Cyclin-Dependent Kinase 5 Is Involved in the Phosphorylation of Gephyrin and Clustering of GABA<sub>A</sub> Receptors at Inhibitory Synapses of Hippocampal Neurons

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Abstract

CDK5 has been implicated in neural functions including growth, neuronal migration, synaptic transmission and plasticity of excitatory chemical synapses. Here we report robust effects of CDK5 on phosphorylation of the postsynaptic scaffold protein gephyrin and clustering of inhibitory GABA<sub>A</sub> receptors in hippocampal neurons. shRNA-mediated knockdown of CDK5 and pharmacological inhibition of cyclin-dependent kinases reduced phosphorylated gephyrin clusters and postsynaptic γ2-containing GABA<sub>A</sub> receptors. Phosphorylation of S270 is antagonized by PP1/PP2a phosphatase and site-directed mutagenesis and in vitro phosphorylation experiments indicate that S270 is a putative CDK5 phosphorylation site of gephyrin. Our data suggest that CDK5 plays an essential role for the stability of gephyrin-dependent GABA<sub>A</sub> receptor clusters in hippocampal neurons.

Introduction

Cyclin-dependent kinase 5 is a proline-directed serine/threonine kinase belonging to the class of CDC2 (CDK1)-like kinases [1]. CDK5 activity is dependent on the association with neuron-specific regulatory proteins p35 or p39 [2,3]. The calpain-dependent cleavage of p35 generates a CDK5 activator (p25) with distinct cellular localization and activity [4]. Interestingly, CDK5 has been implicated in a huge number of neuronal functions, brain development and diseases [5]. CDK5 activity was reported to be crucial for neuronal migration [6], cortex layer formation [7], neurite outgrowth [8,9] and retrograde axonal transport [10]. Phosphorylation of various synaptic substrates was shown to have a major impact on pre- and postsynaptic functions of excitatory synapses. For example, analysis of the CDK5-dependent phosphorylation of PSD95 [11] implies a functional role of CDK5 for regulating synaptic strength by modulating postsynaptic localization and density of glutamate-gated ion channels. Moreover, different studies showed that CDK5-dependent change of postsynaptic NMDA receptor clustering depending on subunit phosphorylation [12]. Consistent with these reports, Li et al. showed that CDK5 inhibition blocks long-term potentiation (LTP) induction in CA1 hippocampal neurons [13]. Similarly, another study revealed that CDK5 loss-of-function in hippocampal circuits results in severe impairments in memory formation and retrieval associated with LTP deficits in hippocampal CA1 neurons [14]. Whereas these and other studies suggest an involvement of CDK5 in the regulation of glutamatergic neurotransmission, almost nothing is known about a putative functional role of CDK5 at inhibitory synapses. We report that knockdown or inhibition of CDK5 in hippocampal neurons results in reduced phosphorylation of postsynaptic clusters of the scaffold protein gephyrin, detected with the phospho-specific antibody mAb7a [15]. Moreover, CDK5 knockdown or inhibition results in a reduced number of γ2 containing GABA<sub>A</sub> receptor clusters, suggesting that CDK5 plays an important role at inhibitory synapses.

Experimental Procedures

Ethics statement

This study was carried out in strict accordance with the European Communities Council Directive (86/609/EEC) to minimize animal pain or discomfort. The local animal care and use committee (Interfakulta¨re Biomedizinische Forschungseinrich- tung (IBF) Heidelberg) gave approval for the study under the number T-81/10.

Construction of expression plasmids and knockdown vectors

DNA fragments encoding the complete coding sequence of rat CDK5 were amplified by PCR from embryonic brain cDNA creating flanking BamHI and HindIII restriction sites and were cloned in the expression vector pCMVTag 3B (Invitrogen) resulting in N-terminal fusion constructs with a myc-epitope (myc-CDK5). Sequences encoding for three shRNA directed against CDK5 and scrambled shRNA were cloned into pFSGW, a modified version of the lentiviral vector pFUGW [16], using a
human synapsin promoter to drive EGFP expression. CDK5-kd1: 5’tattgtacctactgtttaga3’, CDK5-kd2: 5’tttgagtctgtctatcagga 3’, CDK5-kd3: 5’tttgaccaactggccagctaa3’ and a control scrambled sequence (mismatch): 5’tttggttagaatctcagga3’.

