 in the material immunopurified using 804 antibody from rat brain lysate (Figure 3). Custom-made phospho-specific antibody 6808 recognizing the serine-phosphorylated NL3 epitope RRpSPDDIP, was produced by PhosphoSolutions (Aurora, CO, USA). Briefly, antiserum was raised in rabbits immunized with RRpSPDDIP. Antibody was affinity-purified against the immunizing phosphopeptide. This produced antibody RαNL3-pS 6808, which recognizes NL3 phosphorylated at S799, and is likely to recognize any putative phosphorylation at the homologous Gephyrin-binding site serine of NL1 or NL4. 6808 was used for WB at a dilution of 1:3000 in the presence of 2 µg/ml of competing unphosphorylated peptide RRSPDDIP. Peptides were synthesized in-house (Proteomics Group, MPI-EM) by standard solid phase peptide synthesis using Fmoc chemistry.

The following secondary antibody conjugates where used for ICC, ICH, and IUE (listed by host and dye): goat Alexa 488, donkey Alexa 488, goat Alexa 568, donkey Alexa 568 (Molecular Probes, Eugene, OR); goat Cy3, donkey Cy3, goat Cy5, donkey Cy5 (Jackson Immunoresearch, West Grove, PA). Secondary antibodies conjugated with HRP (Jackson ImmunoResearch) or IRDye800 (Rockland) were used for WB. For details and antibody sources see Key Resource Table below.

Immunopurification and Mass Spectrometry (MS)

One adult rat brain (2 g) was homogenized in 9 nine volumes of 320 mM sucrose solution supplemented with cOmplete Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail 1 and 2 (Sigma) at manufacturer recommended concentrations. Homogenates were produced at 4ºC with 13 strokes in a Potter-Elvehjem homogenizer rotating at 900 rpm. Homogenates were centrifuged at 1200 x g for 15 minutes to obtain the supernatant postnuclear homogenate. Membranes were pelleted from the postnuclear homogenate with 100,000 x g for 1h. Membrane proteins were extracted from pellets with 1% SDS in TNE buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA). SDS lysate was centrifuged at 20,000 x g for 30 minutes and supernatants were subsequently diluted with 7 volumes of 1.15% Triton-X 100 in TNE with Phosphatase Inhibitor Cocktail 1 and 2 (Sigma) to produce a mixed-micelle lysate with final detergent concentrations of 0.125% SDS and 1% Triton-X 100, which allow immunopurification procedures (Poulopoulos 2009). Endogenous NL3 was immunopurified from this lysate by using the anti-NL3 antibody 804, preloaded onto Protein G Sepharose beads (Amersham). After overnight incubation at 4°C with the lysate, beads were washed three times with TNE buffer containing 1% Triton-X 100 and once with TNE buffer alone. Proteins bound to beads were eluted in SDS sample buffer, separated by SDS-PAGE on precast 10% Bis-Tris gels (NuPAGE, Thermo Fisher Scientific) with MOPS running buffer, and visualized by colloidal Coomassie staining.

Gel bands of interest were excised and subjected to in-gel digestion with endoproteinase Asp-N to generate a proteolytic peptide that covers the potential phosphorylation sites within the Gephyrin-binding site of NL3 and is of a size readily accessible for mass spectrometric sequencing (which is not possible with trypsin because of the lack of cleavage sites within a ~40 amino acid sequence stretch C-terminally of the Gephyrin-binding site, Figure 3A). Phosphopeptides were enriched by TiO_2 chromatography as described (Oellerich et al., 2009) and the presence of a monophosphorylated species of the peptide NL3 (residues 791-800, DYTLTLRRSP, M_{calc} = 1300.62) in the enriched fraction was confirmed by MS (data not shown). This target phosphopeptide was sequenced by NanoLC-ESI MS analysis on an Ultima API-Q-TOF mass spectrometer (Waters Cooperation) as described (Oellerich et al., 2009).

