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Differential regulation of the Cdk5-dependent phosphorylation sites of inhibitor-1 and DARPP-32 by depolarization

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Abstract

While cyclin-dependent kinase 5 (Cdk5) is of growing importance to neuronal signaling, its regulation remains relatively unexplored. Examination of the mechanism by which NMDA modulates the phosphorylation of protein phosphatase inhibitor-1 at Ser6 and Ser67 and dopamine- and cAMP-regulated phosphoprotein M_r 32 000 at Thr75 revealed that generalized depolarization, rather than specific activation of NMDA receptors, was sufficient to induce decreases in these Cdk5 sites. Although no evidence for the involvement of the Cdk5 cofactors p35 or p39, or for L- and T-type voltage-gated Ca^{2+} channels, was found, evaluation of the role of phosphatases and extracellular cations revealed differential regulation of the three sites. NMDA-induced decreases in the phosphorylation of Thr75 of dopamine- and cAMP-regulated phosphoprotein M_r 32 000 required protein phosphatase 1/2A activity and extracellular Ca^{2+}. In contrast, the effects on Ser6 and Ser67 of inhibitor-1 were not cation specific; either Na^+ or Ca^{2+} sufficed. Furthermore, while the decrease in phosphorylation of Ser6 was partially dependent on protein phosphatase 2B, that of Ser67 was independent of the major protein serine/threonine phosphatases, likely indicating the presence of a pathway by which NMDA inhibits Cdk5 activity. Thus, in the striatum the regulation of phosphorylation of Cdk5-dependent sites by NMDA occurs through multiple distinct pathways.

Keywords: cyclin-dependent kinase 5, depolarization, dopamine- and cAMP-regulated phosphoprotein, *N*-methyl-D-aspartate, protein phosphatase, protein phosphatase inhibitor-1.

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In spite of its name, the proline-directed serine/threonine cyclin-dependent kinase 5 (Cdk5) (Lew *et al.* 1992) is not cyclin dependent. Its activity is instead dependent on the relatively neuron-specific cofactor p35 (Lew *et al.* 1994; Tsai *et al.* 1994), or its homolog p39 (Tang *et al.* 1995; Tang and Wang 1996), thereby largely restricting its activity to post-mitotic neurons (Cheung *et al.* 2006). Cdk5 now possesses an extensive list of substrates, among them two protein kinase A (PKA)-dependent inhibitors of protein phosphatase 1 (PP-1), dopamine- and cAMP-regulated phosphoprotein, M_r 32 000 (DARPP-32) (Bibb *et al.* 1999) and PP inhibitor-1 (I-1) (Bibb *et al.* 2001; Nguyen *et al.* 2007). Cdk5-dependent phosphorylation of DARPP-32 at Thr75 converts it into an inhibitor of PKA (Bibb *et al.* 1999). In direct contrast, Cdk5-dependent phosphorylation of I-1 at Ser6 and Ser67 impairs dephosphorylation and inactivation of the protein, allowing perpetuation of the PKA signal (Nguyen *et al.* 2007).

In striatal slices, levels of phosphorylation of Thr75 of DARPP-32 and Ser6 and Ser67 of I-1 are reduced upon treatment with NMDA (Bibb *et al.* 2001; Nishi *et al.* 2002; Nguyen *et al.* 2007), suggesting potential NMDA-dependent regulation of Cdk5 activity. Interestingly, counter-regulation of the NMDA receptor by Cdk5 has been observed. Phosphorylation of the NR2A subunit of the NMDA receptor by Cdk5 has been suggested to increase NMDA receptor activity required for the induction of long-term potentiation in the Schaffer collateral pathway (Li *et al.* 2001). Moreover, Cdk5 is thought to regulate NMDA receptor constituency by modulating the stability of the NR2B subunit (Hawasli *et al.* 2007).

Mechanisms for the regulation of Cdk5 activity by NMDA have been varied. Ionotropic glutamate receptors, including

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; AP5, 2-amino-5-phosphonopentanoic acid; calyA, calyculin A; Cdk5, cyclin-dependent kinase 5; CK1, casein kinase 1; cyA, cyclosporin A; DARPP-32, dopamine- and cAMP-regulated phosphoprotein, M_r 32 000; I-1, (protein phosphatase) inhibitor-1; OA, okadaic acid; PKA, cAMP-dependent protein kinase; PP, protein phosphatase.

NMDA receptors, mediate cleavage of p35 to p25 (Kerokoski *et al.* 2004) by the Ca^{2+}-activated protease calpain (Kusakawa *et al.* 2000; Lee *et al.* 2000; Nath *et al.* 2000). Cleavage of p35 releases it from the membrane, allowing Cdk5 to aberrently phosphorylate substrates implicated in a wide range of neuropathologies (Patrick *et al.* 1999; Nath *et al.* 2000; Nguyen *et al.* 2001; Wang *et al.* 2003). Ionotropic glutamate receptor-mediated regulation of Cdk5 activity independent of p25 generation has also been observed (Wei *et al.* 2005). Treatment of cortical cultures with NMDA or kainate induces autophosphorylation of p35, which targets it for degradation by the proteasome, ultimately leading to a reduction in Cdk5 activity.

Like other cyclin-dependent kinases, Cdk5 is regulated by the availability of its cofactors (Hisanaga and Saito 2003). The transcriptional up-regulation of p35 by Egr1 results in Cdk5 activation, which is required for nerve growth factor-induced differentiation of PC12 pheochromocytoma cells (Harada *et al.* 2001). In contrast, Cdk5-dependent autophosphorylation of p35 results in its ubiquitin-dependent proteasomal degradation with consequent Cdk5 inhibition (Patrick *et al.* 1998; Saito *et al.* 1998, 2003; Kerokoski *et al.* 2002; Wei *et al.* 2005). Autophosphorylation of p35 also protects it from calpain-dependent conversion to p25 (Kamei *et al.* 2007). Supporting this conclusion is the observation that p35 from adult brains is both less phosphorylated at Thr138 and more susceptible to calpain-dependent cleavage than that from fetal brains (Kamei *et al.* 2007). Much less is known about p39, but it too seems to be converted to p29 by calpain (Patzke and Tsai 2002). Recently, phosphorylation of p35 was shown to affect Cdk5 activity by regulating association of the Cdk5/p35 complex with membranes (Zhu *et al.* 2005; Sato *et al*. in press).

Distinct from the down-regulation of Cdk5 activity by ionotropic glutamate receptors is the transient up-regulation of Cdk5 activity by group I metabotropic glutamate receptors, a process dependent on activation of casein kinase 1 (CK1) (Liu *et al.* 2001). How CK1 mediates activation of Cdk5 remains unclear, as the ability of CK1 to directly phosphorylate and activate the catalytic subunit of Cdk5 is a matter of some debate (Sharma *et al.* 1999; Liu *et al.* 2001). Direct phosphorylation and activation of the catalytic subunit of Cdk5 at Tyr15 by protein tyrosine kinases is another means of Cdk5 activation (Zukerberg *et al.* 2000; Sasaki *et al.* 2002; Fu *et al.* 2007), as is the phosphatidylinositol-linked D_1 dopamine receptor (Zhen *et al.* 2004). Pharmacological inhibition of protein kinase C activity with calphostin C prevented D_1 receptor-mediated activation of Cdk5, suggesting a novel functional link between Cdk5 and protein kinase C (Zhen *et al.* 2004). These and other complex pathways regulating Cdk5 activity are only beginning to be understood. In this study, we examined the mechanism by which NMDA induces decreases in the phosphorylation of three Cdk5-dependent sites on I-1 and DARPP-32.

Materials and methods

Chemicals and reagents

N-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate, and DL-2-amino-5-phosphono-pentanoic acid (AP5) were from Tocris (Ellisville, MO, USA). Okadaic acid (OA) and calyculin A (calyA) were from Alexis (Lausen, Switzerland), cyclosporin A (cyA) from LC Laboratories (Woburn, MA, USA), and 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetomethylester from Calbiochem (San Diego, CA, USA). All other chemicals and reagents were purchased from Sigma (St Louis, MO, USA).

Preparation and incubation of acute dorsal striatal slices

Slices from male C57BL/6 mice (6-10 weeks old) were prepared in Krebs buffer as described (Sahin *et al.* 2006). Each 400-μm slice was transferred to a net-well (Costar, New York, NY, USA) resting in one well of a 12-well plate containing 3 mL of Krebs buffer and allowed to recover at 30°C under constant oxygenation with 95% O_2/5% CO_2 for 45–60 min, with one or two changes of buffer. Slices were subsequently treated with drugs as specified for each experiment, transferred to microfuge tubes, snap-frozen on dry ice, and stored at −80°C until further analysis. Control slices were treated with the solvent used to dissolve the drugs.

