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Regulation of Glycine Receptor Diffusion Properties and Gephyrin Interactions by Protein Kinase C

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 28 January 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees appreciate the finding that PKC-promoted phosphorylation of the glycine receptor affects its diffusion in the plasma membrane. However, there are also a number of different issues that have to be resolved before further consideration here. Referee #3 brings up different concerns. One is that the results are somewhat inconsistent with your previous work (Levi et al. 2008) and that this issue has to be further resolved. Referee #3 also finds that the findings need to be further validated using the full-length beta-subunit. Referee #2 also raises different points. Given the comments provide by the referees, I would like to invite you to submit a suitably revised manuscript for our consideration. In this case it would be productive if you could send me a detailed point-by-point response as soon as possible detailing what you can do, within reason, to resolve the raised issues. We can then discuss further what experiments are exactly needed for publication here.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to seeing your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I and one of my coworkers have read the present paper an we were both impressed by it. As far as we are concerned, the paper can be published as is. It provides important new information on the mechanisms by which inhibitory synapses are formed.

Referee #2 (Remarks to the Author):

The project identified a serine residue on glycine receptor beta-subunits that is target to phosphorylation and regulates gephyrin interactions.
Single residue mutations alter the mobility of glycine receptors at the neuronal plasma membrane and PKC activation/inactivation causes similar results.
The project applies single particle tracking combined with immunofluorescence, mass spectrometry and a cosedimentation assay.

The project is interesting with respect to the synaptic localization of neurotransmitter receptors, however a large amount of the data require major revision to justify the conclusions.

Specific points:

Figure 1F: the authors mention that mRFP-gephyrin serves to identify inhibitory synapses. As gephyrin particles are also found at extrasynaptic and intracellular locations, a presynaptic protein should be detected throughout all experiments to verify a synapse.

PMA treatment causes about 5% less GlyRa1 clusters (1G), however the image in 1F shows more particles (compare with control image).

The authors should test whether PKC colocalizes with GlyR clusters either through immunostaining or EM. Where does phosphorylation occur? Is S403 modified on vesicular or surface membrane GlyRs?

Phosphorylation regulates GABAA receptor endocytosis - are glycine receptor surface levels affected under the different conditions?

How to judge whether GFX and PMA are absolutely specific for PKC and do not modulate related kinases to a lesser extent (e.g. PKA)? A second independent assay would be of help and negative controls are required.
The data do not provide final evidence that PKC phosphorylates residue S403.
In vitro phosphorylation assays might be suitable to show this.
As the main message of the paper and the title claim that PKC is a major regulator of receptor diffusion such evidence should be given and PKA should be used as a negative control.

The receptor surface mobility assays are well done, however a second experiment is required to verify synaptic versus non-synaptic receptor localization. mIPSCs should be analysed under similar conditions.

Figures 2 and 3: as mentioned in the Figure legend most data are based on two experiments only. All data throughout the manuscript should be based on at least three independent experiments.

Figure 4: no n-values are mentioned. Is the cosedimentation assay based on multiple experiments?

The authors claim on page 9 that the amount of gephyrin that was sedimented by the phospho-mimetic variants was Ñsignificantly" lower than with betaLwt. How was significance analysed? What are the p-values?

The loading control in 4A is oversaturated and should be exchanged.

Figures 4B and 4C should be combined - are the differences significant?

Figure 5F: is there less mRFP-gephyrin in the phospho mutant? Does PKC phosphorylation affect gephyrin clustering? The S403A mutant should be included in this experiment.

Minor points:

Combine figures 3 and 4

Referee #3 (Remarks to the Author):

Comments to authors:

The manuscript by Specht et al. presents a follow-up study of previous pioneering work from the Triller laboratory, which showed that Ca2+ influx resulting from excitatory synaptic input decreases the lateral mobility of GlyRs and results in an increased GlyR clustering at inhibitory synapses and enhanced mIPSC amplitudes (Levi et al., 2008). This finding is very important for our understanding of inhibitory synaptic plasticity. Specht et al. now focussed on PKC as a potential down-stream effector that could mediate the observed regulation of synaptic GlyR levels by activity-driven Ca2+ influx. The authors report the identification of a serine residue, S403, located in the large cytoplasmic loop of the GlyR beta-subunit, whose phosphorylation by PKC reduces binding to the GlyR clustering protein gephyrin in vitro and increases the diffusional mobility of a beta-loop construct. In addition, PKC stimulation by PMA is shown to increase the lateral diffusion of neuronal GlyRs, whereas the PKC inhibitor GFX has opposite effects. The authors conclude that PKC negatively regulates synaptic GlyR levels via phosphorylation of S403, which decreases the affinity of receptor binding to subsynaptic gephyrin and thereby reduces receptor immobilization in the postsynaptic membrane.

