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cAMP-EPAC-dependent regulation of gephyrin phosphorylation and GABA_AR trapping at inhibitory synapses

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Summary

GABA_A and glycine receptors are thought to compete for gephyrin binding sites at mixed inhibitory synapses. Changes in the occupancy of one receptor type are therefore expected to have opposite effects on the clustering of the other receptors. This does not explain, however, whether different receptors can be regulated independently from one another. Here, we show that cAMP-dependent signalling reduces gephyrin phosphorylation at residue S270 in spinal cord neurons. Although no ultra-structural changes of the synaptic scaffold were detected using super-resolution imaging, gephyrin de-phosphorylation was associated with a selective increase in GABA_AR diffusion and the loss of the receptors from synapses. As opposed to the PKA-dependent dispersal of $\alpha 3$ -containing GlyRs, the regulation of gephyrin phosphorylation and GABA_AR dynamics acts via non-canonical EPAC signalling. Subtype-specific changes in receptor mobility can thus differentially contribute to changes in inhibitory synaptic strength, such as the disinhibition of spinal cord neurons during inflammatory processes.

Key words

single molecule localisation microscopy (SMLM), single particle tracking (SPT), exchange protein directly activated by cAMP (EPAC), protein kinase A (PKA), cyclic adenosine monophosphate (cAMP), γ -aminobutyric acid type A receptor (GABA_AR), glycine receptor (GlyR), forskolin, 007-AM (8-pCPT-2-O-Me-cAMP-AM)

Introduction

Inhibitory neurotransmission in the spinal cord is shaped by the balance between GABA_A and glycine receptors that co-localise at the majority of synapses (Dumoulin et al., 2000; Geiman et al., 2002; Todd et al., 1996) and that are activated by the co-release of the two neurotransmitters from presynaptic vesicles (Aubrey and Supplisson, 2018; Jonas et al., 1998; Shrivastava et al., 2011). GABA_ARs and GlyRs bind to the same region of the synaptic scaffold protein gephyrin (Kim et al., 2006; Kowalczyk et al., 2013; Maric et al., 2011; Tretter et al., 2011), which is thought to create strong competition between the receptors for synaptic binding sites. As a consequence, the main parameters controlling receptor trapping at mixed inhibitory synapses are the number of receptors, the number of available binding sites, and the relative affinity of the receptors for these sites (discussed in Specht, 2019). Plastic changes in the strength of receptor-gephyrin interactions are expected to shift the equilibrium between GABA_ARs and GlyRs and alter the functional profile of mixed inhibitory synapses.

Post-translational modifications of gephyrin regulate the clustering of the scaffold protein and hence the number of receptor binding sites at synapses (Groeneweg et al., 2018).

Phosphorylation of amino acid residue S270 of gephyrin appears to be particularly important for the clustering of gephyrin at GABAergic synapses, causing a variety of changes in the number, size and intensity of synaptic clusters (Battaglia et al., 2018; Ghosh et al., 2016; Kalbouneh et al., 2014; Kuhse et al., 2012; Tyagarajan et al., 2013; Tyagarajan et al., 2011). Since the synaptic copy numbers of gephyrin and receptor complexes often change in synchrony due to their reciprocal stabilisation at synapses, however, it is unclear whether S270 phosphorylation acts on gephyrin oligomerisation or on receptor-gephyrin binding (Specht, 2019). From a mechanistic point of view, gephyrin is known to assume various conformational states (Sander et al., 2013) that could mediate the downstream effects of S270 phosphorylation.

The intracellular domains (ICDs) of GABA_ARs and GlyRs are subject to post-translational modifications that target the receptors' gephyrin-binding motifs (Mukherjee et al., 2011; Specht et al., 2011). It was recently shown that receptor-gephyrin interactions are also modulated by conformational changes associated with receptor activity (Gouzer et al., 2014; Patrizio et al., 2017). These studies suggest that in addition to the primary interaction domains, the strength of receptor-gephyrin binding is controlled by mechanisms that are

related to the channel function. To test this idea, we set out to investigate whether the cAMP-dependent reduction of glycinergic transmission during inflammation (Harvey et al., 2004) is accompanied by changes in receptor mobility. We found that PKA phosphorylation of GlyR α 3 at amino acid residue S346 indeed disrupted receptor trapping at spinal cord synapses. Unexpectedly, we also identified a non-canonical cAMP signalling pathway that controls the phosphorylation status of gephyrin without affecting the size of gephyrin clusters, and that leads to a specific reduction in GABA_AR clustering. These observations lend support to the concept that diverging signalling processes downstream of cAMP differentially regulate GABA_AR and GlyR numbers at mixed inhibitory synapses.

Results

1. Reduction of GABA_AR levels at spinal cord synapses by forskolin

In order to investigate the regulation of inhibitory synapses by cAMP-dependent mechanisms, we carried out immunocytochemical analysis of endogenous GlyRs, GABA_ARs and the scaffold protein gephyrin in two-week old dissociated rat spinal cord neurons. cAMP-dependent signalling was induced with 20 μM forskolin for 30 min, followed by fixation of the neurons and triple labelling with specific antibodies against GlyRα1, GABA_ARβ3 and gephyrin (Fig. 1A). Quantification of the integrated fluorescence intensity of synaptic puncta did not reveal any significant differences of GlyR levels between control cultures treated with vehicle (0.2% ethanol, see methods for details) and neurons exposed to forskolin (Fig. 1B). We did, however, observe a strong reduction of both GABA_AR and gephyrin immunoreactivity in response to forskolin treatment. The effect of forskolin was similar in magnitude for GABA_ARβ3 and gephyrin ($p > 0.05$, ANOVA), with a mean reduction to $80.2 \pm 2.8\%$ and $76.6 \pm 3.0\%$ (mean \pm SEM, not background-corrected) of the control value, respectively, suggesting that the two effects could be linked (Fig. 1B).

Synapse by synapse analysis of the relative changes in receptor and gephyrin immunoreactivity confirmed that forskolin treatment had little effect on the GABA_ARβ3/gephyrin ratio (Fig. 1C, red traces). However, the apparent increase in the GlyRα1/gephyrin ratio in the presence of forskolin (black dotted line) was difficult to interpret, given that gephyrin levels directly control the number of receptor binding sites (Specht et al., 2013). It appeared doubtful to us how GlyR levels could be maintained in the face of a 25% loss in the number of binding sites. One explanation could be that the internalised fraction of GlyRs is increased (Breitinger et al., 2018), without changing the total fluorescence intensity at synapses. We therefore carried live surface labelling of GlyRα1 (Fig. S1A). However, we did not observe any significant difference between the surface GlyRα1 levels in control conditions and after 30 min of 20 μM forskolin application (Fig. S1B).

