The effect of Cyclin-dependent kinase 5 on voltage-dependent calcium channels in PC12 cells varies according to channel type and cell differentiation state

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Abstract

Cyclin-dependent kinase 5 (Cdk5) is a Ser/Thr kinase that plays an important role in the release of neurotransmitter from pre-synaptic terminals triggered by Ca\(^{2+}\) influx into the pre-synaptic cytoplasm through voltage-dependent Ca\(^{2+}\) channels (VDCCs). It is reported that Cdk5 regulates L-, P/Q-, or N-type VDCC, but there is conflicting data as to the effect of Cdk5 on VDCC activity. To clarify the mechanisms involved, we examined the role of Cdk5 in regulating the Ca\(^{2+}\)-channel property of VDCCs, using PC12 cells expressing endogenous, functional L-, P/Q-, and N-type VDCCs. The Ca\(^{2+}\) influx, induced by membrane depolarization with high K\(^+\), was monitored with a fluorescent Ca\(^{2+}\) indicator protein in both undifferentiated and nerve growth factor (NGF)-differentiated PC12 cells. Overall, Ca\(^{2+}\) influx was increased by expression of Cdk5-p35 in undifferentiated PC12 cells but suppressed in differentiated PC12 cells. Moreover, we found that different VDCCs are distinctly regulated by Cdk5-p35 depending on the differentiation states of PC12 cells. These results indicate that Cdk5-p35 regulates L-, P/Q-, or N-type VDCCs in a cellular context-dependent manner.

Keywords: calcium, cameleon, cyclin-dependent kinase 5, nerve growth factor, PC12 cell, voltage-dependent calcium channel.

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Abbreviations used: CaMKII, Ca\(^{2+}\)/calmodulin-dependent kinase II; Cdk, cyclin-dependent kinase; DIV, days in vitro; FRET, fluorescence resonance energy transfer; knCdk5, kinase-negative cyclin-dependent kinase 5; LTD, long-term depression; LTP, long-term potentiation; NGF, nerve growth factor; VDCC, voltage-dependent calcium channel; YC-Nano, yellow cameleon-Nano.

Synaptic transmission is initiated in the pre-synaptic regions by Ca\(^{2+}\) influx into the cytoplasm through voltage-dependent Ca\(^{2+}\) channels (VDCCs) in response to depolarization, resulting in neurotransmitter release (Catterall and Few 2008). There are five types of VDCCs, the L-, P/Q-, N-, R-, and T-types (Catterall 2011), all of which are expressed in neurons. In particular, P/Q- and N-type VDCCs are enriched in the pre-synaptic regions and are involved in neurotransmitter release (Reid et al. 2003). However, while it is known that the VDCCs are a critical initiator of synaptic transmission and that their activity is regulated at several levels including gene expression, localization, interaction with regulatory proteins, and post-translational modification, the precise mechanisms of this regulation are not yet fully understood.

Cyclin-dependent kinase 5 (Cdk5) is a proline-directed Ser/Thr protein kinase activated by the binding of either a p35 or p39 regulatory subunit (Dhavan and Tsai 2001; Hisanaga and Endo 2010). It plays an important role in a variety of neuronal activities including neuronal migration during brain development (Ohshima et al. 1996; Chae et al. 1997), synaptic signaling (Lai and Ip 2009; Utreras et al. 2009), survival, and neuron death (Cruz and Tsai 2004). Cdk5 is also involved in synaptic activity (Hawasli and Bibb 2007; Lai and Ip 2009) as demonstrated by its effect on induction of long-term potentiation and long-term depression in p35\(^{-}\) mouse hippocampus slices (Ohshima et al. 2005; Wei et al. 2005b). Furthermore, conditional knockout of Cdk5 in the adult mouse brain also enhances hippocampal...
long-term potentiation and NMDA receptor-mediated excitatory post-synaptic currents (Hawasaki et al. 2007), while over-activation of Cdk5 by p25, the N-terminal truncation fragment of p35, increases memory formation and decreases memory extinction (Fischer et al. 2005). We have shown that the excitatory neurotransmitter glutamate down-regulates Cdk5 activity in cortical neurons by stimulating p35 degradation (Wei et al. 2005b), which has been shown to enhance Ca^{2+}/calmodulin-dependent kinase II activity (Hosokawa et al. 2006). Based on these findings, we have proposed a hypothesis that Cdk5 modulates neuronal processes by determining the threshold for synaptic activity (Wei et al. 2005b; Hisanaga and Endo 2010).

