Phosphorylation of Neuroligin-2 by PKA regulates its cell surface level and synaptic stabilization

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Abstract

The trans-synaptic adhesion molecule Neuroligin-2 (NL2) is essential for the development and function of inhibitory synapses. NL2 recruits the postsynaptic scaffold protein gephyrin, which in turn stabilizes γ-aminobutyric acid type A receptors ($GABA_A$Rs) in the postsynaptic domain. NL2 levels at the synapse can thereby control synaptic $GABA_A$R concentration to tune inhibitory neurotransmission efficacy. Here, using biochemistry, imaging, single particle tracking and electrophysiology, we uncovered a key role for cAMP-dependent protein kinase (PKA) in the synaptic stabilization of NL2. We found that PKA-mediated phosphorylation of NL2 at Ser^{714} caused its dispersal from the synapse and reduced NL2 surface levels, leading to a loss of synaptic $GABA_A$Rs. Conversely, abolishing the phosphorylation of NL2 enhanced its stability and led to increased inhibitory signaling. Thus, PKA plays a key role in regulating NL2 function and GABA-mediated synaptic inhibition.

Introduction

Synaptic inhibition through postsynaptic γ-aminobutyric acid (GABA) type-A receptors ($GABA_ARs$) is crucial for controlling the balance between excitation and inhibition (E/I) of neuronal signalling in the brain. Defects in neuronal inhibition underlie several neurological conditions including epilepsy, autism, and schizophrenia (1-3). To maintain the E/I balance during neural activity, the strength of the postsynaptic response is dynamically regulated through altering the number of $GABA_ARs$; this involves processes such as phosphorylation and ubiquitination, which impact on stabilisation *versus* dispersal of receptors from the synapse and receptor internalisation *versus* recycling (4-9).

The postsynaptic membrane-spanning adhesion molecule Neuroligin-2 (NL2), together with its postsynaptic binding partners LHFPL4 (Lipoma HMGIC Fusion Partner-Like 4) and Slitrk3 (Slit- and Trk-like family protein 3), plays a key role in the development and function of GABAergic synapses (10-16). NL2 ensures correct positioning opposite pre-synaptic terminals through interaction with pre-synaptic neurexins (17-19). Crucially, NL2, through a direct interaction with collybistin, drives clustering of a post-synaptic gephyrin scaffold, which stabilises $GABA_ARs$ at synapses (20,21).

NL2, when phosphorylated, recruits Pin1, a peptidyl-prolyl *cis-trans* isomerase that disrupts the interaction between NL2 and gephyrin, thereby reducing inhibitory synapse size and GABAergic signaling (22). Likewise, changes in synaptic levels of NL2 through its internalization and recycling contribute to the modulation of inhibitory synapse size and signaling strength (23-25). In this study, we investigated the molecular mechanisms that regulate the phosphorylation of NL2 as well as its cell surface levels at the synapse. Our findings reveal a role for cAMP-dependent protein kinase (PKA) in regulating the surface level of NL2 and the consequent modulation of inhibitory signaling.

Results

NL2 is uniquely and constitutively phosphorylated at Ser^{714}

Mass spectrometry studies aiming to map tissue-specific phospho-proteomes identified residues Ser^{714}, Ser^{719} and Ser^{721} in the intracellular region of rodent NL2 as putative serine phosphorylation sites (26-28). To investigate the extent and role of phosphorylation at these sites, we created phospho-null (Ser to Ala) and phospho-mimetic (Ser to Asp [D]) mutants of murine HA-tagged NL2 (^{HA}NL2; Fig.1A). As a control we also included a non-related mutant, Y770A; this Tyr is conserved across neuroligins, and phosphorylation of the equivalent site in NL1 (Tyr^{782}) has been shown to prevent gephyrin binding (29). We expressed wild-type (WT) and mutant ^{HA}NL2 in COS-7 cells and analysed the cell lysates by Western blotting using a standard SDS-PAGE gel supplemented with PhosTag. This alkoxide-bridged Mn^{2+}-binding compound, when added to a standard polyacrylamide gel, reduces the migration of phosphorylated proteins compared to their non-phosphorylated counterparts (30), thus enabling detection of protein phosphorylation without the requirement of generating site-specific phospho-antibodies. Using this method, we found that WT and Y770A ^{HA}NL2 were phosphorylated at steady state, whereas mutants of Ser^{714} alone or in combination with Ser^{719} and Ser^{721} were not phosphorylated (Fig.1B). This indicates that ^{HA}NL2 is basally phosphorylated at Ser^{714}, whereas the other sites are not phosphorylated in COS-7 cells.

Given that only a small proportion of ^{HA}NL2-WT is phosphorylated in COS-7 cells (5.1 ± 1.2% from 5 blots; Fig. 1B), we next investigated the extent of phosphorylation of endogenous NL2 in neurons. We incubated cortical and hippocampal lysates with or without lambda phosphatase to dephosphorylate NL2 in vitro and analysed the lysates on SDS-PAGE supplemented with PhosTag. Whereas a standard SDS-PAGE revealed one major band for NL2, the PhosTag gel showed two clearly distinguishable bands (Fig. 1C and fig. S1A). Upon incubation with lambda phosphatase, a strong decrease in intensity of the higher band was observed, whereas intensity of the lower band increased. This suggests that the higher band represents phosphorylated NL2, whereas the lower band represents the non-phosphorylated population, consistent with slower

migration of phosphorylated proteins on a PhosTag gel. Quantification of signal intensity revealed that in cortical neurons ~55% of NL2 is basally phosphorylated (Fig. 1, C and D). Similar levels of basal phosphorylation were observed in cultured hippocampal neurons at different ages (DIV8 to DIV13) (fig. S1B). The additional lower band observed on a standard SDS-PAGE for ^{HA}NL2 overexpressed in COS-7 cells (Fig. 1B), but not for endogenous NL2 in neurons (Fig. 1C), most likely represents immature protein that is not fully glycosylated. Treatment of COS-7 cell lysates containing overexpressed ^{HA}NL2 variants with EndoH, an enzyme that cleaves glycan chains that are only present during protein maturation in the endoplasmatic reticulum (ER) and Golgi, lead to a reduction in molecular weight for the lower band only (fig. S2).

To assess whether NL2 phosphorylation specifically occurs at Ser^{714}, under basal conditions in neurons, we performed a Proximity Ligation Assay (PLA) on rat hippocampal neurons, using an antibody that detects the intracellular domain of NL2, combined with mitotic phosphoprotein monoclonal 2 (MPM2) antibody that specifically recognises phosphorylated Ser/Thr-Pro motifs (31) (Fig. 1E). Among the serine residues located on the intracellular domain of NL2, Ser^{714} is the only serine preceding a proline residue (Fig. 1A) and should therefore be specifically recognised by the MPM2 antibody when phosphorylated. Consistently, this antibody detects WT, but not S714A ^{HA}NL2 (22). PLA signal was visible on the soma and dendritic arbour, consistent with the distribution of inhibitory synapses (32). PLA signals were normalised to background fluorescence obtained from incubating neurons with each antibody separately. Despite the high background for MPM2 alone, we observed a greater than 2-fold increase in signal when both antibodies were combined compared to background fluorescence (Fig. 1F). Although we cannot completely rule out that other nearby postsynaptic proteins or NL2 binding partners containing the same phosphorylation motif contribute to the overall PLA signal, the unique location of this motif in NL2, complemented with further data in this study, suggests that phosphorylated NL2 is a major contributor to the signal. Thus, these findings combined support our hypothesis that NL2 is phosphorylated at Ser^{714} under basal conditions in neurons.