2. Forskolin alters mAb7a immunoreactivity but not the total gephyrin at synapses

The monoclonal gephyrin antibody mAb7a that was used in the above experiments is widely employed to quantify gephyrin levels at inhibitory synapses, despite the fact that it recognises an epitope in the central domain of gephyrin that includes the phosphorylated serine residue

S270 (Kuhse et al., 2012). In order to verify the observed reduction of gephyrin labelling after forskolin treatment we performed experiments with a different monoclonal antibody that binds to the gephyrin E-domain (3B11; Smolinsky et al., 2008). To our surprise, gephyrin immunoreactivity with the antibody 3B11 was not different in control and forskolin treated neurons ($p > 0.05$, ANOVA, Fig. 2A, B), while mAb7a labelling was strongly reduced ($55.3 \pm 4.2\%$ of control, mean \pm SEM, $p < 0.001$). Note that the data were corrected for the background, which explains the stronger reduction than in the previous experiment (Fig. 1B, see methods for data analysis). Endogenous GlyR α 1 labelling was not significantly different between the two conditions ($p > 0.05$), in agreement with our earlier data.

These observations raised our suspicion that the reduction in gephyrin labelling could be due to the mAb7a antibody and not reflect any real change in the total gephyrin amount at synapses. We tested this hypothesis in spinal cord cultures derived from a knock-in mouse strain that expresses endogenous mRFP-tagged gephyrin (Machado et al., 2011). Neither GlyR α 1 nor the total mRFP-gephyrin levels at synapses were altered by bath application of forskolin (Fig. 2C, D; $p_{\text{GlyR}\alpha 1} > 0.05$, $p_{\text{mRFP-Geph}} > 0.05$, ANOVA), confirming that the treatment did not have a noticeable effect on the gephyrin molecule numbers at synapses.

To directly compare the effects of forskolin on mAb7a immunoreactivity and total mRFP-gephyrin levels in a more integrated system, we also conducted experiments in organotypic slice cultures from knock-in animals. Three-week-old spinal cord slice cultures were treated with 20 μ M forskolin for 60 min, fixed, immunolabelled, and analysed with confocal microscopy (Fig. 2E). The mAb7a and mRFP-gephyrin fluorescence at synapses was quantified in single image planes that were taken from the dorsal edge of the slice towards the central region. We observed that the ratio of mAb7a/mRFP-gephyrin was reduced after forskolin treatment across all regions, demonstrating that mAb7a immunoreactivity is systematically reduced in different neuronal populations, and is not representative of the total synaptic gephyrin levels in our experimental paradigm.

3. Forskolin reduces gephyrin phosphorylation of residue S270

On the molecular level, the reduction in mAb7a immunoreactivity could be due either to the de-phosphorylation of gephyrin at residue S270, and/or to conformational changes induced by forskolin that render the epitope inaccessible for the antibody. To distinguish between these

possibilities, we analysed the gephyrin phosphorylation status by Western blotting (Fig. 3). Dissociated rat spinal cord cultures were kept in medium containing 20 μ M forskolin for 30 min, total protein fractions were collected in the presence of phosphatase inhibitors, and samples were separated by SDS-PAGE. Western blot membranes were sequentially probed with mAb7a antibody and with 3B11 that served as a loading control for total gephyrin (Fig. 3A, first two lanes). Quantification of the mAb7a/3B11 ratio showed that forskolin treatment reduces mAb7a immunoreactivity by about 50% compared to the control condition (Fig. 3B). It should be noted, however, that the mAb7a antibody is not well suited for Western blotting and produces only weak chemiluminescence signals.

In order to confirm the identity of the detected 95 kDa bands, we repeated the same experiment using cultures that had been infected with lentivirus driving the expression of mEos4b-gephyrin. The detection of an additional band at 130 kDa in these samples demonstrates the specificity of the mAb7a antibody (Fig. 3A, lanes 3 and 4). What is more, the signals of both bands were weaker in the forskolin treated sample, showing that the phosphorylation of both, endogenous gephyrin as well as recombinant mEos4b-gephyrin are reduced by forskolin (Fig. 3B). Across both pairs of samples and three independent experiments, the reduction in mAb7a/3B11 ratio was highly significant (mean reduction 45.8%, $p < 0.001$, paired t-test, $n = 9$ bands per condition). These data indicate that forskolin treatment reduces S270 phosphorylation, although they do not exclude that gephyrin could undergo concomitant conformational changes (Sander et al., 2013).

4. PKA-independent effect of forskolin on gephyrin phosphorylation

PKA is considered as the main effector downstream of cAMP signalling. In order to characterise the mechanism by which forskolin leads to the de-phosphorylation of residue S270 of gephyrin, we therefore tested the involvement of known *in vivo* PKA phosphorylation sites of gephyrin. Wild-type mEos4b-gephyrin and a PKA-phosphorylation deficient variant carrying the amino acid substitutions S294A/S295A/S303A/S305A/S319A (Eos4-Geph^{PKA-}) were expressed in spinal cord neurons by lentivirus infection (Fig. 4A, S1C), and synaptic gephyrin levels were quantified after treatment of the neurons with forskolin for different durations (15, 30, 60 min).

Immunolabelling with mAb7a antibody confirmed that S270 phosphorylation was decreased relative to the total Eos4-Geph^{wt} levels at synapses (mAb7a/Eos4 ratio, $p < 0.001$ at all time

points versus control, KW test; Fig. 4B, see also supplementary Fig. S1D, E). Surprisingly, Eos4-Geph^{PKA-} expressing neurons showed the same temporal profile, suggesting that forskolin did not act directly via any of the mutated PKA phosphorylation sites. We also applied forskolin together with the PKA inhibitor H-89 (Fig. 4C). The mAb7a labelling was reduced to a similar level as in Eos4-Geph^{wt} expressing neurons treated with forskolin without H-89 ($p > 0.05$, ANOVA). Therefore, the effect of forskolin on gephyrin phosphorylation did not appear to be mediated by PKA.

We then considered the involvement of other cAMP-dependent signalling proteins, namely the exchange proteins directly activated by cAMP (EPAC). Immunolabelling of EPAC shows a punctate distribution in spinal cord neurons that partially overlaps with gephyrin clusters (Fig. S2A, B). Co-expression of N- and C-terminally tagged EPAC2 together with mRFP-gephyrin substantiated the presence of EPAC at inhibitory synapses (Fig. S2C). The EPAC specific agonist 007-AM led to a reduction of mAb7a labelling of endogenous gephyrin and of $\beta 3$ -containing GABA_ARs ($p < 0.01$, ANOVA), but did not have any effect on GlyR $\alpha 1$ levels (Fig. 4D, E). These effects were very similar to what had been observed with forskolin (Fig. 1B). Together, the results indicate that EPAC and not PKA is responsible for the changes in gephyrin phosphorylation.

Since EPAC has been reported to form a complex and act in concert with protein phosphatase PP2a (Hong et al., 2008), we treated cells for 15 min with or without 40 nM okadaic acid, an inhibitor of PP1/PP2a, then added forskolin together with H-89 for further 30 min (Fig. 4F, G). Okadaic acid reversed the forskolin effect, resulting in a significant increase in mAb7a but not 3B11 labelling ($p_{\text{mAb7a}} < 0.001$, $p_{\text{3B11}} = 0.20$, t-test). This is in agreement with an earlier study that has implicated PP1/PP2a in the de-phosphorylation of gephyrin at position S270 (Kalbouneh et al., 2014).