Immunoblot analysis

Lysis and immunoblot analysis of striatal slices were performed essentially as described (Sahin *et al.* 2006). An equal amount of total protein (80–100 μg) from each sample as determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL, USA) was analyzed. The membranes were immunoblotted using antibodies for phospho-Ser6 I-1 (1 : 750) (Nguyen *et al.* 2007), phospho-Ser67 I-1 (1 : 4000) (Bibb *et al.* 2001), total I-1 (1 : 2000) (Gustafson *et al.* 1991), phospho-Thr75 DARPP-32 (1 : 2000) (Bibb *et al.* 1999), total DARPP-32 (1 : 8000) (Hemmings and Greengard 1986), p35 (1 : 1000, C-19; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or p39 (1 : 1000) (Fu *et al.* 2002) following published protocols. All but one antibody (total DARPP-32) were polyclonal. Antibody incubations were in 5% milk + Tris-buffered saline with 0.1% Tween-20, except that for p39, in which bovine serum albumin replaced milk. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Chemicon (Temecula, CA, USA), and the enhanced chemiluminescence immunoblotting detection system from Amersham Biosciences (Piscataway, NJ, USA).

Data analysis

Image J (NIH) was used to quantitate immunoblots. Results are stated as mean ± standard error of the mean. Changes are stated as percentage decrease or fold increase of the mean ± error. Error of the change was calculated from standard errors of the mean using error propagation formulas. Individual bands of representative blots were always taken from the same exposure of the same membrane, and all represented total bands were derived from the same sample on the same membrane as the corresponding phosphoband.

Results

Dose-dependent reduction of three Cdk5-dependent phosphorylation sites by NMDA, independent of changes in levels of p35 and p25

Inhibitor-1 is phosphorylated by Cdk5 at two sites, Ser6 and Ser67 (Bibb *et al.* 2001). The I-1 homolog DARPP-32 serves as a substrate for Cdk5 at Thr75 (Bibb *et al.* 1999). We discovered robust regulation of these phosphorylation sites *in vivo*; treatment of acutely dissected striatal slices with NMDA reduced phosphorylation at all three sites (Fig. 1a). Levels of phospho-Ser6 and phospho-Ser67 I-1 and phospho-Thr75 DARPP-32 were 12 ± 1%, 45 ± 4%, and 32 ± 5% of control levels, respectively, after treatment with 50 μmol/L NMDA for 5 min. Neither doubling the dose of NMDA from 50 to 100 μmol/L nor increasing the exposure time from 5 to 20 min resulted in a substantially larger effect. In spite of previous findings reporting ionotropic glutamate receptor-induced down-regulation of Cdk5 activity via the degradation of p35 (Wei *et al.* 2005), we observed no corresponding changes in levels of p35 in our preparation. Minimal amounts of p25 were detected in control slices, and application of NMDA did not induce further generation of p25 until a dose of 100 μmol/L was reached, even though 25 μmol/L NMDA was enough to induce reductions in all three Cdk5-dependent sites (Fig. 1b). Interestingly, levels of phospho-Ser6 I-1 consistently decreased more than those of phospho-Ser67 I-1, with 25 μmol/L NMDA reducing levels to 23 ± 7% and 39 ± 3% of control, respectively. Thus, NMDA can markedly reduce phosphorylation of Ser6, Ser67, and Thr75 in a dose-dependent manner without affecting levels of p35 or p25.

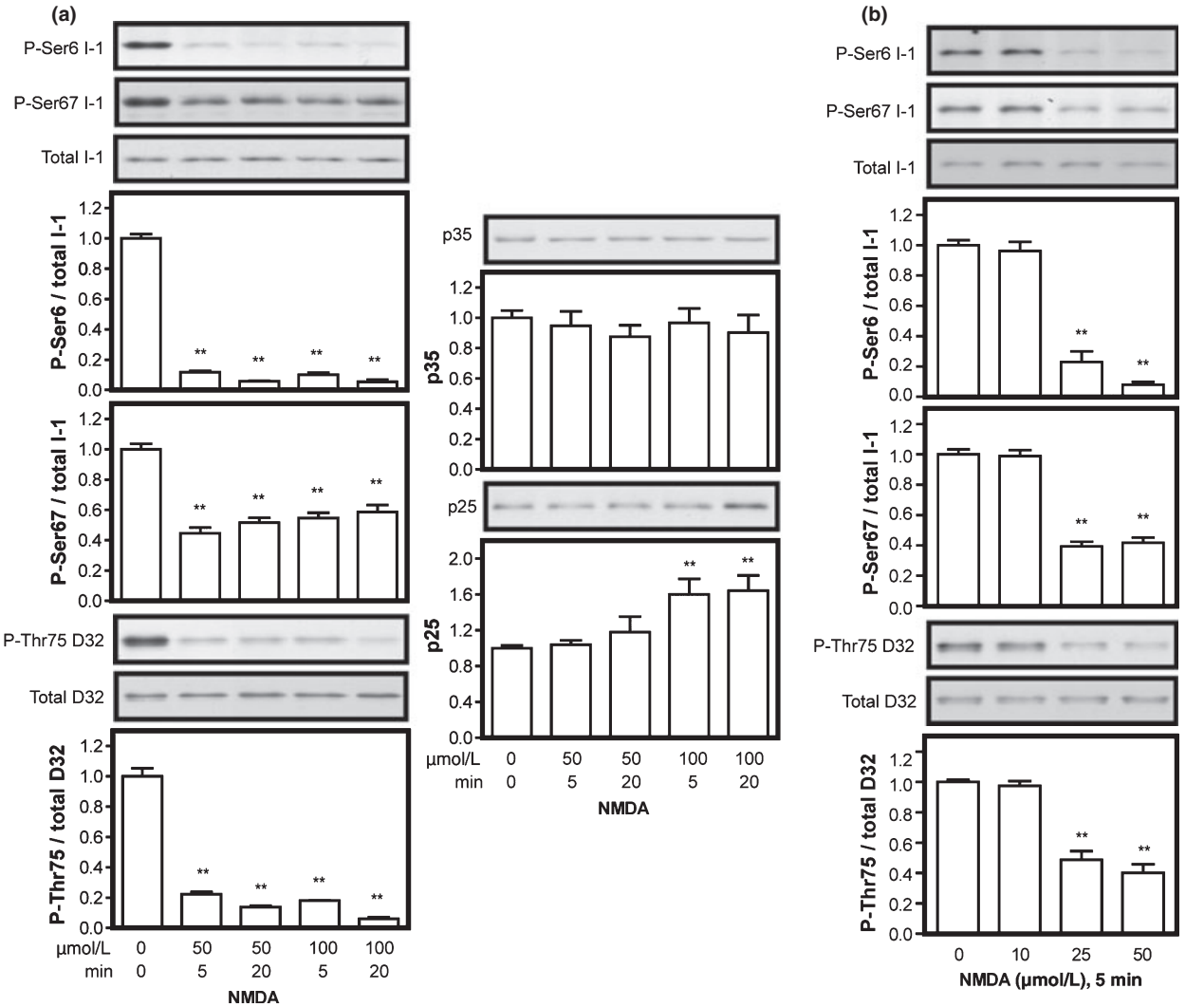

Fig. 1 Reduction in phosphorylation of three Cdk5 sites by NMDA. Quantitative immunoblot analysis of acute striatal slices incubated with NMDA for (a), the doses and times indicated, $n = 3$–6 or (b), the doses indicated for 5 min, $n = 4$–12. $^{**}p < 0.01$ versus control, one-way ANOVA with Dunnett's multiple comparison test.

Evaluation of the role of Cdk5-activating cofactors

In contrast to the apparent lack of effect of low doses of NMDA on p35 and p25, we detected a shift in the electrophoretic mobility of p39 in slices treated with NMDA (Fig. 2a). NMDA treatment caused the p39 doublet to collapse into a single band with the same mobility as the lower band of the doublet. Notably, in some gels, we were able to detect a very slight downward shift in the mobility of p35 upon NMDA treatment (Fig. 2b), but this effect was inconsistent, being highly dependent on stochastic gel conditions, and we did not pursue it further. Small mobility shifts are frequently caused by changes in the state of phosphorylation of a protein. While phosphorylation of p39 has not previously been reported, phosphorylation of p35 has been linked to changes in Cdk5 activity (Sato *et al*. in press). As the threshold for the p39 mobility shift was 25 μmol/L, the same as the threshold for NMDA-induced reduction in levels of phosphorylation of I-1 and DARPP-32, we suspected that NMDA might be reducing levels of phosphorylation at Ser6, Ser67, and Thr75 by inhibiting Cdk5 activity via a dephosphorylation of p39. To determine if the effect of NMDA on the phosphorylation state of I-1 and DARPP-32 was cofactor specific, we treated acutely dissected striatal slices from p35 (Chae *et al.* 1997) and p39 (Ko *et al.* 2001) knockout mice with NMDA. Neither constitutive loss of p35 nor p39 influenced the ability of NMDA to reduce levels of phosphorylation of I-1 or DARPP-32 (Fig. 2c), suggesting either compensation by the remaining cofactor in the knockout mice or simply the lack of a causal relationship.