PKA rapidly phosphorylates NL2 at Ser^{714}

To identify factors that can regulate phosphorylation of Ser^{714}, we investigated a range of treatments that increase neuronal activity by incubating rat neuronal cultures. These treatments include 4-aminopyridine (4AP), glutamate, kainic acid and Mg^{2+}-free medium, as well as regulators of kinases and phosphatases that commonly play a role in neuronal signalling, such as FK506, forskolin, PMA, and SC79, and analysed the cell lysates on PhosTag gels. We found that 25 µM forskolin enhanced NL2 phosphorylation in a time-dependent manner in cortical cultures (Fig. 2A-B), whereas none of the other treatments affected NL2 phosphorylation. Brief incubation of 5 min led to a sizeable increase in the amount of phosphorylated NL2, whereas 15 min incubation resulted in a greater increase, and 1 hour incubation caused near-complete phosphorylation in cortical neurons (Fig. 2A-B) and hippocampal neurons at different ages (DIV8 to DIV13, fig. S3A), without affecting total levels of NL2 (fig. S3B).

Forskolin activates the kinase PKA by increasing intracellular levels of cAMP (33). Indeed, the site Ser^{714} in NL2 is compatible with a strong consensus sequence for PKA-induced phosphorylation, RRXS/TY (Fig. 1A). To verify whether forskolin-induced phosphorylation of NL2 is mediated by PKA, we incubated cortical cultures simultaneously with forskolin and the PKA-specific inhibitor H89. Quantification of the lysates on PhosTag gel (Fig. 2C-D) showed no significant change in the amount of phosphorylated NL2, even after 1 hour of incubation, confirming that NL2 phosphorylation is dependent on PKA.

To verify whether forskolin-induced phosphorylation of NL2 occurs at Ser^{714}, we incubated COS-7 cells expressing WT or S714A ^{HA}NL2 with forskolin. Analysis of cell lysates on PhosTag gel revealed that incubation with forskolin increased levels of phosphorylated ^{HA}NL2-WT, whereas no change was observed for ^{HA}NL2-S714A (Fig. 2E). This confirms that forskolin enhances phosphorylation of NL2 specifically at Ser^{714}. Notably, despite the serine residue at this position being conserved in other Neuroligin isoforms (fig. S4A), we found that forskolin-induced changes

in phosphorylation level are unique for NL2. Incubating COS-7 cells overexpressing ^{HA}NL1, ^{HA}NL2 or ^{HA}NL3 with forskolin, caused a change in banding pattern on PhosTag gel only for ^{HA}NL2-containing lysates (Fig. 2F-G, fig. S4B).

Finally, to verify in vitro whether phosphorylation of NL2 is mediated by PKA, we fused the intracellular C-terminal tail of NL2 to glutathione-S-transferase (GST-NL2^{CT}) and incubated purified GST-NL2^{CT}WT or phospho-mutant GST-NL2^{CT}S714D with purified PKA (Fig. 2H). Analysis on PhosTag gel showed an increase in phosphorylation over time for WT but not S714D GST-NL2^{CT}, further confirming that PKA phosphorylates NL2 at Ser^{714}.

Together, these results show that PKA mediates rapid phosphorylation of NL2 at Ser^{714}, and that this pathway can be induced by forskolin.

Phosphorylation of NL2 leads to dispersal from the synapse

Given the role of phosphorylation in regulating protein interactions and trafficking, we investigated whether NL2 phosphorylation affected its synaptic stabilisation and surface distribution. First, we investigated how phosphorylation affects real-time membrane diffusion of NL2 using single-molecule tracking with quantum dots (QDs). Hippocampal neurons were co-transfected with ^{HA}NL2-WT, ^{HA}NL2-S714A, or ^{HA}NL2-S714D and Gephyrin-FingR, a GFP-conjugated intracellularly expressed IgG-like domain that specifically recognizes gephyrin clusters (34). This allows for simultaneous identification of inhibitory synapses using Gephyrin-FingR, and QD tracking of surface-exposed ^{HA}NL2 molecules using anti-HA primary and QD-conjugated secondary antibodies (Fig. 3A). WT, S714A and S714D ^{HA}NL2 diffused on the plasma membrane and localized to synaptic and extrasynaptic microdomains (Fig. 3B). In addition, some ^{HA}NL2s exchanged localization between synaptic and extrasynaptic areas, suggesting that ^{HA}NL2s are recruited to inhibitory synapses by lateral diffusion. As expected, from the mean square displacement (MSD) plots, synaptic ^{HA}NL2s diffused slower and were more confined

compared to extrasynaptic ^{HA}NL2s (Fig. 3C-F). These parameters suggest that ^{HA}NL2 is showing similar dynamics to that expected for endogenous NL2.

The effect of PKA-mediated phosphorylation of Ser^{714} on mobility of NL2 was studied by incubating neurons in vehicle (DMSO, Ctrl) or 25 µM forskolin (Fsk) for 10 min before imaging. Synaptic trajectories of ^{HA}NL2-WT showed a clear increase of diffusion coefficient *D* upon treatment with forskolin, whereas *D* of ^{HA}NL2-S714A and ^{HA}NL2-S714D trajectories was unaffected by forskolin treatment, consistent with removal of the PKA-dependent phosphorylation site (Fig. 3G-H). ^{HA}NL2-S714D trajectories in either control or forskolin-treated conditions did not significantly differ from forskolin-treated ^{HA}NL2-WT trajectories, suggesting that the S714D mutant acts as a phospho-mimetic. Consistently, the basal synaptic confinement area for ^{HA}NL2-S714D was significantly higher than for WT or S714A, and forskolin treatment only increased the confinement area for WT ^{HA}NL2 but not the phospho-mutants (Fig. 3I-J). These results indicate a phosphorylation-dependent lateral mobility of synaptic NL2.

Forskolin marginally increased extrasynaptic lateral diffusion of ^{HA}NL2-WT and the diffusion coefficient but not confinement area of ^{HA}NL2-S714A. Neither *D* nor confinement area of ^{HA}NL2-S714D were affected by forskolin (Fig. 3K-N). These results suggest that phosphorylation of NL2 at Ser^{714} does not play a major role in its extrasynaptic lateral diffusion. Indeed, *D* of forskolin-treated WT and S714A ^{HA}NL2 extrasynaptic trajectories were not significantly different while at the synapse WT ^{HA}NL2 mobility was higher compared to S714A due to phosphorylation (Fig. 3H). Whether phosphorylation at other sites than Ser^{714} underlie the increase in extrasynaptic lateral diffusion of ^{HA}NL2 (WT and S714A) upon forskolin treatment remains to be investigated. Notably, these results suggest that upon phosphorylation at Ser^{714}, synaptic NL2 is dispersed from inhibitory synapses.

To investigate whether phosphorylation-induced dispersal of NL2 from the synapse coincides with a reduced proximity to gephyrin, we investigated the interaction between endogenous NL2 and gephyrin using PLA (Fig. 4A-B). In DMSO-treated cells, the PLA signal in the presence of

both antibodies was significantly higher than background, indicating specific interaction. Forskolin treatment reduced this fluorescence by ~24% to background levels. Thus, phosphorylation of NL2 at Ser^{714} decreases proximity between NL2 and gephyrin, presumably by decreased interaction.