Unfortunately this conclusion and the data presented are incompatible with the previous results of Levi et al., who demonstrated enhanced GlyR clustering upon activity-driven Ca2+ influx. If PKC would mediate the homeostatic regulation of synaptic GlyR levels reported in the Levi paper, it should reduce rather than increase GlyR diffusion. Alternatively, the PKC regulation demonstrated here would be irrelevant for the homeostatic regulation described earlier. Surprisingly, the authors do not even discuss this contradiction to their previous high-profile publication, nor is an attempt made to correlate the present biochemical results and diffusion measurements to functional data, e. g. mIPSC recordings as done previously. This in my opinion is essential to support the idea that "the regulation of glycine receptor levels by PKC contributes to the plasticity of inhibitory synapses". In its present form, the manuscript just confuses rather than clarifies our current picture of GlyR diffusional control of inhibitory synaptic strength. Without an answer to the question whether the Ca2+ dependent increase in GlyR clustering found earlier involves PKC activation or not, this study appears incomplete to me.

Other points:

- Data in Fig. 2: All the beta-loop substitution experiments examining lateral diffusion in COS7 cells cotransfected with mRFP-gephyrin were performed on a construct harboring a single TMD. In contrast, the beta subunit has four TMDs, and in the native pentameric receptor a total of 20 TMDs is found. One therefore wonders whether the diffusion behaviour of the constructs used indeed is representative of the intact receptor. Why were the mutations not introduced into the tagged beta-subunit and the surface mobility of the latter examined in spinal neurons (see below)?

- Similarly I am puzzled by the use of a fully soluble Myc-His-tagged beta-loop construct for determining the in vivo utilized PKC phosphorylation site in the GlyR beta-loop sequence upon

heterologous expression in HEK293 cells. The authors present a lengthy discussion of the single PKC site they detected with this approach. The possibility that an important site may not have been found because not the intact membrane-bound GlyR and neuronal cells were examined, is not even discussed.

- Fig. 4: The Western blot data presented in Fig. 4A are not convincing. The reduced gephyrin binding capacities of the S403D and S403E mutants are barely detectable with the pellets but inferred from increased gephyrin immunoreactivities in the supernatants. In case of the S403A mutant, more gephyrin is found in the pellet but the supernatant also contains more gephyrin than the wt sup. In conclusion, there seems to be considerable variation between the incubations. These experiments should be repeated several times, and quantitative data including a solid statistical evaluation should be presented. This also appears crucial in view of the fact that the differences in pellet intensities seen in Fig. 4A between wt and S403D/E are small and difficult to reconcile with the full loss of high-affinity binding inferred from the ITC data shown in panels B and C (p. 10).

- Fig. 5: Cf. comments to Fig. 2. Of course it is essential to demonstrate the relevance of S403 phosphorylation for GlyR diffusion in intact spinal neurons. But again, why was this studied with a beta reporter construct containing only a single TMD and not in the intact GlyR??? Of course this construct facilitates detection but obviously its diffusion behaviour must be very different from the native receptor containing 20 TMDs. Furthermore, PKC sites may have quite different accessibilities. It appears essential to me to validate the conclusions given by using the full-length beta-subunit.

Minor:

- Fig. 1: These data are professionally collected. However, I doubt whether all the different parameters that were extracted from the particle tracking experiments have to be shown here. Also, according to Fig. 1G, the effect of GFX on synaptic GlyR clusters is not significant.

- Fig. 3: This display is not very informative and should be removed or shown in the supplement.

1st Revision - authors' response 10 June 2011

Please find attached our revised manuscript on the Regulation of Glycine Receptor Diffusion Properties and Gephyrin Interactions by Protein Kinase C (EMBOJ-2010-76809R). In this study, we have combined single particle tracking of glycine receptor (GlyR) complexes and membrane constructs with a detailed biochemical characterisation of the GlyR-gephyrin interaction and its regulation by protein kinase C (PKC). More precisely, we have shown that the PKC-dependent phosphorylation of the GlyR subunit at amino acid residue S403 decreases the GlyR-gephyrin interaction, causing an increased rate of receptor diffusion and a reduced GlyR accumulation at inhibitory synapses. In our view, the most exciting and novel aspect of our study is the remarkable relationship between the gephyrin-binding affinities and the diffusion properties of GlyR membrane constructs, which we have used to dissect the molecular mechanism underlying the PKC-dependent regulation of GlyR diffusion and synaptic localisation.

