

## MOLECULAR AND DEVELOPMENTAL NEUROSCIENCE

# Involvement of cyclin-dependent kinase-5 in the kainic acid-mediated degeneration of glutamatergic synapses in the rat hippocampus

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## Abstract

Increased levels of glutamate causing excitotoxic damage accompany neurological disorders such as ischemia/stroke, epilepsy and some neurodegenerative diseases. Cyclin-dependent kinase-5 (Cdk5) is important for synaptic plasticity and is deregulated in neurodegenerative diseases. However, the mechanisms by which kainic acid (KA)-induced excitotoxic damage involves Cdk5 in neuronal injury are not fully understood. In this work, we have thus studied involvement of Cdk5 in the KA-mediated degeneration of glutamatergic synapses in the rat hippocampus. KA induced degeneration of mossy fiber synapses and decreased glutamate receptor (GluR)6/7 and post-synaptic density protein 95 (PSD95) levels in rat hippocampus *in vivo* after intraventricular injection of KA. KA also increased the cleavage of Cdk5 regulatory protein p35, and Cdk5 phosphorylation in the hippocampus at 12 h after treatment. Studies with hippocampal neurons *in vitro* showed a rapid decline in GluR6/7 and PSD95 levels after KA treatment with the breakdown of p35 protein and phosphorylation of Cdk5. These changes depended on an increase in calcium as shown by the chelators 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid acetoxymethyl ester (BAPTA-AM) and glycol-bis (2-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid. Inhibition of Cdk5 using roscovitine or employing dominant-negative Cdk5 and Cdk5 silencing RNA constructs counteracted the decreases in GluR6/7 and PSD95 levels induced by KA in hippocampal neurons. The dominant-negative Cdk5 was also able to decrease neuronal degeneration induced by KA in cultured neurons. The results show that Cdk5 is essentially involved in the KA-mediated alterations in synaptic proteins and in cell degeneration in hippocampal neurons after an excitotoxic injury. Inhibition of pathways activated by Cdk5 may be beneficial for treatment of synaptic degeneration and excitotoxicity observed in various brain diseases.

## Introduction

Excitotoxicity with overactivation of ionotropic glutamate receptors (GluRs) is a major cause of cell death in several neurological diseases. Excitotoxicity is accompanied by an increase in calcium levels in neurons with activation of various proteases, such as calpains (Vanderklish & Bahr, 2000) and caspases (Mattson, 2003). However, the precise signaling pathways for cell demise in excitotoxicity are not fully understood. We have previously shown that excitotoxicity induced by the GluR agonist kainic acid (KA) leads to endoplasmic reticulum stress in hippocampal neurons with subsequent cell degeneration (Sokka *et al.*, 2007). Concomitant with changes in

signaling pathways, synaptic proteins are affected in many neurological disorders (Korhonen & Lindholm, 2004; Korhonen *et al.*, 2005). In this work, we have studied the role of cyclin-dependent kinase-5 (Cdk5) in the alterations of synaptic proteins by KA as a means of understanding early signals mediating excitotoxicity in hippocampal neurons.

The proline-directed serine/threonine protein kinase Cdk5 is an atypical cyclin-dependent kinase expressed primarily in post-mitotic neurons and activated by binding to the p35 and p39 activator proteins (Tsai *et al.*, 1994; Cai *et al.*, 1997). Deregulation of Cdk5 secondary to cleavage of p35 and p39 proteins occurs in some neurodegenerative diseases (Weishaupt *et al.*, 2003), such as Alzheimer's disease (Kobayashi *et al.*, 1993; Noble *et al.*, 2003) and Huntington's disease (Paoletti *et al.*, 2008). Lewy bodies, accumulating in Parkinson's disease, are also immunoreactive for Cdk5 and p35 proteins (Nakamura *et al.*, 1997).

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It has been shown that Cdk5 is important for synaptic plasticity and learning, and for normal development of the brain (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Zhang *et al.*, 1997; Hawasli & Bibb, 2007; Lai & Ip, 2009). Cdk5 influences the clustering of the post-synaptic density protein 95 (PSD95) complex (Morabito *et al.*, 2004) and regulates trafficking of metabotropic GluRs (Orlando *et al.*, 2009) and *N*-methyl-D-aspartate (NMDA) receptor subunits (Li *et al.*, 2001). These observations suggest that Cdk5 is important for the regulation of GluRs and proper synaptic function in the brain.

Kainate receptors (KARs) are involved in both pre- and post-synaptic functions in the nervous system, and are important in synaptic plasticity and learning (Jane *et al.*, 2009; Vincent & Mulle, 2009). KARs are homo- or heteromeric combinations of five types of subunits, GluR5 [glutamate receptor ionotropic kainate (GIRK)1] GluR6 (GRIK2), GluR7 (GRIK3), KA1 (GRIK4) and KA2 (GRIK5) (Jaskolski *et al.*, 2005). As shown using gene-deleted animals, GluR6 is essentially involved in KA-induced epileptic seizures and in neuronal degeneration in the cornu ammonis (CA)3 region in the hippocampus (Mulle *et al.*, 1998). In this work, we found that KA induces an activation of Cdk5 in cultured hippocampal neurons as well as in the hippocampus *in vivo*. KA treatment decreased the levels of GluR6/7 and PSD95 in the hippocampal neurons. Inhibition of Cdk5 using roscovitine, or the use of silencing RNA (siRNA) or dominant-negative (DN) Cdk5 constructs counteracted the decreases in these proteins in cultured hippocampal neurons, indicating an important role of Cdk5 in KA-mediated synaptic degeneration and excitotoxicity.