Yeast-two-hybrid

Yeast-two-hybrid (YTH) assays were performed in the *Saccharomyces cerevisiae* L40 reporter strain using small-scale Li-acetate cotransformations with pLexN bait constructs encoding the intracellular domains of NL1-NL4 and either empty pVP16-3 or pVP16-3 vectors encoding full-length Gephyrin or S-SCAM fragment PDZ 1-3 (residues 422–497). Transformed yeast were

incubated at 30°C for three days to allow prototropic colonies to emerge as described previously (Betz et al., 1997). Clones were tested in duplicate for activation of the β-galactosidase reporter gene by filter assays (Vojtek et al., 1993). The readout corresponds to LacZ activity using standard X-gal chromogenic assay.

***In vitro* kinase assays**

For Kinase Hot-Spot assays, a 21 amino acid peptide spanning residues 785-805 of rat NL3 was used as substrate for *in vitro* kinase assays. Streamlined filtration binding assay was performed on a commercial basis by the Reaction Biology Corporation (PA, USA) in buffer containing 20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 1 mM EGTA, 0.02% Brij35, 0.02 mg/ml BSA, 0.1 mM Na_3VO_4, 2 mM DTT, 1% DMSO in the presence of ATP-γ-^{33}P and substrate peptide. Experiments were carried out in single dose duplicates with results expressed as percent incorporation relative to internal control, or as nM phosphate transferred to 10 µM peptide (see Figure S2).

ADP Glo assays (Promega) were performed according to manufacturer's recommendations. WT rat NL3 peptide representing residues 785-805, and the three mutant variants Y793F, T795A, and S799A were examined.

All peptides were synthesized in-house (Proteomics Group, MPI-EM) by standard solid phase peptide synthesis using Fmoc chemistry.

Western blot

Adult mouse brains from WT and KO littermates were dissected into regions of interest and manually homogenized in Lysis Buffer (0.32 M Sucrose, 5 mM $MgCl_2$ supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktails, 200 U/mL benzonase) using a Teflon pestle in microcentrifuge tubes. Total protein concentrations were determined using Bio-Rad protein assay according to the manufacturer's instructions. Homogenate samples were prepared in Laemmli Buffer (10% Glycerol, 50 mM Tris-HCl pH 6.8, 2 mM EDTA, 2% SDS, 100 mM DTT, 0.05% Bromophenol blue) at 65 °C for 20 min. Samples corresponding to 40 µg total protein were loaded for SDS-PAGE. Proteins separated on gels were electroblotted onto nitrocellulose (Protran 0.2 µm, GE Healthcare) with a constant current of 100 mA for 10 h using wet transfer. For Immunoblot, membranes were incubated in Blocking Buffer (5% BSA in TBS-T) for 1 h at RT. Standard western blot procedures were followed with overnight incubations of primary antibody solutions in Blocking Buffer at 4 °C. For NL3-pS WB, primary antibody 6808 solution was pre-incubated with 2 mg/mL unphosphorylated antigen peptide (RRSPDDIP) for 1h at RT prior to primary antibody incubation. HRP or IRDye800 conjugated secondary antibodies were incubated with membranes for 1h at RT in Blocking Buffer. Immunoblot signal was detected using enhanced chemiluminescence (ECL, GE Healthcare) or with the Odyssey Infrared Imaging System (LI-COR Biosciences), respectively. Experiments were repeated and confirmed using WT rat brain as well.

Spot blot

Peptide stock solutions containing 2 mg/mL of NL3 antigenic peptides (unphosphorylated RRSPDDIP or phosphorylated RRpSPDDIP) were

prepared in TBS (20 mM Tris-HCl pH 7.5, 137 mM NaCl). Peptide stock solutions were serially diluted into 1000 ng/µL, 250 ng/µL, 100 ng/µL and 50 ng/µL. 1 µL of each serial dilution of phosphorylated and non-phosphorylated peptides was spotted onto nitrocellulose membranes. Immunoblot against NL3-pS was performed using the protocol described above for western blots.

