CdK5 Phosphorylation of Munc18-1 Enhances Synaptic Transmission

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Abstract

Cyclin-dependent kinase 5 (Cdk5) functions in many neuronal processes and has recently been implicated in homeostatic plasticity. Cdk5 activation results in reallocation of synaptic strength, including weakening of some connections while strengthening others. The molecular mechanisms underlying these phenomena are thus far not understood. We show that Cdk5 phosphorylation of the presynaptic protein Munc18-1 plays an important role in the regulation of synaptic strength. In the absence of phosphorylation, neurons show reduced spontaneous and evoked release, RRP size and release probability. Hence, Cdk5 phosphorylation of Munc18-1 does not underlie Cdk5-dependent silencing of synapses, but may play a role in neuronal silencing induced increase of presynaptic strength.
Introduction
Cyclin-dependent kinase 5 (Cdk5), a proline-directed serine/threonine kinase, is highly expressed in postmitotic neurons (Cruz and Tsai, 2004; Meyerson et al., 1992; Tsai et al., 1993). Activated by small regulatory proteins p35 and p39, Cdk5 functions in many neuronal processes, ranging from neuronal development and outgrowth (Ko et al., 2001; Nikolic et al., 1996; Ohshima et al., 1996), neurogenesis (Lagace et al., 2008), protein trafficking (Samuels et al., 2007), synaptic transmission (Kim and Ryan, 2010), addiction (Meyer et al., 2008; Takahashi et al., 2005; Taylor et al., 2007) and learning (Fischer et al., 2005; Fischer et al., 2002, 2003; Hawasli et al., 2007; Sananbenesi et al., 2007). In recent studies, Cdk5 has been implicated in homeostatic plasticity (Kim and Ryan, 2010; Mitra et al., 2011). Homeostatic plasticity commonly describes cellular processes by which a neuron regulates its synaptic strength to stabilize network activity (Davis, 2006; Pozo and Goda, 2010; Turrigiano, 2011). Cdk5 activity increases upon presynaptic neuronal silencing (Mitra et al., 2011). Increased Cdk5 activity upon chronic inhibition results in non-uniform homeostatic adaptation of synaptic strength: On the one hand, Cdk5 activity decreases functional connectivity between CA3-CA3 pyramidal cells without obvious loss of synapses. On the other hand, synaptic strength is increased in the remaining recurrent connections. Whereas loss of connectivity could be explained by Cdk5-dependent silencing of synapses and reserve pool modulation (Chergui et al., 2004; Kim and Ryan, 2010), the mechanisms underlying increased synaptic strength upon Cdk5 activation remain entirely unknown. Although many presynaptic targets of Cdk5, including Synapsin (Matsubara, 1996), Septin 5 (Amin et al., 2008), CASK (Samuels et al., 2007), CRMP2 (Cole et al., 2008), Munc18-1 (Shuang et al., 1998) and the CDK5-like kinase Pctaire (Cheng et al., 2002; Fu et al., 2011; Liu et al., 2006), have been described, the effect of their Cdk5 phosphorylation on neurotransmitter release mostly remains elusive. Hence, it is currently not understood which downstream targets mediate the effect of Cdk5-dependent reallocation of synaptic strength upon presynaptic neuronal silencing.
Here, we show that Cdk5 phosphorylation of the presynaptic protein Munc18-1 regulates synaptic strength. Munc18-1, a member of the SM protein family is essential for synaptic vesicle fusion and functions in
various steps of the synaptic vesicle cycle (Toonen and Verhage, 2007; Verhage et al., 2000). Cdk5 phosphorylates Munc18-1 at threonine 574 (T574) (Shuang et al., 1998). This phosphorylation underlies Cdk5’s release enhancing effect in secretory cells (Chergui et al., 2004; Fletcher et al., 1999; Lilja et al., 2001), but it’s role in neurons remains elusive.

We investigated the role of Munc18-1 phosphorylation by Cdk5 in munc18-1 null mutant hippocampal neurons infected with either non-phosphorylatable Munc18 T574A or Munc18WT as control. Inhibiting Cdk5 phosphorylation of Munc18-1 does not significantly influence neuronal morphology, but results in a strong decrease of both spontaneous and evoked release. Munc18 T574A neurons show paired-pulse facilitation and slower rundown kinetics during action potential trains. Hence, Cdk5 phosphorylation of Munc18-1 enhances synaptic vesicle release and might underlie the Cdk5-dependent strengthening of synapses after presynaptic inhibition.