## Materials and methods

### Animals

Experiments were approved by the ethical committees at the University of Helsinki and the University of Palermo, and carried out in accordance with the European Communities Council Directive (86/609/EEC). Adult male Wistar rats (200–300 g) (B&K, Hull, UK) were injected with KA (Sigma, Helsinki, Finland) into the lateral ventricle (0.35  $\mu\text{g}/\mu\text{L}$ ) in a volume of 0.5  $\mu\text{L}$  per side as described previously (Sokka *et al.*, 2007), and killed at different time-points from 15 min to 48 h. Controls received an equal volume of saline. Rats were decapitated under deep anesthesia with chloral hydrate, and brains were dissected, frozen in isopentane, cooled in liquid nitrogen and stored at  $-70^\circ\text{C}$  until analysis. The right side of the brain was used for histology and the left for western blot analyses.

### Neuronal cultures

Hippocampal neurons were prepared from embryonic day 17 Wistar rats (Harlan, Holland) and plated on poly-ornithine (Sigma)-coated six-well plates at a density of  $2 \times 10^6$  cells (2.0  $\text{cm}^2$ ). Cells were cultured in Neurobasal medium containing 2% B27 as described previously (Korhonen *et al.*, 2001; Sokka *et al.*, 2007; Kairisalo *et al.*, 2009). Cell death was induced by treating neurons for different times with 100  $\mu\text{M}$  KA (Calbiochem, Espoo, Finland), which induces cell death in approximately 40–50% of the neurons within 24 h as reported previously (Korhonen *et al.*, 2001; Sokka *et al.*, 2007). In some experiments, 1–10  $\mu\text{M}$  1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester (BAPTA-AM) (Sigma), 20  $\mu\text{M}$  roscovitine (Sigma) or 2 mM glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetra-acetic acid (EGTA) was added 10–60 min before KA.

### Modulation of cyclin-dependent kinase-5 in neurons

To downregulate Cdk5,  $5 \times 10^6$  neurons were transfected with 100 nM siRNA constructs against Cdk5 (On TargetPlus Smartpool, Dharmacon) using the Rat Neuron Nucleofector system (Amaxa GmbH, Germany) essentially as described previously (Kairisalo *et al.*, 2009). A scrambled siRNA construct was used as control. Cdk5 activity was also inhibited using the DN-Cdk5 expression plasmid with mutation in the active site (D144N) linked to the hemagglutinin epitope (van den Heuvel & Harlow, 1993) or to enhanced green fluorescent protein (EGFP) (courtesy of Dr Tsai, Massachusetts Institute of Technology, USA). The DN-Cdk5-EGFP was transfected into hippocampal neurons as above, and the vector containing EGFP was used as control. After 5 days in culture, neurons were treated with 100  $\mu\text{M}$  KA for 1–24 h and immunoblots were made as described below.

### Calcium imaging

Hippocampal neurons plated on polyornithine-coated glass coverslips were loaded with 4  $\mu\text{M}$  fura-2 acetoxymethyl ester (Invitrogen, Carlsbad, CA, USA) for 20 min at  $37^\circ\text{C}$  in Hepes-buffered medium (137 mM NaCl, 5 mM KCl, 1 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgCl}_2$ , 0.44 mM  $\text{KH}_2\text{PO}_4$ , 4.2 mM  $\text{NaHCO}_3$ , 10 mM glucose, 20 mM Hepes, pH 7.4), rinsed once and used immediately.  $\text{Ca}^{2+}$  measurements were performed at  $35^\circ\text{C}$  using a Nikon TE2000 fluorescence microscope [magnification 20 $\times$  and numerical aperture 0.75 (20 $\times$  /0.75) air objective] and Andor iXon 885 EM-CCD camera under the control of Nikon NIS ELEMENTS AR software with 6D extension essentially as described previously (Korhonen *et al.*, 2005; Reijonen *et al.*, 2010). For  $\text{Ca}^{2+}$  imaging, the cells were excited with alternating 340 and 380 nm light (Sutter DG4 Plus) and the emitted light collected through a 400 nm dichroic mirror and a 450 nm long-pass filter. Transfected cells were selected based upon EGFP fluorescence. Additions were made by constant perfusion (Hepes-buffered medium). Regions of interest (cell bodies) were defined in NIS software and the data extracted to Microsoft Excel for visualization and quantification. A total of 19–70 cells were measured in each experiment, and each experiment was repeated four times or more.