Membrane co-clustering assay

Membrane co-clustering assays were performed in COS7 cells transfected with GFP Gephyrin, myc-CB2^{SH3-} and HA-NL3 variants as described previously (Poulopoulos et al., 2009). Briefly, cells were fixed 10 h after transfection in 4% PFA and blocked with 5% normal goat serum and 0.1% gelatin in 0.1 M phosphate buffer (PB). Prior to permeabilization, cells were stained with polyclonal rabbit anti-HA antibody in blocking solution for 2 h at RT to detect the surface pool of HA-tagged NL3. After three washes with PB, cells were permeabilized with 0.1% Triton X-100, 5% normal goat serum, and 0.1% gelatin in PB, and stained with mouse-anti-myc antibody (clone 9E10, 1:1,000, Sigma-Aldrich) in the same buffer for 2 h at RT, followed by the appropriate Alexa-conjugated secondary antibodies.

Samples were imaged using a Leica DMIRE2 microscope equipped with a 63× oil-immersion objective connected to a Leica TCS SP2 AOBS confocal laser scanning setup. Intensity correlation analysis was performed on multichannel images using ImageJ. Briefly, a Gaussian blur was applied, and GFP and HA channels were thresholded. A standard Pearson's correlation coefficient was evaluated between the HA and GFP channels in the thresholded fields using the Intensity Correlation Analysis plugin for ImageJ (http://rsb.info.nih.gov/ij/) from T. Collins and E. Stanley (Toronto, ON, Canada).

Synaptic and morphological analyses in cultured neurons

Quantification of synaptic puncta and neuron morphology were carried out in cultured mouse cortical neurons at DIV7-14 transfected with HA-NL3 variants and stained for HA and endogenous synaptic markers. Images for pre- or postsynaptic quantifications were captured and analyzed in a double-blind manner wherever possible. For quantification of presynaptic VGluT1, VIAAT, and postsynaptic Gephyrin or PSD-95, each image was manually thresholded in ImageJ to generate a binary image in which individual clusters of synaptic molecule markers could be observed. In order to isolate puncta of transfected neurons only, a mask was created from a co-stained image captured for HA. This mask allowed quantification of synaptic puncta selectively in transfected neurons. The number of clusters per neuron was counted using the 'Analyze Particles' function with Watershed Segmentation. The total mask area was measured with ‘measure’ function. Puncta were expressed as number of puncta per given area. For DIV7 cultures where synaptic puncta have not fully matured, we assessed total intensity of Gephyrin and PSD-95 within the HA mask of transfected neurons. The intensity was normalized to total image intensity. Results were expressed as means of four independent experiments.

Neuronal arborization was assessed by thresholding the HA-stained image, creating a binary image in which all neuronal branches are visible and distinguishable. Axons were manually removed using selection tools, as were

any other dendrites or axons not originating from the selected neuron. Sholl analysis (Sholl, 1953) was carried out with a radius of 10 μm to 100 μm from the soma. The number of intersections was plotted against distance from the soma. Analysis of filopodia was performed by manual counting of filopodial protrusions along 10 μm segments of 2nd and 3rd order dendrites. Three dendrites were assessed per image with at least 15 images per condition.

Immunohistochemistry on brain sections

Adult mice were anesthetized with intraperitoneal ketamine-xylazine 1:1 (0.1 ml/kg) and decapitated. Brains were dissected out and cut into sagittal or coronal slabs of ≈1 mm and fixed by immersion in ice-cold formaldehyde (4% in 0.1 M phosphate buffer) for 20–30 min as described previously (Schneider Gasser et al., 2006; Viltono et al., 2008). The slabs were cryoprotected in ascending sucrose solutions (10%, 20%, and 30%) and sectioned with a cryostat. Following a blocking step in normal goat (or donkey) serum (3% in PBS with 0.5% Triton X-100), sections were incubated overnight with combinations of two or three primary antibodies raised in different species. The sections were then rinsed in PBS, incubated with appropriate secondary antibodies, rinsed again and coverslipped with Dako fluorescence mounting medium (Dako Italia, Italy).

Immunolabeled sections were imaged using a laser scanning confocal microscope (Zeiss LSM5 Pascal) using the multichannel acquisition mode to avoid fluorescence crosstalk. Images (512 × 512 pixels) were acquired with a 100× oil-immersion objective (1.4 numerical aperture) at a magnification of 8.1 x 10^{-3} or 2 x 10^{-3} μm^2/pixel (zoom 2 and zoom 4, respectively), and the pinhole set at 1 Airy unit.