5. Single molecule localisation microscopy (SMLM) of synaptic gephyrin clusters

To explore the downstream consequences of S270 de-phosphorylation on the organisation of the gephyrin scaffold at inhibitory synapses, we did super-resolution SMLM imaging of recombinant gephyrin tagged with the photoconvertible protein mEos4b in fixed spinal cord neurons (Fig. 5A). Due to the high spatial resolution of the technique, differences in the ultra-structure of the synapse and in the distribution of scaffold proteins can be identified on the nanometre scale. As judged from pointillist images of the single fluorophore detections, Eos4-

Geph^{wt} is densely packed in synaptic clusters along the dendrites and on the somata of infected neurons. These clusters can be easily distinguished by their size and the number of detections from non-synaptic gephyrin molecules that are diffusely distributed throughout the cell (Fig. 5A, arrowheads).

The quantification of gephyrin cluster sizes did not reveal any differences between control and forskolin treated neurons (synapse by synapse analysis, median area_{Ctrl}: 0.041 μm^2 , area_{For}: 0.042 μm^2 , $p = 0.20$, MW test). Similarly, the number of mEos4b detections per cluster was not altered (Fig. 5B). These data show that forskolin treatment did not affect the clustering of gephyrin molecules at synapses. The fact that the number of detections was the same in both experimental conditions was expected, since the mEos4b detections reflect the total gephyrin content of the synapse. Due to the large sample size ($n > 800$ synapses per condition), there was a significant difference in the detection density (Fig. 5B, right panel). However, cell by cell analysis of the same data did not reveal any obvious difference of the absolute detection densities (Fig. S3A).

Experiments were also carried out using dSTORM to identify changes in the distribution of phosphorylated pS270-gephyrin (Fig. 5C). Here, endogenous gephyrin in fixed spinal cord neurons was labelled with mAb7a and Alexa Fluor 647-coupled secondary antibodies. Pointillist images of Alexa 647 detections were analysed in the same way as the mEos4b images, but with an adjusted threshold for cluster detection (see methods). Both cluster size and detection numbers showed a significant reduction after forskolin treatment (median area_{control}: 0.018 μm^2 , area_{forskolin}: 0.014 μm^2 , $p < 0.001$, MW test, see Table S2). Interestingly, the detection density of pS270-gephyrin clusters was not noticeably changed by forskolin (Fig. 5D, S3B). Our interpretation of these data is that the de-phosphorylation of gephyrin occurs within specific sub-synaptic domains, but that it has no influence on the overall organisation of the gephyrin scaffold.

6. cAMP-dependent regulation of GABA_AR diffusion

Since the S270 phosphorylation status did not appear to have any effect on gephyrin clustering as such, we explored possible consequences of S270 de-phosphorylation on receptor binding at synapses. Single particle tracking with quantum dots (QD-SPT) was carried out in infected rat spinal cord neurons expressing different Dendra2-tagged receptor

subunits. Diffusion coefficients were calculated from receptor trajectories at synapses as well as in the extrasynaptic plasma membrane (Fig. 6A).

In the presence of forskolin, the diffusion of Dendra2-GABA_AR γ 2 receptors at synapses was considerably faster compared to control neurons (Fig. 6B; $p < 0.001$, KW test). The same was true for neurons treated with the EPAC agonist 007-AM ($p < 0.001$). In contrast, we did not observe any changes in the diffusion of GlyR α 1-containing receptors following a 30-minute exposure to forskolin (Fig. 6C). These data indicate that cAMP-dependent signalling via EPAC acts specifically on GABA_AR-gephyrin binding, which is reflected in an increased mobility of the receptor at synapses.

Neurons in the dorsal horn of the spinal cord relay nociceptive signals from primary sensory neurons to the brain. The release of prostaglandin PGE₂ during inflammation leads to the central disinhibition of the nociceptive pathways by blocking GlyR α 3-containing receptor complexes in a PKA-dependent manner (Harvey et al., 2004). Given the known regulation of GlyR α 3 by PKA in the context of inflammatory pain, we therefore looked at receptor diffusion in neurons expressing the splice variants Dendra2-GlyR α 3L and α 3K. The variant GlyR α 3L contains a 15 amino acid insertion in the proximity of the PKA phosphorylation site S346 (Harvey et al., 2004; Nikolic et al., 1998). While the mobility of GlyR α 3K-containing receptors was not changed, the GlyR α 3L variant was accelerated significantly after forskolin application ($p < 0.001$, KW test; Fig. 6D), suggesting that forskolin weakened the gephyrin binding of GlyR α 3L-containing receptor complexes. This fits with immunocytochemical data that showed a reduction of Dendra2-GlyR α 3L levels at synapses in response to forskolin (supplementary Fig. S4). Moreover, mutagenesis of GlyR α 3L at position S346 abolished the PKA-dependent reduction of synaptic receptor levels (Fig. S5). In conclusion, separate cAMP pathways act on GABA_ARs and GlyR α 3L/ β complexes at mixed inhibitory synapses through post-translational modifications targeting gephyrin as well as individual receptor subtypes (Fig. 6E).

Discussion

The main result of this study is that cAMP-dependent EPAC signalling reduces gephyrin phosphorylation at the amino acid residue S270, weakening the GABA_AR-gephyrin

interaction and leading to the selective dispersal of GABA_ARs from mixed inhibitory synapses in spinal cord neurons.

Gephyrin clustering and the role of S270 phosphorylation

Labelling of gephyrin with the widely used antibody mAb7a showed that immunoreactivity of synaptic gephyrin clusters was substantially reduced in response to forskolin. Since the antibody is specific for an epitope that includes the phosphorylated residue S270 of gephyrin (Kuhse et al., 2012), these data indicated that forskolin treatment causes a marked change in the phosphorylation status of endogenous and recombinant gephyrin in cultured spinal cord neurons as well as in organotypic slices.

The phosphorylation of gephyrin at residue S270 has been implicated in the regulation of gephyrin clustering at GABAergic synapses (Groeneweg et al., 2018). Several studies suggested that the phosphorylation-deficient gephyrin variant S270A gives rise to a higher number (Tyagarajan et al., 2013; Tyagarajan et al., 2011) or greater intensity and size of synaptic gephyrin clusters (Battaglia et al., 2018). We did not observe any significant changes in the total synaptic gephyrin levels, suggesting that S270 phosphorylation does not affect gephyrin clustering as such. This is consistent with the data of Kuhse and colleagues, who did not see any obvious changes of gephyrin clustering by S270 de-phosphorylation following the inhibition of Cdk5 (Kalbouneh et al., 2014; Kuhse et al., 2012).