In response to the criticisms raised by the reviewers we have conducted additional experiments that generally confirm our initial conclusions and that provide closer insights into the regulation of GlyR dynamics by PKC (data shown in the new Figures 1H, 3, 4, 6, S1, S2, S4). Furthermore, we have re-analysed some data and substantially rewritten the manuscript in order to discuss some of our results in more detail, as requested by the reviewers. We thus believe that our revised manuscript addresses most of the reviewers' concerns and hope that it is now suitable for publication at The EMBO Journal.

Referee #1:

I and one of my coworkers have read the present paper an we were both impressed by it. As far as we are concerned, the paper can be published as is. It provides important new information on the mechanisms by which inhibitory synapses are formed.

Referee #2:

The project identified a serine residue on glycine receptor beta-subunits that is target to phosphorylation and regulates gephyrin interactions. Single residue mutations alter the mobility of glycine receptors at the neuronal plasma membrane and PKC activation/inactivation causes similar results. The project applies single particle tracking combined with immunofluorescence, mass spectrometry and a cosedimentation assay. The project is interesting with respect to the synaptic localization of neurotransmitter receptors, however a large amount of the data require major revision to justify the conclusions.

Specific points:
Figure 1F: the authors mention that mRFP-gephyrin serves to identify inhibitory synapses. As gephyrin particles are also found at extrasynaptic and intracellular locations, a presynaptic protein should be detected throughout all experiments to verify a synapse.

We have previously shown that most endogenous mRFP-gephyrin clusters represent active inhibitory synapses (FM4-64 co-localisation, see Calamai et al. 2009, J Neurosci 29:7639 - 7648). We have now carried out additional immuno-labelling experiments, which confirm that the overwhelming majority of the gephyrin clusters identified by MIA analysis indeed represent inhibitory glycinergic synapses, as judged by co-localisation with the presynaptic marker VGAT (new Fig. S2). The percentage of gephyrin and GlyRα1-positive puncta co-localising with VGAT in spinal cord neurons is as high as 96%.

PMA treatment causes about 5% less GlyRa1 clusters (1G), however the image in 1F shows more particles (compare with control image).

We have revised the results section of the manuscript to clarify our findings and to avoid this misunderstanding: treatments with PMA and GFX do not cause changes in the number of synaptic clusters, but alter the accumulation of receptors at synapses (Fig. 1F,G). The quantification of the synaptic GlyR enrichment was done by dividing the GlyR fluorescence at synaptic locations (defined by the presence of endogenous mRFP-gephyrin clusters) by the diffuse GlyR fluorescence in dendrites from the same field of view. This type of analysis was chosen to limit the large cell-to-cell variability of the GlyR expression, and thus reflects the equilibrium between synaptic and extrasynaptic GlyR populations. Our data show that GlyRs are more strongly enriched at synapses following PKC inhibition (with 50 nM GFX). In contrast, the PKC agonist PMA (100 nM) has an opposite effect, meaning that endogenous GlyRs are slightly less accumulated at synapses than under control conditions, and significantly less than with GFX. It should be noted that the relative efficacy of the two PKC drugs in this assay ultimately depends on the steady-state activity of PKC in the neurons.

The authors should test whether PKC colocalizes with GlyR clusters either through immunostaining or EM. Where does phosphorylation occur? Is S403 modified on vesicular or surface membrane GlyRs?

Immuno-labelling of neurons and COS-7 cells showed that PKC is widely distributed throughout the cytosol (new Fig. S1). Upon application of the PKC agonist PMA, translocation of PKC to the plasma membrane was observed. No specific enrichment of PKC at synaptic gephyrin clusters was seen under control conditions or after PMA treatment in neurons (Fig. S1). We therefore conclude that PKC phosphorylation of GlyRβ would mostly occur in the extrasynaptic membrane compartment. Although a phosphorylation of GlyRβ at synapses cannot be ruled out, the tight binding of the β-loop to gephyrin is likely to interfere with PKC phosphorylation at synapses.
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Phosphorylation regulates GABAA receptor endocytosis - are glycine receptor surface levels affected under the different conditions?

Recent studies provide compelling evidence that the surface delivery and the internalisation of neurotransmitter receptors occur mainly in the extrasynaptic membrane compartment and that lateral diffusion and receptor trafficking during synaptic plasticity are relatively independent from one another (e.g. Petrini et al. 2009, Neuron 63:92-105; Makino and Malinow 2009, Neuron 2009 64:381-390). For example, in the case of the GlyR we have recently shown that the CaMKII-dependent regulation of receptor diffusion and synaptic clustering by integrins is independent of receptor endocytosis or exocytosis (Charrier et al. 2010, Nat Neurosci 13:1388-1395). In the current study we have focused on the particular question in what way PKC phosphorylation regulates the interaction between GlyRs and the gephyrin scaffold and how this shifts the dynamic equilibrium of synaptic versus extrasynaptic GlyRs. It is possible that the rate of constitutive receptor endocytosis is indeed altered downstream to this mechanism due to the GlyR redistribution. However, whether or not this is dependent on the phosphorylation of GlyRs at residue S403 does not change the interpretation of our results. We therefore feel that internalisation experiments would not add relevant information to our mechanistic study of GlyR membrane diffusion.

