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

Supplementary information

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Figure S1

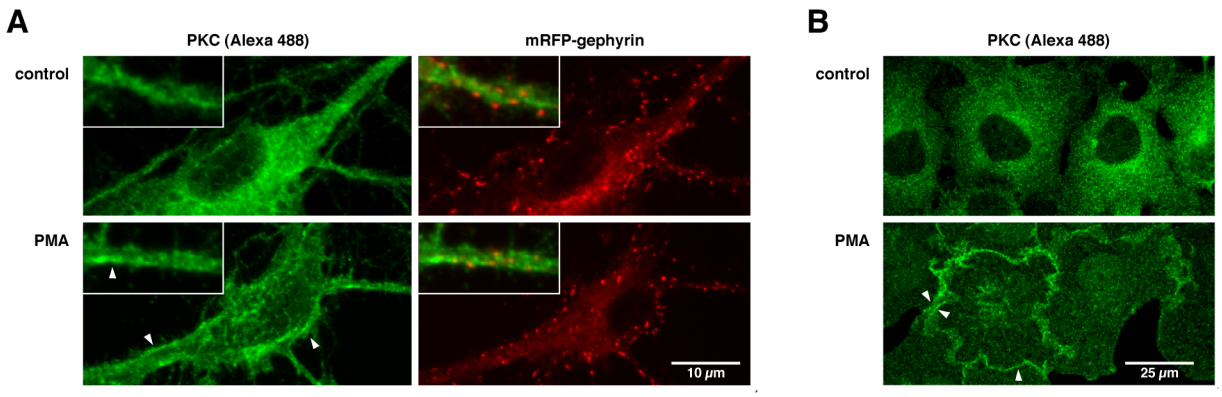

Figure S1: PKC translocation to the plasma membrane in neurons and COS-7 cells.

A. Cultured spinal cord neurons from mRFP-gephyrin expressing knock-in animals were fixed at day *in vitro* (DIV) 16, permeabilised and labelled with an antibody that recognises the α, β and γ isoforms of PKC (Abcam, clone M110, #ab23511; 1:500 dilution), followed by Alexa Fluor 488 conjugated secondary antibody (Invitrogen, 1:500; maximal intensity projections of confocal image stacks). PKC is widely distributed throughout the cytosol, but notably excluded from the nucleus. No specific accumulation of PKC at inhibitory gephyrin clusters is apparent (see magnified insets, 10 µm width). In neurons treated with 100 nM phorbol 12-myristate 13-acetate (PMA) for 15 minutes prior to fixation, the translocation of PKC to the plasma membrane can be observed (arrowheads). Yet, no enrichment of PKC was detected at inhibitory gephyrin clusters.

B. COS-7 cells were fixed and labelled with PKC antibody (as above). Under control conditions, PKC is present throughout the cell and excluded from the cell nucleus. Treatment with the PKC agonist PMA (100 nM, 15 min) causes the translocation of PKC to the plasma membrane (arrowheads), as described previously (Kazi & Soh, 2007; Shirai & Saito, 2002).

Figure S2

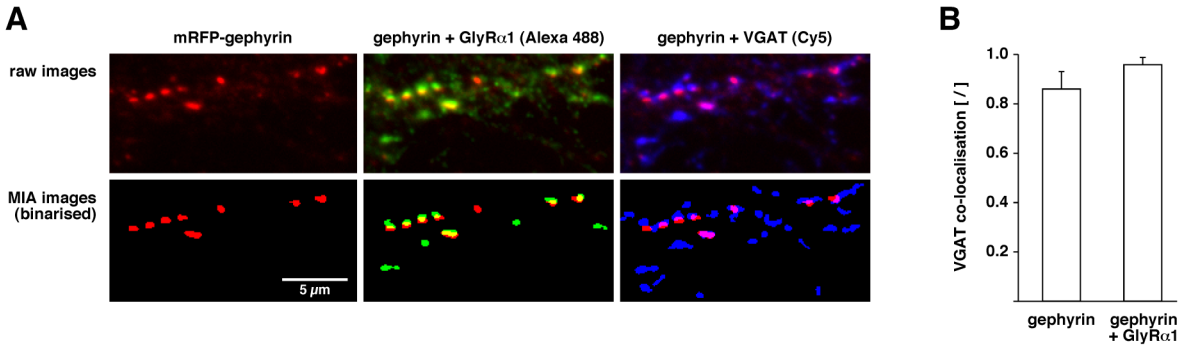

Figure S2: Synaptic localisation of GlyRs in spinal cord neurons.