### Western blotting

Immunoblotting was performed essentially as described previously (Korhonen *et al.*, 2001; Sokka *et al.*, 2007; Reijonen *et al.*, 2008; Kairisalo *et al.*, 2009). In brief, hippocampal tissue and neurons were lysed using ice-cold radioimmunoprecipitation assay buffer (150 mM NaCl, 1% Triton-X-100, 0.5% sodium deoxycholate, 1% sodium dodecyl sulfate, 50 mM Tris-HCl, pH 7.4) supplemented with protease inhibitor cocktail (Roche, Espoo, Finland) and phosphatase inhibitors (PhoStop; Roche). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto nitrocellulose filters (Amersham, Helsinki, Finland), which were incubated for 1 h in 5% skimmed milk or bovine serum albumin, in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20 and then with primary antibodies overnight at  $4^\circ\text{C}$ . These included antibodies against GluR6/7 (diluted 1 : 1000, no. 04-921; Millipore/Upstate), PSD95 (1 : 2000, no. 610496; BD Biosciences, VWR International Oy, Espoo, Finland), Cdk5 (1:2000, no. 05-364; Millipore/Upstate), [phospho-cyclin-dependent-kinase-5 (p-Cdk5)(tyrosine 15)](Y15) (1:1000, no. sc-12918; Santa Cruz Biotechnologies, AH Diagnostics, Helsinki, Finland), p35/25 (1 : 1000, no. C64B10, Cell Signaling Technologies, Finnzymes, Helsinki, Finland) and  $\beta$ -actin (1 : 1000, no. 2066; Sigma). After washing, the filter was incubated with horseradish

peroxidase-conjugated secondary antibodies (1 : 2500; Jackson ImmunoResearch Laboratories, Espoo, Finland), followed by detection using enhanced chemiluminescence (Pierce, Helsinki, Finland). Quantifications were performed using the IMAGEJ software (version 1.41o).

### Immunocytochemistry

#### *In vivo*

Rat brain sections (10  $\mu$ m) were fixed for 10 min at  $-20^{\circ}\text{C}$  using acetone:methanol (1 : 1). Slides were incubated for 1 h with phosphate-buffered saline/5% bovine serum albumin (Sigma)/0.1% Triton-X-100 at room temperature ( $20^{\circ}\text{C}$ ), and then overnight at  $4^{\circ}\text{C}$  with primary antibodies [PSD95 (1 : 500; no. 12093; Abcam), GluR6/7 (1 : 100), neuronal nuclei (1 : 100; MAB377, Millipore/Chemicon) or  $\beta$ -tubulin (1 : 500; MMS-4351, Nordic Biosite/Covance)] followed by secondary Alexa 488- or 594-conjugated antibodies (1 : 500; Jackson ImmunoResearch Laboratories). The nuclei were counterstained with bisbenzamide/Hoechst 33258 (Sigma).

#### *In vitro*

Neurons were fixed for 20 min using 4% paraformaldehyde, incubated for 1 h using phosphate-buffered saline/5% bovine serum albumin/0.1% Triton-X and stained as above with antibodies for GluR6/7, PSD95, p-Cdk5 and synapsin I (1 : 500, no. 61392; Nordic Biosite/Covance). Stainings were visualized by fluorescent microscope (Leica DM4500B, Espoo, Finland).

### Cell degeneration assay

#### *In vivo*

Fluoro-Jade staining for degenerating nerve cells was performed essentially as described previously (Korhonen *et al.*, 2001, 2005; Sokka *et al.*, 2007).

#### *In vitro*

Hippocampal neurons were transfected with DN-Cdk5-EGFP or with EGFP expression vector as above. KA (100  $\mu\text{M}$ ) was added for 24 h and the nuclei were labeled with Hoechst 33258 blue (Sigma). The number of cells with condensed or fragmented chromatin was counted as an index of degenerating neurons.

### Confocal imaging

Neurons were analyzed at room temperature with a confocal microscope (LSM 510 Meta; Carl Zeiss, Inc.) using LSM AIM software and a magnification 63 $\times$  and numerical aperture 1.4 (63 $\times$ /1.4) Plan-Apochromat oil objective. Images were processed using IMAGEJ and ADOBE PHOTOSHOP CS software.

### Quantification and statistics

Statistical comparisons were performed using one-way ANOVA followed by a Bonferroni or Tukey's (if more than five groups) *post-hoc* test. The Student's unpaired *t*-test was used in experiments with two groups.  $P \leq 0.05$  was considered significant. Four to eight rats were used for each time point *in vivo*, and the *in vitro* assays were repeated more than three times. Values are expressed as mean  $\pm$  SEM.