Cdk5-p35 regulates synaptic transmission in pre-synaptic terminals at several targets, including both neurotransmitter release and uptake (Hawasaki and Bibb 2007; Lai and Ip 2009). Among these targets, VDCCs may be critical for initiating the synaptic transmission cascade (Catterall and Few 2008). However, the role of Cdk5 is complex as it has different effects on the different type of VDCCs. For example, Cdk5-p35 phosphorylates the α1 subunit of P/Q-type VDCC, down-regulating its channel activity (Tomizawa et al. 2002), while phosphorylation of the α1 subunit of N-type VDCC by Cdk5-p35 enhances its activity by facilitating the probability of its Ca^{2+} channel being open (Su et al. 2012). Also, a more recent study indicates that the suppression of Cdk5 activity leads to large potentiation of N-type VDCC activity, whereas P/Q-type VDCC activity is not affected (Kim and Ryan 2013). Thus, while it looks evident that Cdk5 regulates VDCC activity, this effect is complex and remains to be clarified.

Pheochromocytoma (PC12) cells, which have been frequently used as a model for nerve growth factor (NGF)-induced differentiation into neuron-like cells (Greene and Tischler 1976), are also used as a model of high K+ depolarization-induced Ca^{2+} influx (Adams et al. 2010; Gruol et al. 2012). Because PC12 cells express functional N-, L-, and P/Q-type VDCCs (Usowicz et al. 1990; Avidor et al. 1994; Liu et al. 1996), they provide a suitable experimental system to characterize the effect of Cdk5 on VDCC activity in similar cellular conditions. In this study, we measured Ca^{2+} influx into the cytoplasm through VDCCs in the absence and presence of Cdk5-p35 in both undifferentiated and NGF-induced differentiated PC12 cells and found that the above-mentioned three types of VDCCs were distinctly regulated by Cdk5 depending on differentiation states.

Materials and methods

Antibodies, chemicals, and plasmids

Antibodies directed against p35 (C19) and Cdk5 (DC17) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), while anti-actin antibody was purchased from Sigma (St. Louis, MO, USA). Anti-Cav1.2 antibody (clone L57/46) was obtained from Millipore (Billerica, MA, USA). Anti-Cav2.1 antibody (#ACC-001) and anti-Cav2.2 antibody (#ACC-002) were purchased from Alomone Labs (Jerusalem, Israel). Recombinant human NGF-β and Nifedipine were purchased from Wako (Osaka, Japan), while α-Agatoxin IVA and α-Conotoxin GVIA were obtained from Peptide Institute (Osaka, Japan). Yellow Cameleon-Nano (YC-Nano) in pcDNA3 (Horikawa et al. 2010) was provided by Takeharu Nagai at Osaka University. Mouse p35-myc and Cdk5-HA in pcDNA3 were used as described previously (Asada et al. 2012). Kinase-negative Cdk5-HA D144N in pcDNA3 was used as a template. The primers used were as follows: 5'-TTGAAATTTGCTAATTCTTGCCGTGCGC-3' and 5'-GCCAGGCGAAAAATTAGCAGCAATTCCG-3'.
recorded using a Zeiss confocal microscope LSM710 both with (Fig. 2a and b) and without (Figs 2c, 5, and 6) perfusion of recording buffer. YC-Nano was excited at 458 nm and the fluorescent images were captured at 519–621 nm (yellow) and 463–509 nm (cyan) at intervals of 2 s. The intracellular Ca\textsuperscript{2+} concentration was estimated by the ratio of yellow to cyan fluorescence resonance energy transfer (FRET). The concentrations of Ca\textsuperscript{2+} were measured by \(\Delta F/\text{F}\), which is the ratio of maximum to minimum FRET, reflecting calcium entry into the cytoplasm.