A. Spinal cord cultures derived from an mRFP-gephyrin expressing knock-in mouse model were fixed at DIV 16, permeabilised and labelled with antibodies against GlyRα1 (Synaptic Systems, mAb2b, #146111; 1:400 dilution) and vesicular inhibitory amino acid transporter VGAT (guinea pig, Synaptic Systems, #131004; 1:500), followed by Alexa Fluor 488 and Cy5-conjugated secondary antibodies, respectively (Invitrogen and Jackson Immunoresearch; 1:500). Putative synaptic clusters were identified by multidimensional image analysis (MIA) in the three fluorescence channels (mRFP, Alexa 488 and Cy5) and overlaid to determine the degree of their co-localisation.

B. Quantification of the images shows a high degree of co-localisation of endogenous gephyrin clusters with the presynaptic marker VGAT (86 ± 7%, mean ± standard deviation, n = 25 cells from 2 coverslips). The percentage of gephyrin- and GlyRα1-positive puncta co-localising with VGAT was as high as 96 ± 3%. These data suggest that the overwhelming majority of the gephyrin clusters identified by MIA analysis indeed represent inhibitory glycinergic synapses (see also Calamai et al, 2009).

Figure S3

Figure S3: PKC phosphorylates S403 of the GlyR β-loop.

Sequence alignment (residues N334-A454, input sequence) of the murine GlyR β-loop with peptides identified by mass spectrometry (from two independent experiments). The wild-type βL^{wt}-Myc-6xHis construct was expressed in HEK 293 cells, affinity purified, digested with trypsin, chymotrypsin and Lys-C and subjected to LC-MS. A sequence coverage of 90% was achieved and two peptides containing the phosphorylated residue S403 were identified (highlighted in red), namely SIVG**S***LPRDF (identified in both LC-MS runs) and SNDFSIVG**S***LPR.

Figure S4

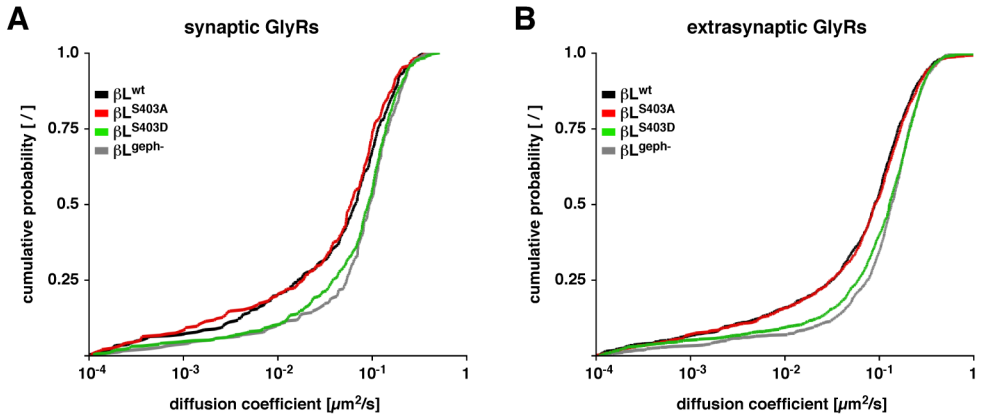

Figure S4: GlyRβ mutagenesis at S403 modulates membrane diffusion.

Single particle tracking (SPT) of βL-TMD-pHluorin constructs (wild-type construct βL^{wt}, phosphorylation-deficient variant βL^{S403A}, phospho-mimetic variant βL^{S403D} and gephyrin-binding deficient variant βL^{geph-}) in spinal cord neurons (DIV 12-13; n > 40 transfected neurons from ≥ 4 coverslips and two independent experiments).

A. Synaptic diffusion coefficients (n > 240 quantum dot trajectories; KS: βL^{S403D} vs βL^{wt}, $p < 0.001$; βL^{geph-} vs βL^{wt}, $p < 10^{-4}$; βL^{S403A} vs βL^{wt}, $p = 0.66$).

B. Extrasynaptic diffusion coefficients (n > 800 QD trajectories; KS: βL^{S403D} vs βL^{wt}, $p < 10^{-4}$; βL^{geph-} vs βL^{wt}, $p < 10^{-4}$; βL^{S403A} vs βL^{wt}, $p = 0.68$).

References

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