Acquired images were processed with the image-analysis program Imaris (release 4.2; Bitplane, Zurich, Switzerland). To analyze colocalization between NL3 and gephyrin or PSD-95, images were first segmented using a threshold that maximized the selection of immunofluorescent puncta over background labeling, and then processed with the “colocalization” module, in which a mask is generated from the comparison of two different confocal channels. The number of puncta was then calculated with NIH ImageJ software (http://rsb.info.nih.gov/nih-image) as previously described (Viltono et al., 2008).

In Utero Electroporation (IUE)

Mouse embryos were subjected to IUE at embryonic day (E) 14.5 to target layer 2/3 neurons of the cerebral cortex, as previously described (Ripamonti et al., 2017). A laparotomy was carried out on deeply anesthetized mice exposing the uterus and embryos, which were subsequently moistened with warm PBS supplemented with antibiotics (Penicillin and Streptavidin, Gibco). GFP expression plasmid pFUGW (0.2 mg/ml) was mixed together with HA-NL3 expression plasmid F(SYN)UGW-RBN (0.5 mg/ml) in 0.05% Fast Green solution. Plasmid solution was injected into the lateral ventricle of the embryonic brain through a glass micropipette connected to a pneumatic pump (PV820). Injected embryos were electroporated with three pulses of 40 V delivered through tweezertrodes (CUY650P1, NepaGene) by a square pulse generator (ECM830BTX Harvard Apparatus).

Electroporated animals were sacrificed at P21 and brains were dissected

and postfixed with 4% PFA overnight, and cryoprotected with 30% w/v sucrose for a further 16-24 h. Brains were subsequently frozen in isopentane at -40 ºC, sectioned coronally at 20 µm, and transferred onto glass slides. Slides were incubated with 4% PFA for 12 min followed by 30 min incubation at 90 °C in sodium citrate buffer. After further washes, slides were blocked in 10% goat serum, 0.2% Triton X-100, and 0.1% fish gelatin in PBS for 1 h, prior to immunolabeling. The slides were then incubated with mouse-anti-Gephyrin and guinea pig-anti-VIAAT antibodies overnight at 4 °C, and subsequently with corresponding secondary antibodies for 1-2 h. Images were captured on a Leica SP2 confocal microscope with a 40-x objective.

QUANTIFICATION AND STATISTICAL ANALYSIS

Puncta were defined by intensity thresholding of the immunoreactive signals, and the proportion of overlapping puncta was determined as previously described (Viltono et al., 2008). Due to the different densities with which PSD-95 and Gephyrin puncta present in the brain, we followed distinct approaches to quantify the degree and specificity of overlap in each case. PSD-95, displays high punctum densities covering much of grey matter neuropil. As such, colocalization alone is a poor readout of specific association due to the high incidence of random overlap. Thus random overlap with PSD-95 immunoreactivity was assessed and compared to specific overlap.

Statistical comparisons in this study were made using the unpaired, two-tailed Student's *t*-test when comparing two variable means. In the case of three variables, we used the one-way ANOVA with Bonferroni posthoc test. *p* values < 0.05 were considered significant. p value outcomes were indicated by asterisks in the figures as follows: *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-beta-tubulin	Sigma-Aldrich	Cat #T4026 RRID:AB_477577
Rabbit polyclonal anti-NL3	Varoqueaux, 2006	
Rabbit polyclonal anti-NL3-pS (6808)	This study	
Mouse anti-Actin (AC40)	Sigma	Cat#A4700, RRID:AB_476730
Mouse-anti-myc (9E10)	Sigma	Cat#M4439, RRID:AB_439694