Our results are in accord with a destabilization of NL2 from the inhibitory postsynaptic scaffold upon Ser^{714} phosphorylation.

Phosphorylation of NL2 reduces surface expression and affects inhibitory signaling

Dispersal of NL2 from the synapse and reduced interaction of NL2 with gephyrin upon PKA-induced phosphorylation suggests that phosphorylation may affect the surface distribution of NL2. We used surface protein biotinylation to investigate the effect of PKA-mediated NL2 phosphorylation on cell surface levels and found that phosphorylation reduced surface-exposed NL2 in cortical neurons (Fig. 5A-B); a similar decrease in NL2 surface levels was observed in hippocampal neurons (fig. S5A-B). This suggests that phosphorylation of NL2 at Ser^{714} may enhance its internalization. To further investigate the role of Ser^{714} in internalization, we overexpressed ^{HA}NL2-WT, ^{HA}NL2-S714A or ^{HA}NL2-S714D in hippocampal neurons and assessed basal internalization using antibody feeding, making use of the extracellular HA-tag to live-label NL2 with antibody and allow time for internalization (Fig. 5C). Quantification of internalized *vs* surface ^{HA}NL2 revealed that internalization of both ^{HA}NL2-S714A and ^{HA}NL2-S714D was reduced compared to ^{HA}NL2-WT, with S714D being less impaired than S714A (Fig. 5D). Thus, an intact phosphorylation site at Ser^{714} is required for internalisation of NL2, suggesting internalization is phosphorylation dependent. Furthermore, whereas the S714D mutant acted as phospho-mimetic in the lateral diffusion experiments, it appears to act as a partial phospho-null mutant in the internalization assay.

To investigate how internalization of NL2 upon phosphorylation affects the clustering of synaptic $GABA_A$Rs, we quantified surface staining of synaptic clusters in hippocampal neurons treated with DMSO or forskolin, using antibodies recognising extracellular regions of endogenous

$GABA_AR$ γ2 subunits and NL2, respectively (Fig. 5E-F). A trend towards reduced surface NL2 cluster numbers was observed (*P*=0.061) along with a significant reduction in γ2 cluster number, and NL2 and γ2 cluster area and fluorescence intensity, suggesting a decrease in the concentration of both markers from synapses. This implies that a loss of synaptic NL2 coincides with destabilization of $GABA_ARs$ at the inhibitory synapse, consistent with our earlier study (23).

Finally, to test whether phosphorylation of NL2 at Ser^{714}, and altered levels of synaptic NL2 and $GABA_ARs$, impacts on inhibitory signalling, we used electrophysiology to record spontaneous inhibitory postsynaptic currents (sIPSCs) from cultured hippocampal neurons expressing either WT, S714A, or S714D ^{HA}NL2 (Fig. 6A). We found no change in sIPSC frequency with either mutant compared to ^{HA}NL2-WT, suggesting presynaptic release remained unaffected (Fig. 6B). By contrast, both S714A and S714D ^{HA}NL2 overexpression increased sIPSC amplitude compared to WT ^{HA}NL2 (Fig. 6A,C-D), with S714D showing a smaller increase than S714A. This suggests that the inability of NL2 to be phosphorylated, and consequently its increased stability at the synapse and reduced internalization, results in enhanced inhibitory signaling, possibly indirectly through increased stabilization of postsynaptic complexes incorporating $GABA_ARs$. ^{HA}NL2-S714D exhibited an intermediate phenotype, as observed for the internalization assay (Fig. 5D). This is consistent with behaviour of the S714D mutant as a partial phospho-null mutation, despite showing increased membrane dynamics consistent with phospho-mimetic behaviour (Fig. 3). Whereas the latter could result from increased affinity for Pin1 and, consequently, disrupted interaction with gephyrin (22,35), the S714D mutation could disrupt interaction with endocytic machinery, preventing its internalization. It could also be that ^{HA}NL2 overexpression in itself augments synapse formation and $GABA_AR$ synaptic levels (36). Future work could include inhibiting or activating PKA during NL2 overexpression to enable separating the effects of phosphorylation from overexpression.

Notably, analysis of sIPSC amplitude histograms revealed three gaussian-distributed peaks for WT and S714D ^{HA}NL2 whereas a fourth peak was observed for ^{HA}NL2-S714A at -1771 pA

(Fig. 6E). Moreover, the high-amplitude peaks were notably increased for NL2 mutants compared to WT, with the shift being more pronounced for S714A than S714D. This suggests that the overall increase in sIPSC amplitude (Fig. 6D) is mainly caused by the occurrence of larger GABAergic synapses upon overexpression of the S714A mutant. Overall, these data suggest that interfering with NL2 phosphorylation leads to enhanced inhibitory signaling, which may be through stabilization of postsynaptic components at the inhibitory synapse.

Together, these experiments suggest that NL2 surface levels are modulated by PKA-mediated phosphorylation at Ser^{714}, consequently altering synaptic levels of $GABA_A$Rs and impacting on inhibitory signalling strength.

Discussion

Neuroligin-2 (NL2) is essential for the maintenance and stabilization of the inhibitory postsynaptic domain (17,19,37). Changes in synaptic NL2 levels modulate inhibitory signalling (23,24), however, the mechanisms regulating surface levels and synaptic stabilisation of NL2 remained unresolved. In this study, we identified a role for PKA in these processes. We showed that PKA-mediated phosphorylation of NL2 at Ser^{714} causes rapid dispersal of NL2 from inhibitory synaptic clusters, reduced NL2 surface levels and, consequently, a reduced concentration of synaptic $GABA_A$Rs. Conversely, abolishing NL2 phosphorylation by mutating Ser^{714} reduced its internalisation and enhanced inhibitory signalling. Thus, PKA-mediated phosphorylation of NL2 affects synaptic inhibition by modulating NL2 synaptic stability (Fig.7).

Lateral diffusion of receptors and trans-membrane proteins allows their rapid (in seconds) recruitment into and dispersal from the synapse (38,39). Our data suggests that NL2 phosphorylation enables rapid downregulation of inhibitory signalling as a first step in destabilising the GABAergic synapse. Conversely, stabilization of unphosphorylated NL2 at the synapse may

lead to an increase in synapse size (Fig. 7). Thus, up- and downregulation of NL2 phosphorylation in response to neuronal signalling can fine-tune inhibitory strength to maintain the E/I balance.

We found here that 50-60% of NL2 is basally phosphorylated in neuronal cultures. Whether this population mostly resides intracellularly or remains at the cells surface extrasynaptically remains to be investigated. We also found that PKA activation only partially reduced NL2 surface levels, despite near-complete phosphorylation at this time-point. This suggests that the majority of phosphorylated NL2 may remain at the cell surface, where it can be dephosphorylated and rapidly re-join the synapse. Alternatively, phosphorylated and internalized NL2 could be replenished by membrane insertion of non-phosphorylated NL2. A long incubation with forskolin, as used in our biotinylation experiment (1 hour), may push the balance towards enhanced internalization. NL2 phosphorylation remained stable upon addition of PKA-specific inhibitor H89, suggesting that basal dephosphorylation of NL2 is low and may require specific activation of a yet to be identified mechanism. Alternatively, kinases other than PKA may phosphorylate NL2 during different stages of its trafficking, whereas the role of PKA may be restricted to the synapse. In either scenario, NL2 phosphorylation provides a putative mechanism for transient downregulation of inhibitory synapses, which can be quickly restored upon dephosphorylation of extrasynaptic NL2, or membrane reinsertion followed by rapid lateral diffusion into the synapse.