Evaluation of the role of protein phosphatases

Decreases in the level of phosphorylation of a protein can be due to either a reduction in kinase activity or an enhancement of phosphatase activity. Ser6 of I-1 is a rather promiscuous site for phosphatases, serving as a substrate for PP-1 and PP-2A basally, and additionally for PP-2B with increased intracellular Ca^{2+} (Nguyen *et al.* 2007). PP-2A and PP-2B both contribute to the dephosphorylation of Ser67 of I-1 (Bibb *et al.* 2001), while PP-2A, and to a lesser extent, PP-1 and PP-2C contribute to that of Thr75 of DARPP-32 (Nishi *et al.* 2000). To examine the possibility that NMDA reduces levels of phosphorylation of these three Cdk5 sites by activating a phosphatase, we treated striatal slices with various phosphatase inhibitors alone and in combination with NMDA.

Calyculin A inhibits PP-1 and PP-2A with equal potency, while 1 μmol/L OA inhibits 95% of PP-2A activity and 35% of PP-1 activity in the striatum (Nishi *et al.* 1999). Treatment with the PP-1/PP-2A inhibitors OA and calyA raised basal levels of phosphorylation at Ser6 (Fig. 3a). NMDA eliminated the OA- and calyA-induced increases and further reduced levels of phospho-Ser6 I-1 to 20 ± 2% and 54 ± 15% of control levels, respectively. In contrast, these phosphatase inhibitors did not change levels of phospho-Ser67 I-1, either basally or in combination with treatment with NMDA. Importantly, NMDA was unable to attenuate OA- and calyA-induced increases in basal levels of phospho-Thr75 DARPP-32, indicating a role for PP-1/PP-2A in mediating the effects of NMDA on DARPP-32. Thus, the regulation of Cdk5 sites by NMDA receptors occurs by at least two distinct mechanisms. An enhancement of PP-1/PP-2A activity is responsible for NMDA-induced reductions in the phosphorylation of DARPP-32. An alternate pathway, independent of PP-1/PP-2A, allows NMDA to reduce levels of phospho-Ser6 and phospho-Ser67 I-1 in the face of treatment with OA and calyA.

In contrast to the PP-1/PP-2A inhibitors, the PP-2B inhibitor cyA did not alter basal levels of phosphorylation of Ser6 or Ser67 (Fig. 3b). It did, however, partially reverse

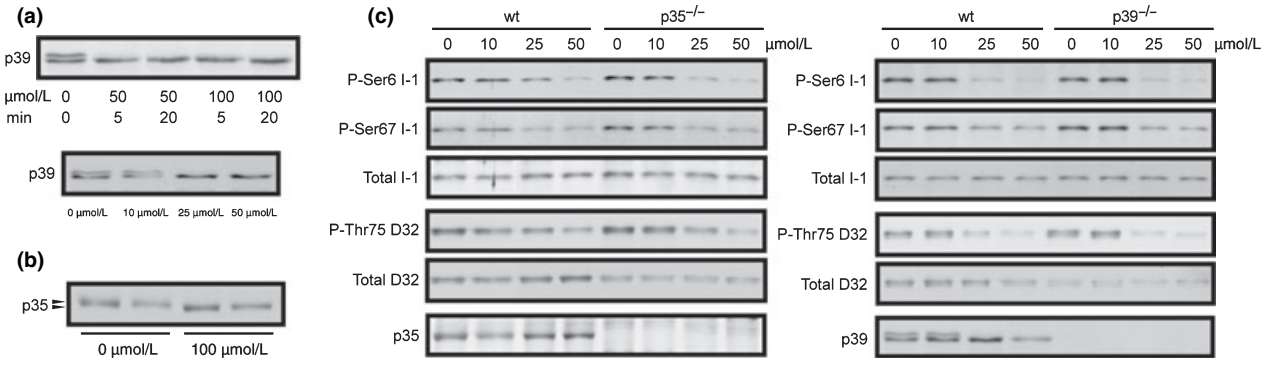

Fig. 2 Evaluation of the role of cyclin-dependent kinase 5 (Cdk5)-activating cofactors. (a and b) Immunoblots of acute striatal slices treated with NMDA for the doses and times indicated (a, top, $n = 2–3$) or for the doses indicated for 5 min (a, bottom, $n = 4$, b). (c) Immunoblots of acute striatal slices from wild-type and p35−/− (left) or p39−/− (right) mice treated with NMDA at the indicated doses for 5 min, $n = 2$.

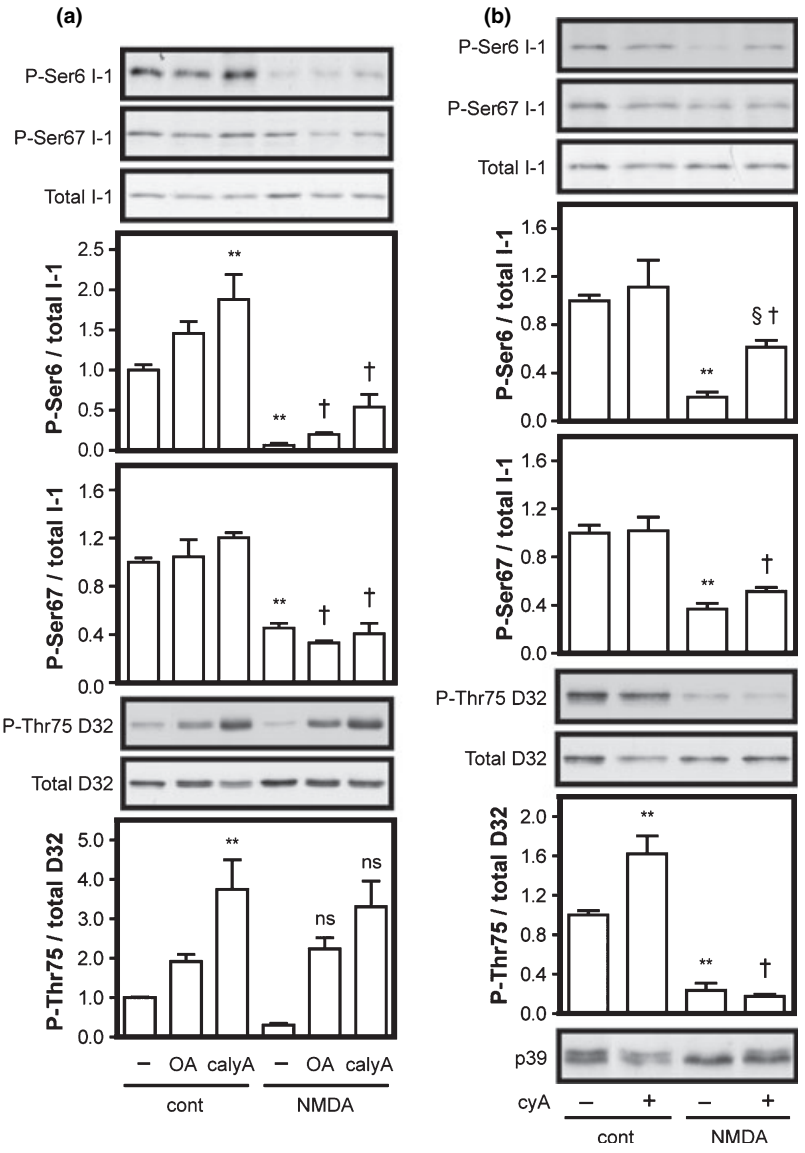

Fig. 3 Evaluation of the role of protein phosphatases. Quantitative immunoblot analysis of acute striatal slices incubated in the absence or presence of 50 μmol/L NMDA for 5 min with or without 60 min pre-incubation with (a), okadaic acid (OA, 1 μmol/L) or calyculin A (calyA, 1 μmol/L), $n = 3$–5 or (b), cyclosporin A (cyA, 10 μmol/L), $n = 2$–7. $^{**}p < 0.01$ versus control; §$p < 0.001$ versus NMDA alone; †$p < 0.01$ and NS, not significant versus corresponding treatment without NMDA; one-way ANOVA with Bonferroni's multiple comparison test.

the ability of NMDA to reduce levels of phospho-Ser6 I-1 (from 20 ± 4% to 61 ± 6% of control). cyA also raised basal levels of phospho-Thr75 DARPP-32 without attenuating NMDA-mediated decreases in this site or in Ser67. Thus, while phosphatases are somewhat or majorly responsible for the reduction in levels of phosphorylation of Ser6 and Thr75, there remains a mechanism by which NMDA reduces the phosphorylation of Ser67 independent of the major serine/threonine phosphatases, possibly via a reduction in Cdk5 activity.

Interestingly, cyA also attenuated the NMDA-induced p39 mobility shift (Fig. 3b), indicating that the shift represents a dephosphorylation. However, as this treatment did not block NMDA-induced decreases in the phosphorylation of Ser67 or Thr75, the p39 shift probably represents an epiphenomenon that is not causally related to the reductions in phosphorylation.