Results

To investigate the role of Munc18-1 phosphorylation by Cdk5 in neurons, we infected hippocampal autaptic neurons from munc18-1 null mutant mice with Lentiviruses expressing either the non-phosphorylatable Munc18-1 variant Munc18 T574A (M18 T574A) or Munc18WT (M18WT) as control and studied their electrophysiological properties.

Cdk5 phosphorylation of Munc18-1 enhances evoked and spontaneous release.

Neurons rescued with M18 T574A showed a significantly smaller evoked postsynaptic current (EPSC) amplitude compared to neurons rescued with M18WT (Figure 4.1A-B). To distinguish between pre- and postsynaptic defects underlying the changes in EPSC amplitude, we investigated the effects of Cdk5 phosphorylation of Munc18-1 on spontaneous release (Figure 4.1C). Similarly to EPSC size, spontaneous miniature EPSC frequency was lower in M18 T574A (Figure 4.1D), while mEPSC amplitudes and kinetics were unchanged (Figure 4.1E-G). Hence, Cdk5 phosphorylation of Munc18-1 promotes spontaneous and evoked release by a presynaptic mechanism in hippocampal neurons.
Figure 4.1 Cdk5 phosphorylation of Munc18-1 enhances evoked and spontaneous synaptic vesicle release.

Hippocampal autaptic neurons of munc18-1 null mutant mice were rescued with Lentiviruses expressing M18WT or M18T574A. (A) Amplitude of excitatory synapse response evoked by an action potential \((M18_{WT}: 2.09 \pm 0.20\text{nA}, n=65; M18_{T574A}: 1.44 \pm 0.16\text{nA}, n=79; **, Mann-Whitney test, p=0.005)\). (B) Typical example of evoked release. Scale bar represents 10ms and 0.5nA. (C) Typical example of spontaneous vesicle release. Scale bar represents 0.5s and 40pA. (D) Spontaneous release frequency \((M18_{WT}: 3.41 \pm 0.78\text{Hz}, n=57; M18_{T574A}: 11.71 \pm 0.45\text{Hz}, n=82; **, Mann-Whitney test, p=0.001)\). (E) Amplitude of spontaneous release events \((M18_{WT}: 16.36 \pm 0.90\text{pA}, n=52; M18_{T574A}: 15.36 \pm 0.64\text{pA}, n=61; \text{ns., Mann-Whitney test, p}>0.05)\). (F) Mean rise time of spontaneous release events \((M18_{WT}: 1.34 \pm 0.08\text{ms}, n=52; M18_{T574A}: 1.14 \pm 0.06\text{ms}, n=61; \text{ns., Mann-Whitney test, p}=0.071)\). (G) Mean decay time of spontaneous release events \((M18_{WT}: 1.60 \pm 0.05\text{ms}, n=27; M18_{T574A}: 1.53 \pm 0.06\text{ms}, n=41; \text{ns., Mann-Whitney test, p}>0.05)\). All data are expressed as mean ± SEM.

Changes in neuronal morphology do not account for changes in neurotransmitter release observed in Munc18T574A neurons.

To investigate whether the observed changes in neurotransmitter release are caused by underlying changes in neuronal morphology, autaptic neurons were fixed and immunostained for MAP2 as dendrite marker and VAMP2 as synapse marker (Figure 4.2A). No significant changes in
Figure 4.2 Neuronal morphology cannot account for the electrophysiological effects of Cdk5 phosphorylation of Munc18.

Hippocampal autaptic neurons of munc18-1 null mutant mice were rescued with Lentiviruses expressing M18\textsubscript{WT} or M18\textsubscript{T574A} and stained for the dendritic marker MAP2 (red) and synapse marker VAMP2 (green). (A) Typical examples.

Scale bar represents 50μm. (B) Mean dendrite length per cell (M18\textsubscript{WT}: 1336 ± 60μm, n=38; M18\textsubscript{T574A}: 1497 ± 83μm, n=43; ns., Mann-Whitney test, p>0.05).

(C) Number of dendritic branches as a function of radial distance from the soma quantified by Sholl analysis.

(D) Mean synapse number per cell (M18\textsubscript{WT}: 250 ± 15, n=38; M18\textsubscript{T574A}: 307 ± 27, n=43; ns., Mann-Whitney test, p>0.05).