**Phosphorylation of a1 subunits of VDCCs in PC12 cells**

PC12 cells transfected with p35 and Cdk5 or knCdk5 were cultured in the presence of 3.7 MBq of \(^{32}\text{P}\)orthophosphate for 3 h as described previously (Kamei et al. 2006). Briefly, after washing with phosphate-buffered saline, cells were lysed in radio-immunoprecipitation assay buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM MgCl\textsubscript{2}, 1 mM EDTA, and 0.5 mM EGTA) containing 0.4 mM pefabloc, 10 \(\mu\text{g/mL}\) leupeptin, 1 mM dithiothreitol, and 0.5 \(\mu\text{M}\) microcystin on ice for 10 min. After centrifugation at 17 000 \(\times\) g for 20 min, the supernatant was incubated with anti-Cav2.1 antibody or anti-Cav2.2 antibody for 1 h on ice and then with protein A agarose beads. After washing four times with radio-immunoprecipitation assay buffer, proteins bound to beads were subjected to SDS–polyacrylamide gel electrophoresis (PAGE). \(^{32}\text{P}\) incorporation into Cav2.1 or Cav2.2 was detected by an FLA 7000 image analyzer (GE Healthcare, Buckinghamshire, UK).

**SDS–PAGE and immunoblotting**

PC12 cells were harvested by centrifugation at 1100 \(\times\) g for 5 min. Cells were dissolved in Laemmli’s sample buffer. Proteins in the whole cell lysates were separated by Laemmli’s SDS–PAGE using 5 or 12.5% polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore) using a submerged or semi-dry blotting apparatus. Membranes were probed with the primary antibodies and then anti-mouse or anti-rabbit IgG secondary antibodies conjugated with horseradish peroxidase (Dako, Glostrup, Denmark). The reactions were detected using an Immobilon western chemiluminescent horseradish peroxidase substrate (Millipore) or an enhanced chemiluminescence detection kit (GE Healthcare).

**Statistical analysis**

Immunoreaction was visualized as digital images by scanning X-ray film, and band intensities were measured using Image J software (NIH, Bethesda, MD, USA). All quantitative data were represented as mean ± SEM and were subjected to Student’s \(t\)-test for single comparison.

**Results**

**Protein expression of Cdk5, p35, and a1 subunit of L-, P/Q-, and N-type VDCCs in PC12 cells**

PC12 cells treated with NGF for 3 days showed a neuron-like phenotype with long processes (Fig. 1a), which we designated ‘differentiated’, whereas the untreated PC12 cells were designated ‘undifferentiated’. We found increased expression of p35 in PC12 cells after the treatment with NGF, confirming previous reports (Harada et al. 2001). However, the expression levels were much lower compared to what is seen in neurons. When expression levels were normalized to actin, even after NGF treatment p35 was considerably lower in PC12 cells compared to what is found in primary cortical neuronal culture (Fig. 1b). It is reported that functional L-, P/Q-, and N-type VDCCs are expressed in PC12 cells (Usowicz et al. 1990; Avidor et al. 1994; Liu et al. 1996), but it is not known whether their protein expression is affected by NGF treatment. As previous reports already examined the expression both at the transcriptional level (Liu et al. 1996) and using electrophysiological experiments (Usowicz et al. 1990; Liu et al. 1996), we examined protein expression of the pore-forming a1 subunits of L-, P/Q-, and N-type VDCCs, known respectively as Cav1.2, 2.1, and 2.2, in undifferentiated and differentiated PC12 cells by immunoblotting. We found that the protein levels of Cav1.2 and 2.2 were not changed by the NGF treatment, but the expression of Cav2.1 was increased upon differentiation (Fig. 1c).