Site-directed mutagenesis

Site-directed mutagenesis of 6xHis-tagged gephyrin was performed using the Quickchange Lightning mutagenesis kit from Stratagene following the suppliers’ instructions. Mutated sequences were verified by sequence analysis.

Lentivirus preparation

Recombinant lentiviral particles were produced as described earlier [15] and references therein.

Cell culture

Primary cultures of rat hippocampal neurons were prepared from E19 embryos plated at a density of 60,000 cells/cm² as described previously [17]. Infection with lentivirus dilutions was performed six days after plating (div6). Fixation and immunocytochemistry was done at div14.

Pharmacological treatment of cultured hippocampal neurons

Treatment of cells was done as described earlier [15].

Immunocytochemistry

Immunocytochemistry was performed as described earlier [15]. For Ab-175 immunolabeling cultured neurons were washed once with PBS at 37°C and fixed with cold methanol (−20°C) for 10 min. Cells were incubated with the primary antibody dilutions of mouse monoclonal anti-gephyrin mAb7a [18] and rabbit anti-gephyrin Ab-175 [19] as described earlier [15]. Cells were incubated with the following additional antibodies: chicken anti-GABAAR gamma2-subunit antibody (1:100) (BD Transduction Labs, Cat. No.: 612076), rabbit anti-vesicular inhibitory amino acid transporter (VIAAT) antibody (1:2000) (Sigma, No.: V 5764), rabbit anti-CDK5 antibody (1:200) (Santa Cruz, No. sc-173). Images were taken with a Leica confocal TCS SP8 microscope using a 63× Plan-Apochromat (1.4 NA) objective. To evaluate the number of synaptic puncta, mAb7a and Ab-175 fluorescence images were processed as described earlier [15]. Proteins were separated on 4–12% polyacrylamide gradient SDS-gels (NuPAGE, Novex Bis-Tris mini gels, Invitrogen). Gels were stained with Coomassie blue as proof of equal protein loading, dried on a vacuum gel dryer and exposed to X-ray Kodak films.

Statistical analysis

Experimental data were evaluated of at least three independent experiments without knowledge of the experimental conditions used. Statistical analysis was performed using the GraphPad-prism IV software with non-parametric ANOVA followed by Tukey’s or Bonferroni’s multiple comparison tests, other data were analyzed with Student’s t-test. Data are shown as mean ± standard error of the mean (SE) or in the analysis of the in vitro 32P-phosphorylation assay as mean ± SD. Values of P<0.05 were considered as significant.

Results

Reduction of CDK5 expression in hippocampal neurons reduces a number of immunoreactive gephyrin puncta

To investigate the functional role of CDK5 for the phosphorylation of gephyrin clusters at GABAergic synapses of cultured hippocampal neurons, we first established a knockdown of CDK5 with three different shRNA expressing lentiviruses. After having established that these sequences efficiently reduced heterologously expressed, myc-tagged CDK5 in HEK293T cells (data not shown), the reduction of CDK5 expression by these shRNAs in cultured hippocampal neurons was analyzed by immunofluorescence microscopy. As control, a scrambled shRNA (mismatch) was used.

Cells infected by the CDK5-kd viruses - as indicated by GFP expression (Fig. 1) - revealed about 50% reduction in CDK5 expression (Fig. 1B). Neurons with reduced CDK5 expression (Fig. 1A’) showed a robust reduction of phosphorylated gephyrin puncta (Fig. 1C), as demonstrated with the phospho-specific antibody mAb7a [15], whereas a control shRNA (Fig. 1A”) did neither reduce CDK5 expression nor mAb7a staining of the infected cells. The quantitative analysis of mAb7a-specific immunoreactive puncta of three independent CDK5-specific shRNAs revealed a comparable reduction of gephyrin puncta with kd1 (knockdown1): 2.2±0.77 puncta/30 μm (mean ± SE), 1.58±0.43 puncta (mean ± SE) for kd2, and 2.24±0.39 puncta (mean ± SE) for kd3 compared to non-infected neurons: 8.47±0.74 puncta, or scrambled control shRNA (mm): 7.8±0.74 puncta, each per 30 μm dendrite (Fig. 1C). These data support the conclusion, that the CDK5 knockdown is functionally linked to a reduction in gephyrin cluster phosphorylation.