Moreover, we did not detect any ultra-structural changes of the synaptic gephyrin scaffold using SMLM of mEos4b-tagged gephyrin in spinal cord cultures treated with forskolin. Endogenous pS270-gephyrin occupied a smaller synaptic area than total Eos4-Geph^{wt} as judged by dSTORM imaging. Upon forskolin treatment, the pS270 area was further reduced, while the detection density remained unchanged. These data are at odds with the observed differences in the density of phosphorylation variants of gephyrin that were overexpressed in primary hippocampal neurons (Battaglia et al., 2018). Even though the overall clustering of gephyrin was not dependent on its phosphorylation, our super-resolution images suggest that the pS270 form is concentrated in specialised sub-synaptic domains (Pennacchiotti et al., 2017). However, it cannot be ruled out that these results are influenced by the stochasticity of dSTORM imaging (Yang and Specht, in press 2020).

A possible explanation for the conflicting findings is that gephyrin residue S270 is the target of converging signalling pathways, which means that its phosphorylation could involve additional, as yet unidentified post-translational modifications. After all, gephyrin contains more than 50 known *in vivo* modifications (see PhosphoSitePlus database, www.phosphosite.org; Hornbeck et al., 2015). For example, the phosphorylation of S268 by ERK1/2 (Tyagarajan et al., 2013), SUMOylation, or acetylation of gephyrin (Ghosh et al., 2016) could shape the responses downstream of S270 phosphorylation. In our experiments, the silencing of multiple putative PKA sites of gephyrin (including residues S303 and S305; Flores et al., 2015) did not induce any changes in cluster intensity ($I_{\text{Geph PKA}^-} = 1.001 \pm 0.087$ of the wildtype construct, mean \pm SEM, $n = 42$ cells, 2 experiments), ruling out that the phosphorylation of these sites by PKA could have counteracted an effect of pS270 on gephyrin clustering.

We therefore conclude that S270 phosphorylation does not regulate gephyrin clustering, that is to say that it has no effect on gephyrin-gephyrin binding. Instead, our data point to a model whereby S270 phosphorylation promotes the GABA_AR-gephyrin interaction (Fig. 6E). This is in line with the loss of GABA_AR clustering in response to S270 de-phosphorylation after Cdk5 inhibition (Kalbouneh et al., 2014). The mobilisation of GABA_ARs does not have major structural consequences at mixed inhibitory synapses in the spinal cord, where GlyR-gephyrin interactions maintain the stability of the synaptic scaffold. In contrast, GABA_AR dispersal is followed by a rapid loss of gephyrin at purely GABAergic synapses due to the reciprocal stabilisation of receptors and scaffold proteins (e.g. Niwa et al., 2012). A higher fraction of pS270 may also explain how synaptic GABA_AR levels can be maintained during long-term application of diazepam, despite a substantial reduction in total gephyrin content (Lorenz-Guertin et al., 2019). In our view, this model can therefore reconcile some of the variable experimental results that have been reported.

Independence of GABA_AR and GlyR binding at mixed inhibitory synapses

The reduction of GABA_AR-gephyrin binding by pS270 de-phosphorylation is remarkable in that it regulates receptor diffusion in a subtype-specific manner by targeting the gephyrin scaffold itself. Post-translational modifications of the ICDs of GABA_ARs and GlyRs have been shown to control the strength of individual receptor-gephyrin interactions (Mukherjee et al., 2011; Petrini et al., 2014; Specht et al., 2011). Since the inhibitory receptors bind to overlapping sites of gephyrin (Maric et al., 2011; Tretter et al., 2011) they directly compete

for synaptic binding sites. This is exemplified by the opposite changes of $\alpha 2$ versus $\alpha 5$ -containing GABA_AR dynamics at hippocampal synapses (Gerrow and Triller, 2014), where the reduction in the affinity of one receptor type allows another receptor to occupy the liberated binding sites.

The fact that forskolin treatment specifically interfered with the clustering of GABA_ARs (and GlyR $\alpha 3$) at synapses but did not affect GlyR $\alpha 1$ shows that receptor levels can also be regulated separately, without prompting a compensatory effect (Fig. 6E). Long-term blockade of network activity by tetrodotoxin likewise reduced GABA_AR levels independently of GlyRs (Specht et al., 2013). These observations imply that there is only a limited direct competition between receptors at mixed inhibitory synapses. Given the non-identity of the gephyrin binding motifs (e.g. Grunewald et al., 2018; Kowalczyk et al., 2013; Maric et al., 2014), it is feasible that S270 de-phosphorylation and potentially associated conformational changes could only affect the binding of certain receptor subtypes. This raises the new concept that the synaptic gephyrin scaffold displays distinct, receptor-specific binding modes.

If different receptors can be regulated independently from one another, what then is the fate of the liberated binding sites? Although we cannot exclude that GABA_ARs with a different subunit composition replace the $\beta 3$ -containing receptors, the most likely explanation is that the excessive binding sites are lost over time through the dissociation of gephyrin molecules from the synaptic cluster. This is consistent with the observation of a delayed reduction in mEos4b-gephyrin levels during long forskolin applications (supplementary Fig. S1D, E). There was also a trend that GlyRs decline after prolonged exposure to forskolin, suggesting that S270 de-phosphorylation could possibly affect GlyR-gephyrin binding. In view of the high affinity of the GlyR β -gephyrin interaction, however, these processes are expected take place on a much longer timescale and to a lesser degree (Specht, 2019).

Another possibility for the relative independence of GABA_AR clustering is the implication of alternative binding mechanisms. GABA_AR $\gamma 2$ -containing receptors for instance can be recruited by neuroligin-2 via Lhfpl4 binding (Davenport et al., 2017; Yamasaki et al., 2017). The slight mismatch in the relative changes of GABA_ARs and pS270 levels (Fig. 1C, red traces) could indeed point to the existence of a gephyrin-independent mechanism. Yet the overall correspondence between GABA_AR and S270 phosphorylation levels suggests that

GABA_AR clustering at mixed inhibitory synapses in spinal cord neurons relies predominantly on a gephyrin-related mechanism.

Integration of cAMP-dependent pathways regulating receptor trapping at synapses

The effect of forskolin on GABA_AR diffusion at synapses led us to assume initially that the mechanism would be dependent on protein kinase A. There is ample evidence that PKA-dependent phosphorylation regulates GABA_AR function and trafficking, often producing divergent effects (reviewed in Nakamura et al., 2015). Unexpectedly, we found that neither mutagenesis of PKA phosphorylation sites of gephyrin nor the PKA blocker H-89 inhibited pS270 de-phosphorylation and GABA_AR dispersal. It turned out that the GABA_AR loss was in fact caused by a different process, namely the activation of EPAC (Robichaux and Cheng, 2018). The presence of this cAMP-regulated guanine nucleotide exchange factor at inhibitory synapses (Fig. S2) acting in parallel to PKA, could have an important role in the regulation of inhibitory neurotransmission. In other words, cAMP could simultaneously trigger PKA and EPAC signalling processes, leading to complex downstream effects at inhibitory synapses.