How to judge whether GFX and PMA are absolutely specific for PKC and do not modulate related kinases to a lesser extent (e.g. PKA)? A second independent assay would be of help and negative controls are required. The data do not provide final evidence that PKC phosphorylates residue S403. In vitro phosphorylation assays might be suitable to show this. As the main message of the paper and the title claim that PKC is a major regulator of receptor diffusion such evidence should be given and PKA should be used as a negative control.

In response to the reviewer's question, our pharmacological experiments were conducted with highly specific PKC modulators that have not been reported to act on PKA at the concentrations used (GFX: used at 50 nM, specific for PKC-α and PKC-β_I; PMA: used at 100 nM, specific for conventional and novel PKC isoforms in the low nanomolar range). We have additionally carried out in vitro phosphorylation of the GlyR β-loop with purified kinases (new Fig. 3). These data confirm that different PKC isoforms phosphorylate residue S403 of GlyR β, including conventional (PKC-β_I, PKC-γ) and novel PKC isoforms (PKC-δ). On the other hand, we did not observe any GlyRβ phosphorylation by GSK-3β kinase. These data fully support our model by which PKC phosphorylation of residue S403 regulates the GlyR diffusion properties by reducing the strength of the GlyR-gephyrin interaction.

The receptor surface mobility assays are well done, however a second experiment is required to verify synaptic versus non-synaptic receptor localization. mIPSCs should be analysed under similar conditions.

We have carried out a limited set of electrophysiological measurements to test the functional consequences of PKC modulation on glycinergic neurotransmission. Given the effect of PKC inhibition on the diffusion of endogenous GlyRs (Fig. 1B,D,E) and the increased accumulation of GlyRs at inhibititory synapses (Fig. 1G), we have measured mIPSCs before and during perfusion with 50 nM GFX for up to 12 minutes (new Fig. 1H). Indeed, we observed an increase of the mIPSC amplitudes within this time window, in agreement with the changes of GlyR dynamics and synaptic distribution observed in the imaging experiments. However, we also found that PKC blockade altered the channel properties (decay times) of endogenous GlyR complexes, suggesting that PKC affects GlyRs in several ways, which complicates the interpretation of pharmacological data. It is precisely for this reason that we have subsequently analysed the molecular mechanism that links PKC phosphorylation with the regulation of GlyR diffusion and gephyrin interaction in a reduced cellular system (Figs. 2, 4, 5).

Figures 2 and 3: as mentioned in the Figure legend most data are based on two experiments only. All data throughout the manuscript should be based on at least three independent experiments.

Additional experiments have been carried out where required.

Figure 4: no n-values are mentioned. Is the cosedimentation assay based on multiple experiments? The authors claim on page 9 that the amount of gephyrin that was sedimented by the phospho-mimetic variants was "significantly" lower than with betaLwt. How was significance analysed? What are the p-values?

Additional experiments and a more extensive analysis has been carried out (new Fig. 4A), including a statistical evaluation of the data (new Fig. 4B).

The loading control in 4A is oversaturated and should be exchanged.

Done.

Figures 4B and 4C should be combined - are the differences significant?

The ITC runs with the purified β-loop fragments (wild-type and S403D) have been combined in a single graph (Fig. 4C). These experiments have now been carried out six times with comparable results and a statistical evaluation of the data has been included (new Fig. 4D).

Figure 5F: is there less mRFP-gephyrin in the phospho mutant? Does PKC phosphorylation affect gephyrin clustering? The S403A mutant should be included in this experiment.

Endogenous gephyrin levels were not significantly altered by the different expression constructs (new Fig. 5G, centre). However, minor trends were observed, that could be related to the strong accumulation of some constructs at inhibitory synapses (βL^{wt}, βL^{S403A} and to a lesser extent βL^{S403D}) compared to the gephyrin binding-deficient construct βL^{geph-}. Thus, the overexpression of constructs capable of binding to gephyrin may indeed recruit additional gephyrin molecules and thus slightly alter the synaptic scaffold. Data on the phosphorylation-deficient construct βL^{S403A} have also been included (new Fig. 5F,G, see also new Fig. S4).

Minor points:
Combine figures 3 and 4.

The old Fig. 3 has been moved to the supplement (new Fig. S3), as suggested by reviewer 3.