Reduction of CDK5 expression results in a reduced number of GABA<sub>A</sub> receptor γ2 clusters

To determine whether reduced number of phosphorylated gephyrin clusters detected with mAb7a at postsynaptic sites could affect the clustering of endogenous GABA<sub>A</sub> receptors, the
Figure 1. CDK5 knockdown virus infection results in reduced CDK5 expression and reduced numbers of phospho-gephyrin clusters in cultured hippocampal neurons. Hippocampal neurons (div14) were stained with anti-GFP antibodies to detect infected neurons (upper panel), with anti-CDK5 antibody to quantify CDK5 expression levels (middle panel) and with the phosphospecific anti-gephyrin mAb7a antibody (lower panel). (A) Non-infected cells; (A’) CDK5-knockdown; (A”) control shRNA (CDK5-mismatch). Neurons were infected with the indicated viruses at div6.
The subcellular localization of GABA\(_\alpha\) receptor \(\gamma\)\(_2\) subunits was investigated. GABA\(_\alpha\) receptors harboring this subunit are known to colocalize with and to depend on gephyrin for synaptic localization in hippocampal neurons \[20,21\].

As shown in Fig. 2A and 2B, infection of cultured hippocampal neurons with CDK5-kd2 resulted in a strong reduction of mAb7a-immunoreactive puncta. In addition, a reduction of GABA\(_\alpha\) receptor \(\gamma\)\(_2\)-immunoreactive puncta along dendrites could be seen, whereas the immunoreactivity within the soma was preserved. The quantification shows that the number of GABA\(_\alpha\) receptor \(\gamma\)\(_2\) clusters is significantly reduced (Fig. 2B) kd2: 4.64 ± 0.38 puncta/30 \(\mu\)m (mean ± SE) compared to 8.54 ± 0.41 (mean ± SE) puncta for control shRNA (mm) and 9.67 ± 0.45 puncta (mean ± SE) for non-infected hippocampal neurons. Moreover, the average fluorescence intensities of clusters were reduced upon CDK5 knockdown (Fig. 2C). Thus, a knockdown of CDK5 results in a reduced number and density of \(\gamma\)\(_2\)-containing GABA\(_\alpha\) receptor clusters in hippocampal neurons.

As a control for the specificity of the effect of CDK5 knockdown on GABA\(_\alpha\) receptor cluster number and density the immunofluorescence of the presynaptic marker vesicular inhibitory amino acid transporter (VIAAT) was analyzed. Although a small reduction of the number of VIAAT puncta could be observed in kd2 infected cells (Fig. 3A and B), the VIAAT puncta showed no significant changes within the proximal dendrites analyzed (Fig. 3B). The VIAAT and mAb7a double staining was also used to determine the number of VIAAT-apposed mAb7a-positive gephyrin clusters. As shown in Fig. 3, the CDK5 knockdown reduced the number of synaptic mAb7a puncta above a certain intensity threshold drastically. Hence, we conclude that CDK5 is specifically involved in the phosphorylation of synaptic gephyrin clusters.