**High K+ treatment induces Ca\textsuperscript{2+} influx in PC12 cells**

Treatment of PC12 cells with high concentrations of KCl depolarizes membranes, which induces Ca\textsuperscript{2+} influx through
VDCCs into the cytoplasm (Adams et al. 2010; Gruol et al. 2012). This Ca$^{2+}$ influx was measured by FRET of YC-Nano, calmodulin fused with yellow fluorescent protein and cyan fluorescent protein (Horikawa et al. 2010), and representative images of the changes in cytoplasmic Ca$^{2+}$ concentration induced by high K$^+$ solution are shown in Fig. 2a. Increases in intracellular Ca$^{2+}$ were observed immediately after KCl treatment, which then decayed slowly until ~140 s, when the Ca$^{2+}$ concentration returned to levels before KCl treatment (Fig. 2b). This time course is almost similar to that observed in cortical neurons using Fura-2AM, a chemical Ca$^{2+}$ indicator (Brittain et al. 2012).

The peak value of Ca$^{2+}$ influx ($\Delta$FRET) was increased with KCl concentrations and reached a plateau at over 40 mM KCl (Fig. 2c). In following experiments, we used 50 mM KCl for depolarization to obtain saturated and constant FRET values. Because these changes in $\Delta$FRET were not detected when Ca$^{2+}$ in the culture medium was chelated with EGTA, we concluded that $\Delta$FRET was owing to Ca$^{2+}$ influx through plasma membranes (data not shown).

Cdk5-p35 modulates the Ca$^{2+}$ influx into PC12 cells
To examine the effect of Cdk5 on VDCC activity, we transfected p35 and knCdk5 or Cdk5 together with YC-Nano in PC12 cells and measured $\Delta$FRET induced by treatment with 50 mM KCl. While $\Delta$FRET was increased in undifferentiated PC12 cells by expression of Cdk5 (Fig. 3a), Cdk5 expression suppressed $\Delta$FRET in differentiated cells (Fig. 3b). These results suggest that Cdk5 modulates VDCC activity but that this effect is different between undifferentiated and differentiated PC12 cells.

Cdk5-p35 does not affect the protein expression of VDCCs in PC12 cells
The effect of Cdk5 on the expression of VDCCs was examined by immunoblotting using antibodies against Cav1.2, 2.1, and 2.2 in undifferentiated (Fig. 4a) and NGF-treated (Fig. 4b) PC12 cells. The expression of these pore-forming $\alpha$ subunits was not changed whether Cdk5-p35 or knCdk5-p35 was expressed. These results suggest that Cdk5-p35 does not affect expression levels of $\alpha$1 subunit of VDCCs.

The Ca$^{2+}$ channel activity of L-, P/Q-, or N-type VDCC in PC12 cells before and after NGF treatment
The high K$^+$-evoked Ca$^{2+}$ influx was not different between undifferentiated and differentiated PC12 cells (Fig. 5a), which should represent a total activity of VDCCs expressed in PC12 cells. To evaluate the activity of respective VDCCs in undifferentiated and differentiated PC12 cells, we used blockers specific to each type of VDCCs, o-Agatoxin IVA for P/Q-type VDCC, o-conotoxin GVIA for N-type VDCC, and nifedipine for L-type VDCC. When the Ca$^{2+}$ influx was measured in the presence of all three blockers, it was reduced to 20–30% of what was found in the absence of blockers, confirming that L-, P/Q-, and N-type VDCCs comprise a large proportion of VDCCs in PC12 cells (data not shown). The Ca$^{2+}$ influx through L-type VDCC was measured in the