## Results

### *Kainic acid induces synaptic degeneration and decreases post-synaptic density protein 95 and glutamate receptor 6/7 levels in rat hippocampal neurons in vivo*

To study the hippocampal pathways altered in excitotoxicity, we treated rats with KA *in vivo* as described in Materials and methods. Intraventricular injections of KA have been shown to mainly cause cell degeneration of neurons in the CA3 region of the hippocampus as well as in the amygdala and less cell degeneration of CA1 neurons, which is observed more with intraperitoneal injections. To study neuronal degeneration, we used Fluoro-Jade, which has been shown to detect both degenerating cell bodies and dendrites after excitotoxicity-induced injuries in rat retina (Chidlow *et al.*, 2009) and dopaminergic neurons (El-Khodori & Burke, 2002). Fluoro-Jade D staining revealed a degeneration of neurites in the hippocampal CA3 area in the KA-treated rats (Fig. 1A) and a reduction in PSD95-positive synaptic terminals in the CA3 area in the hippocampus (Fig. 1B). Immunoblotting of tissue lysates showed a reduction in PSD95 levels in the hippocampus at 12–48 h after KA administration (Fig. 1C; one-way ANOVA with Bonferroni *post-hoc* test and Student's unpaired *t*-test,  $P < 0.01$  for control vs. 12 h KA and  $P < 0.05$  for control vs. 48 h and 72 h KA).

We then studied the effect of KA on GluR6, which is a subtype of GluR in the brain that is important for KA-mediated effects (Mulle *et al.*, 1998). For this we used an antibody that recognizes both GluR6 and GluR7 receptor subtypes, and therefore we here use the term GluR6/7. It is of note that the expression of GluR7 is very low in the hippocampus particularly in the CA3 area (Bureau *et al.*, 1999), and thus our data may primarily reflect changes in GluR6. Immunostaining data showed that GluR6/7 decreased in the CA3 area of the hippocampus at 12–24 h after KA administration (Fig. 1D). Double staining using antibodies against neuronal nuclei, microtubuli ( $\beta$ -tubulin) and GluR6/7 indicated that the decrease in GluR6/7 in the mossy fiber area after KA occurred with the preservation of the cell soma of many CA3 neurons (Fig. 1E). Immunoblotting of tissue lysates also showed a downregulation of GluR6/7 in the hippocampus at 3 h after KA administration with a rebound at longer times that may reflect new synthesis of the receptor during the recovery phase (Fig. 1F; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  for control vs. 6 h KA and  $P < 0.05$  for control vs. 3, 12, 24 and 72 h KA).

### *Kainic acid also decreases post-synaptic density protein 95 and glutamate receptor 6/7 levels in hippocampal neurons in vivo*

The effects of KA *in vivo* are rather complex and reflect changes in individual neurons, as well as alterations in neuronal circuits and in activation of glial cells that occur with time. To obtain a more detailed and mechanistic insight into the KA-mediated effects, we studied cultured hippocampal neurons prepared from rat hippocampus. Data showed that KA also induced a decrease in PSD95 levels in cultured hippocampal neurons that occurred within the first hours of incubation, as shown by immunostaining and immunoblots (Fig. 2A and B; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  control vs. 1 and 3 h KA).

Immunostaining of GluR6/7 in the hippocampal neurons showed that the addition of KA also rapidly reduced GluR6/7, observed particularly within the dendrites of hippocampal neurons (Fig. 2C). Immunoblotting confirmed that the levels of GluR6/7 in the hippocampal neurons decreased markedly within the first 30 min