**Figure 2.** CDK5 knockdown correlates with reduced numbers of GABA\(_\alpha\) receptor clusters containing \(\gamma\)\(_2\)-subunits. Hippocampal neurons (div14) were immunolabeled with anti GFP antibodies to detect infected neurons (upper panel), with phosphospecific anti gephyrin mAb7a antibody (middle panel), and with anti-GABA\(_\alpha\) receptors \(\gamma\)\(_2\)-subunit of (lower panel). (A) Non-infected cells; (A') CDK5-kd2 knockdown; (A'') control shRNA (CDK5-mismatch). Neurons were infected with the indicated viruses at div6. The scale bar represents 15 \(\mu\)m. (B) Quantification of the number of GABA\(_\alpha\) receptor \(\gamma\)\(_2\) puncta on three proximal dendritic segments of 30 \(\mu\)m. 20 or 18 cells from n = 3 independent cultures of control or mismatch neurons and 25 cells (69 dendrites) from n = 4 independent cultures of kd2-infected neurons, mean ± SE. ANOVA with post-hoc test, ***P < 0.001. (C) Quantification of the relative fluorescence intensities of GABA\(_\alpha\) receptor \(\gamma\)\(_2\) puncta. Mean ± SE. Each value was calculated from data from the same cell numbers as in B from n = 3 independent cultures. ANOVA with post-hoc test: ***P < 0.001, **P < 0.01.

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CDK5 inhibition results in a reduced number of mAb7a-positive gephyrin clusters and GABA_\text{\textsubscript{A}} \gamma_2 clusters

To confirm these conclusions in a different experimental approach and to demonstrate that CDKs' enzyme activity is involved, we inhibited CDK enzymes using the antagonists aminopurvalanol A (highest inhibitory activity against CDK 5) [22] or SU9516 (highest inhibitory activity against CDK 5) [22]. We inhibited CDKs for two or three days with 5 \text{mM} aminopurvalanol A or SU9516 in hippocampal neurons. As shown in Fig. 4, under these experimental conditions the number of gephyrin clusters detected with mAb7a was reduced from 17.6 ± 2.28 puncta/30 \text{\mu m} dendrite (mean ± SE) to 2.91 ± 1.53 puncta/30 \text{\mu m} and 1.0 ± 0.62 puncta/30 \text{\mu m} upon 2 or 3 days inhibition, respectively. Similarly, GABA_\text{\textsubscript{A}} receptor \gamma_2 puncta were reduced from 10.9 ± 0.97 puncta/30 \text{\mu m} dendrite (mean ± SE) to 4.33 ± 0.96 puncta/30 \text{\mu m} dendrite (Fig. 4B, 4C) after three days treatment with 5 \text{mM} aminopurvalanol A. Similar results were obtained with SU9516 inhibition (data not shown). These results support the conclusions from our CDK5 knockdown experiments, suggesting that CDKs' enzyme activity is crucial for gephyrin phosphorylation and clustering of \gamma_2-containing GABA_\text{\textsubscript{A}} receptors in hippocampal neurons.

Gephyrin phosphorylation is dependent on PP1/PP2a phosphatases

To investigate the removal of gephyrin phosphorylation seen upon kinase inhibition we performed experiments inhibiting PP1/PP2a phosphatases known to be involved in the regulation of phosphorylation of gephyrin [23]. As shown in Fig. 5A and 5B, the supplementation of the cell culture medium with 5 \text{mM} aminopurvalanol A for six hours before cell fixation reduced mAb7a immunoreactivity. As shown in Fig. 5B and 5E, the number of gephyrin clusters detected with mAb7a above a certain intensity threshold was reduced from 16.4 ± 1.70 puncta/30 \text{\mu m} dendrite (mean ± SE) to 4.87 ± 1.36 puncta/30 \text{\mu m} upon six hours inhibition. However, the simultaneous inhibition of CDKs with aminopurvalanol A (5 \text{mM}) and of PP1/PP2a phosphatases with okadaic acid (0.25 \text{mM}) for six hours abolished the reduction of mAb7a immunoreactivity observed by inhibiting CDK activity only (Fig. 5C, E). The inhibition of PP1/PP2a phosphatases with okadaic acid (0.25 \text{mM}) without kinase inhibition (six hours) resulted in an increase of somatic mAb7a immunoreactivity (Fig. 5D). However, alterations of presynaptic VIAAT staining were obvious (data not shown). Thus, these results indicate that the reduction of mAb7a immunoreactivity observed upon CDK inhibition is due to the activity of PP1/PP2a phosphatases.
suggesting that a given level of mAb7a immunoreactivity seen at inhibitory synapses or in the cell soma is the result of a certain balance of CDK kinases and PP1/PP2a phosphatase activities.