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The presence of α-Agatoxin IVA and α-conotoxin GVIA with no difference between undifferentiated and differentiated PC12 cells (Fig. 5b). Similarly, Ca^{2+} influx through P/Q-type VDCC was observed after application of nifedipine and α-conotoxin GVIA. In this case, Ca^{2+} influx was increased by NGF treatment (Fig. 5c), which might be caused by the increased expression level of Cav2.1 found in NGF-differentiated PC12 cells (Fig. 1b). In contrast, Ca^{2+} influx through N-type VDCC, which was measured in the presence of α-agatoxin IVA and α-conotoxin GVIA, the Ca^{2+} influx through L-type VDCCs was suppressed by expression of Cdk5-p35 in undifferentiated cells (Fig. 6a), while no effect was observed in differentiated PC12 cells (Fig. 6b). On the other hand, Ca^{2+} influx through P/Q-type VDCCs was suppressed in the presence of nifedipine and α-conotoxin GVIA by Cdk5 in both undifferentiated and differentiated PC12 cells (Fig. 6c and d). In addition, the activity of N-type VDCC in the presence of α-agatoxin IVA and nifedipine was increased by Cdk5-p35 in undifferentiated PC12 cells (Fig. 6e), whereas it responded to Cdk5-p35 negatively in differentiated PC12 cells, although the effect was small (Fig. 6f). These results suggest that Cdk5 regulates the activity of VDCCs distinctly depending on type and differentiation states.

Cdk5-dependent phosphorylation of α1 subunits of Cav2.1 and Cav2.2 in PC12 cells
We confirmed that Cdk5-p35 phosphorylates α1 subunits of Cav2.1 and Cav2.2 in PC12 cells. Phosphorylation of α1 subunit of Cav2.1 or Cav2.2 was examined by autoradiogram after their immunopreparation from PC12 cells, which were metabolically labeled with ^{32}P in the presence of Cdk5-p35 or knCdk5-p35 (Fig. 7). Cav2.1 and Cav2.2 were indeed phosphorylated in PC12 cells (lane 1). Expression of Cdk5-p35 increased (lane 2), and knCdk5-p35 greatly suppressed their phosphorylation (lane 3). These results indicate that α1 subunits of Cav2.1 and Cav2.2 are phosphorylated in PC12 cells in a Cdk5-dependent manner.

Discussion
Using PC12 cells, we examined the effect of Cdk5-p35 on Ca^{2+} channel activity of VDCCs. We found that Cdk5-p35 regulated L-, P/Q-, and N-type VDCCs differently depending on cell differentiation states. In undifferentiated PC12 cells, Cdk5 suppressed Ca^{2+} influx through L- and P/Q-type VDCC, while Ca^{2+} influx was increased in N-type VDCC. In contrast, in differentiated PC12 cells, Cdk5 suppressed P/Q-type VDCC while showing no effect on the activity of L- and N-type VDCCs. These results suggest that Cdk5-p35 regulates VDCCs in a type- and context-dependent manner.

PC12 cells express Cdk5 and p35 endogenously, which are involved in differentiation (Harada et al. 2001). To avoid a possible effect of up- or down-regulation of Cdk5 activity on NGF-dependent differentiation, we treated PC12 cells with NGF for 3 days, then transfected them with Cdk5 and p35, then assayed them the next day. Even though the expression of p35 increased in differentiated PC12 cells (Fig. 1b,
we introduced Cdk5-p35 to maximize its effect because the expression levels of p35 were much lower than those in primary neurons (Fig. 1b). While the Cdk5 inhibitor roscovitine or olomoucine was used to inhibit Cdk5 activity in some previous reports (Tomizawa et al. 2002; Wei et al. 2005a; Kim and Ryan 2013), we used knCdk5 as a more specific inhibitor because roscovitine is shown to inhibit L-type VDCC by slowing activation and enhancing deactivation (Yarotskyy and Elmslie 2007), and P/Q- and N-type VDCCs by slowing deactivation (Yan et al. 2002; Buraei et al. 2005).

We found that Cdk5-p35 suppressed Cdk5 activity in undifferentiated PC12 cells (Fig. 1b). While the Cdk5 inhibitor roscovitine or olomoucine was used to inhibit Cdk5 activity in some previous reports (Tomizawa et al. 2002; Wei et al. 2005a; Kim and Ryan 2013), we used knCdk5 as a more specific inhibitor because roscovitine is shown to inhibit L-type VDCC by slowing activation and enhancing deactivation (Yarotskyy and Elmslie 2007), and P/Q- and N-type VDCCs by slowing deactivation (Yan et al. 2002; Buraei et al. 2005).