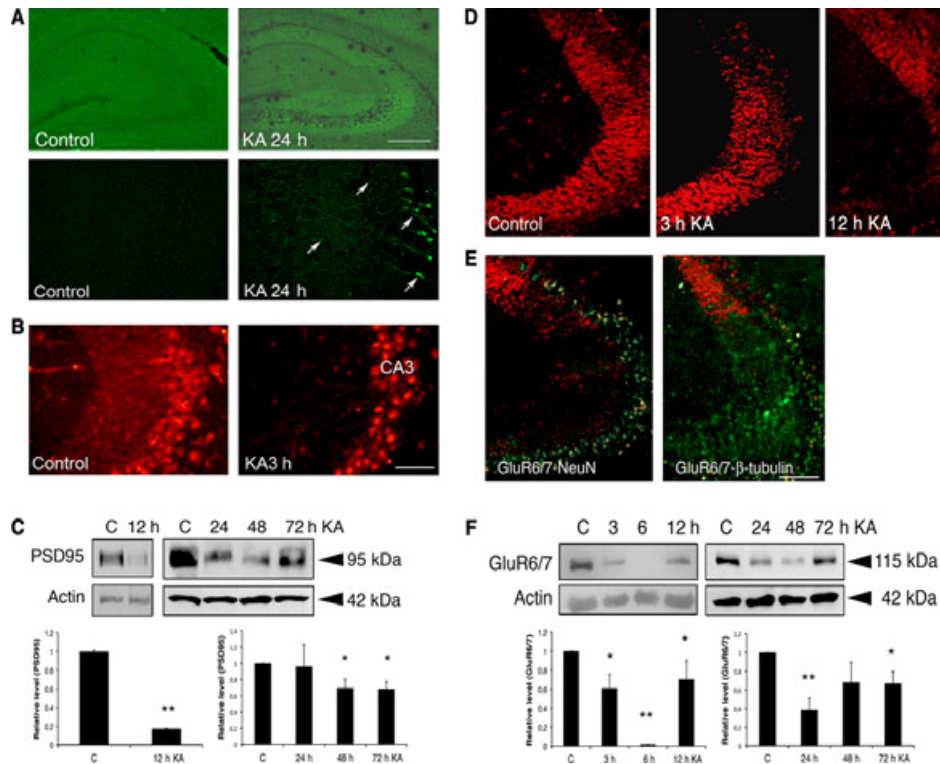


FIG. 1. KA treatment induces degeneration of hippocampal synapses and reduces levels of PSD95 and GluR6/7 *in vivo*. (A) Rats were treated with KA for 24 h as described in Materials and methods. Upper panel: whole hippocampus. Lower panel: higher magnification. Fluoro-Jade staining (green fluorescence) showed cell degeneration in the rat hippocampal CA3 area by KA. Arrows indicate some of the degenerating neurons. Scale bar: upper panel, 500  $\mu\text{m}$ ; lower panel, 100  $\mu\text{m}$ . (B) Immunostaining was performed as described in Materials and methods. PSD95 immunoreactivity was reduced in the CA3 region. Scale bar, 100  $\mu\text{m}$ . (C) Immunoblots were made as described in Materials and methods. PSD95 was downregulated after KA and  $\beta$ -actin was used as control (C). Values are means  $\pm$  SEM,  $n = 3$ . \*\* $P < 0.01$  or \* $P < 0.05$  for KA vs. C. (D) GluR6/7 immunoreactivity (red) in the CA3 area was reduced by KA treatment. Scale bar, 100  $\mu\text{m}$ . (E) Immunostaining of GluR6/7 (red) in conjunction with the nuclear marker, neuronal nuclei (NeuN), or the microtubule marker,  $\beta$ -tubulin (green), at 12 h of KA treatment. Note reduction in GluR6/7 with the preservation of neuronal soma. Scale bar, 100  $\mu\text{m}$ . (F) Immunoblot. Note a decrease in GluR6/7 after KA.  $\beta$ -actin was used as control (C). Values are means  $\pm$  SEM,  $n = 3$ . \*\* $P < 0.01$  or \* $P < 0.05$  for KA vs. C.

after KA addition (Fig. 2D and E; one-way ANOVA with Tukey's *post-hoc* test,  $P < 0.01$  for control vs. 30 min–24 h KA). This decrease in GluR6/7 by KA *in vitro* was more robust and long lasting than that observed *in vivo* (cf. Fig. 1F), suggesting that there is a more fine-tuned regulation of GluR6/7 levels in brain compared with the *in vitro* situation.

#### Kainic acid induces a calcium- and calpain-dependent cleavage of p35 protein in cultured hippocampal neurons

The activation of GluRs is linked to increases in intracellular calcium that in turn affect various signaling molecules in neurons. We have previously shown that KA induces endoplasmic reticulum stress and increases calcium levels in cultured hippocampal neurons (Sokka *et al.*, 2007). In a search for changes in upstream molecules, we observed that treatment of cultured hippocampal neurons with 100  $\mu\text{M}$  KA caused a cleavage of the Cdk5 activator protein p35 into the p25 fragment that started at about 15 min after KA addition (Fig. 3A and B; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  control vs. 15 min–3 h KA). To study whether the breakdown of p35 by KA was linked to changes in calcium levels, we analyzed the dose- and time-dependent calcium movements in KA-treated hippocampal neurons. Data showed that KA treatment resulted in a dose-dependent increase in intracellular calcium level (Fig. 3C) with the activation of the calcium-dependent protease calpain, shown by the accumulation of

calpain-specific 145 kDa cleavage products of  $\alpha$ -spectrin (Fig. 3D). Inhibition of the intracellular calcium increase by the chelation of intracellular or extracellular calcium using BAPTA-AM (Fig. 3E and F; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  for control vs. KA and  $P < 0.01$  for KA vs. KA+BAPTA-AM) or EGTA (Fig. 3G and H), respectively, attenuated the accumulation of p25 brought about by KA. In addition, the calpain inhibitor XI prevented the p35 cleavage by KA, indicating that calpain is involved in this process (Fig. 3G and H; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  for control vs. KA and  $P < 0.01$  for KA vs. EGTA+KA and  $P < 0.01$  for KA vs. XI+KA).

#### Kainic acid induces an increase in cyclin-dependent kinase-5 phosphorylation in hippocampal neurons in culture and in vivo

Previous studies have shown that the cleavage of p35 into p25 results in the deregulation of Cdk5 with changes in the localization and stability of Cdk5 (Kusakawa *et al.*, 2000; Lee *et al.*, 2000; Asada *et al.*, 2008). To study the role of Cdk5 in the excitotoxicity induced by KA, we analyzed p-Cdk5, as a measure of the increased activation of this kinase. Data showed that 100  $\mu\text{M}$  KA induced a time-dependent increase in Cdk5 phosphorylation in the hippocampal neurons after 30 min of incubation (Fig. 4A and B; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  for KA vs. 1 h-KA). Immunostaining further revealed that an accumulation of phosphor-