Referee #3:

The manuscript by Specht et al. presents a follow-up study of previous pioneering work from the Triller laboratory, which showed that Ca2+ influx resulting from excitatory synaptic input decreases the lateral mobility of GlyRs and results in an increased GlyR clustering at inhibitory synapses and enhanced mIPSC amplitudes (Levi et al., 2008). This finding is very important for our understanding of inhibitory synaptic plasticity. Specht et al. now focussed on PKC as a potential down-stream effector that could mediate the observed regulation of synaptic GlyR levels by activity-driven Ca2+ influx. The authors report the identification of a serine residue, S403, located in the large cytoplasmic loop of the GlyR beta-subunit, whose phosphorylation by PKC reduces binding to the GlyR clustering protein gephyrin in vitro and increases the diffusional mobility of a beta-loop construct. In addition, PKC stimulation by PMA is shown to increase the lateral diffusion of neuronal GlyRs, whereas the PKC inhibitor GFX has opposite effects. The authors conclude that PKC negatively regulates synaptic GlyR levels via phosphorylation of S403, which decreases the affinity of receptor binding to subsynaptic gephyrin and thereby reduces receptor immobilization in the postsynaptic membrane.

Unfortunately this conclusion and the data presented are incompatible with the previous results of Levi et al., who demonstrated enhanced GlyR clustering upon activity-driven Ca2+ influx. If PKC would mediate the homeostatic regulation of synaptic GlyR levels reported in the Levi paper, it should reduce rather than increase GlyR diffusion. Alternatively, the PKC regulation demonstrated here would be irrelevant for the homeostatic regulation described earlier. Surprisingly, the authors do not even discuss this contradiction to their previous high-profile publication, nor is an attempt made to correlate the present biochemical results and diffusion measurements to functional data, e. g. mIPSC recordings as done previously. This in my opinion is essential to support the idea that "the regulation of glycine receptor levels by PKC contributes to the plasticity of inhibitory synapses". In its present form, the manuscript just confuses rather than clarifies our current picture of GlyR diffusional control of inhibitory synaptic strength. Without an answer to the question whether the Ca2+ dependent increase in GlyR clustering found earlier involves PKC activation or not, this study

appears incomplete to me.

We acknowledge that we have not been sufficiently specific on this point. A detailed discussion of the raised issues has been included in the revised manuscript. Furthermore, we have conducted a limited set of electrophysiological experiments as requested (see response to reviewer 2).

Other points:
Data in Fig. 2: All the beta-loop substitution experiments examining lateral diffusion in COS7 cells cotransfected with mRFP-gephyrin were performed on a construct harboring a single TMD. In contrast, the beta subunit has four TMDs, and in the native pentameric receptor a total of 20 TMDs is found. One therefore wonders whether the diffusion behaviour of the constructs used indeed is representative of the intact receptor. Why were the mutations not introduced into the tagged beta-subunit and the surface mobility of the latter examined in spinal neurons (see below)?

We have carried out additional experiments with full-length GlyRs (new Fig. 6). Briefly, we have generated phosphomimetic and phosphorylation-deficient variants of a full-length chimeric GlyRα1 construct containing the gephyrin-binding sequence of GlyRβ in its cytoplasmic M3-M4 region (construct GlyRα1βgb, see Meier *et al*. 2000, J Cell Sci 113:2783-2795). These constructs were used for single particle tracking in mature spinal cord neurons. As the reviewer had anticipated, the diffusion coefficients of these full-length constructs were notably lower than those of single transmembrane constructs or indeed as endogenous GlyRs, due to the presence of multiple transmembrane and gephyrin-binding domains (see also Ehrensperger *et al.* 2007, Biophys J 92:3706-3718; Charrier *et al.* 2006, J Neurosci 26:8502-8511). Despite these differences, however, the diffusion of GlyRα1βgb was greatly accelerated by the S403D phosphomimetic mutation, in close correspondence with the effect of the S403D mutation on the diffusion of single transmembrane constructs in COS-7 cells as well as in neurons (βL-TMD-pHluorin, see Fig. 2 and Fig. 5). These findings clearly confirm that our observations are valid in more general terms.

Similarly I am puzzled by the use of a fully soluble Myc-His-tagged beta-loop construct for determining the in vivo utilized PKC phosphorylation site in the GlyR beta-loop sequence upon heterologous expression in HEK293 cells. The authors present a lengthy discussion of the single PKC site they detected with this approach. The possibility that an important site may not have been found because not the intact membrane-bound GlyR and neuronal cells were examined, is not even discussed.