We found that Cdk5-p35 suppressed Ca^{2+} influx through L-type VDCCs in both undifferentiated and differentiated PC12 cells (Fig. 6c and d). These results are in agreement with reports in rat hippocampal neurons that Cdk5 down-regulates P/Q-type VDCC activity by phosphorylation of the α1 subunit of Cav2.1 at the intracellular domain between the II and III loops (Tomizawa et al. 2002). This phosphorylation also inhibits the interaction of P/Q-type VDCCs with SNAP-25 and synaptotagmin I, leading to decreased neurotransmitter release (Tomizawa et al. 2002). This is in contrast to what we found in L-type VDCC, where NGF-induced differentiation blocked the effect of Cdk5-p35. This may be caused by different SNARE proteins interacting with L- or P/Q-type VDCCs, as the loop II–III amino acid sequences are quite different between L and P/Q types (Kohn et al. 2001).
The Ca\(^{2+}\) influx activity of N-type VDCC was decreased in differentiated compared to undifferentiated PC12 cells (Fig. 5d). This is probably not because of the expression levels of N-type VDCCs, as we found no difference in the levels of \(\alpha_1\) subunit of Cav2.2 protein between undifferentiated and differentiated PC12 cells (Fig. 1e). This suggests that the regulation mechanism is changed by NGF-induced differentiation. In addition to SNARE proteins (Yokoyama et al. 1997), G proteins are also reported to inhibit N-type VDCC (Zamponi et al. 1997). While we found differentiation-dependent alteration of basal activity, N-type VDCC activity was stimulated by Cdk5-p35 in undifferentiated PC12 cells (Fig. 6e), in differentiated PC12 cells Cdk5 reduced activity (Fig. 6f). As reported elsewhere, the effects of Cdk5 on N-type VDCC are complex. For example, Cdk5 is shown to phosphorylate the pre-synaptic scaffold protein CASK to induce interaction with \(\alpha_1\) subunit of N-type VDCC, resulting in increased Ca\(^{2+}\) channel activity (Samuels et al. 2007). Furthermore, a recent study has reported that Cdk5 directly stimulates N-type VDCC activity by phosphorylating the C-terminal tail region of the \(\alpha_1\) subunit (Su et al. 2014).

![Fig. 5 Ca\(^{2+}\) influx in PC12 cells through L-, P/Q-, or N-type Ca\(^{2+}\) channels. PC12 cells were transfected with YC-Nano and then cultured for 3 days without (U) or with nerve growth factor (NGF) (D). (a) fluorescence resonance energy transfer (FRET) was measured in PC12 cells following treatment with 50 mM KCl. \(\Delta\text{FRET}\) in differentiated PC12 cells (D) was expressed as the ratio to that of undifferentiated PC12 cells (U) (mean \(\pm\) SEM, \(n = 17\) for undifferentiated and \(n = 16\) for differentiated; * \(p < 0.05\), Student’s t-test). (b) \(\Delta\text{FRET}\) for L-type voltage-dependent calcium channels (VDCCs) measured in the presence of \(\alpha\)-agatoxin IVA and \(\alpha\)-conotoxin GVIA (mean \(\pm\) SEM, \(n = 16\) for undifferentiated and \(n = 7\) for differentiated; * \(p < 0.05\), Student’s t-test), (c) P/Q-type VDCCs measured in the presence of \(\alpha\)-conotoxin GVIA and nifedipine (mean \(\pm\) SEM, \(n = 25\) for undifferentiated and \(n = 24\) for differentiated; * \(p < 0.05\), Student’s t-test) and (d) N-type VDCC in the presence of \(\alpha\)-agatoxin IVA and nifedipine (mean \(\pm\) SEM, \(n = 16\) for undifferentiated and \(n = 18\) for differentiated; * \(p < 0.05\), Student’s t-test).](image1)