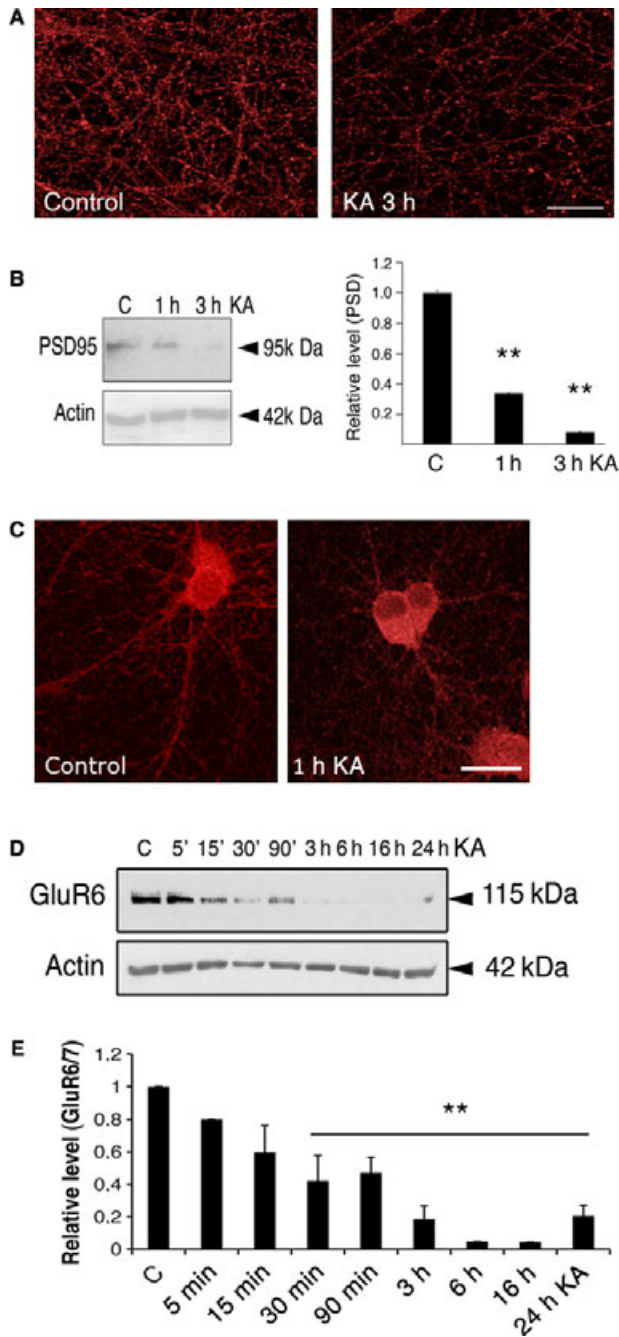


FIG. 2. KA downregulates PSD95 and GluR6/7 in cultured hippocampal neurons. Hippocampal neurons were cultured from embryonic day 17 rat hippocampus and treated with 100  $\mu$ M KA and analyzed further as described in Materials and methods. (A) Immunostaining. PSD95 immunoreactivity was reduced in hippocampal neurons at 3 h after KA treatment. Scale bar, 5  $\mu$ m. (B) Immunoblot shows downregulation of PSD95. Values are means  $\pm$  SEM,  $n = 3$ . \*\* $P < 0.01$  for KA vs. control (C). (C) Immunostaining. GluR6/7 was decreased within dendrites after KA stimulation. Scale bar, 20  $\mu$ m. (D) Immunoblots. Neurons were incubated with KA for various periods of time. GluR6/7 decreased starting after 15 min of incubation.  $\beta$ -actin was used as control (C). (E) Values are means  $\pm$  SEM,  $n = 3$ . \*\* $P < 0.01$  for KA vs. C. PSD, postsynaptic density.

ylated Cdk5 protein occurred within neurites in KA-treated neurons (Fig. 4C). Pre-treatment of cells with BAPTA-AM or EGTA reduced the Cdk5 phosphorylation induced by KA (Fig. 4D and E; one-way ANOVA with Tukey's *post-hoc* test,  $P < 0.01$  for control vs. KA,

$P < 0.01$  for KA vs. EGTA-KA and  $P < 0.01$  for KA vs. BAPTA-AM+KA), showing an involvement of calcium in the regulation of Cdk5 by KA. Immunoblots using tissue lysates from KA-treated rats substantiated the data obtained *in vitro*, and showed an increase in p-Cdk5 and in the breakdown of p35 by KA in the hippocampus occurring mainly at 12 h after KA administration (Fig. 4F and G; one-way ANOVA with Tukey's *post-hoc* test,  $P < 0.01$  for control vs. 12 h KA vs. control in the case of p-Cdk5 and p25, and  $P < 0.05$  for control vs. 24–72 h KA in the case of p35).