Mouse anti-Gephyrin (3B11)	Synaptic System	Cat#147111, RRID:AB_887719
mouse anti-Gephyrin (mAb7a)	Synaptic System	Cat#147011, RRID:AB_887717
mouse anti-PSD-95 (clone K28/43)	NeuroMab	Cat#75-028, RRID:AB_2292909
mouse anti-PSD-95 (6G6-1C9)	Abcam	Cat#ab2723, RRID:AB_303248
rabbit anti-GFP	MBL	Cat#598, RRID:AB_591819
rabbit anti-GFP	Synaptic System	Cat#132003, RRID:AB_1834147
guinea pig anti-$GABA_AR\square 2$	provided by Dr. Jean-Marc Fritschy, Zurich	
mouse anti-GlyRα1 (Mab4a)	provided by Dr. Heinrich Betz, Frankfurt	
guinea pig anti-VIAAT	Synaptic System	Cat#131004, RRID:AB_887873
guinea pig anti-VGluT1	Synaptic System	Cat#135304, RRID:AB_887878
rabbit anti-HA	Thermo Fisher Scientific	Cat#71-5500, RRID:AB_2533988
Goat anti-Rabbit IgG-HRP	Dianova	Cat#111-035-144; RRID:AB_2307391
Goat anti-Rabbit IgG-IRDye800	Rockland	Cat#611-132-122; RRID:AB_220152
Goat anti-Mouse IgG-IRDye800	Rockland	Cat#610-132-121; RRID:AB_220125
Goat anti-Mouse IgG-Alexa 488	Thermo Fisher Scientific	Cat#A-11029, RRID:AB_138404
Goat anti-Rabbit IgG-Alexa 488	Thermo Fisher Scientific	Cat#A-11034, RRID:AB_2576217
Goat anti-Guinea Pig IgG-Alexa 488	Thermo Fisher Scientific	Cat#A-11073, RRID:AB_142018
Goat anti-Mouse IgG-	Thermo Fisher Scientific	Cat#A-11031,

Alexa 568		RRID:AB_144696
Goat anti-Rabbit IgG-Alexa 568	Thermo Fisher Scientific	Cat#A-11036, RRID:AB_143011
Goat anti-Guinea Pig IgG-Alexa 568	Thermo Fisher Scientific	Cat#A-11075, RRID:AB_141954
Donkey anti-Mouse IgG-Alexa 488	Thermo Fisher Scientific	Cat#A32766, RRID:AB_2762823
Donkey anti-Rabbit IgG-Alexa 488	Thermo Fisher Scientific	Cat#A32790, RRID:AB_2762833
Donkey anti-Mouse IgG-Alexa 568	Thermo Fisher Scientific	Cat#A10037, RRID:AB_2534013
Donkey anti-Rabbit IgG-Alexa 568	Thermo Fisher Scientific	Cat#A10042, RRID:AB_2534017
Goat anti-Mouse IgG-Cy3	Jackson Immunoresearch	Cat#115-165-146, RRID:AB_2338690
Goat anti-Rabbit IgG-Cy3	Jackson Immunoresearch	Cat#111-165-144, RRID:AB_2338006
Goat anti-Guinea Pig IgG-Cy3	Jackson Immunoresearch	Cat#106-165-003, RRID:AB_2337423
Goat anti-Mouse IgG-Cy5	Jackson Immunoresearch	Cat#115-175-146, RRID:AB_2338713
Goat anti-Rabbit IgG-Cy5	Jackson Immunoresearch	Cat#111-175-144, RRID:AB_2338013
Donkey anti-Mouse IgG-Cy3	Jackson Immunoresearch	Cat#715-165-151, RRID:AB_2315777
Donkey anti-Rabbit IgG-Cy3	Jackson Immunoresearch	Cat#711-165-152, RRID:AB_2307443
Donkey anti-Guinea Pig IgG-Cy3	Jackson Immunoresearch	Cat#706-165-148, RRID:AB_2340460
Donkey anti-Mouse IgG-Cy5	Jackson Immunoresearch	Cat#715-175-151, RRID:AB_2340820
Donkey anti-Rabbit IgG-Cy5	Jackson Immunoresearch	Cat#711-175-152, RRID:AB_2340607
Donkey anti-Guinea Pig IgG-Cy5	Jackson Immunoresearch	Cat#706-175-148, RRID:AB_2340462
Chemicals, Peptides,		