For instance, prostaglandin PGE₂ is known to inhibit glycinergic currents in a PKA-dependent manner during inflammatory pain (Harvey et al., 2004). The GlyR α 3-specificity of this effect comes from the presence of a PKA phosphorylation site at residue S346 in the ICD that is not present in GlyR α 1. Phosphorylation of this site was shown to induce global conformational changes that extend to the agonist binding pocket (Han et al., 2013). We found that forskolin treatment of Dendra2-GlyR α 3L-expressing spinal cord neurons increased the mobility of the receptors, whereas no change was observed for GlyR α 1. This suggests that changes in receptor-scaffold interaction parallel the changes in receptor activity (Harvey et al., 2004; Rajalu et al., 2009). In other words, the PKA-dependent inhibition of α 3-containing GlyRs is associated with a weakening of the receptor-gephyrin interaction, lending further support to the hypothesis that the α -subunits of the GlyR can influence the strength of GlyR β -gephyrin binding (Patrizio et al., 2017).

In addition, GABA_AR β subunits contain conserved PKA sites in their ICDs, the phosphorylation of which regulates GABA_AR function (McDonald et al., 1998), internalisation (Kittler et al., 2005), and possibly gephyrin binding (Bohnsack et al., 2016; Kowalczyk et al., 2013). How these different signalling processes are integrated at mixed

inhibitory synapses is not yet understood. Nonetheless, our data demonstrate that cAMP signalling has a significant impact on the trapping of GABA_AR γ 2 and GlyR α 3L-containing receptors (Fig. 6E). Given that α 1-containing GlyRs cannot compensate for this loss, a reduction of inhibitory neurotransmission seems inevitable. The dispersal of GABA_ARs is likely to shift the inhibitory phenotype from a mixed to a purely glycinergic profile, with a fast, monophasic decay and lower charge transfer (Aubrey and Supplisson, 2018). This effect could exacerbate the disinhibition observed during pathological processes such as inflammatory pain. On a more positive note, the independence of GABA_AR and GlyR regulation may also enable the development of specific pharmacological approaches that promote functional compensation.

Limitations of our study

Different regulatory processes at inhibitory synapses may engage multiple and overlapping phosphorylation sites in the central domain of gephyrin. The phospho-specific antibody mAb7a therefore has to be seen as a sensitive tool that reports on one of these modifications that could be implicated in several regulatory mechanisms with different downstream consequences. This also raises concerns about the use of mAb7a antibody as a synaptic marker.

Methods

Complete methods can be found in the supplementary information under Transparent Methods.

Supplementary information

Supplementary data include five supplementary figures, two tables and detailed methods.

Data availability

The raw data of this article are available from the corresponding author upon request.

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Author contributions

F.N., A.P., A.T. and C.G.S. planned the experiments; F.N., A.P. and C.G.S. performed the experiments and analysed the data; F.N., A.P. and C.G.S. wrote the manuscript.

Competing interests statement

The authors declare no competing interests.

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Figure legends

Figure 1. Forskolin reduces GABA_AR and gephyrin immunoreactivity at spinal cord synapses.

(A) Triple immunolabelling of endogenous GlyRs, GABA_ARs and gephyrin in cultured rat spinal cord neurons (DIV16). Cells were treated with 20 μM forskolin (For) or kept under control conditions for 30 min (Ctr; vehicle 0.2% ethanol), fixed, and stained with antibodies against the GlyRα1 and GABA_ARβ3 subunits, and with the rat gephyrin antibody mAb7a (Geph 7a, Synaptic Systems). Scale: 5 μm. (B) Quantification of the integrated fluorescence intensity at synaptic GlyR puncta in the three channels (cell by cell analysis, arbitrary units normalised to the control condition in each channel; data are represented as 10%, 25%, 50% (median), 75% and 90% percentiles; the mean is indicated as a cross; n_{Ctr} = 86, n_{For} = 79 cells from 6 coverslips and 3 independent experiments; ***p < 0.001, ANOVA). (C) Cumulative probability of the ratio of the GlyR (black traces) or GABA_AR fluorescence intensity (red traces) relative to the Geph 7a immunoreactivity at synapses (control: solid lines, n_{Ctr} = 1.4 x 10⁴; forskolin: dotted lines, n_{For} = 1.2 x 10⁴ synapses).

Figure 2. Gephyrin protein levels at synapses are not altered by forskolin.

(A) Sample images showing the effect of forskolin treatment on GlyRα1 (magenta in the merged image) and two different gephyrin antibodies (mAb7a and 3B11, green). Fluorescent signals from Geph 7a were reduced by forskolin while GlyRα1 and Geph 3B11 were not affected. Scale: 5 μm. (B) Integrated fluorescence intensity of endogenous gephyrin at rat spinal cord synapses was quantified in GlyRα1-positive puncta (background-corrected data). Geph 7a labelling was decreased by 45 ± 4% after 30 min treatment with 20 μM forskolin (For) compared to the control (***p < 0.001, ANOVA), whereas no significant changes (NS) were observed with Geph 3B11 (GlyRα1: n_{Ctr} = 149, n_{For} = 144 cells, 5 experiments; Geph 7a: n_{Ctr} = 60, n_{For} = 56, 2 experiments; Geph 3B11: n_{Ctr} = 89, n_{For} = 88 cells, 3 experiments). Data are represented as 10%, 25%, 50%, 75% and 90% percentiles; the mean is indicated as a cross. (C) Dissociated spinal cord cultures prepared from an mRFP-gephyrin knock-in mouse strain (mRFP-Geph KI, magenta) were treated with/without forskolin and then immunolabelled for GlyRα1 (green). Scale: 5 μm. (D) No significant change in mRFP-Geph KI fluorescence and GlyRα1 immunoreactivity was observed (n_{Ctr} = 39; n_{For} = 40 cells, 1

experiment). **(E)** Organotypic spinal cord cultures made from mRFP-gephyrin KI mice were treated at DIV21 with 20 μ M forskolin for 60 min and fixed, labelled and analysed by confocal microscopy. Geph 7a immunoreactivity (green in merged image) and mRFP fluorescence (magenta) were quantified in a consecutive series of nine single plane images taken from the dorsal edge of the slice towards the centre ($n_{\text{Ctr}} = 44$, $n_{\text{For}} = 57$ images from 7-8 slices and 2 independent experiments; $***p < 0.0001$ for the pooled data points for each condition, t-test; data are shown as mean \pm SD). The sample images represent a non-treated control slice, the grey outline illustrates the shape of the complete organotypic slice. Scale: 100 μ m, zoomed images: 5 μ m.

Figure 3. Forskolin reduces gephyrin phosphorylation of residue S270.

(A) Western blotting of total protein fractions of rat spinal cord cultures exposed to forskolin (For) or kept in control condition (Ctr). Sequential labelling of the membranes with the gephyrin antibodies mAb7a and 3B11 identified the endogenous protein (95 kDa) and lentivirus expressed mEos4b-gephyrin (130 kDa). **(B)** For each Western blot membrane, we measured the intensity ratio of mAb7a/3B11 for all corresponding bands (endogenous gephyrin and recombinant mEos4b-gephyrin). Forskolin treatment reduced S270 phosphorylation of gephyrin by 45.8% as judged by the lower mAb7a/3B11 ratio ($n = 9$ bands per condition from 3 independent experiments, $p < 0.001$, paired t-test).