![Fig. 6 Effect of cyclin-dependent kinase 5 (Cdk5)-p35 on the Ca\(^{2+}\) channel activity of L-, P/Q-, or N-type voltage-dependent calcium channels (VDCCs) in PC12 cells. PC12 cells were cotransfected with YC-Nano, p35, and either kinase-negative cyclin-dependent kinase 5 (knCdk5) or Cdk5, followed by treatment with 50 ng/mL nerve growth factor (NGF) for 3 days. (a) and (b) change in fluorescence resonance energy transfer (\(\Delta\text{FRET}\)) for L-type VDCC in the presence of \(\alpha\)-agatoxin IVA and \(\alpha\)-conotoxin GVIA in undifferentiated PC12 cells (a, U) (mean \(\pm\) SEM, \(n = 39\) for knCdk5-p35 and \(n = 33\) for Cdk5-p35; * \(p < 0.05\), ** \(p < 0.01\), Student’s t-test) and in differentiated PC12 cells (b, D) (mean \(\pm\) SEM, \(n = 20\) for knCdk5-p35 and \(n = 20\) for Cdk5-p35; * \(p < 0.05\), ** \(p < 0.01\), Student’s t-test). (c) and (d) \(\Delta\text{FRET}\) for P/Q-type VDCC in the presence of \(\alpha\)-conotoxin GVIA and nifedipine in undifferentiated PC12 cells (c, U) (mean \(\pm\) SEM, \(n = 32\) for knCdk5-p35 and \(n = 38\) for Cdk5-p35; * \(p < 0.05\), ** \(p < 0.01\), Student’s t-test) and in differentiated PC12 cells (d, D) (mean \(\pm\) SEM, \(n = 18\) for knCdk5-p35 and \(n = 21\) for Cdk5-p35; * \(p < 0.05\), ** \(p < 0.01\), Student’s t-test). (e) and (f) \(\Delta\text{FRET}\) for N-type VDCC in the presence of \(\alpha\)-agatoxin IVA and nifedipine in undifferentiated PC12 cells (e, U) (mean \(\pm\) SEM, \(n = 25\) for knCdk5-p35 and \(n = 23\) for Cdk5-p35; Student’s t-test) and in differentiated PC12 cells (f, D) (mean \(\pm\) SEM, \(n = 17\) for knCdk5-p35 and \(n = 18\) for Cdk5-p35; * \(p < 0.05\), ** \(p < 0.01\), Student’s t-test).](image2)
et al. 2012), which modulates the interaction with Rab3-interacting molecule 1 (RIM1). In contrast, another study demonstrates down-regulation of the N-type VDCC activity by Cdk5 (Kim and Ryan 2013). These results are apparently contradictory, but our results indicate that both activation and inactivation can occur in a single type of cell if the cellular conditions are different. It is possible that the observed changes in the effect of N-type VDCC on Cdk5-p35 may be caused by changes in the interaction of phosphorylated α1 subunit of Cav2.2 with regulatory proteins. Future studies could further explore this problem by analyzing levels of Cdk5 and p35. Upper panels are immunoblots of α1 subunits in inputs.

Cdk5 is a multifunctional neuronal protein kinase located in the pre-synaptic region and is reported to phosphorylate a number of proteins such as Munc-18, Septin 5, Dynamin 1, synapsin I, Amphiphysin, and Synaptotagmin 1 (Matsubara et al. 1999; Tomizawa et al. 2003; Lee et al. 2004; Taniguchi et al. 2007), suppressing their effect on synaptic transmission. Those results are in agreement with our hypothesis that Cdk5 suppresses synaptic transmission. However, our results show that in the case of VDCCs, Cdk5-p35 can act as either an activator or inhibitor in PC12 cells depending on differentiation states. As well as supporting previous results (Tomizawa et al. 2002; Wei et al. 2005a; Su et al. 2012; Kim and Ryan 2013), the present results raise several questions; such as what differences between undifferentiated and differentiated PC12 cells underlie VDCCs’ activity and how the activation of N-type VDCC by Cdk5-p35 is coupled to exocytosis in PC12 cells. It is important to understand the function and regulation of these VDCCs in various types of neurons and PC12 cells provide a good model system for studying the direct effect of Cdk5 on excitation-induced Ca2+ channel activation subsequent exocytosis.

Acknowledgements and conflict of interest disclosure

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All experiments were conducted in compliance with the ARRIVE guidelines.

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