and Recombinant Proteins		
FuGENE 6	Roche	Cat# 11 814 443
Lipofectamine 2000	Invitrogen	Cat# 11668
NL3 antigen peptide (RRSPDDIP)	This study	
NL3 antigen phosphopeptide (RRpSPDDIP)	This study	
Phosphatase Inhibitor Cocktail 2	Sigma	P5726
Phosphatase Inhibitor Cocktail 3	Sigma	P0044
PhosSTOP™	Roche	PHOSS-RO
cOmplete™, EDTA-free Protease Inhibitor Cocktail	Roche	*04693132001*
Benzonase nuclease	Sigma	E1014-25KU
Critical Commercial Assays		
ADP-Glo™ Kinase Assay	Promega	V6930
Bio-Rad Protein Assay	Bio-Rad	#5000006
Experimental Models: Cell Lines		
COS-7 cells	ATCC	ATCC CRL-1651; RRID:CVCL_0224
Experimental Models: Organisms/Strains		
L40 *Saccharomyces cerevisiae*	ATCC	MYA-3332
C57BL/6J wild-type mice	Jackson Laboratory	JAX 000664
Sprague-Dawley rats	Charles River Laboratory	400
Nlgn3 knockout mice	Varoqueaux, 2006	*Nlgn3* -/-
Recombinant DNA		
HA-NL3 in pCMV	Poulopoulos et al., 2009	
pRaichu plasmid vector	Mochizuki et al., 2001	

pLexN plasmid vector	Vojtek et al., 1993	
F(SYN)UGW-RBN plasmid vector	Weston et al., 2011	
NL3 expression plasmids subcloned into the above vectors	This study	
pEGFPN1	Clontech	6085-1
pFUGW	Addgene	Plasmid #14883
S-SCAM (PDZ1–3) in pLexN	Meyer et al., 2004	
Gephyrin in pLexN	Poulopoulos et al. 2009	
GFP-Gephyrin	Fuhrmann et al., 2002	
myc-CB2^{SH3-}	Harvey et al., 2004	
Sequence-Based Reagents		
Software and Algorithms		
Image Studio™ Lite	LI-COR	https://www.licor.com/bio/products/software/image_studio_lite/
Microsoft Excel	Microsoft	https://products.office.com/de-de/excel
ImageJ	NIH	https://imagej.net

SUPPLEMENTARY FIGURE TITLES AND LEGENDS

Supplementary Figure 1. NL3 synaptic localization in cerebellar glomeruli and brainstem.
(A) NL3 localizes to both glutamatergic and GABAergic synapses in cerebellar glomeruli. Confocal images showing colocalization of NL3 with both PSD-95 (A_1) and Gephyrin (A_2) at glomerular synapses. Scale bar 3 µm.
(B) Triple-labeling for NL3 (red), GlyR1 (green) and $GABA_AR\gamma2$ (blue) in the brainstem. Note that NL3 is present at synapses containing both glycine and $GABA_A$ receptors. In some cases, NL3 was associated with purely glycinergic synapses lacking GABA receptors (arrows). Scale bar 7 µm.

Supplementary Figure 2. *In vitro* kinase assays do not identify selective

NL3 S799 kinase.
(A) *In vitro* colorimetric kinase activity assays testing a selection of purified kinases (CaMKII, PKA, PKC, and c-kit) on a 21-residue substrate peptide corresponding to NL3 785-805 (RLTALPDYTLTLRRSPDDIPL) and control peptides with corresponding alanine mutations of putative phosphorylatable positions S799, T795, and Y792. Baseline signal indicated as “-ve control”. Results are means of three data sets. No specific phosphorylation of position S799 was detected.
(B) High-throughput *in vitro* radiolabeled phosphate kinase assays measuring phosphate transfer onto the NL3 785-805 peptide and corresponding S799A negative control against 365 individual kinases. The graph shows top kinases ranked in order of phosphate transferred. Experiment was carried out in single dose duplicates. None of the kinases displayed specific phosphorylation of S799.

Supplementary Figure 3. Yeast-two-hybrid of NL intracellular domains versus PDZ domains**.**
Yeast-two-hybrid culture plates (left) and β-galactosidase filter assays (right) of NL1-4 intracellular domain bait constructs in their WT forms (S) and their respective phosphomimetic (D) and phospho-null (A) mutants of the Gephyrin-binding site serine residue (S799 in NL3) versus empty control prey construct (upper) and prey construct with an S-SCAM fragment encompassing PDZ domains 1-3 (PDZ, bottom row). PDZ domain interactions remain unaffected by serine mutation in all NLs.

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