Figure 4. PKA-independent effect of forskolin on gephyrin phosphorylation.

(A) mEos4b-tagged wildtype (wt) and PKA-insensitive (PKA-) gephyrin were expressed in rat spinal cord neurons using lentiviral infection (green in merged image). Cells treated without (Ctr) or with forskolin (For) were labelled with Geph 7a antibody (magenta) and GlyR α 1 (shown in Fig. S1D, E). Scale: 5 μ m. **(B)** Timecourse of the ratio of Geph 7a / Eos4-Geph fluorescence intensity (normalised for each construct to the control condition in each experiment) after forskolin application of up to 60 min ($n > 40$ cells for each construct and time point from 2 experiments, $***p < 0.001$ against control, KW test). **(C)** After 30 min exposure to forskolin, the Geph 7a / Eos4-Geph ratio was similarly reduced, regardless of the presence of the PKA inhibitor H-89 ($n = 60$ cells per condition, 2 experiments, $***p < 0.001$ against control, ANOVA). **(D)** Triple immunostaining of GlyR α 1, Geph 7a and GABA $_A$ R β 3 with/without the EPAC agonist 007 for 30 min. Scale: 5 μ m. **(E)** Normalised fluorescence intensity of 007 treated neurons ($n_{\text{Ctr}} = 90$, $n_{007} = 83$ cells from 3 experiments). EPAC activity

significantly reduced Geph 7a and GABA_ARβ3 labelling but not GlyRα1 (**p < 0.01, ANOVA). (F, G) Spinal cord neurons were treated for 30 min with forskolin and H-89 in the presence or absence of 40 nM okadaic acid (Oka). Blockade of phosphatase PP1/PP2A increased the Geph 7a signal (magenta) but not Geph 3B11 (green) at synapses (n_{For/H89} = 86 and n_{For/H89+Oka} = 84 cells from 3 experiments, ***p < 0.001, t-test). Scale: 5 μm. Data are represented as 10%, 25%, 50%, 75% and 90% percentiles; the mean is indicated as a cross.

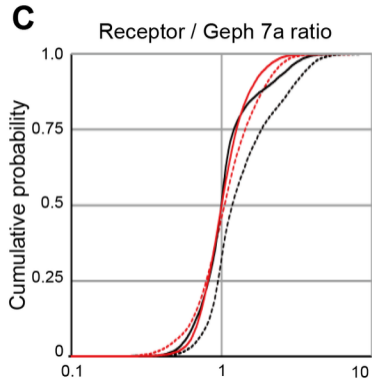
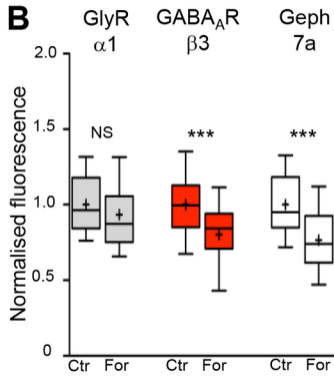
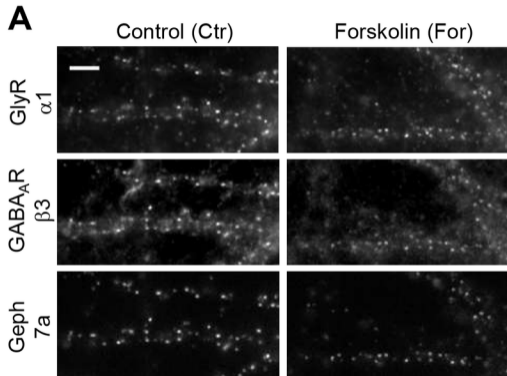
Figure 5. SMLM super-resolution imaging of synaptic gephyrin clusters.

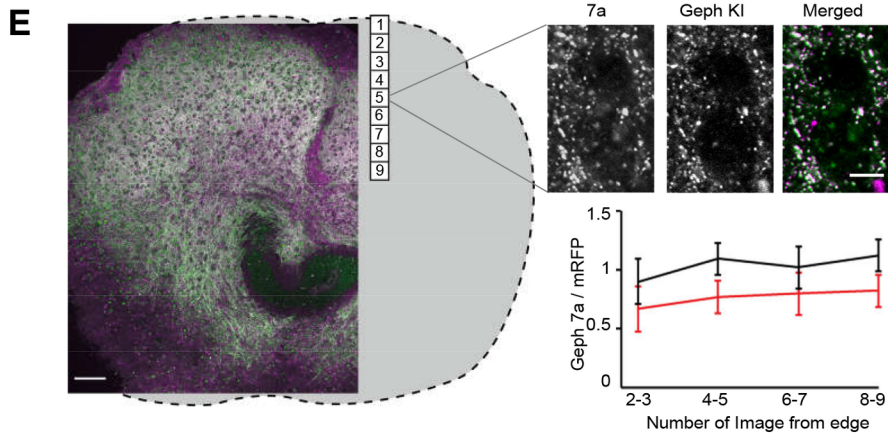
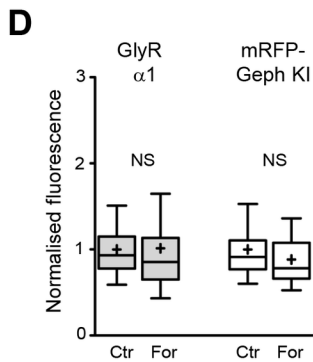
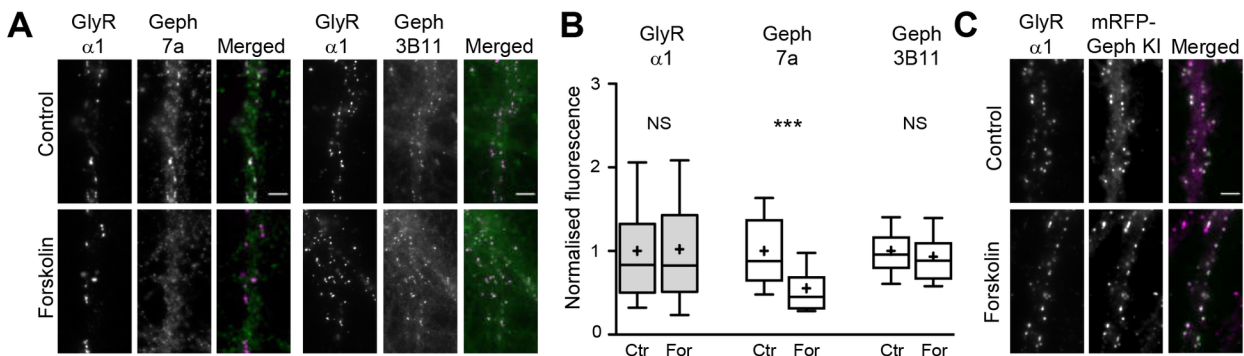
(A) Single molecule detections (shown as black dots, or blue in the zoomed images) of recombinant mEos4b-gephyrin expressed in spinal cord neurons in control condition (Ctr) or after forskolin application (For). Arrowheads indicate the positions of small, non-synaptic clusters arising from the repetitive detection of single mEos4b fluorophores. Dense synaptic clusters were identified based on cluster size and detection number in the pointillist images (yellow areas in the zoomed images, see methods). Clusters with less than 200 detections were not considered (green areas). Scale: 1 μm, insert 100 nm. (B) Quantification of synaptic cluster areas, detection numbers, and detection densities (n_{Ctr} = 1237, n_{For} = 894 clusters from 32 cells per condition and 3 independent experiments, *p < 0.05, MW test). (C) dSTORM imaging of endogenous gephyrin labelled with mAb7a and secondary Alexa Fluor 647-coupled antibodies. Cluster analysis was done as in (A), with a lower threshold of 50 detections per cluster. Scale: 1 μm, insert 100 nm. (D) The quantification of the cluster area and the number of Alexa 647 detections shows a correlated decrease in the forskolin treated neurons compared to control cells (n_{Ctr} = 8162 clusters in 69 cells, n_{For} = 7010 clusters in 68 cells, 3 experiments, ***p < 0.001, MW test). Data are shown as 10%, 25%, 50%, 75% and 90% percentiles and the mean (cross).