Similar effects on the three Cdk5-dependent sites by other ionotropic glutamate receptor agonists and by direct depolarization

The ability of other ionotropic glutamate receptor agonists to reduce phosphorylation of I-1 and DARPP-32 at the Cdk5 sites was next assessed. When applied at a concentration of 25 μmol/L for 5 min, both AMPA and kainate reduced levels of phospho-Ser6 I-1 (22 ± 8% and 28 ± 14% of control, respectively), phospho-Ser67 I-1 (66 ± 1% and 60 ± 3% of control), and phospho-Thr75 DARPP-32 (51 ± 1% and 48 ± 13% of control) (Fig. 4a). Doubling the concentration of AMPA or kainate did not further reduce phosphorylation at any of the sites.

As the application of ionotropic glutamate receptor agonists can have numerous consequences, one of which is generalized membrane depolarization, the effect of direct depolarization using KCl was tested. KCl dose-dependently

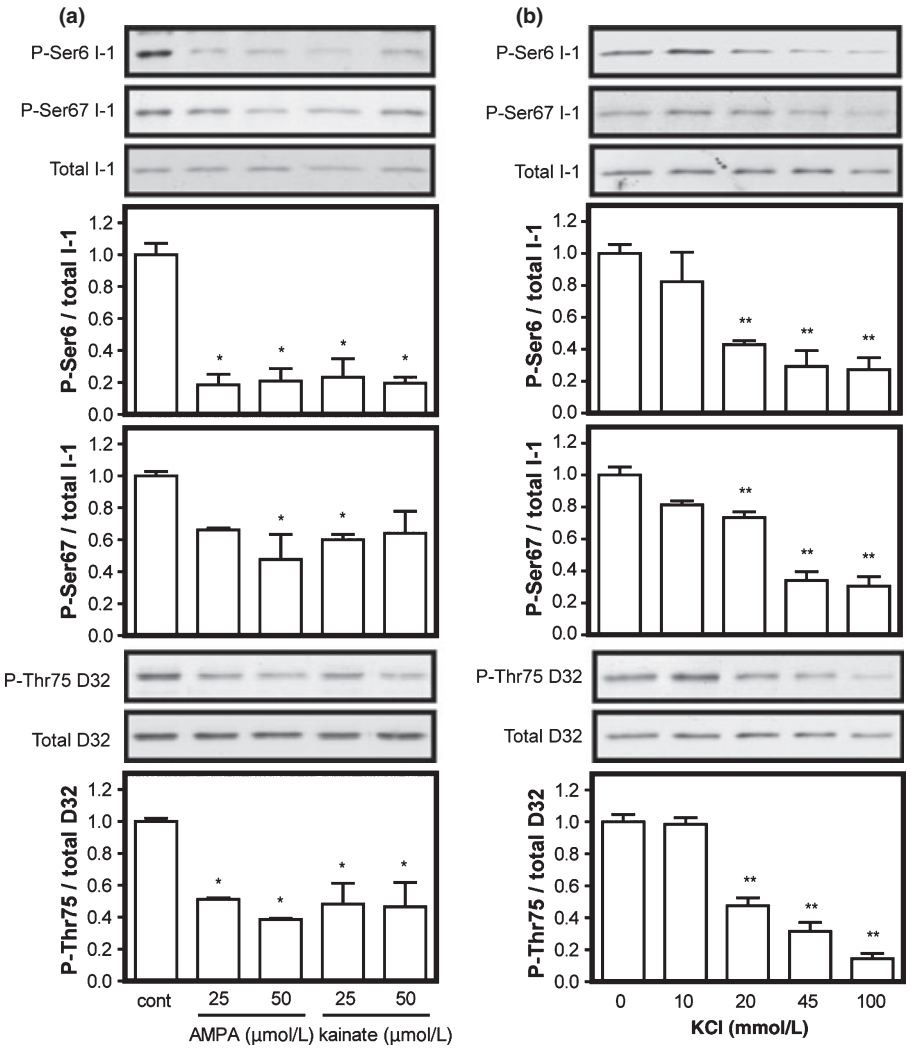

Fig. 4 Similar reductions in phosphorylation of three cyclin-dependent kinase 5 (Cdk5) sites by α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), kainate, and KCl. Quantitative immunoblot analysis of acute striatal slices incubated with (a), AMPA or kainate at the doses indicated for 5 min, $n = 2$-3 or (b), KCl at the doses indicated for 5 min, $n = 3$–5. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control, one-way ANOVA with Dunnett's multiple comparison test.

reduced levels of phosphorylation at Ser6, Ser67, and Thr75, with 45 mmol/L reducing levels to 29 ± 10%, 34 ± 6%, and 31 ± 6% of control, respectively (Fig. 4b). The minimal effective dose for all three sites was 20 mmol/L. Increasing the dose beyond 45 mmol/L to 100 mmol/L further reduced levels of phospho-Thr75 DARPP-32 to 14 ± 3% of control, but not of phospho-Ser6 or phospho-Ser67 I-1. These findings indicate that simple depolarization is sufficient for the reduction in phosphorylation of these Cdk5 sites.

Evaluation of the role of NMDA receptors

Relief of the Mg^{2+} block by depolarization is a pre-requisite for NMDA receptor activity. Furthermore, NR2B subunits of the NMDA receptor physically associate with Cdk5 (Hawasli *et al.* 2007). To determine if the reductions in phosphorylation caused by AMPA and KCl resulted from secondary activation of NMDA receptors, we employed the activity-dependent and activity-independent NMDA receptor antagonists MK801 and AP5. Neither MK801 (Fig. 5a) nor AP5 (Fig. 5b) blocked the AMPA-induced decrease in phosphorylation of Ser6, Ser67, and Thr75. AP5 was similarly ineffective at blocking the effect of KCl (Fig. 5b). It was, however, able to block the effect of NMDA, returning phospho-Ser6 and phospho-Ser67 I-1 and phospho-Thr75 DARPP-32 to 79 ± 5%, 86 ± 11%, and 106 ± 3% of control levels, respectively (Fig. 5c). Thus, while the effects of NMDA were specifically mediated by NMDA receptors, the effects of AMPA and KCl did not result from secondary activation of NMDA receptors.