(E) Number of synapses as a function of radial distance from the soma. Significance was tested by Mann-Whitney test at the following distances: 0μm: ***, p<0.001; 10μm: *, p=0.025; 20μm: ns., p>0.05; 30μm: ns., p>0.05; 40μm: ns., p>0.05; 50μm: ns.
Cdk5 phosphorylation of Munc18-1 influences RRP size

The amount of neurotransmitter release is dependent on the size of the readily releasable pool (RRP) and the release probability of an individual vesicle within that pool ($P_{ves}$). To distinguish between the two, we probed the RRP by application of 500mM hyperosmotic sucrose solution (Rosenmund and Stevens, 1996). RRP$_{sucrose}$ size in M18$_{T574A}$ neurons was smaller compared to M18$_{WT}$ neurons (Figure 4.3A-B). Vesicular release probability calculated as quotient of EPSC and RRP$_{sucrose}$ charge was not significantly changed even though a trend towards lower release probability in M18$_{T574A}$ neurons was observed (Figure 4.3C). Alternatively, one can deplete the RRP by a high frequency train of action potentials (100 APs at 40Hz). We plotted cumulative EPSC charge, fitted the last 20 APs (steady state) with linear regression and back-extrapolated to the time point 0.
Figure 4.3 Cdk5 phosphorylation of Munc18-1 affects sucrose pool.
Hippocampal autaptic neurons of munc18-1 null mutant mice were rescued with Lentiviruses expressing M18<sub>WT</sub> or M18<sub>T574A</sub>. (A) Typical response to a single hyperosmotic sucrose application (500mM, 3.5s). Scale bar represents 0.2nA and 1s. (B) Mean RRP size as assessed with 500mM sucrose application (M18<sub>WT</sub>: 0.90 ± 0.09nC, n=21; M18<sub>T574A</sub>: 0.65 ± 0.08nC, n=21; *, Mann-Whitney test, p=0.039). (C) Vesicular release probability P<sub>v</sub> calculated as quotient of EPSC charge by sucrose triggered charge (M18<sub>WT</sub>: 2.1 ± 0.3%, n=21; M18<sub>T574A</sub>: 1.6 ± 0.4%, n=21; ns., Mann-Whitney test, p>0.05). (D) Cumulative plot of the total EPSC charge during a 40 Hz train (100APs). Yellow lines indicate back-extrapolation from the steady-state phase (AP 80-100) to y-axis intercept, which can be used as a RRP estimate. (E) RRP estimate as measured with back-extrapolation of cumulative total charge from 40Hz train (M18<sub>WT</sub>: 138.04 ± 17.15pC, n=32; M18<sub>T574A</sub>: 192.24 ± 33.33pC, n=35; ns., Mann-Whitney test, p>0.05). (F) Slope of 40Hz cumulative charge (M18<sub>WT</sub>: 170.53 ± 28.49pC/s, n=32; M18<sub>T574A</sub>: 238.33 ± 52.91pC/s, n=35; ns., Mann-Whitney test, p=0.002). All data are expressed as mean ± SEM.

The intercept on the Y-axis can be used to estimate the initial RRP<sub>APS</sub> size (Schneggenburger et al., 1999). RRP<sub>APS</sub> size was not significantly different between M18<sub>T574A</sub> and M18<sub>WT</sub> neurons (Figure 4.3D-E). Furthermore, RRP refilling, as measured by the slope of the cumulative plot, was similar in both genotypes (Figure 4.3F). Thus, phosphorylation of Munc18-1 is needed to set RRP size but Ca<sup>2+</sup>-dependent processes can rescue the initial reduction of RRP size in M18<sub>T574A</sub> neurons.
Figure 4.4 Cdk5 phosphorylation of Munc18-1 sets release probability.

Hippocampal autaptic neurons of munc18-1 null mutant mice were rescued with Lentiviruses expressing M18\textsubscript{WT} or M18\textsubscript{T574A}. (A) Paired pulse ratio (ratio of the second to the first synaptic response plotted as a function of interstimulus interval) (n=33 (M18\textsubscript{WT}), n=37 (M18\textsubscript{T574A})). Mann-Whitney tests: PP(20ms): *, p=0.034; PP(50ms): *, p=0.020; PP(100ms): ns., p=0.056; PP(200ms): ns., p>0.05; PP(500ms): ns., p>0.05; PP(1000ms): *, p=0.011). (B) Typical examples of paired pulse responses of a M18\textsubscript{WT} and M18\textsubscript{T574A} neuron. Scale bars represent 0.5nA and 100ms. (C) EPSC amplitudes during a 5Hz train normalized to first EPSC (100 APs). Insert shows zoom of the first 10 APs (n=25 (M18\textsubscript{WT}), n=34 (M18\textsubscript{T574A}). (D) EPSC amplitudes during a 10Hz train normalized to first EPSC (100
Cdk5 phosphorylation of Munc18-1 sets release probability.
To investigate the role of Cdk5 phosphorylation of Munc18-1 on release probability, we stimulated cells with paired pulses (PP) of varying interstimulus intervals ranging from 20 to 1000ms. M18\textsubscript{T574A} neurons showed paired-pulse facilitation compared to M18\textsubscript{WT} neurons, arguing for a lower initial synaptic release probability in those neurons (Figure 4.4A-B). In line with the observed paired-pulse facilitation, rundown kinetics during action potential trains at 5Hz were slower in M18\textsubscript{T574A} neurons (Figure 4.4C). However, no significant differences were observed in rundown kinetics after higher frequency (10Hz or 40Hz) stimulation (Figure 4.4D-E). Furthermore, both synchronous and asynchronous release components scaled similarly with the effect on EPSC size (Figure 4.4F). Hence, Cdk5 phosphorylation is a determinant of synaptic release probability and short-term plasticity, but does not affect the ratio of synchronous and asynchronous release components.