#### Inhibition of cyclin-dependent kinase-5 counteracts the effects of kainic acid in cultured hippocampal neurons

To study the role of Cdk5 in more detail we sought to inhibit its action in the hippocampal neurons. Pre-treatment of the neurons with roscovitine, a known inhibitor for various cyclin-dependent kinases including Cdk5 (Meijer *et al.*, 1997), reduced the downregulation of GluR6/7 and PSD95 observed with KA at 3 h (Fig. 5A and B; one-way ANOVA with Tukey's *post-hoc* test,  $P < 0.01$  for 3 h KA vs. roscovitine +3 h KA). Immunolabeling of GluR6/7 in roscovitine-treated neurons revealed a localization of the receptor adjacent to the cell membrane, which was more pronounced in cells treated with KA (Fig. 5C). Apart from roscovitine, we modulated the level or activity of Cdk5 in hippocampal neurons using siRNA and DN constructs for Cdk5. Data showed that the DN-Cdk5 counteracted the decrease in PSD95 and GluR6 induced by KA in the neurons (Fig. 5D and E; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.05$  for KA vs. DN-Cdk5+KA in the case of GluR6/7 and  $P < 0.01$  for KA vs. DN-Cdk5+KA in the case of PSD95). Similarly, the downregulation of Cdk5 using siRNA prevented the decrease in GluR6/7 caused by KA (Fig. 5F and G; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.05$  for KA vs. DN-Cdk5+KA). As the inhibition of Cdk5 affected the levels of GluR6/7 we hypothesized that this might affect the calcium movements in the neurons. Thus, we used calcium imaging as described in Materials and methods to distinguish between DN-Cdk5-expressing or roscovitine-treated and control neurons. However, there was no significant difference in calcium movements between control and Cdk5-inhibited neurons (data not shown).

To study the long-term effects of Cdk5 inhibition, we analyzed the neuronal cell death induced by KA after 24 h. Data showed that the number of degenerating neurons was reduced in cultures treated with DN-Cdk5 compared with KA-treated controls (Fig. 5H; one-way ANOVA with Bonferroni *post-hoc* test,  $P < 0.01$  for control vs. KA and  $P < 0.05$  for KA vs. DN-Cdk5+KA). This shows that the neuronal damage induced by KA is less in the presence of the DN-Cdk5 construct inhibiting Cdk5 activity.

#### Discussion

In this work, we show that treatment with an excitotoxic dose of KA causes a rapid degeneration of mossy fiber synapses and a decrease in GluR6/7 levels in rat hippocampus *in vivo* as well as in cultured hippocampal neurons. The results obtained support the view that Cdk5 deregulation is instrumental in this process, as it occurs subsequent to the cleavage of the activator protein p35 that is induced by calpain and by the elevation of intracellular calcium levels in hippocampal neurons after KA stimulation. In keeping with this, inhibition of Cdk5 using roscovitine or employing DN-Cdk5 and siRNA constructs counteracted the decreases in GluR6/7 and PSD95 observed after KA in cultured hippocampal neurons. The present results on the involvement of Cdk5 in KA-mediated excitotoxicity add to previous findings on the