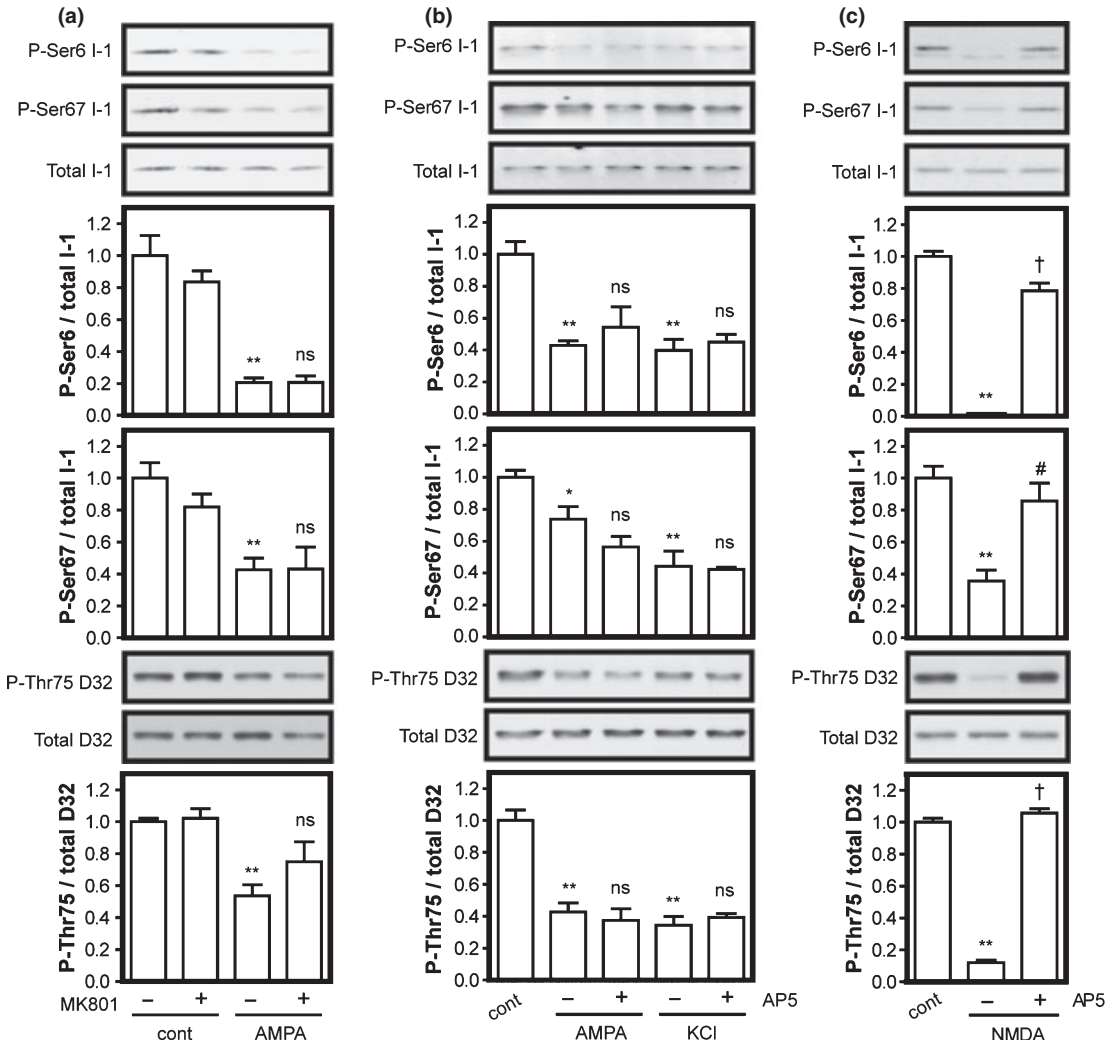

Fig. 5 Evaluation of the role of NMDA receptors. Quantitative immunoblot analysis of acute striatal slices incubated in the absence or presence of NMDA (50 μmol/L), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (50 μmol/L), or KCl (45 mmol/L) for 5 min with or without 7 min pre-incubation with (a), MK801, n = 5-6, (b), 2-amino-5-phosphonopentanoic acid (AP5), n = 5–6, or (c), AP5, n = 3. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control; $\#p < 0.05$, $\dagger p < 0.001$ and NS, not significant versus corresponding treatment without NMDA receptor antagonist; one-way ANOVA with Bonferroni's multiple comparison test.

Evaluation of the role of voltage-gated Ca^{2+} channels

Depolarization results in the activation of voltage-gated Ca^{2+} channels, which may set off a whole host of intracellular signaling cascades. As an initial screen for the involvement of these channels, the ability of NMDA to reduce phosphorylation of I-1 and DARPP-32 in the presence of 1 mmol/L Ni^{2+} was assessed (Fig. 6a). At this concentration, Ni^{2+} acts as a non-specific Ca^{2+}-channel blocker. Application of 1 mmol/L Ni^{2+} ablated the effect of NMDA on phospho-Ser6 and phospho-Ser67 I-1 and phospho-Thr75 DARPP-32, returning levels to 94 ± 12%, 88 ± 12%, and 115 ± 7% of control, respectively. However, neither nimodipine, a T-type voltage-gated Ca^{2+}-channel antagonist, nor 100 μmol/L Ni^{2+}, a specific T-type voltage-gated Ca^{2+}-channel antagonist at this concentration, had any effect on the ability of NMDA to reduce phosphorylation of I-1 or DARPP-32 (Fig. 6b). These results suggest either the involvement of another type of Ca^{2+} channel or a non-selective action of 1 mmol/L Ni^{2+}.

Evaluation of the role of Ca^{2+} and Na^{+}

Depolarization generally results from the influx of cations into the cytoplasm. Thus, we assessed the contribution of extracellular Ca^{2+} by removing Ca^{2+} from the buffer during the period of NMDA treatment and replacing it with Mg^{2+} to preserve divalency and avoid surface charge issues. The effect of NMDA on the phosphorylation of I-1 was marginally affected under these conditions, possibly because of enhanced Mg^{2+}-dependent block of the NMDA receptor (Fig. 7a). In contrast, the effect of NMDA on the phosphorylation of DARPP-32 was greatly affected, changing from 22.5 ± 0.8%

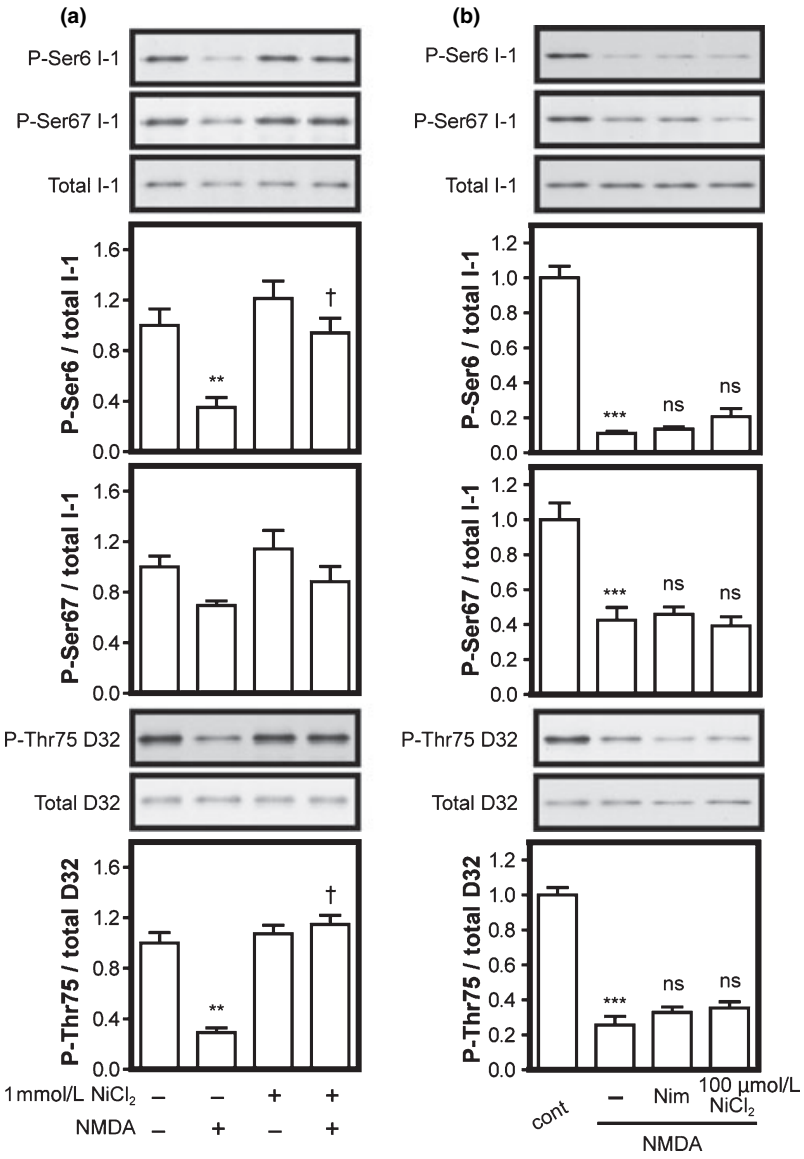

Fig. 6 Evaluation of the role of voltage-gated Ca^{2+} channels. Quantitative immunoblot analysis of acute striatal slices incubated in the absence or presence of 50 μmol/L NMDA for 5 min with or without (a), 5 min pre-incubation with 1 mmol/L $NiCl_2$, $n = 4–6$ or (b), 20 min pre-incubation with 15 μmol/L nimodipine (Nim) or 5 min pre-incubation with 100 μmol/L $NiCl_2$, $n = 5–6$. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus control; †$p < 0.01$ and NS, not significant versus NMDA alone; one-way ANOVA with Bonferroni's multiple comparison test.

of control levels in normal buffer to 70.5 ± 3.1% of control levels in minimal Ca^{2+} buffer. Incomplete reversal of the effect of NMDA on DARPP-32 may have resulted from residual Ca^{2+} in the extracellular matrix.

In a more stringent Ca^{2+}-free treatment paradigm, inclusion of the Ca^{2+} chelator EGTA in minimal Ca^{2+} buffer produced similar results for I-1 (Fig. 7b). Surprisingly, this treatment did not reverse the effect of NMDA on the phosphorylation of DARPP-32, unlike the minimal Ca^{2+} treatment. One explanation is enhanced release of Ca^{2+} from intracellular stores caused by increased influx of Na^+ through voltage-gated Ca^{2+} channels normally guarded by bound Ca^{2+} (Bernath 1992). However, pre-treatment with the calcium store depleter thapsigargin (20 min) did not render EGTA effective in reversing the effect of NMDA on Thr75 (data not shown).

Increases in intracellular Ca^{2+} may result from the influx of extracellular Ca^{2+} or the release of Ca^{2+} from internal stores. No role for the regulation of Ser6, Ser67, or Thr75 by internal stores of Ca^{2+} was found, as pre-treatment of striatal slices with thapsigargin (20 min) did not attenuate the effect of NMDA on levels of phosphorylation of these sites when NMDA was applied under minimal Ca^{2+} conditions (data not shown).

To assess the contribution of Na^+, we replaced Na^+ in the extracellular buffer with the impermeant ion choline during the period of NMDA treatment. Even in the absence of extracellular Na^+, NMDA was able to reduce phosphorylation of all three Cdk5 sites (Fig. 7b). Thus, specific removal of either Ca^{2+} or Na^+ was ineffective in reversing the effect of NMDA on I-1.