In the meantime, the in vivo phosphorylation of S403 (first reported in Trinidad et al. 2008, Mol Cell Proteomics 7:684-96) has been confirmed in a recent characterisation of the mouse brain phosphoproteome (Wisniewski et al. 2010, J Prot Res 9:3280-3289). This study identified only one other in vivo phosphorylation site in the β−loop, namely T369. However, this new site is rather distant from the precise gephyrin-binding domain and thus unlikely to be involved in the regulation of the GlyR-gephyrin interaction. The fact that our MS analysis does not rule out the presence of additional in vivo phosphorylation sites in the β−loop has been discussed in the revised version of the manuscript.

Furthermore, we have validated our MS analysis by in vitro phosphorylation of the GlyR β−loop (new Fig. 3). These experiments showed that different PKC isoforms phosphorylate residue S403 of the GlyRβ subunit. However, since the phosphorylation-deficient variant S403D was also phosphorylated by PKC-β_I (though at a reduced level) additional PKC sites may indeed be present in the β-loop. Nonetheless, it is unlikely that any of these additional sites are involved in the regulation of the GlyR-gephyrin interaction as shown by our SPT/mutagenesis experiments of all putative PKC sites (Fig. 2: phosphorylation-deficient $\beta L^{T381A/T388A/S389A/S403A} = \beta L^{PKC-}$; Fig. 5: phosphomimetic constructs βL^{T381D} and $\beta L^{T388D/S389D}$).

Fig. 4: The Western blot data presented in Fig. 4A are not convincing. The reduced gephyrin binding capacities of the S403D and S403E mutants are barely detectable with the pellets but inferred from increased gephyrin immunoreactivities in the supernatants. In case of the S403A mutant, more gephyrin is found in the pellet but the supernatant also contains more gephyrin than the wt sup. In conclusion, there seems to be considerable variation between the incubations. These experiments should be repeated several times, and quantitative data including a solid statistical

evaluation should be presented. This also appears crucial in view of the fact that the differences in pellet intensities seen in Fig. 4A between wt and S403D/E are small and difficult to reconcile with the full loss of high-affinity binding inferred from the ITC data shown in panels B and C (p. 10).

We have repeated the co-sedimentation experiments several times, using both endogenous and recombinant gephyrin, and have included a thorough statistical analysis of the data (new Fig. 4B). We have also exchanged the Western blots shown in Fig. 4A for a better representation of our findings.

Fig. 5: Cf. comments to Fig. 2. Of course it is essential to demonstrate the relevance of S403 phosphorylation for GlyR diffusion in intact spinal neurons. But again, why was this studied with a beta reporter construct containing only a single TMD and not in the intact GlyR??? Of course this construct facilitates detection but obviously its diffusion behaviour must be very different from the native receptor containing 20 TMDs. Furthermore, PKC sites may have quite different accessibilities. It appears essential to me to validate the conclusions given by using the full-length beta-subunit.

As described above the proposed experiments with full-length GlyR complexes have been carried out and fully support our conclusions (new Fig. 6).

Minor:
Fig. 1: These data are professionally collected. However, I doubt whether all the different parameters that were extracted from the particle tracking experiments have to be shown here. Also, according to Fig. 1G, the effect of GFX on synaptic GlyR clusters is not significant.

Despite the fact that single particle tracking has been increasingly used in recent years to study receptor diffusion in neurons (reviewed in Renner et al. 2008, Curr Opin Neurobiol 18:532ñ540) we feel that the detailed description of the most important diffusion parameters is helpful for the understanding of our experimental strategy. However, as requested by reviewer 3 and due to space limitations we have kept the SPT data to a minimum in the new Figs. 6 and S4.
Regarding Fig. 1G, we have done additional experiments and re-analysed the data for a better understanding of the observed changes of the GlyR distribution after PKC modulation (see response to reviewer 2).

Fig. 3: This display is not very informative and should be removed or shown in the supplement.

Done (Fig. S3).

2nd Editorial Decision 05 July 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original referees # 2 and 3 to review the revised manuscript and I have now heard back from them.

Referee #2 is satisfied with the revised version, while referee #3 has some remaining issues with the analysis that mostly can be addressed with appropriate text changes. Regarding the first issue (physiological relevance) raise by referee #1. The referee refers to the added Fig 1H and wonders why PMA has not been tested. Have you done the experiment also using PMA? If so it would seem useful to add that to the manuscript. If not, then please comment on that in the point-by-point response.

Given the comments, I would like to ask you to respond to the last remaining issues in a final revision.

I am looking forward to seeing the final version.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have addressed most of my comments.
They carried out a number of additional experiments, improved the statistical analysis, included electrophysiological analysis of mIPSCs and added additional controls (for instance the S403A mutant in Fig. 5).