Mutations in neuroligins associated with neurological and cognitive disorders have been suggested to affect their surface levels and trafficking (17,37,40), therefore a better understanding of mechanisms that regulate neuroligin trafficking may improve our insight into the molecular basis of these disorders. NL2 surface levels can be modulated by internalization to endosomes (41) and recycling by sorting-nexin 27 (SNX27) (23). Our current study demonstrates that phosphorylation of NL2 at Ser^{714} controls its synaptic stability and internalization. Loss of NL2 from the synapse, either by increased phosphorylation and internalisation or blocked recycling, reduces synaptic $GABA_A$Rs, and, conversely, stabilising NL2 at the synapse by abolishing phosphorylation and internalisation or increasing recycling enhances inhibitory signalling.

Our data are consistent with and complementary to an earlier study, which showed that NL2 requires an intact Ser^{714} phosphorylation site to recruit the peptidyl-prolyl isomerase Pin1, and that Pin1 disrupts interaction between NL2 and gephyrin (22). In *Pin1*$^{-/-}$ animals, where downstream effects of NL2 phosphorylation are abolished, synaptic $GABA_AR$ levels and, consequently, sIPSC amplitude were increased. Likewise, overexpression of ^{HA}NL2-S714A or ^{HA}NL2-S714D, which cannot be phosphorylated, enhanced inhibitory signaling, consistent with another study, which found that overexpressing either ^{HA}NL2-S714A or ^{HA}NL2-S714D in a NL1-3-null background restores inhibitory signaling (42). Our current work demonstrated the reverse of this mechanism, whereby phosphorylation induces a dispersal of NL2 from the synapse to reduce synaptic $GABA_AR$ clusters. These studies combined show that NL2 phosphorylation, either directly through dispersal from the synapse, or indirectly through Pin1 activation, disrupts interactions between inhibitory postsynaptic proteins to reduce inhibitory synaptic signaling (Fig.7).

Notably, forskolin-induced changes in phosphorylation levels uniquely occurred for NL2, despite the serine residue being conserved in NL1 and NL3. This may be due to a unique motif downstream of Ser^{714} in NL2, which may promote PKA binding or, conversely, motifs downstream of this serine in other NL isoforms that may hinder PKA-mediated phosphorylation. Just downstream of this serine, the kinase CaMKII phosphorylates NL1 at Thr^{739} in response to synaptic activity, enhancing its surface expression and strengthening postsynaptic excitatory signalling (43). A conserved tyrosine phosphorylation site in NL1 (Tyr^{782}) and NL2 (Tyr^{770}) regulates their specialization for excitatory synapses and postsynaptic density protein 95 (PSD95), or inhibitory synapses and gephyrin, respectively (20,29,44). PKA-mediated phosphorylation of NL1 at Ser^{839}, a residue directly preceding the highly conserved C-terminal PDZ-binding motif, abolishes its interaction with PSD95 and enhances NL1 internalization (45). A similar mechanism involving this residue has not yet been identified for other NL isoforms. Thus, despite relatively high sequence conservation of the Neuroligin intracellular domains, differential

phosphorylation of Neuroligin isoforms may determine their specialization at specific synapses (46).

The neuromodulatory signaling pathways resulting in PKA-mediated phosphorylation of NL2, as well as the pathways mediating dephosphorylation and synaptic stabilization of NL2, remain to be established. Many signalling pathways, involving a wide range of hormones and neurotransmitters, increase intracellular cAMP levels, subsequently activating PKA. PKA is an important element for regulating synaptic plasticity (33). $GABA_AR$ subunits β1 and β3 contain PKA phosphorylation sites, where phosphorylation of β1 inhibits GABAergic signaling, whereas phosphorylation of β3 has the opposite effect (8,47). Gephyrin contains at least 26 putative phospho-sites, which regulate its ability to oligomerise and stabilize $GABA_ARs$ (21,48). Specifically, one of these sites is phosphorylated by PKA, and affects $GABA_AR$ confinement at the synapse (49,50). Excitatory targets of PKA include subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and N-methyl-D-aspartate (NMDA) receptors (51). The role of PKA-mediated phosphorylation of NL2 was not investigated in these studies. Thus, the net effect of PKA activity on neuronal signalling may be a summation of many factors, including compartmentalization of PKA and accessibility of its binding sites. Due to the complex interplay between these pathways involving PKA we cannot predict how PKA-mediated phosphorylation of other synaptic components plays a role in our experiments, or how NL2 phosphorylation and synaptic destabilization contributed to effects of PKA modulation on neuronal signaling reported in earlier studies. However, considering all our data together, we have shown a direct connection between PKA-mediated phosphorylation of NL2, its molecular surface dynamics and surface levels, and inhibitory signaling. This research thus furthers our understanding of how NL2 function is regulated, and how this modulates inhibitory signaling strength.

Materials and methods

Animals

All procedures for the care and treatment of animals were in accordance with the Animals (Scientific Procedures) Act 1986 and had full Home Office ethical approval. Animals were maintained under controlled conditions (temperature 20 ± 2°C; 12-hour light-dark cycle), were group housed in conventional cages and had not been subject to previous procedures. Food and water were provided ad libitum. Wild-type E18 Sprague-Dawley rats were generated through wild-type breeding; pregnant females were single housed. Embryos of either sex were used for generating primary neuronal cultures.

Primary Hippocampal Culture and transfection

Cultures of hippocampal and cortical neurons were prepared from E18 WT Sprague-Dawley rat embryos of either sex as previously described (52,53). In brief, rat hippocampi and cortices were dissected from embryonic brains in ice-cold Hanks's Balanced Salt Solution (HBSS, GIBCO) supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, GIBCO). Dissected hippocampi were incubated in the presence of 0.25% trypsin and 5 Units/ml DNase (Sigma) for 15 mins at 37°C, washed twice in HBSS with HEPES, and triturated to a single cell suspension in attachment media (Minimum Essential Media, MEM; GIBCO) containing 10% horse serum, 10 mM sodium pyruvate, and 0.6% glucose, all GIBCO) using a fire-polished glass Pasteur pipette. Dissociated cortical neurons were plated in attachment media on poly-L-lysine (PLL, Sigma) coated dishes at a density of $2x10^6$ cell/6 cm dish. Dissociated hippocampal neurons were plated in attachment media on PLL coated coverslips at a density of $5x10^5$ cells/6 cm dish (PLA, cluster analysis, antibody feeding), $1.5x10^6$ cells/6 well dish (single particle tracking, electrophysiology), or PLL coated dishes at a density of $4x10^6$ cells/6 cm dish (lambda phosphatase treatment and surface biotinylation). After 6 hours, serum-containing medium was replaced with Neurobasal medium (GIBCO) containing 2% B-27, 2 mM glutaMAX, 100 U/ml penicillin and 100 µg/ml streptomycin (all GIBCO). Cultures were maintained at 37°C in humidified atmosphere with 5% CO_2. Hippocampal neurons were transfected using a calcium phosphate method (53) or Lipofectamine 2000 (Invitrogen) at DIV7 (days in vitro 7) for cluster analysis and electrophysiology experiments, or at DIV10 for antibody feeding and single particle tracking, and maintained until DIV13-15.

COS-7 cell culture and transfection

COS-7 cells (RRID:CVCL_0224, ATCC) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO), supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all GIBCO), at 37°C in humidified atmosphere with 5% CO_2. Cells were transfected 24 hours before further processing using the Amaxa Nucleofector® device (Lonza) following the manufacturer's protocol.