However, concurrent removal of both ions completely ablated the effect of NMDA (Fig. 7c). Slices were prepared and recovered in Na^+-free media containing normal amounts of Ca^{2+} then pre-treated for 20 min with Ca^{2+}- and Na^+-free

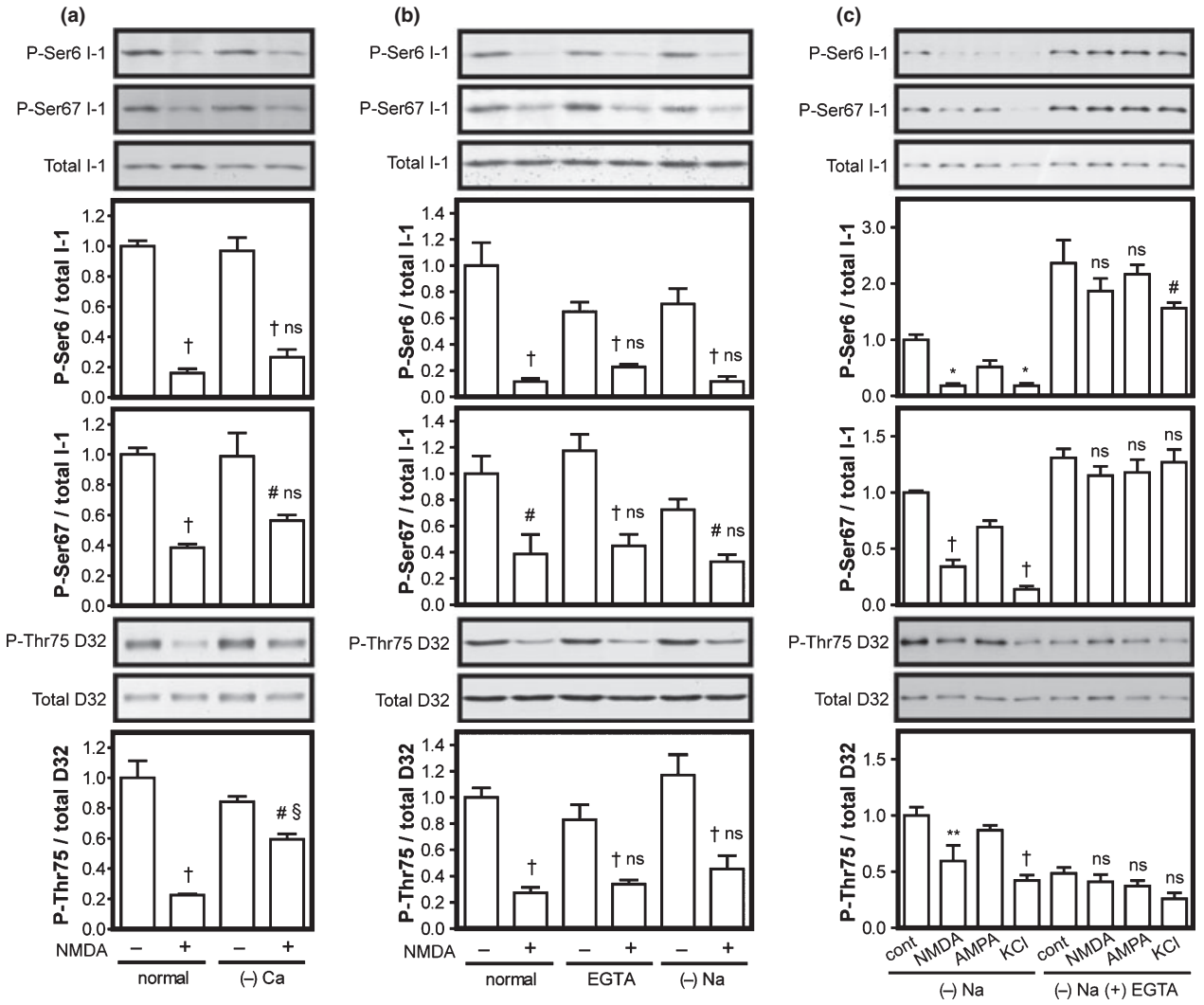

Fig. 7 Evaluation of the role of extracellular cations. Quantitative immunoblot analysis of acute striatal slices incubated in the absence or presence of NMDA (50 μmol/L) for 5 min in normal buffer or in buffer in which (a), Ca^{2+} is replaced by Mg^{2+} [(–) Ca], $n = 4$ or (b), extracellular Ca^{2+} is chelated with 1 mmol/L EGTA (and Ca^{2+} is replaced by Mg^{2+}) or Na^+ is replaced by choline [(–) Na], $n = 3$–6. #$p < 0.05$ and †$p < 0.01$ versus corresponding buffer without NMDA; §$p < 0.01$ and NS, not significant versus normal buffer with NMDA; one-way ANOVA with Bonferroni's multiple comparison test. (c) Quantitative immunoblot analysis of acute striatal slices recovered in choline buffer and treated with NMDA (50 μmol/L), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (50 μmol/L), or KCl (45 mmol/L) for 5 min in choline buffer [(–) Na] or in choline buffer lacking Ca^{2+} and containing 10 mmol/L EGTA [(–) Na (+) EGTA]. *$p < 0.05$, **$p < 0.01$, †$p < 0.001$, and NS, not significant versus corresponding buffer without depolarizing agent; one-way ANOVA with Bonferroni's multiple comparison test.

media containing EGTA before the addition of NMDA, AMPA, or KCl. In contrast to previous experiments in which EGTA was applied only during the period of NMDA treatment, pre-treatment with media containing EGTA caused a large increase in levels of phospho-Ser6 I-1 (2.4 ± 0.6-fold), which was not significantly reversed by NMDA or AMPA. KCl caused a small reversal. Levels of phospho-Ser67 I-1 were unaltered by pre-treatment with EGTA alone or in combination with any of the depolarizing agents, while levels of phospho-Thr75 DARPP-32 were lowered to 48 ± 5% of control by EGTA, with no further reduction upon addition of any of the depolarizing agents. Considered as a whole, these data suggest that simple depolarization, in a non-cation-specific manner, regulates Cdk5-dependent phosphorylation of I-1. In contrast, the regulation of phospho-Thr75 DARPP-32 by depolarization is Ca^{2+}-specific.

Discussion

The regulation of any phosphorylation site depends on the balance of activity between the kinase(s) and the phosphatase(s) acting on the site. In this study, we explored the

mechanism by which NMDA causes a decrease in phosphorylation of two inhibitors of PP-1 at three Cdk5-dependent phosphorylation sites, Ser6 and Ser67 of I-1 and Thr75 of DARPP-32. NMDA-induced reductions in these phosphorylation sites has been reported previously (Bibb *et al.* 2001; Nishi *et al.* 2002; Wei *et al.* 2005; Ahn *et al.* 2007; Nguyen *et al.* 2007).

Studies by Wei *et al.* (2005) support the notion that NMDA and kainate globally induce reduction of phosphorylation at Cdk5 sites in primary cortical neurons and in hippocampal slices by causing the degradation of p35 and the down-regulation of Cdk5 activity. In contrast, studies by Nishi *et al.* (2002) found that levels of Cdk5 activity in striatal slices were unchanged by treatment with NMDA, AMPA, or KCl. These traditional Cdk5 activity assays were conducted as immunoprecipitation-kinase assays in which Cdk5 immunoprecipitated from a detergent-solubilized lysate was allowed to phosphorylate histone H1 in the presence of [γ-^{32}P]-ATP. Given the recent finding that detergent-induced membrane dissociation leads to the activation of latent Cdk5/p35 complexes (Zhu *et al.* 2005; Sato *et al*. in press), the results of these types of Cdk5 assays should be interpreted carefully.

Regardless, the use of phosphatase inhibitors allowed Nishi *et al.* to attribute reductions in levels of phospho-Thr75 DARPP-32 caused by NMDA, AMPA, and KCl to a Ca^{2+}-dependent enhancement of PP-2A activity, the mechanism of which was recently reported (Ahn *et al.* 2007). Supporting the involvement of phosphatases in the NMDA-mediated regulation of phospho-Thr75 DARPP-32 was the finding that NMDA decreased levels of this site to the same extent regardless of the absence or presence of the Cdk5 inhibitor butyrolactone. The apparently contradictory results of Wei *et al.* and Nishi *et al.* might be due to differences in the preparation used (cultured cortical and acute hippocampal vs. acute striatal) and/or the duration of NMDA treatment. The time course of the effect of NMDA on Cdk5 activity in primary cortical neurons conducted by Wei *et al.* reveals transient stimulation of Cdk5 activity at 1 min, a return to control levels at 5 min, and a down-regulation thereafter, with levels reaching a nadir at the longest time point, 60 min. As Nishi *et al.* examined only the 5 min time point, they would not have observed a subsequent decrease in Cdk5 activity. Thus, our studies and those by Nishi *et al.*, both conducted at 5 min of treatment, relate to a shorter NMDA exposure than the studies by Wei *et al.*

We found that 5 min of treatment of striatal slices with NMDA, AMPA, kainate, or KCl all reduced phosphorylation of the Cdk5 sites on I-1 and DARPP-32. While levels of p35 and p25 were unaffected by 5 min of 50 μmol/L NMDA, p39 exhibited an electrophoretic mobility shift consistent with dephosphorylation. The threshold for this p39 shift was the same as the threshold for decreased phosphorylation of I-1 and DARPP-32, but we concluded that the events were probably not causally related, as cyA was able to dissociate them.