The phosphorylation of gephyrin is cell compartment specific

In order to study the level of mAb7a immunoreactivity and thus the phosphorylation of S270 more directly, we performed CDK activity recovery experiments. For this, we inhibited CDK5 with 5 μM SU9516 for 6 hrs, replaced the culture medium by conditioned medium from sister cultures to terminate CDK inhibition, thus allowing a putative increase of gephyrin phosphorylation for 1 or 2 hours. The incubation of cells was stopped by fixation and cells were double stained for gephyrin with Ab-175

Figure 4. Gephyrin and GABA<sub>A</sub> receptor γ2 puncta are reduced upon CDK5/2/1 inhibition. (A) Hippocampal neurons were double-immunolabeled for gephyrin with antibody mAb7a (red, upper panel) and with anti-VIAAT antibody (green, middle panel). Superposition of both immunolabelings (lower panel, merge). Neurons were fixed and immunolabeled at div16. (A) Control cells, non-treated; (A') cultures treated with aminopurvalanol A (5 μM) for 2 days; (A'') cultures treated with aminopurvalanol A (5 μM) for 3 days. Note a higher number of VIAAT-opposed gephyrin puncta (yellow, merge) under A, compared to A' and A''. The scale bar represents 15 μm. (B) Hippocampal neurons were double-immunolabeled for GABA<sub>A</sub> receptors with anti-γ2-subunit antibody (red, upper panel) and with anti-VIAAT antibodies (green, middle panel). Superposition of both immunolabelings (lower panel, merge). Neurons were fixed and immunolabeled at div16. (A) Control cells, non-treated; (A') cultures treated with aminopurvalanol A (5 μM) for 2 days; (A'') cultures treated with aminopurvalanol A (5 μM) for 3 days. Note a higher number of VIAAT-opposed γ2-subunit puncta (yellow, merge) under B, compared to B' and B''. (C) Quantification of the number of gephyrin (mAb7a) puncta. Quantification was done with about 10 cells from 4 independent cultures. Mean ± S.E.; ANOVA with post-hoc test: * P<0.05. (D) Quantification of the number of GABA<sub>A</sub> receptors with anti-γ2-subunit antibody. Mean ± S.E. Quantification was done with 49 cells from n=4 independent cultures (control), 34 cells from n=3 independent cultures (2 days) and 18 cells from n=3 independent cultures (3 days). ANOVA with post-hoc test: ** P<0.01.

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and mAb7a. Next, relative protein levels of somatic and dendritic gephyrin clusters were estimated by quantification of Ab-175 immunofluorescence intensities in focal sections of stained cells by confocal microscopy. The same focus plane and cluster regions were also analyzed for relative fluorescence intensities of mAb7a immunofluorescence (Fig. 6 A–D). The ratio of mAb7a intensities compared to Ab-175 intensities were calculated for each cluster and a total of about 660 gephyrin clusters (52 to 118 for the single conditions) of nine cells from three different cell cultures were analyzed. The results indicate that the relative amount of gephyrin phosphorylation at different gephyrin clusters is different. Interestingly mAb7a/Ab175 relative fluorescence intensities were not randomly distributed from somatic to dendritic gephyrin clusters. However, the relative phosphorylation of gephyrin clusters revealed to be higher at somatic clusters compared to dendritic clusters (Fig 6E). The comparison of the mAb7a/Ab175 ratio after SU9516 treatment showed a robust reduction of phosphorylation. After medium exchange and incubation of cells in an incubator for one or two hours, kinase activity leading to an increase of mAb7a immunoreactivity was clearly evident, however, it did not reach the level of control cells (Fig 6E). Thus, the level of gephyrin phosphorylation by CDK5 might be under distinct regulation in different subcellular compartments.

Gephyrin is phosphorylated by CDK5 at serine residue S270 in vitro.