Together the revised version significantly improved the manuscript and I can now support publication in EMBO Journal.

Referee #3 (Remarks to the Author):

The authors have made considerable efforts to amend their manuscript, and principally I would be very happy to be convinced of their proposal that PKC phosphorylation regulates GlyR-gephyrin interaction. However, the question whether the observations made are relevant for the in vivo regulation of synaptic efficacy is in my opinion still unsolved. In response to my previous concern that the present data are incompatible with the calcium-dependent homeostatic regulation of synaptic GlyR levels reported by the same lab (Levi et al., 2009), only a short para has been added to the discussion which now mentions this discrepancy but does not offer a true explanation. Furthermore, the new electrophysiological data shown in Fig. 1H relate only to a short-term treatment with GFX, and the modest increase in mIPSC amplitude observed seems not even to be significant (p value > 0.05). The authors give arguments why no further electrophysiology was done, but I wonder why PMA was not tested using the same application protocol as described for the QD experiments. In conclusion, convincing evidence for PKC regulating inhibitory synaptic efficacy via phosphorylation of S403 is still lacking.

The other points raised in my previous review have been mostly addressed by the authors although the majority of the results still is on soluble betaL constructs and not functional GlyR (4 out of 6 figures). The new QD results with the chimeric alpha1betagb construct (new Figure 6) show that similar mobility changes as induced by mutating S403 in the betaL-TDM-pHluorin construct are similarly seen in a mutated pentameric receptor. However, the MS phosphorylation site analysis is still only with soluble tagged betaL, and the newly added in vitro phosphorylation data demonstrate only that this peptide is differentially phosphorylated by different PKC isoforms (any idea which of these isoforms are expressed in spinal neurons?). The cosedimenation data for gephyrin binding have been improved as requested (what is the loading control shown in A for? Not specified).

Minor:
- In order to respond to requests of referee #2, the authors added additional Supplementary figures which I consider not necessary. Why show the PMA-induced translocation of PKC to the plasma membrane in COS cells (Figure S1B)? This is amply documented in the literature. Similarly, the results given in Figure S2 have all been published previously. It would suffice to just mention these confirmatory data in the text.

- The abstract is too general and does not describe the results obtained.

2nd Revision - authors' response 13 July 2011

We have indeed performed electrophysiological experiments with the PKC agonist PMA in spinal cord neurons. These experiments did not show a significant change of the mIPSC amplitude, although some cells displayed a clear reduction of glycinergic mIPSCs and others a more variable response. Previous studies had also reported conflicting results with PMA on GlyR currents (discussed in Legendre 2001, Cell. Mol. Life Sci. 58: 760-793). These observations are not surprising, given that the agonist PMA initiates pleiotropic effects due to different PKC targets. In contrast, PKC inhibition was expected to lead to more consistent effects, as shown with the antagonist GFX (Fig. 1H). We have now added our results with PMA to the results section of the revised manuscript.
We have also introduced some changes and clarifications to the manuscript, which address the additional comments of reviewer #3. Please find attached a point-by-point response to the comments of reviewer #3. We believe that most of these issues had already been addressed in the manuscript. However, please let us know if you feel that a more detailed discussion of any of these points should be added to the manuscript. We hope that you will find the revised version suitable for publication in the EMBO Journal.

Referee #2 (Remarks to the Author):

The authors have addressed most of my comments.They carried out a number of additional experiments, improved the statistical analysis, included electrophysiological analysis of mIPSCs and added additional controls (for instance the S403A mutant in Fig. 5).
Together the revised version significantly improved the manuscript and I can now support publication in EMBO Journal.

Referee #3 (Remarks to the Author):

The authors have made considerable efforts to amend their manuscript, and principally I would be very happy to be convinced of their proposal that PKC phosphorylation regulates GlyR-gephyrin interaction. However, the question whether the observations made are relevant for the in vivo regulation of synaptic efficacy is in my opinion still unsolved. In response to my previous concern that the present data are incompatible with the calcium-dependent homeostatic regulation of synaptic GlyR levels reported by the same lab (Levi et al., 2009), only a short para has been added to the discussion which now mentions this discrepancy but does not offer a true explanation. Furthermore, the new electrophysiological data shown in Fig. 1H relate only to a short-term treatment with GFX, and the modest increase in mIPSC amplitude observed seems not even to be significant (p value > 0.05). The authors give arguments why no further electrophysiology was done, but I wonder why PMA was not tested using the same application protocol as described for the QD experiments. In conclusion, convincing evidence for PKC regulating inhibitory synaptic efficacy via phosphorylation of S403 is still lacking.