DNA Constructs

Constructs of HA-tagged murine NL1, NL2 and NL3 were a gift from Peter Scheiffele (Addgene plasmid #15260, #15246, and #59318, respectively (40,54)). All NL2 phosphorylation mutants (^{HA}NL2-S714A, S714D, S714/719/721A, S714/719/721D, Y770A) were generated using this NL2 cDNA as a template, where subsequent mutagenesis was performed by DNAExpress (Canada). To create GST-NL2^{CT} WT and S714D, a PCR product of the intracellular domain flanked by *Bam*HI and *Xho*I restriction sites was created from the equivalent full-length construct (forward primer catcatggatcctacaagcgggaccggcgcc; reverse primer catcatctcgagctatacccgagtggtggagtg) and subcloned into pGEX4T3 (GE Healthcare). Green fluorescent protein (GFP) tagged Gephyrin-FingR was a gift from Don Arnold (Addgene plasmid #46296 (34)). eGFP-C1 used to identify transfected cells in electrophysiology experiments has been described previously (53).

Purification of GST-fusion protein and in vitro phosphorylation

Glutathione-S-transferase (GST) fusion proteins were produced as described (55). In brief, BL21 *E.Coli* (Invitrogen, cat# C600003) containing pGEX4T3-NL2^{CT} WT or S714D were grown in Luria Broth until an OD600 of 0.5-0.6. Protein production was induced by addition of 1mM isopropyl β-d-1-thiogalactopyranoside (IPTG, Melford) for 3 hours. Cells were harvested by centrifugation for 30 min at 4°C, washed in buffer containing 50 mM Tris pH8.0, 25% sucrose, 10 mM ethylenediamine tetra-acetic acid (EDTA), and pelleted by centrifugation for 30 min at 4°C. Cells were then lysed by sonication in buffer containing 1% Triton-X100, 10 mM Tris pH7.4, 1 mM EDTA, 1 mM Dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), antipain, pepstatin and leupeptin at 10 µg/ml. Lysates were further incubated for 30 min upon addition of 12.5 mM HEPES pH 7.6, 75 mM KCl, 125 mM EDTA, 12.5% glycerol, and then spun for 1 hour at 12,000x*g* and 4°C. GST protein was purified by adding Sepharose 4B beads (GE Healthcare) and incubating for 2 hours at 4°C. Beads were washed and stored at 4°C in buffer containing 20 mM HEPES pH7.6, 100 mM KCl, 0.2M EDTA, 20% glycerol, 1 mM DTT, and 1 mM PMSF.

To perform in vitro phosphorylation, 5 µg of purified GST-NL2^{CT} (WT or S714D) fusion protein was incubated with 2.5kUnits purified catalytic subunit of PKA (NEB) supplemented with 200 µM ATP at 30°C for the indicated time points.

Protein sample preparation and Western blotting

Neuronal and COS-7 cell cultures were treated with dimethyl sulfoxide (DMSO; vehicle control, 1:1000 culture volume), 25 µM forskolin (Calbiochem), and/or 50 µM H89 (Sigma) for the time points indicated. Cell lysates were obtained by direct lysis in sodium dodecyl sulfate (SDS) sample buffer and boiling for 30 min prior to separation by gel electrophoresis. For in vitro dephosphorylation, untreated cultures of hippocampal and cortical neurons were lysed in lambda phosphatase buffer (NEB), incubated for 20 min at 4°C to ensure total lysis, and spun for 10 min at 14,000x*g* to remove cell membranes. The supernatant was incubated with 500 Units lambda phosphatase (NEB) or phosphatase inhibitors (1 mM NaVO4, 5 mM NaF) for 1 hour at 30°C. The reaction was stopped by addition of SDS sample buffer. For in vitro deglycosylation, COS-7 cells transfected with WT and mutant ^{HA}NL2 were lysed in phosphate buffered saline (PBS) supplemented with 1% NP40, 1 mM PMSF, antipain, pepstatin and leupeptin at 10 µg/ml, incubated for 30 min at 4°C to ensure total lysis, and spun for 10 min at 14,000x*g* to remove cell membranes. Proteins in the supernatant were denatured by addition of 0.5% SDS and 40 mM DTT and incubation for 10 min at 100°C, and subsequently deglycosylated upon addition of 50 mM sodium acetate (pH 6) and 250 Units EndoH (NEB), and incubation for 30 min at 37°C. The reaction was stopped by addition of SDS sample buffer.

For surface biotinylation, cortical and hippocampal cultures were treated for 1h with DMSO or forskolin. Biotinylation was performed as described (56). In brief, cells were washed 3x with ice-cold PBS supplemented with Mg^{2+} and Ca^{2+}, then incubated with 0.5 mg/ml EZ-link Sulfo-NHS-LC-Biotin (ThermoFisher) for 12 min on ice. The reaction was quenched using 1 mg/ml bovine serum albumin (BSA) in PBS, and then the cells were washed with PBS. Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% Nonidet-P40, 0.5% DOC, 0.1% SDS, 1 mM EDTA, 2 mM EGTA, 1 mM PMSF, 50 mM Tris pH 7.5 supplemented with antipain, pepstatin and leupeptin at 10 µg/ml), and left to rotate at 4°C for 1 hour. Membranes were pelleted by centrifugation at 14,000x*g* for 10 min at 4°C. To isolate biotinylated surface proteins, supernatants were then incubated with 25 µl of a 50% Neutravidin bead slurry (Pierce, ThermoFisher) for 2 hours at 4°C. Beads were washed 3x in RIPA buffer before resuspending in sample buffer and analyzed by SDS-PAGE and Western blotting.

Protein samples were separated by standard Laemmli sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 9% Tris-Glycine gels and transferred onto nitrocellulose membrane (GE Healthcare Bio-Sciences). PhosTag gels were prepared by adding 200 µM PhosTag™ (WAKO chemicals, Alpha Laboratories) and 40 µM $MnCl_2$ to a 5% standard Laemli SDS-PAGE gel. For detection by Western blot, PhosTag gels were transferred to polyvinylidene difluoride (PVDF membrane, GE Healthcare). Membranes were blocked for 1 hour in milk (PBS, 0.1% Tween, 4% w/v skimmed milkpowder), and then incubated overnight at 4°C with shaking in primary antibodies diluted in milk (1:1000 Rabbit-anti-Neuroligin2 (Synaptic Systems 129-202, RRID: AB_993011), 1:200 Mouse-anti-HA-tag (supernatant, clone 12CA5, produced in house, RRID:AB_2532070), or 1:1000 Mouse-anti-β-Tubulin (Sigma T5293, RRID:AB_477580)). Blots were then incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody (Goat-anti-rabbit HRP, RRID:AB_2313567, or goat-anti-mouse HRP, RRID:AB_10015289, both Jackson ImmunoResearch) for 1 hour at room temperature (RT), and developed using Luminate Crescendo Western HRP substrate (Millipore). Signal was detected using an ImageQuant LAS4000 mini (GE Life Sciences).