Cdk5 phosphorylation of Munc18-1 increases synaptic Munc18-1 and Syntaxin-1 protein levels
Synaptic strength strongly correlates with Munc18-1 protein levels (Toonen et al., 2006). To investigate whether the observed electrophysiological changes in the Cdk5 phosphorylation deficient variant of Munc18-1 could be explained by changes in Munc18-1 protein levels, we immunostained neurons for Munc18-1 together with the dendritic marker MAP2 and the synapse marker VAMP2. No significant changes in synaptic Munc18-1 levels were observed between M18\textsubscript{T574A} and M18\textsubscript{WT} neurons (Figure 4.5A-B). Protein levels of syntaxin are known to strongly depend on the presence of its binding partner Munc18-1 (Verhage et al., 2000). In addition, Cdk5 phosphorylation of Munc18-1 decreases the affinity for monomeric Syntaxin in secretory cells (Fletcher et al., 1999; Shuang et al., 1998). To investigate the effect of a lack of Cdk5 phosphorylation of Munc18-1 on the transport of syntaxin, we immunostained neurons for...
Figure 4.5
Cdk5 phosphorylation of Munc18-1 influences synaptic Munc18-1 and syntaxin protein levels.

Hippocampal neurons of munc18-1 null mutant mice were rescued with Lentiviruses expressing M18\textsubscript{WT} or M18\textsubscript{T574A} and were immunostained for MAP2, VAMP2 and Munc18-1 or Syntaxin-1. (A) Mean synaptic Munc18-1 intensity (M18\textsubscript{WT}: 1232 ± 127, n=38; M18\textsubscript{T574A}: 1148 ± 622, n=37; ns., Mann-Whitney test, p>0.05). (B) Cumulative probability plot of synaptic Munc18-1 intensities. (C) Mean synaptic Munc18-1 intensity as a function of radial distance from the soma. (D) Mean synaptic syntaxin intensity (Munc18\textsubscript{WT}: 582 ± 102, n=7; Munc18\textsubscript{T574A}: 588 ± 65, n=11; ns., Mann-Whitney test, p>0.05). (E) Cumulative probability plot of synaptic syntaxin intensities. (F) Mean synaptic syntaxin intensity as a function of radial distance from the soma. All data are expressed as mean ± SEM.
syntaxin and analyzed its amount and localization. In line with Munc18-1 levels, synaptic Syntaxin-1 levels were similar in M18<sub>T574A</sub> and M18<sub>WT</sub> neurons (Figure 4.5D-E). Furthermore, we did not observe any distance dependence of Munc18-1 and Syntaxin-1 localization (Figure 4.5C,F). Thus, Syntaxin-1 levels highly correlate with Munc18-1 levels and both proteins are trafficked independently of Cdk5 phosphorylation of Munc18-1.

**Discussion**

This study shows that in hippocampal neurons Cdk5 phosphorylation of Munc18-1 is important for synaptic transmission. Neurons in which Cdk5 cannot phosphorylate Munc18-1 have normal neuronal morphology, but show a strong reduction in evoked and spontaneous release. Lack of phosphorylation decreases RRP size, release probability and influences short-term plasticity. Levels and localization of Munc18-1 or its binding partner Syntaxin-1 are independent of Cdk5 phosphorylation.