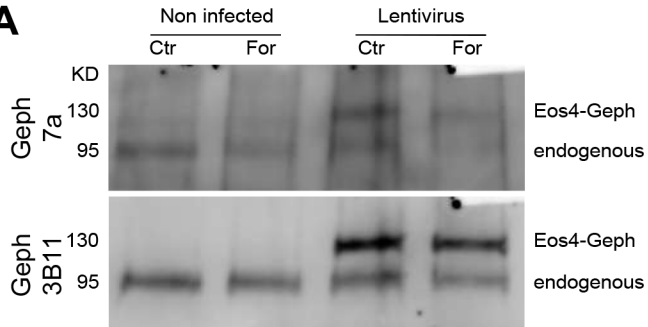
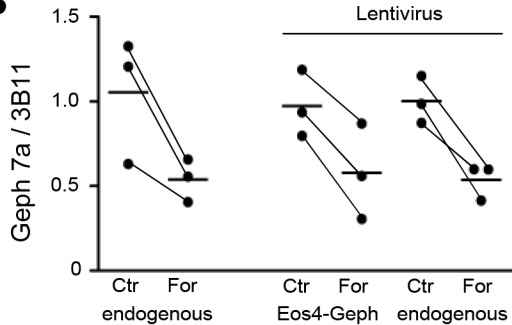
Figure 6. cAMP-dependent regulation of GABA_AR and GlyR diffusion.

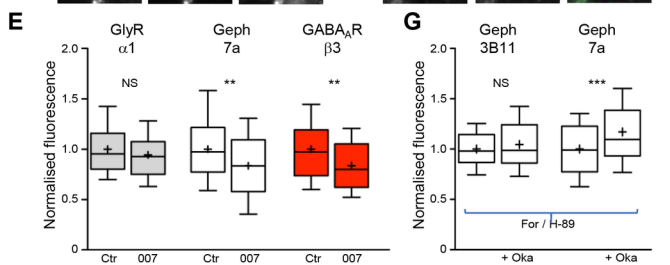
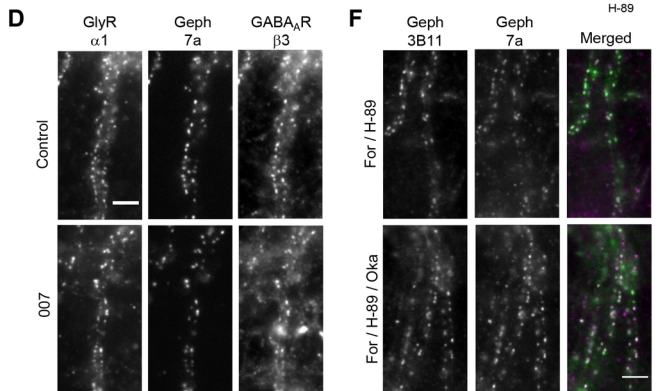
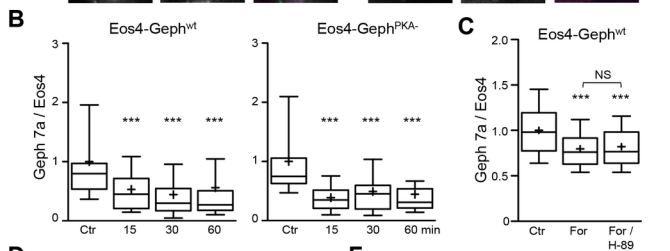
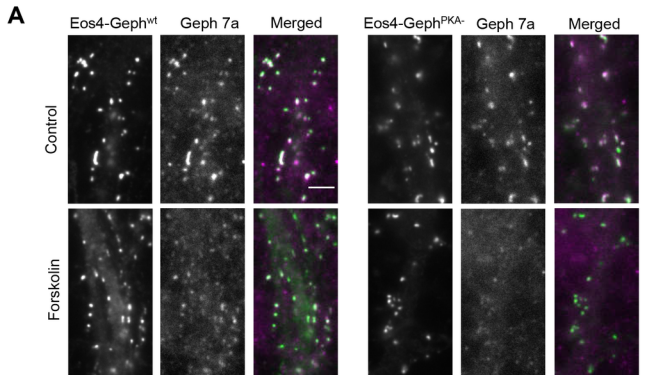
(A) QD-trajectories of Dendra2-GABA_ARγ2 containing receptor complexes (red traces) in control neurons and in the presence of either forskolin or 007-AM. Synapses were identified with FM4-64 labelling (white areas). Scale: 5 μm. (B) Cumulative probabilities of QD-tagged Dendra2-GABA_ARγ2 diffusion coefficients at synapses in the three experimental conditions (median D values: D_{Ctr} = 0.0022 μm²/s, D_{For} = 0.0031, D₀₀₇ = 0.0028, n_{Ctr} = 730 trajectories from 41 cells, n_{For} = 318 trajectories from 42 cells, n₀₀₇ = 384 trajectories from 40 cells, 7 coverslips per condition, 3 experiments). (C) The median diffusion coefficients of Dendra2-