Immunocytochemistry and Proximity Ligation Assay

Antibody feeding in hippocampal neurons (DIV13-14) was performed by adding 1:50 Mouse-anti-HA (supernatant, clone 12CA5, produced in house, RRID:AB_2532070) directly to neuronal maintenance medium and allowing internalisation for 40 min at 37°C. Neurons were washed twice in medium before fixation for 7 min in 4% PFA (PBS, 4% paraformaldehyde, 4% sucrose, pH 7) and blocking for 10 min (PBS, 10% horse serum, 0.5% BSA). Surface ^{HA}NL2 was stained for 45 min with 1:300 goat-anti-mouse Alexa555 (RRID:AB_141780, ThermoFisher). Cells were then permeabilised by incubation in block solution supplemented with 0.2% Triton X-100 for 10 min, and subsequently incubated for 45 min with 1:500 donkey-anti-mouse Alexa488 (RRID: AB_2341099, ThermoFisher), and finally mounted using ProLong Gold antifade reagent (Invitrogen).

For cluster staining of surface-exposed NL2 and $GABA_AR$ γ2 subunit, hippocampal neurons (DIV13-14) were fixed and incubated in block solution as described above. Neurons were then stained for 1 hour at RT with primary antibody diluted in block solution [1:100 rabbit-anti-NL2 (Alomone Labs, ANR-036, RRID:AB_2341007) or 1:500 guinea pig-anti-γ2 (Synaptic Systems, 224-004, RRID:AB_10594245)]. Cells were then permeabilised in block solution supplemented with 0.2% Triton X-100, and incubated for 1 hour at RT with 1:1000 mouse anti-MAP2 (microtubule-associated protein 2; Synaptic Systems, 188-011, RRID:AB_2147096), followed by 45-60 min incubation with the

appropriate Alexa-fluorophore conjugated secondary antibodies (goat-anti-mouse Alexa405, RRID:AB_221604, goat-anti-rabbit Alexa555, RRID:AB_2535851, and goat-anti-guinea pig Alexa647, RRID:AB_2535867, all ThermoFisher). Finally, coverslips were mounted onto glass slides using ProLong Gold antifade reagent (Invitrogen). For proximity ligation assays (PLA) hippocampal neurons (DIV13-14), where applicable, were treated with DMSO or forskolin for 1 hour, fixed as described above, washed 3 times in PBS, and blocked for 10 min in blocking buffer (10% Horse Serum, 5 mg/ml BSA, and 0.2% Triton in PBS). Coverslips were then incubated for 1 hour at RT with primary antibodies in blocking buffer [1:500 rabbit anti-NL2 (Synaptic Systems, 129-203, RRID:AB_993014), or 1:400 mouse anti-MPM2 (Millipore, 05-368, RRID:AB_309698), or 1:500 mouse anti-gephyrin (Synaptic Systems, 147-011, RRID:AB_887717), or a combination of NL2 with MPM2 or NL2 with gephyrin, in all cases combined with 1:1000 guinea pig anti-MAP2 (Synaptic Systems, 188-004, RRID:AB_2138181)]. The remaining of the staining was performed according to the manufacturer's protocol (Duolink® PLA). In brief, coverslips were incubated in a humidity chamber with anti-mouse MINUS and anti-rabbit PLUS probes (Sigma Aldrich) plus 1:500 goat anti-guinea pig Alexa647 (RRID:AB_2535867, ThermoFisher) for 1 hour at 37 ^{0}C, hybridized for 30 min at 37 ^{0}C, and amplified for 100 min at 37 ^{0}C using the red fluorophore (equivalent to TexasRed). Coverslips were then allowed to dry at room temperature for 20 min in the dark and mounted using Duolink® *In Situ* mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI).

Image acquisition and analysis

Confocal images were acquired on a Zeiss LSM700 upright confocal microscope using a 63x oil objective [numerical aperture (NA): 1.4] and digitally captured using ZEN LSM software (version 2.3), with excitation at 405 nm for DAPI and Alexa-Fluor405, 488nm for GFP and Alexa-Fluor488, 555 nm for Alexa-Fluor555 and PLA red fluorophore (equivalent to TexasRed), and 633 nm for Alexa-Fluor647 conjugated secondary antibodies. Image analysis was performed using in-house written scripts for ImageJ (*https://imageJ.net/Welcome*), details of which are given below. For antibody feeding, a single image with an optimal thickness of 1 µm was acquired for entire neurons (image size 203 x 203 µm) or a zoomed image of the soma (40.6 x 40.6 µm). Laser settings were adjusted to ensure channels were not saturated. Acquisition settings and laser power were kept constant within experiments. For quantification of antibody feeding, zoomed soma images of surface and internal staining were thresholded to generate a mask, which

was then used to measure total intensity of surface and internal signal in the original image. The ratio between internal and total (surface plus internal) intensity was calculated as a relative measure for internalization.

For PLA images, a single image with an optimal thickness of 1 µm was acquired for entire neurons (image size 203 x 203 µm). The field of view was selected based on MAP2 staining, ensuring each image contains a similar cell density and avoiding bias. Using ImageJ, a suitable threshold was selected for MAP2 and PLA signal and kept constant for all data sets. To estimate background in absence of MPM2-NL2 or gephyrin-NL2 interaction, the number of PLA dots per MAP2 area in control conditions (incubated with individual antibodies and in DMSO) were summed; all values were then normalized to this estimated background

For cluster analysis, 3 sections of dendrite per hippocampal neuron, ~50 µm from the soma, were imaged with a 3.4x zoom (equating to 30 µm length of dendrite). Cluster analysis was performed as previously described (10,23,57). In brief, a suitable threshold was selected for each channel using Metamorph software (version 7.8; Molecular Devices) and applied to all images within the same dataset. Clusters with an intensity above this threshold and size of at least 0.04 μm^2 were quantified. Quantification was performed on 5 to 8 cells per experiment.

Single particle tracking

Single particle analysis was carried out by tagging ^{HA}NL2 with quantum dot (QD) nanocrystals in primary hippocampal neurons (DIV13). Coverslips containing neurons expressing WT, S714A or S714D ^{HA}NL2 with GFP-Gephyrin-FingR were pre-incubated for 5 min with DMSO (vehicle control, 1:1000 culture volume) or 25 µM forskolin directly added to the culture medium. Coverslips were then washed in Krebs solution (containing in mM: 140 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.2 $MgCl_2$, 11 glucose, and 5 HEPES; pH 7.4) and subsequently incubated for 5 min at 37°C with 1:50 mouse anti-HA primary antibody (supernatant, clone 12CA5, produced in house, RRID:AB_2532070) supplemented with DMSO or 25 µM forskolin in Krebs solution. Coverslips were then washed and incubated for 1 min at RT with 1:5000 goat-anti-mouse secondary antibody conjugated via Streptavidin with Qdot655 (Q-11021MP, RRID:AB_2556462, Invitrogen) in Krebs solution supplemented with 10% Horse Serum and DMSO or forskolin. After washes the coverslips were loaded in an environmental chamber (Solent Scientific) and imaged at 37°C within 5 min of mounting in a Krebs solution supplemented with DMSO or forskolin.

^{HA}NL2-QD complexes were imaged using a wide-field inverted microscope (Olympus IX71) with a high magnification objective (60X; NA – 1.35; Olympus), a halogen light source (PhotoFluor-II Metal Halide illumination

system) and a back-illuminated cooled electron-multiplying charge coupled device (EMCCD; iXon3 885, Andor Technology). GFP-Gephyrin-FingR was imaged with a 457–487 nm band-pass excitation filter (all filters / mirrors were acquired from Semrock), a 496 nm long-pass emission filter, and 495 nm dichroic mirror. QD655 was imaged with a 415-455 nm band-pass excitation filter, a 647.5-662.5 nm band-pass emission filter, and a 510 nm dichroic mirror. Real-time movement of NL2-QDs was captured by first imaging GFP-Gephyrin-FingR, followed by QD655s in the same region of interest and plane of view at 33 Hz for 300 frames.