**Cdk5 phosphorylation of Munc18-1 enhances release in neurons and secretory cells**

Our data in hippocampal neurons are in line with the phenotype observed in secretory cells where Cdk5 promotes vesicle fusion via a Munc18-dependent mechanism (Chergui et al., 2004; Fletcher et al., 1999; Lilja et al., 2001). In pancreatic beta cells, the non-phosphorylatable variant Munc18<sub>T574A</sub> cannot support Cdk5-dependent enhancement of secretion (Lilja et al., 2004). In addition, overexpression of Munc18<sub>T574A</sub> in bovine adrenal chromaffin cells significantly slows fusion kinetics. In secretory cells, Cdk5 phosphorylation of Munc18 lowers its affinity to monomeric Syntaxin-1 (Fletcher et al., 1999; Shuang et al., 1998). Although not affecting Syntaxin-1 levels and localization in neurons, Cdk5 phosphorylation of Munc18-1 may dissociate Munc18-1 from closed Syntaxin-1, allowing Syntaxin-1 to participate in SNARE complex formation and therewith enhance vesicle fusion. Cdk5 phosphorylation thus changes intrinsic molecular properties of Munc18-1 that enhance vesicle fusion independent of the cell-type used.
Munc18-1 as presynaptic target in Cdk5-dependent regulation of synaptic strength

Cdk5 is activated upon presynaptic neuronal silencing resulting in differential regulation of synaptic strength in recurrent networks. Whereas some CA3-CA3 neuron pairs loose their functional connectivity upon TTX treatment, other synapses are strengthened (Mitra et al., 2011). Loss of connectivity upon chronic activity deprivation might be explained by Cdk5 mediated synaptic silencing and restriction of recycling pool size (Kim and Ryan, 2010) or phosphorylation of P/Q-type calcium channels (Tomizawa et al., 2002). In the remaining active synapses, the enhanced synaptic strength is due to a marked increase in release probability (Mitra et al., 2011). Though in different synapses, both loss of connectivity and increase in synaptic strength are mediated by Cdk5 activation. So far, the exact molecular mechanisms and presynaptic Cdk5 targets involved are missing. Here, we propose a role for Cdk5 phosphorylation of Munc18-1 in the regulation of synaptic strength and exclude Munc18-1 as a major target involved in presynaptic silencing. Neurons expressing non-phosphorylatable Munc18\textsubscript{T574A} show smaller RRP\textsuperscript{sucrose} and reduced release probability, leading to decreased spontaneous and evoked release. In line with the effect on release probability, action potential trains at 5Hz have slower rundown kinetics in the absence of Cdk5 phosphorylation. In contrast, rundown kinetics at higher frequency trains (10Hz or 40Hz) or RRP\textsubscript{APS} were more similar in the two genotypes. This might be due to the fact that our measurements were performed in autaptic neurons. These neurons are grown in complete isolation and are thus devoid of network activity, arguing for initial high Cdk5 activity. Ca\textsuperscript{2+}-dependent activation of phosphatases might lead to dephosphorylation of Munc18-1 upon high frequency stimulation. The balance between Cdk5 and the phosphatase calcineurin has already shown to be important for the regulation of recycling pool size (Kim and Ryan, 2010). Likewise, Cdk5-dependent regulation of synaptic strength might also be influenced by the equilibrium between kinase and phosphatase activities.

Heterogeneous effect of Cdk5 mediated homeostatic adaptation

Cdk5 activation upon chronic activity deprivation triggers loss of connectivity or increased synaptic strength. The differential effects in different synapse might be explained by independent mechanisms depending on expression
and relative amounts of Cdk5 targets. We identify Munc18-1 as one important target in Cdk5-mediated increase in synaptic strength, while other presynaptic Cdk5 targets must exist to mediate Cdk5’s effects on synaptic silencing and/or recycling pool size. Presynaptic protein levels, and Munc18-1 is no exception, are highly variable between synapses (SK Schmitz, unpublished observation), as has been observed for release probability (Branco and Staras, 2009). The amounts of Munc18-1 relative to one or multiple other Cdk5 targets might therefore determine the combinatorial outcome of Cdk5 activity on functional connectivity.

Munc18’s involvement in epilepsy
Recently, Munc18-1 has been linked to early-infantile epilepsy syndromes, such as Ohtahara syndrome and West syndrome (Saitsu and Matsumoto, 2011). One patient carries a mutation in STXBP1 gene (1720A>C) that leads to an amino acid change in the Cdk5 phosphorylation site on Munc18-1 (574T>P). The patient suffered from seizures from the first day of life until the age of 1, displayed tremor, jerks stereotypic movements and ataxic walk. Furthermore, the patient showed autistic features and was unable to speak (Milh et al., 2011). Due to the mutation, Cdk5 cannot phosphorylate Munc18 at T574. We identified Munc18-1 as major Cdk5 target that contributes to the regulation of synaptic strength. It is tempting to speculate that dysfunctional homeostatic adaptation due to the lack to Munc18-1 phosphorylation by Cdk5 underlies the disease etiology.

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