As we had stated in the discussion, the differences between our results and the data described by Levi et al. (2008, Neuron 59:261-273) are the consequence of the pleiotropic effects of Ca^{2+} in neurons. Whereas a global change in the Ca^{2+} homeostasis was produced in Levi's study (TTX and NMDA applications), here we have specifically altered the activity of protein kinase C. Thus, it is likely that the homeostatic regulation described earlier also involves other Ca^{2+}-dependent pathways. This has been clearly pointed out in the discussion.

Electrophysiological experiments with PMA have also been carried out. We did not observe a significant change in the mIPSC amplitudes after 10-12 min of PMA application. Yet, we found a relatively high cell-to-cell variability, with some cells displaying a clear reduction of glycinergic mIPSCs and others showing a more variable response. This observation may be related to the conflicting results that had been obtained in previous studies with PMA (discussed in Legendre 2001, Cell. Mol. Life Sci. 58:760-793), since PKC activation implicates multiple targets and effects. We have added our data with PMA to the results section of the manuscript.

The other points raised in my previous review have been mostly addressed by the authors although the majority of the results still is on soluble betaL constructs and not functional GlyR (4 out of 6 figures). The new QD results with the chimeric alpha1betagb construct (new Figure 6) show that similar mobility changes as induced by mutating S403 in the betaL-TDM-pHluorin construct are similarly seen in a mutated pentameric receptor. However, the MS phosphorylation site analysis is still only with soluble tagged betaL, and the newly added in vitro phosphorylation data demonstrate only that this peptide is differentially phosphorylated by different PKC isoforms (any idea which of these isoforms are expressed in spinal neurons?). The cosedimenation data for gephyrin binding have been improved as requested (what is the loading control shown in A for? Not specified).

There is ample evidence in the scientific literature that truncated or soluble constructs of membrane proteins can be successfully used to study molecular interactions in reduced cellular systems (e.g. Sola et al. 2004, EMBO J. 23:2510-2519, Zita et al. 2007, EMBO J. 26:1761-1771, Saiepour et al. 2010, JBC 285:29623-29631). We believe that a particular strength of our study is precisely the fact that we have been able to reproduce the behaviour of endogenous GlyR complexes in a reduced system, in the absence of functional receptors. In the context of phosphorylation, it was particularly important to work in a system that does not interfere through non-controlled phosphorylation processes.

This approach allowed us for the first time to relate the diffusion properties of membrane-associated constructs containing a monovalent gephyrin-binding domain to the strength of the GlyR-gephyrin interaction using co-sedimentation and isothermal titration calorimetry. In addition, we have provided evidence on the behaviour of recombinant full-length GlyR complexes as requested explicitly by reviewer #3 (see Fig. 6). These data fully support our model of PKC-dependent regulation of the GlyR-gephyrin interaction and GlyR diffusion.

The β_I and δ PKC isoforms tested in Figure 3A are known to be expressed in most tissues including brain and spinal cord, and PKCγ appears to be specific to brain and spinal cord neurons (see Akinori 1998, Prog. Neurobiol. 54:499-530, Liu and Heckman 1998, Cell. Signal. 10:529-542, Hug and Sarre 1993, Biochem. J. 291:329-343, and references cited therein). We have shown that all three PKC isoforms phosphorylate the GlyR β-loop at residue S403 (Fig. 3A).

The loading control in Figure 4A shows equal loading of the GST fusion proteins on the glutathione resin that was used in the co-sedimentation experiments (stained with Coomassie blue). This information has been added to the legend of Figure 4A.

Minor:
- In order to respond to requests of referee #2, the authors added additional Supplementary figures which I consider not necessary. Why show the PMA-induced translocation of PKC to the plasma membrane in COS cells (Figure S1B)? This is amply documented in the literature. Similarly, the results given in Figure S2 have all been published previously. It would suffice to just mention these confirmatory data in the text.

The mentioned experiments (Fig. S1B, S2) have been specifically requested by reviewer #2 and we agree with this reviewer that they provide useful additional information. The translocation of PKC to the plasma membrane in COS-7 cells shows that endogenous PKC is present in this cell type and is activated by our pharmacological treatment within the time window used in our experiments (100 nM PMA, 15 min). This information was needed for COS-7 cells. The co-localisation of GlyRs and gephyrin with the presynaptic marker VGAT illustrates the way in which synaptic gephyrin clusters are detected, binarised to produce image masks, and used to define inhibitory synapses, which had not previously been shown for the mRFP-gephyrin knock-in mouse model. Yet, since both figures are not necessarily required to understand the article we have chosen to show them as supplementary information.

- The abstract is too general and does not describe the results obtained.

We have rephrased the abstract to make it more precise. In particular, we have added the amino acid residue that is phosphorylated by PKC (residue S403).