Previous work has shown that gephyrin can be phosphorylated in vitro at serine and/or threonine residues by endogenous protein kinases [24]. Moreover, S270 was identified as one putative phosphorylation site sensitive to the phospho-specific antibody mAb7a [15]. To verify whether S270 of gephyrin can serve as a CDK5 phosphorylation site, purified 6xHis-tagged wild type and mutant gephyrin^S270A was subjected to in vitro kinase assays using CDK5/p25 and [γ-32P] ATP. Wild type gephyrin was labeled strongly with 32P after incubation with CDK5/p25 (Fig. 7). Importantly, gephyrin mutant gephyrin^S270A revealed a robust reduction in 32P-incorporation (Fig. 7A, B), suggesting that this site is phosphorylated by CDK5 in wild type gephyrin.

Discussion

We report the following major findings: First, CDK5 activity is involved in the phosphorylation of gephyrin detected with the phospho-specific antibody mAb7a. Second, CDK5 is important for the stability of GABA<sub>A</sub> receptor clusters in hippocampal neurons. Third, gephyrin is dephosphorylated by PP1/PP2A antagonizing the CDK5-dependent phosphorylation. Fourth, the relative level of phosphorylation detected with mAb7a is cell
Figure 6. **S270 phosphorylation is different at different synapses.** Relative fluorescence intensities of double labelling experiments using the pan-gephyrin antibody Ab-175 and the phospho-specific antibody mAb7a were measured in focal planes of confocal microscopy images. (A) Focal plane showing dendritic gephyrin clusters detected with Ab-175 or (B) with the phospho-specific mAb7a antibody. The scale bar represents 10 μm. (A’, B’, E) Upon treatment with CDK5 inhibitor SU9516 (5 μM) for six hours the phosphorylation of gephyrin is significantly reduced. (A’’) After replacement of medium with SU9516 by conditioned medium without inhibitor, cells were fixed and stained after a “recovery” time of two hours.
Note increasing phosphorylation as indicated by mAb7a staining seen in B‘ compared to B‘. The same analysis in C, C‘, C′, and D, D’, D‘, D’‘ for “somatic” gephyrin clusters, revealing decrease and increase of gephyrin phosphorylation upon SU9516 treatment and “recovery”, respectively (D‘ compared to D‘). (E) Quantification of mAb7a-specific phosphorylation comparing the fluorescence intensities of single clusters stained with mAb7a and Ab175 (ratios). c.d: control dendritic clusters; c.s: control somatic clusters; i.d: CDK5 inhibitor treatment for 6 h, dendritic clusters; i.s: CDK5 inhibitor treatment for 6 h, somatic clusters; 1d: 1 hour recovery from inhibition, dendritic clusters; 1s: 1 hour recovery from inhibition, somatic clusters; 2d: 2 hour recovery from inhibition, dendritic clusters; 2s: 2 hour recovery from inhibition, somatic clusters. Quantification was done with 660 clusters (total) from nine cells from three (n = 3) independent cultures. Mean ± SE. ANOVA with post-hoc test: ** P < 0.01.
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Figure 7. Substitution of S270 by alanine reduces phosphorylation of gephyrin by CDK5/p25. (A) Coomassie blue staining of SDS-PAGE gels (left panel) to control for equal input amounts of wild type 6xHis-gephyrin (wt) and S270A mutant proteins and representative autoradiogram (right panel) of wt and S270A mutant proteins subjected to in vitro kinase assay with recombinant CDK5/p25 in the presence of [γ-32P] ATP and separated with SDS-PAGE. (B) Quantification of band intensities of autoradiograms shown in A of three independent experiments (n = 3). Mean ± SD. Student’s t-test, *P < 0.05.

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CDK5 Determines Gephyrin Phosphorylation

CDK5 is a member of the cyclin-dependent kinase family that is widely expressed in the nervous system. Its activity is regulated by the expression of its regulatory subunits, such as p55 and p25. In hippocampal neurons, CDK5 plays a role in the phosphorylation of gephyrin, a protein that forms a complex with GABAA receptors to cluster postsynaptic channels. The phosphorylation of gephyrin at specific sites, such as serine 270, is thought to regulate the formation and distribution of GABAA receptor clusters. This study aimed to investigate the role of CDK5 in gephyrin phosphorylation.

The first part of the study showed that CDK5 knockdown or inhibition by pharmacological treatment decreased the number and fluorescence intensity of mAb7a immunoreactive puncta in hippocampal neurons. These findings suggest that CDK5 plays a functional role in gephyrin phosphorylation at postsynaptic compartments.