Single particle analysis of ^{HA}NL2-QDs was carried out as described previously (58,59). A custom Matlab (MathWorks) plugin, SPTrack (Ver5), was used to analyse QD movement by first identifying the centre of each QD using a 2-D Gaussian fit (spatial resolution ~10–20 nm). The centre of the Gaussian peaks of each QD in successive image frames were track-connected, based on estimated diffusion coefficients and the likelihood of the two Gaussian peaks in consecutive frames belonging to the same QD track. QDs trajectories with less than 15 consecutive frames were discarded.

The mean square displacement (MSD) of each QD was calculated using the following equation:

$$\mathrm{MSD}\,(ndt) = (N - n)^{-1} . \Sigma_{I=1}^{N-n} ((x_{I+n} - x_i)^2 + ((y_{I+n} - y_i)^2)$$

where x_i and y_i are the spatial co-ordinates of a single QD identified in a single image frame i. N is the total number of points in the QD trajectory, dt is the time interval between two successive frames (33 ms), and ndt is the time interval over which the mean square displacement is averaged. From the MSD plot, the diffusion coefficient, D, for a QD was calculated by fitting the first two to five points of the MSD plot against time with the following expression:

$$\mathrm{MSD}(t) = 4D_{2-5}\,t + 4\sigma_x^2$$

where σ_x is the QD localization accuracy in one dimension. D was determined from the slope of the relationship. Given the inherent noise in CCD imaging systems, and the errors in accurately locating single QDs that results, trajectories with $D < 10^{-4}$ μm^2 / s were considered immobile and excluded from calculation of median D.

Synaptic terminals, identified by GFP-Gephyrin-FingR puncta, were thresholded using ImageJ, and co-localised QD trajectories were defined as synaptic.

Patch-clamp electrophysiology

Hippocampal neurons expressing ^{HA}NL2 (WT or S714A or S714D) were identified by their co-expression with eGFP. Spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded at DIV13–15 using patch clamp

electrophysiology. Patch electrodes (4–5 MΩ) were filled with an internal solution containing (in mM): 120 CsCl 1 $MgCl_2$, 11 EGTA, 30 KOH, 10 HEPES, 1 $CaCl_2$, and 2 K_2ATP; pH – 7.2. Neurons were superfused with a saline solution containing (mM): 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 2.52 $CaCl_2$, 11 Glucose, and 5 HEPES; pH 7.4 supplemented with 2 mM kynurenic acid to block all spontaneous excitatory post-synaptic currents. Membrane currents were filtered at 5 kHz (−3 dB, 6th pole Bessel, 36 dB/octave) with optimised series resistance (Rs, <10 MΩ) and whole-cell membrane capacitance compensation. Membrane currents were recorded at a holding potential of −60 mV. Changes of series resistance greater than 10% during the experiment resulted in the recording being excluded from analysis.

sIPSCs were analysed using WinEDR ver 3.4.3 and WinWCP 5.4.6 (University of Strathclyde, UK). Spontaneous IPSC amplitude distributions were fitted with a sum of 1–4 Gaussian components according to the function:

$$f\ (x) = A\ \frac{e^{-\frac{(x-\mu)^2}{2\sigma i}}}{\sigma\sqrt{2\pi}} + C$$

Where A is the amplitude and C defines the pedestal of the histogram. This function provided the Gaussian mean amplitude current (μ) and standard deviation (σ). All the distributions were fitted using this function in Origin (Ver 6). The accuracy of the fits was checked by repeating the iterative non-linear fitting procedure after substituting the best-fit.

Statistical analysis

For all quantified fixed imaging experiments the experimenters were blind to the condition of the sample analysed. All quantified experiments were performed 3 times from independent cell preparations, treatments, and transfections, unless indicated otherwise in the figure legends. N-numbers indicate number of experiments (Western blots, PLA), number of trajectories (QD experiments) or number of cells (all other cases). Data are given as mean ± standard error of the mean (SEM) or median with Inter-quartile range (25-75%) as indicated in the figure legends. Error bars represent SEM.

Statistical analysis was performed in GraphPad Prism (version 8; GraphPad Software, CA, USA) or Microsoft Excel. Data was tested for statistical outliers using ROUT test, Q=0.1%; no statistical outliers were identified. All data was tested for normal distribution with Kolmogorov-Smirnov (QD, electrophysiology) or D'Agostino & Pearson test (all other experiments) to determine the use of parametric (student's *t*-test, one-way analysis of variance (ANOVA), two-

way ANOVA) or non-parametric (Mann-Whitney, Kruskal-Wallis) tests. When at least one of the experimental groups was not normally distributed, a non-parametric test was applied for the whole experiment. When $P<0.05$, appropriate *post-hoc* tests were carried out in analyses with multiple comparisons and are stated in the figure legends.

Supplementary Materials

Figures S1 – S5

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Figure Legends

Figure 1: Neuroligin-2 is constitutively phosphorylated at Ser^{714}. (A) Schematic domain structure of murine NL2 showing putative serine phosphorylation sites in the intracellular region. Phospho-null and phospho-mimetic mutations of Ser phosphorylation sites were created as indicated. TM, trans-membrane; IC, intracellular. **(B)** Western blot of COS-7 cell lysates expressing WT or mutant ^{HA}NL2, separated on standard SDS-PAGE gel or SDS-PAGE supplemented with PhosTag. Arrows indicate phosphorylated NL2 (pNL2). Molecular weight in kD is indicated on the right. IB, Immunoblot. Blots are representative of n=3 experiments. **(C and D)** Western blot of cortical lysates (DIV8) incubated with buffer (Ctrl) or lambda phosphatase (LP), separated on standard SDS-PAGE gel (Std) or SDS-PAGE supplemented with PhosTag. A representative blot is shown in (C), and quantification of phosphorylated and unphosphorylated fractions of total NL2, as mean $\pm$ SEM from n=3 experiments, is shown in (D). $^{**}P<0.01$ by unpaired two-tailed t-test. **(E and F)** Representative confocal microscopy images (E) and analysis (F) of proximity ligation assay (PLA) in DIV13 hippocampal cultures using NL2 and MPM2 antibodies and immunostained for MAP2

(gray). PLA signal (red dots) was normalized to summed background (total bgd) and indicates proximity between NL2 and MPM2 antibodies; nuclei are stained with DAPI (blue). Scale bars, 25 µm (overview) and 6 µm (zoomed insets). Data are mean ± SEM; n=3 experiments (averaged results from 5, 14, and 14 cells, respectively); ***P*<0.01 by two-tailed ratio-paired t test between total background and NL2+MPM2.