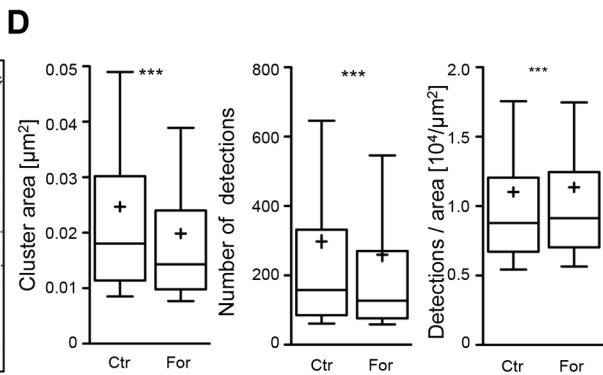
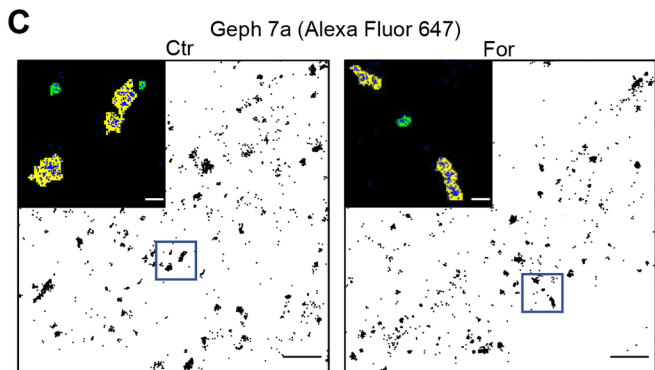
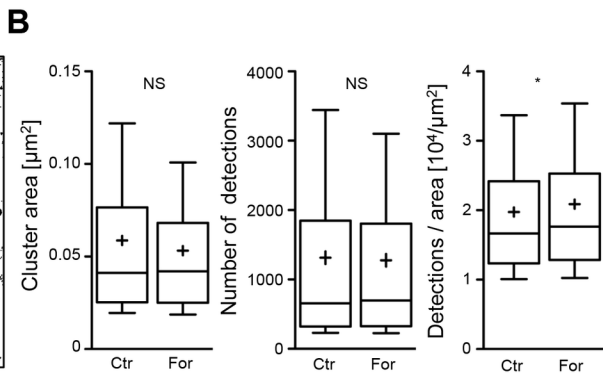
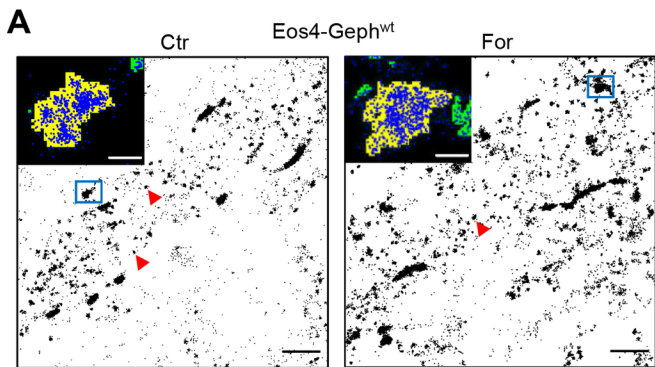
GlyR α 1 at spinal cord synapses were similar in the control and forskolin condition (in the absence of the drug, after a 30 min forskolin incubation; $D_{\text{Ctr}} = 0.0083 \mu\text{m}^2/\text{s}$, $D_{\text{For}} = 0.0076$, $n > 1000$ trajectories from 5 experiments). **(D)** Synaptic diffusion coefficients of Dendra2-GlyR α 3L and α 3K splice variants. Forskolin application specifically increased the speed of diffusion of Dendra2-GlyR α 3L at synapses (α 3L: $D_{\text{Ctr}} = 0.0041$, $D_{\text{For}} = 0.0058$, $n > 1000$; α 3K: $D_{\text{Ctr}} = 0.0060 \mu\text{m}^2/\text{s}$, $D_{\text{For}} = 0.0066$, $n > 900$ QD trajectories; 5 experiments). **(E)** Model of cAMP-dependent pathways regulating inhibitory receptor dynamics at mixed spinal cord synapses, including the pharmacological agents used in this study (see text for details).

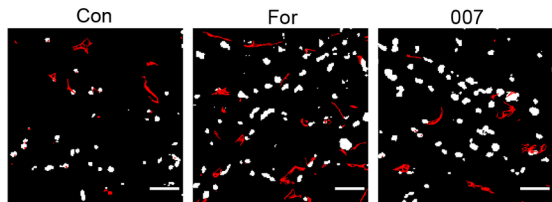
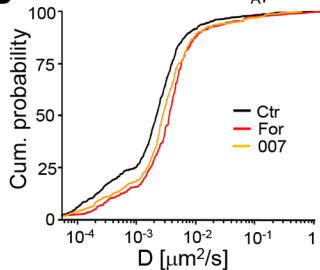
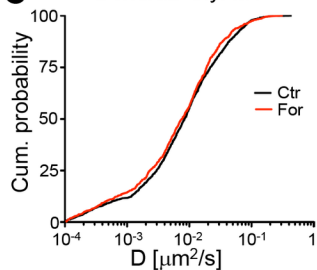
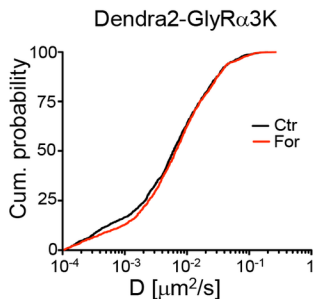
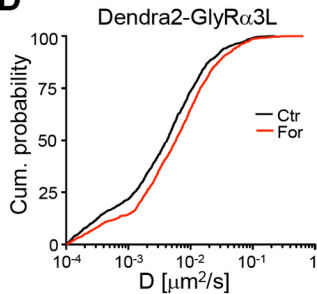
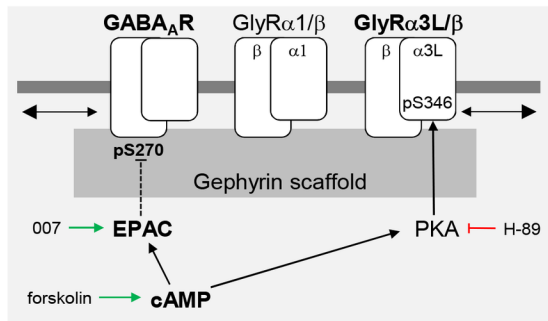


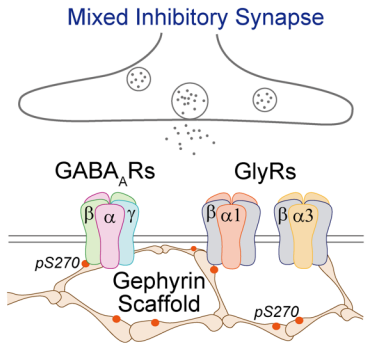
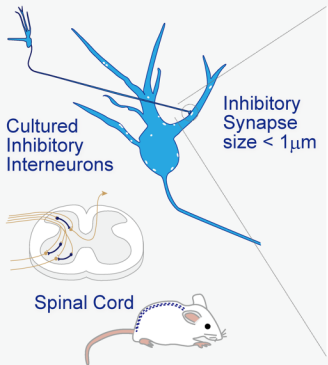


A**B**





A Dendra2-GABA_Aγ2**B** Dendra2-GABA_Aγ2**C** Dendra2-GlyRα1**D****E**



cAMP Dependent Regulation of Inhibitory Synapses