N-methyl-D-aspartate receptors were involved in the regulation of phosphorylation by NMDA, but not by AMPA or KCl. Even though 1 mmol/L Ni^{2+} blocked the effect of NMDA on the three Cdk5 sites, L- and T-type voltage-gated Ca^{2+} channels were found to be uninvolved. While 1 mmol/L Ni^{2+} is generally regarded as a non-specific Ca^{2+} channel blocker, it can have a number of other actions. At a concentration of 1 mmol/L, Ni^{2+} alters the properties of numerous channels, including but not limited to NMDA receptors (Gavazzo *et al.* 2006), epithelial Na^{+} channels (Sheng *et al.* 2002), cardiac (Perchenet and Clement-Chomienne 2001) and smooth muscle (Stockand *et al.* 1993) K^{+} channels, and acid-sensing ion channels (Staruschenko *et al.* 2006). Furthermore, increasing the concentration of extracellular divalent cations by addition of Ni^{2+} reduces the negative potential at the surface of the cell, thereby effectively increasing the transmembrane potential (Piccolino and Pignatelli 1996). Stronger depolarizations would then be required to activate voltage-dependent channels. In addition, the direct binding of Ni^{2+} to acidic ligands such as glutamate and kainate has been suggested to compete with free ligands for binding to AMPA receptors (Dorofeeva *et al.* 2005). Thus, blockade of the NMDA effect by 1 mmol/L Ni^{2+} could have resulted from a number of factors.

Assessment of the dependence of the NMDA effect on phosphatases and extracellular Na^{+} and Ca^{2+} revealed differential regulation of the three Cdk5 sites. Entirely consistent with Nishi *et al.* and a subsequent study (Ahn *et al.* 2007), we observed Ca^{2+}- and PP-1/PP-2A-dependent regulation of phospho-Thr75 DARPP-32 by NMDA. In contrast, the regulation of the Cdk5 sites on I-1 was not cation specific. Individual removal of extracellular Na^{+} or Ca^{2+} had minimal effects on the ability of NMDA to decrease the levels of phospho-Ser6 and phospho-Ser67 I-1; however, simultaneous removal of both cations completely blocked the effect of NMDA. Moreover, NMDA-induced decreases in the phosphorylation of Ser6 were partially PP-2B dependent, but those for Ser67 were not PP-1-, PP-2A, or PP-2B dependent. Thus, NMDA appears to regulate the phosphorylation of Cdk5-dependent sites via pathways involving both enhanced phosphatase activity and reduced kinase activity.

The regulation of Cdk5 activity by depolarization may be of importance to a variety of normal and abnormal processes in the central nervous system. PP-1 activity in the striatum, under the regulation of DARPP-32, is crucial in the learning of addictive behaviors (Nairn *et al.* 2004). Similarly, PP-1 activity in the hippocampus, thought to be under the regulation of I-1, is important in the establishment of long-term depression (Mulkey *et al.* 1994; Morishita *et al.* 2001). Thus, Cdk5-dependent modulation of the function of these two inhibitors of PP-1 represents one way by which Cdk5

might affect learning and memory. Indeed, the conditional loss of Cdk5 results in alterations in synaptic plasticity and hippocampal-dependent learning and memory (Hawasli *et al.* 2007).

In conclusion, by altering levels of phosphorylation at the Cdk5-dependent sites of I-1 and DARPP-32, depolarization can positively regulate striatal PP-1 and PKA activity, thereby influencing higher order processes. The multiple distinct mechanisms by which depolarization regulates the phosphorylation of I-1 and DARPP-32 highlight the complexity of the cascades that can originate from a single signal.

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References

Ahn J. H., Sung J. Y., McAvoy T., Nishi A., Janssens V., Goris J., Greengard P. and Nairn A. C. (2007) The B″/PR72 subunit mediates $Ca2^{+}$-dependent dephosphorylation of DARPP-32 by protein phosphatase 2A. *Proc. Natl Acad. Sci. USA* **104**, 9876–9881.

Bernath S. (1992) Calcium-independent release of amino acid neurotransmitters: fact or artifact? *Prog. Neurobiol.* **38**, 57–91.

Bibb J. A., Snyder G. L., Nishi A. *et al.* (1999) Phosphorylation of DARPP-32 by Cdk5 modulates dopamine signalling in neurons. *Nature* **402**, 669–671.

Bibb J. A., Nishi A., O'Callaghan J. P. *et al.* (2001) Phosphorylation of protein phosphatase inhibitor-1 by Cdk5. *J. Biol. Chem.* **276**, 14490–14497.

Chae T., Kwon Y. T., Bronson R., Dikkes P., Li E. and Tsai L. H. (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* **18**, 29–42.

Cheung Z. H., Fu A. K. and Ip N. Y. (2006) Synaptic roles of Cdk5: implications in higher cognitive functions and neurodegenerative diseases. *Neuron* **50**, 13–18.

Dorofeeva N. A., Tikhonov D. B., Barygin O. I., Tikhonova T. B., Salnikov Y. I. and Magazanik L. G. (2005) Action of extracellular divalent cations on native alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors. *J. Neurochem.* **95**, 1704–1712.

Fu W. Y., Wang J. H. and Ip N. Y. (2002) Expression of Cdk5 and its activators in NT2 cells during neuronal differentiation. *J. Neurochem.* **81**, 646–654.

Fu W. Y., Chen Y., Sahin M. *et al.* (2007) Cdk5 regulates EphA4-mediated dendritic spine retraction through an ephexin1-dependent mechanism. *Nat. Neurosci.* **10**, 67–76.

Gavazzo P., Mazzolini M., Tedesco M. and Marchetti C. (2006) Nickel differentially affects NMDA receptor channels in developing cultured rat neurons. *Brain Res.* **1078**, 71–79.

Gustafson E. L., Girault J. A., Hemmings H. C. Jr, Nairn A. C. and Greengard P. (1991) Immunocytochemical localization of phosphatase inhibitor-1 in rat brain. *J. Comp. Neurol.* **310**, 170–188.

Harada T., Morooka T., Ogawa S. and Nishida E. (2001) ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat. Cell Biol.* **3**, 453–459.

Hawasli A. H., Benavides D. R., Nguyen C., Kansy J. W., Hayashi K., Chambon P., Greengard P., Powell C. M., Cooper D. C. and Bibb J. A. (2007) Cyclin-dependent kinase 5 governs learning and synaptic plasticity via control of NMDAR degradation. *Nat. Neurosci.* **10**, 880–886.

Hemmings H. C. Jr and Greengard P. (1986) DARPP-32, a dopamine- and adenosine 3′:5′-monophosphate-regulated phosphoprotein: regional, tissue, and phylogenetic distribution. *J. Neurosci.* **6**, 1469–1481.

Hisanaga S. and Saito T. (2003) The regulation of cyclin-dependent kinase 5 activity through the metabolism of p35 or p39 Cdk5 activator. *Neurosignals* **12**, 221–229.

Kamei H., Saito T., Ozawa M., Fujita Y., Asada A., Bibb J. A., Saido T. C., Sorimachi H. and Hisanaga S. (2007) Suppression of calpain-dependent cleavage of the CDK5 activator p35 to p25 by site-specific phosphorylation. *J. Biol. Chem.* **282**, 1687–1694.

Kerokoski P., Suuronen T., Salminen A., Soininen H. and Pirttila T. (2002) Influence of phosphorylation of p35, an activator of cyclin-dependent kinase 5 (cdk5), on the proteolysis of p35. *Brain Res. Mol. Brain Res.* **106**, 50–56.

Kerokoski P., Suuronen T., Salminen A., Soininen H. and Pirttila T. (2004) Both N-methyl-D-aspartate (NMDA) and non-NMDA receptors mediate glutamate-induced cleavage of the cyclin-dependent kinase 5 (cdk5) activator p35 in cultured rat hippocampal neurons. *Neurosci. Lett.* **368**, 181–185.

Ko J., Humbert S., Bronson R. T., Takahashi S., Kulkarni A. B., Li E. and Tsai L. H. (2001) p35 and p39 are essential for cyclin-dependent kinase 5 function during neurodevelopment. *J. Neurosci.* **21**, 6758–6771.

Kusakawa G., Saito T., Onuki R., Ishiguro K., Kishimoto T. and Hisanaga S. (2000) Calpain-dependent proteolytic cleavage of the p35 cyclin-dependent kinase 5 activator to p25. *J. Biol. Chem.* **275**, 17166–17172.