Figure 2: Neuroligin-2 is phosphorylated at Ser^{714} by PKA. (A to D) Representative images and analysis of Western blots of cortical lysates (DIV8) separated on SDS-PAGE supplemented with PhosTag after cell cultures were incubated with forskolin (FSK; A and B, n=4), or 25 µM FSK + 50 µM H89 (C and D, n=3) for the indicated time points. IB, immunoblot. Data are mean ± SEM. ns, not significant (*P*>0.05), **P*<0.05, and ****P*<0.001 by one-way ANOVA with Dunnett's multiple comparison vs t=0. **(E to G)** Western blotting of COS-7 cell lysates overexpressing WT or S714A ^{HA}NL2 (E) or ^{HA}NL1, ^{HA}NL2 or ^{HA}NL3 (F, with analysis in G) separated on standard SDS-PAGE gel (Std) or SDS-PAGE supplemented with PhosTag after cultures were treated with 25 µM FSK or DMSO (- / Ctrl). The arrowhead indicates phosphorylated ^{HA}NL2. Molecular weight in kD is indicated on the right. IB, Immunoblot. Data in (G) are mean ± SEM, n=4; ***P*<0.01 by two-tailed paired t-tests. **(H)** Western blot of purified GST-NL2^{CT}WT or GST-NL2^{CT}S714D incubated with purified catalytic subunit of PKA (50 U/µl) for the indicated time points and separated on SDS-PAGE supplemented with PhosTag. The arrowhead indicates phosphorylated GST-NL2^{CT}WT. Blot is representative of 2 experiments.

Figure 3: Phosphorylation of Neuroligin-2 at Ser^{714} causes its synaptic dispersion. (A) Representative fluorescence microscopy image of live DIV13 hippocampal culture, co-expressing gephyrin-FingR (Geph-FngR, cyan) and ^{HA}NL2 (magenta, QD trajectories summed from 300 frames at 33 Hz). Scale bar, 5 µm. Image is representative of n=3 experiments. **(B)** Representative images of example trajectories of overexpressed WT, S714A or S714D ^{HA}NL2 (magenta) treated with DMSO (Ctrl) or 25 µM Forskolin (Fsk); trajectories were classified as synaptic (top) or extrasynaptic (bottom) based on colocalization with Geph-FngR (cyan). Scale bar 0.5 µm. Images are representative of n=3 experiments. **(C to F)** Cumulative probability plots (C, main graph; E), plot of mean square displacement (MSD) (C, inset) and box plot of the median and inter-quartile range (IQR, 25%–75%) for diffusion coefficients *D* (D) or Confinement Area (F)

for synaptic (Syn, black, n=277) and extrasynaptic (Esyn, gray, n=13962) trajectories of ^{HA}NL2-WT; ***P<0.001 by two-tailed Mann-Whitney test. **(G to J)** Cumulative probability plots (G, main graph; I), plot of mean square displacement (MSD) (G, inset), and box plots of the median and IQR of diffusion coefficients D (H) or Confinement Area (J) for synaptic trajectories of ^{HA}NL2-WT, ^{HA}NL2-S714A and ^{HA}NL2-S714D, upon treatment with DMSO (Ctrl) or Forskolin (Fsk); WT Ctrl, n=277; WT Fsk n=297; A Ctrl, n=343; A Fsk, n=174; D Ctrl, n=108; D Fsk, n=64 trajectories; ns, not significant (P>0.05), *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis tests with Dunn's multiple comparison. **(K to N)** As for (G to J) but displaying extrasynaptic trajectories; WT Ctrl, n=13962; WT Fsk n=10615; A Ctrl, n=13317; A Fsk, n=10699; D Ctrl, n=10403; D Fsk, n=7640 trajectories.

Figure 4: Phosphorylation of Neuroligin-2 at Ser^{714} disrupts gephyrin binding. (A and B). Representative confocal microscopy images (A) and analysis (B) of proximity ligation assay (PLA) in DIV13 hippocampal cultures, treated with DMSO (Ctrl) or Forskolin (Fsk), incubated with NL2 and Gephyrin antibodies, separately or combined, and immunostained for MAP2 (gray); nuclei are stained with DAPI (blue). PLA signal (red dots) was normalized to MAP2 area and summed background (total bgd). Scale bars, 25 µm (overview) and 6 µm (zoomed insets). Data are mean ± SEM; n=13-20 images per condition from 3 independent experiments; ns, not significant (P>0.05), **P<0.01 by two-way ANOVA with Bonferroni's multiple comparisons test.

Figure 5: Phosphorylation of Neuroligin-2 at Ser^{714} reduces the surface levels of Neuroligin-2 and GABA$_A$R. (A and B). Representative Western blot (A) and analysis (B) of DIV8 cortical lysates and purified surface protein upon treatment with DMSO (Ctrl) or Forskolin (Fsk), separated on standard SDS-PAGE gel (Std) or SDS-PAGE supplemented with PhosTag (PTag). Data are mean ± SEM from n=3 experiments. *P<0.05 by paired two-tailed t test. (**C and D)**. Representative confocal microscopy images (C) and analysis (D) of antibody feeding in DIV13 hippocampal neurons overexpressing ^{HA}NL2WT (WT), ^{HA}NL2S714A (A) or ^{HA}NL2S714D (D). Confocal images show surface-exposed (cyan) and internalised ^{HA}NL2 (magenta). Scale bars, 25 µm (overview, top) and 5 µm (zoom, bottom). Data are mean ± SEM from n=33 (WT), n=28 (S714A) and n=44 (S714D) cells from 3 independent cell cultures. *P<0.05, ***P<0.001 by Kruskal-Wallis

test with Dunn's multiple comparison test. **(E and F)** Representative Confocal microscopy images of 30 µm dendritic sections (E) and analysis of cluster number, area and normalised intensity (F) from DIV13 hippocampal neurons, immunostained for surface NL2 ($NL2^{EXT}$, red), $GABA_AR$ subunit γ2 (blue) and MAP2 (gray), and treated with DMSO (Ctrl) or Forskolin (Fsk). Arrowheads indicate synaptic clusters of co-localising $NL2^{EXT}$ and γ2. Scale bar, 4 µm. Data are mean ± SEM from n=19 (Ctrl) and n=20 (Fsk) neurons from 3 independent experiments; ns, not significant (P>0.05), *P<0.05, **P<0.01, ***P<0.001 by unpaired two-tailed *t* tests.

Figure 6: Phosphorylation of Neuroligin-2 at Ser^{714} affects inhibitory signalling. (A to E). Representative sIPSC patch-clamp recordings (A) and analysis (B to E) from hippocampal cultures expressing ^{HA}NL2WT, ^{HA}NL2S714A or ^{HA}NL2S714D combined with eGFP. Data are derived from n=16 (WT), n=18 (A) and n=15 (D) cells from 3 independent cultures. **(B)** Pooled data of sIPSC frequencies. Data are mean ± SEM, P>0.05 and not significant by Kruskal-Wallis test. **(C and D)** Cumulative probability plot (C) and box plot of median and Inter-quartile range (25-75%, D) of sIPSC amplitudes; ***P<0.001 by Kruskal-Wallis test with Dunn's multiple comparison. **(E)** Histograms of sIPSC amplitudes showing individual Gaussian-distributed peaks at distinct amplitudes (green lines and table) compared to the overall distribution (red line) upon expression of ^{HA}NL2 WT and mutants.

Figure 7: PKA-mediated phosphorylation site in Neuroligin-2 mediates its surface and synaptic stability. Schematic representation of the proposed molecular model showing the effects of NL2 phosphorylation at Ser^{714}. Forskolin (right-hand side) activates PKA through increasing intracellular cAMP levels; as a result, NL2 is phosphorylated, recruits Pin1, disperses from the synapse and is internalised. Either through loss of synaptic NL2 directly or through Pin1 activity, other components of the inhibitory postsynaptic site are destabilised, including loss of synaptic $GABA_ARs$. Abolishing NL2 phosphorylation through mutation of Ser^{714} (left-hand side) reduces internalisation and stabilises NL2 at the synapse, leading to increased $GABA_AR$ concentration and enhanced sIPSC amplitude.