To verify whether gephyrin serves as a CDK5 substrate, the study used RNA interference to target CDK5, as one candidate kinase, and evaluated its effect on gephyrin phosphorylation. Immunoblotting with a phospho-specific antibody revealed a reduction of gephyrin phosphorylation at S270 upon CDK5 knockdown or pharmacological inhibition.

The study also investigated whether other substrates of CDK5 may be involved in gephyrin phosphorylation. Pharmacological inhibition of cyclin-dependent kinases, including CDK5, strongly reduced upon CDK5 knockdown or inhibition with aminopurvalanol A or SU9516 for three days coincides with a reduced number of mAb7a immunoreactive puncta. These results may help to resolve the discrepancy in the literature concerning the role of CDK5 in gephyrin phosphorylation.

Finally, the study showed that gephyrin can be phosphorylated by CDK5 at position S270 in vitro. The phosphorylation of this site is strongly reduced upon CDK5 knockdown and pharmacological inhibition.

In conclusion, this study provides evidence that CDK5 plays a functional role in gephyrin phosphorylation at postsynaptic compartments. These findings may help to resolve the discrepancy in the literature concerning the role of CDK5 in gephyrin phosphorylation and point to the involvement of other substrates of CDK5 in gephyrin phosphorylation.
unknown sites, which, together might determine gephyrin conformation, gephyrin protein interactions and gephyrin cluster formation and stability. For example, gephyrin proline-directed phosphorylation at S186, S194 and S200 was shown to be important for the recruitment of the peptidyl-prolyl isomerase Pin1. The deletion of the entire proline-rich domain or point mutations at S186, S194 and S200 residues almost completely ablated Pin1 binding to gephyrin and Pin1−/− mice revealed reduced glycine receptor cluster numbers [32].

Other studies have also shown that gephyrin is phosphorylated on several serine and threonine residues, mostly in the central region (C-domain) [31–34]. In particular GSK3β was shown to phosphorylate S270, thereby decreasing the number of gephyrin clusters [33]. In addition, a neighboring serine at position 268 was also shown to reduce cluster formation upon phosphorylation by ERK1 [34]. One possible explanation for these discrepancies could be that these sites might be phosphorylated by different kinases and a specific combinatorial pattern of phosphorylations might have divergent functional consequences. Moreover, GSK3β-mediated phosphorylation of gephyrin might be primed by CDK5, thus explaining that the phosphorylation at S270 is dependent on both, CDK5 and GSK3β activity.

Posttranslational modification of receptor subunits is another mechanism to determine the formation of postsynaptic assemblies of ligand-gated ion channels and scaffold proteins. For example, protein kinase C was shown to regulate the glycinic receptor (GlyR) diffusion properties and gephyrin interaction by phosphorylation. The membrane insertion and turnover of different GABAAR receptor subunits are regulated by phosphorylation of subunits [36]. For example, S383 within the β3 subunit is phosphorylated by Ca2+ calmodulin-dependent protein kinase II, resulting in a rapid insertion of GABAAR receptors in the cell membrane [37]. Moreover, GABAAR receptor γ subunits are phosphorylated at Y365 and Y367, reducing receptor internalization and increasing tonic inhibition [38]. Thus, CDK5-dependent alteration of receptor subunit phosphorylation might contribute to GABAAR receptor cluster formation or stability independent of gephyrin phosphorylation.

In spinal cord neurons, gephyrin clustering is regulated by GlyR activity [39]. More recently, integrin signaling was identified to determine GlyR and gephyrin trafficking at synapses [40]. In addition, BDNF receptor signaling was shown to determine gephyrin clustering [41]. Thus, these surface signaling molecules might be active upstream of CDK5 [9] as a putative regulator of gephyrin and/or GABAAR receptor clustering at inhibitory synapses.

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Author Contributions

Conceived and designed the experiments: J. Kuhse HK J. Kirsch.
Performed the experiments: HK AS J. Kuhse. Analyzed the data: HK J. Kuhse. Wrote the paper: J. Kuhse HK.

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