Lee M. S., Kwon Y. T., Li M., Peng J., Friedlander R. M. and Tsai L. H. (2000) Neurotoxicity induces cleavage of p35 to p25 by calpain. *Nature* **405**, 360–364.

Lew J., Beaudette K., Litwin C. M. and Wang J. H. (1992) Purification and characterization of a novel proline-directed protein kinase from bovine brain. *J. Biol. Chem.* **267**, 13383–13390.

Lew J., Huang Q. Q., Qi Z., Winkfein R. J., Aebersold R., Hunt T. and Wang J. H. (1994) A brain-specific activator of cyclin-dependent kinase 5. *Nature* **371**, 423–426.

Li B. S., Sun M. K., Zhang L., Takahashi S., Ma W., Vinade L., Kulkarni A. B., Brady R. O. and Pant H. C. (2001) Regulation of NMDA receptors by cyclin-dependent kinase-5. *Proc. Natl Acad. Sci. USA* **98**, 12742–12747.

Liu F., Ma X. H., Ule J., Bibb J. A., Nishi A., DeMaggio A. J., Yan Z., Nairn A. C. and Greengard P. (2001) Regulation of cyclin-dependent kinase 5 and casein kinase 1 by metabotropic glutamate receptors. *Proc. Natl Acad. Sci. USA* **98**, 11062–11068.

Morishita W., Connor J. H., Xia H., Quinlan E. M., Shenolikar S. and Malenka R. C. (2001) Regulation of synaptic strength by protein phosphatase 1. *Neuron* **32**, 1133–1148.

Mulkey R. M., Endo S., Shenolikar S. and Malenka R. C. (1994) Involvement of a calcineurin/inhibitor-1 phosphatase cascade in hippocampal long-term depression. *Nature* **369**, 486–488.

Nairn A. C., Svenningsson P., Nishi A., Fisone G., Girault J. A. and Greengard P. (2004) The role of DARPP-32 in the actions of drugs of abuse. *Neuropharmacology* **47** (Suppl. 1), 14–23.

Nath R., Davis M., Probert A. W., Kupina N. C., Ren X., Schielke G. P. and Wang K. K. (2000) Processing of cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. *Biochem. Biophys. Res. Commun.* **274**, 16–21.

Nguyen M. D., Lariviere R. C. and Julien J. P. (2001) Deregulation of Cdk5 in a mouse model of ALS: toxicity alleviated by perikaryal neurofilament inclusions. *Neuron* **30**, 135–147.

Nguyen C., Nishi A., Kansy J. W., Fernandez J., Hayashi K., Gillardon F., Hemmings H. C. Jr, Nairn A. C. and Bibb J. A. (2007) Regulation of protein phosphatase inhibitor-1 by cyclin-dependent kinase 5. *J. Biol. Chem.* **282**, 16511–16520.

Nishi A., Snyder G. L., Nairn A. C. and Greengard P. (1999) Role of calcineurin and protein phosphatase-2A in the regulation of DARPP-32 dephosphorylation in neostriatal neurons. *J. Neurochem.* **72**, 2015–2021.

Nishi A., Bibb J. A., Snyder G. L., Higashi H., Nairn A. C. and Greengard P. (2000) Amplification of dopaminergic signaling by a positive feedback loop. *Proc. Natl Acad. Sci. USA* **97**, 12840–12845.

Nishi A., Bibb J. A., Matsuyama S., Hamada M., Higashi H., Nairn A. C. and Greengard P. (2002) Regulation of DARPP-32 dephosphorylation at PKA- and Cdk5-sites by NMDA and AMPA receptors: distinct roles of calcineurin and protein phosphatase-2A. *J. Neurochem.* **81**, 832–841.

Patrick G. N., Zhou P., Kwon Y. T., Howley P. M. and Tsai L. H. (1998) p35, the neuronal-specific activator of cyclin-dependent kinase 5 (Cdk5) is degraded by the ubiquitin-proteasome pathway. *J. Biol. Chem.* **273**, 24057–24064.

Patrick G. N., Zukerberg L., Nikolic M., de la Monte S., Dikkes P. and Tsai L. H. (1999) Conversion of p35 to p25 deregulates Cdk5 activity and promotes neurodegeneration. *Nature* **402**, 615–622.

Patzke H. and Tsai L. H. (2002) Calpain-mediated cleavage of the cyclin-dependent kinase-5 activator p39 to p29. *J. Biol. Chem.* **277**, 8054–8060.

Perchenet L. and Clement-Chomienne O. (2001) External nickel blocks human Kv1.5 channels stably expressed in CHO cells. *J. Membr. Biol.* **183**, 51–60.

Piccolino M. and Pignatelli A. (1996) Calcium-independent synaptic transmission: artifact or fact? *Trends Neurosci.* **19**, 120–125.

Sahin B., Shu H., Fernandez J., El-Armouche A., Molkentin J. D., Nairn A. C. and Bibb J. A. (2006) Phosphorylation of protein phosphatase inhibitor-1 by protein kinase C. *J. Biol. Chem.* **281**, 24322–24335.

Saito T., Ishiguro K., Onuki R., Nagai Y., Kishimoto T. and Hisanaga S. (1998) Okadaic acid-stimulated degradation of p35, an activator of CDK5, by proteasome in cultured neurons. *Biochem. Biophys. Res. Commun.* **252**, 775–778.

Saito T., Onuki R., Fujita Y., Kusakawa G., Ishiguro K., Bibb J. A., Kishimoto T. and Hisanaga S. (2003) Developmental regulation of the proteolysis of the p35 cyclin-dependent kinase 5 activator by phosphorylation. *J. Neurosci.* **23**, 1189–1197.

Sasaki Y., Cheng C., Uchida Y. *et al.* (2002) Fyn and Cdk5 mediate semaphorin-3A signaling, which is involved in regulation of dendrite orientation in cerebral cortex. *Neuron* **35**, 907–920.

Sato K., Zhu Y. S., Saito T., Yotsumoto K., Asada A., Hasegawa M. and Hisanaga S. (2007) Regulation of the membrane association and kinase activity of Cdk5-p35 by phosphorylation of p35. *J. Neurosci. Res.* in press.

Sharma P., Sharma M., Amin N. D., Albers R. W. and Pant H. C. (1999) Regulation of cyclin-dependent kinase 5 catalytic activity by phosphorylation. *Proc. Natl Acad. Sci. USA* **96**, 11156–11160.

Sheng S., Perry C. J. and Kleyman T. R. (2002) External nickel inhibits epithelial sodium channel by binding to histidine residues within the extracellular domains of alpha and gamma subunits and reducing channel open probability. *J. Biol. Chem.* **277**, 50098–50111.

Staruschenko A., Dorofeeva N. A., Bolshakov K. V. and Stockand J. D. (2006) Subunit-dependent cadmium and nickel inhibition of acid-sensing ion channels. *J. Neurobiol.* **67**, 97–107.

Stockand J., Sultan A., Molony D., DuBose T. Jr and Sansom S. (1993) Interactions of cadmium and nickel with K channels of vascular smooth muscle. *Toxicol. Appl. Pharmacol.* **121**, 30–35.

Tang D. and Wang J. H. (1996) Cyclin-dependent kinase 5 (Cdk5) and neuron-specific Cdk5 activators. *Prog. Cell Cycle Res.* **2**, 205–216.

Tang D., Yeung J., Lee K. Y., Matsushita M., Matsui H., Tomizawa K., Hatase O. and Wang J. H. (1995) An isoform of the neuronal cyclin-dependent kinase 5 (Cdk5) activator. *J. Biol. Chem.* **270**, 26897–26903.

Tsai L. H., Delalle I., Caviness V. S. Jr, Chae T. and Harlow E. (1994) p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* **371**, 419–423.

Wang J., Liu S., Fu Y., Wang J. H. and Lu Y. (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. *Nat. Neurosci.* **6**, 1039–1047.

Wei F. Y., Tomizawa K., Ohshima T. *et al.* (2005) Control of cyclin-dependent kinase 5 (Cdk5) activity by glutamatergic regulation of p35 stability. *J. Neurochem.* **93**, 502–512.

Zhen X., Goswami S., Abdali S. A., Gil M., Bakshi K. and Friedman E. (2004) Regulation of cyclin-dependent kinase 5 and calcium/calmodulin-dependent protein kinase II by phosphatidylinositol-linked dopamine receptor in rat brain. *Mol. Pharmacol.* **66**, 1500–1507.

Zhu Y. S., Saito T., Asada A., Maekawa S. and Hisanaga S. (2005) Activation of latent cyclin-dependent kinase 5 (Cdk5)-p35 complexes by membrane dissociation. *J. Neurochem.* **94**, 1535–1545.

Zukerberg L. R., Patrick G. N., Nikolic M., Humbert S., Wu C. L., Lanier L. M., Gertler F. B., Vidal M., Van Etten R. A. and Tsai L. H. (2000) Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation, and neurite outgrowth. *Neuron* **26**, 633–646.