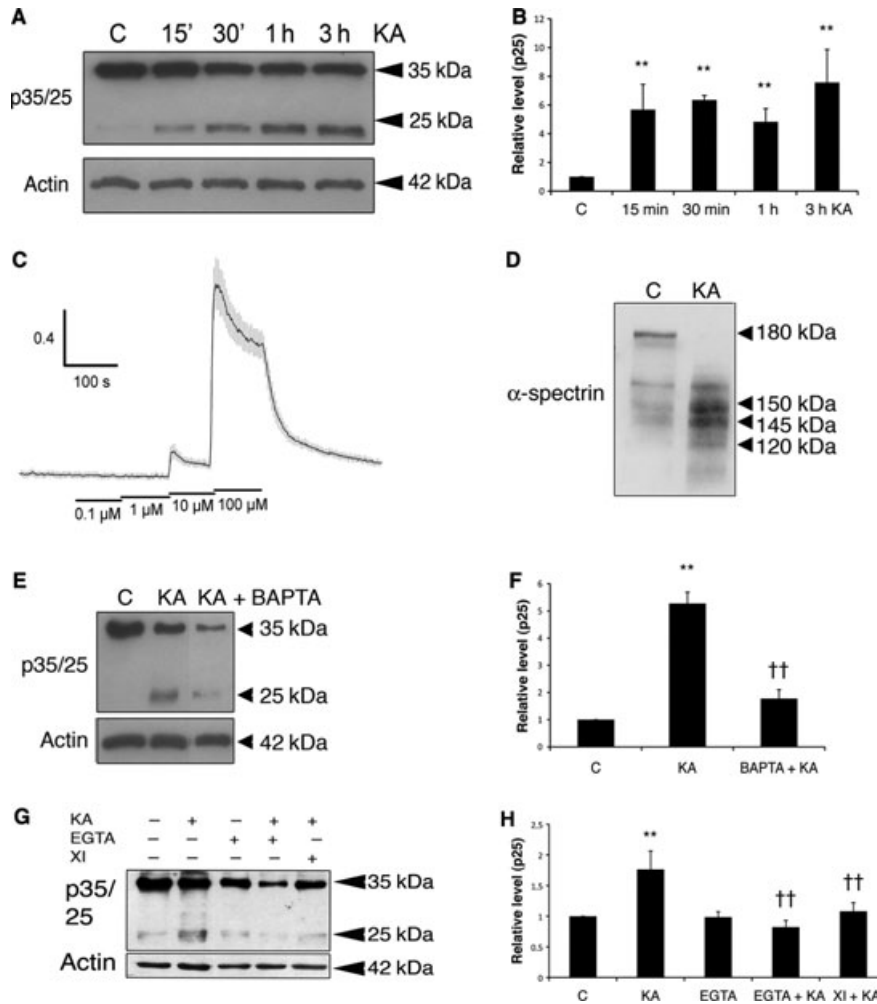


FIG. 3. KA induces a calcium- and calpain-dependent cleavage of the p35 protein in cultured hippocampal neurons. (A and B) Immunoblots. Hippocampal neurons were treated with 100  $\mu$ M KA for different times. The Cdk5 activator p35 was cleaved into the p25 fragment after KA.  $\beta$ -actin was used as control (C). Values are means  $\pm$  SEM,  $n = 4$ .  $**P < 0.01$  for KA treatment vs. C. (C) Fura-2 calcium imaging was performed as described in Materials and methods. Typical  $\text{Ca}^{2+}$  response to KA is shown as an average trace  $\pm$  SEM (a single experiment with 35 cells). For the sake of clarity, error bars are only drawn for every other point. Cells were stimulated with increasing concentrations of KA as indicated. The vertical calibration bar indicates the ratio 340 : 380. (D) Immunoblots. Activation of calpain produces a specific cleavage of  $\alpha$ -spectrin shown here as the 145 kDa protein fragment. A typical experiment is shown and was repeated three times. (E and F) Immunoblots. Hippocampal neurons were treated with the calcium chelator (1 mM BAPTA-AM) at 30 min prior to KA. BAPTA-AM reduced the cleavage of p35 to the p25 protein.  $\beta$ -actin was used as control (C). Values are means  $\pm$  SEM,  $n = 4$ .  $**P < 0.01$  for KA vs. C and  $\dagger\dagger P < 0.01$  for KA+BAPTA-AM vs. KA. (G and H) The calcium chelator EGTA and the calpain inhibitor XI decreased the KA-induced increase in p25.  $\beta$ -actin was used as control (C). Values are means  $\pm$  SEM,  $n = 4$ .  $**P < 0.01$  for KA vs. C. and  $\dagger\dagger P < 0.01$  for EGTA+KA and XI+KA vs. KA.

importance of Cdk5 in Alzheimer's disease (Kobayashi *et al.*, 1993; Noble *et al.*, 2003) and other neurological disorders (reviewed in Dhariwala & Rajadhyaksha, 2008).

Cyclin-dependent kinase-5 has been implicated in numerous processes in the nervous system ranging from developmental effects in neurons to modulation of synaptic plasticity and learning in adults (Ohshima *et al.*, 1996; Chae *et al.*, 1997; Zhang *et al.*, 1997; Hawasli & Bibb, 2007; Lai & Ip, 2009). Activation of Cdk5 requires the binding of the activator proteins, p35 or p39, which have a rather restricted expression in brain tissue (Tsai *et al.*, 1994; Cai *et al.*, 1997). At the subcellular level, Cdk5 localizes to the cytoplasm and nucleus (Ino & Chiba, 1996). However, the interaction of Cdk5 with myristoylated p35 or p39 leads to the recruitment of Cdk5 to cellular membranes both in the cell soma and within dendrites (Asada *et al.*, 2008). It has been shown that the cleavage of p35 into p25 affects Cdk5 in several ways, by influencing the activity, localization and protein-protein interactive network of Cdk5 (Kusakawa *et al.*, 2000;

Lee *et al.*, 2000; Patzke & Tsai, 2002). Recently, inhibition of Cdk5 activity was shown to potentiate the release of neurotransmitters and thereby unmask so-called 'silent' synapses in neurons (Kim & Ryan, 2010). In the post-synaptic compartment, Cdk5 regulates dendritic spine formation through phosphorylation of cytoskeleton-binding proteins, including neurofilaments and microtubule-associated proteins, as well as Tau and other signaling molecules (reviewed in Lai & Ip, 2009). Activation of Cdk5 after increased neuronal activity was shown to phosphorylate the N-terminal domain of PSD95 and decrease the clustering of PSD95 and NMDA receptors at synapses, whereas the inhibition of Cdk5 had the opposite effects (Morabito *et al.*, 2004). Cdk5 was further shown to phosphorylate the NMDA receptor subunit 2A and to enhance NMDA-mediated currents in hippocampal neurons (Li *et al.*, 2001). Studies of conditionally Cdk5 gene-deleted mice showed that the degradation of NMDA receptor subunit NMDA receptor subunit 2B and the threshold for induction of long-term potentiation were altered in brain (Hawasli *et al.*, 2007).



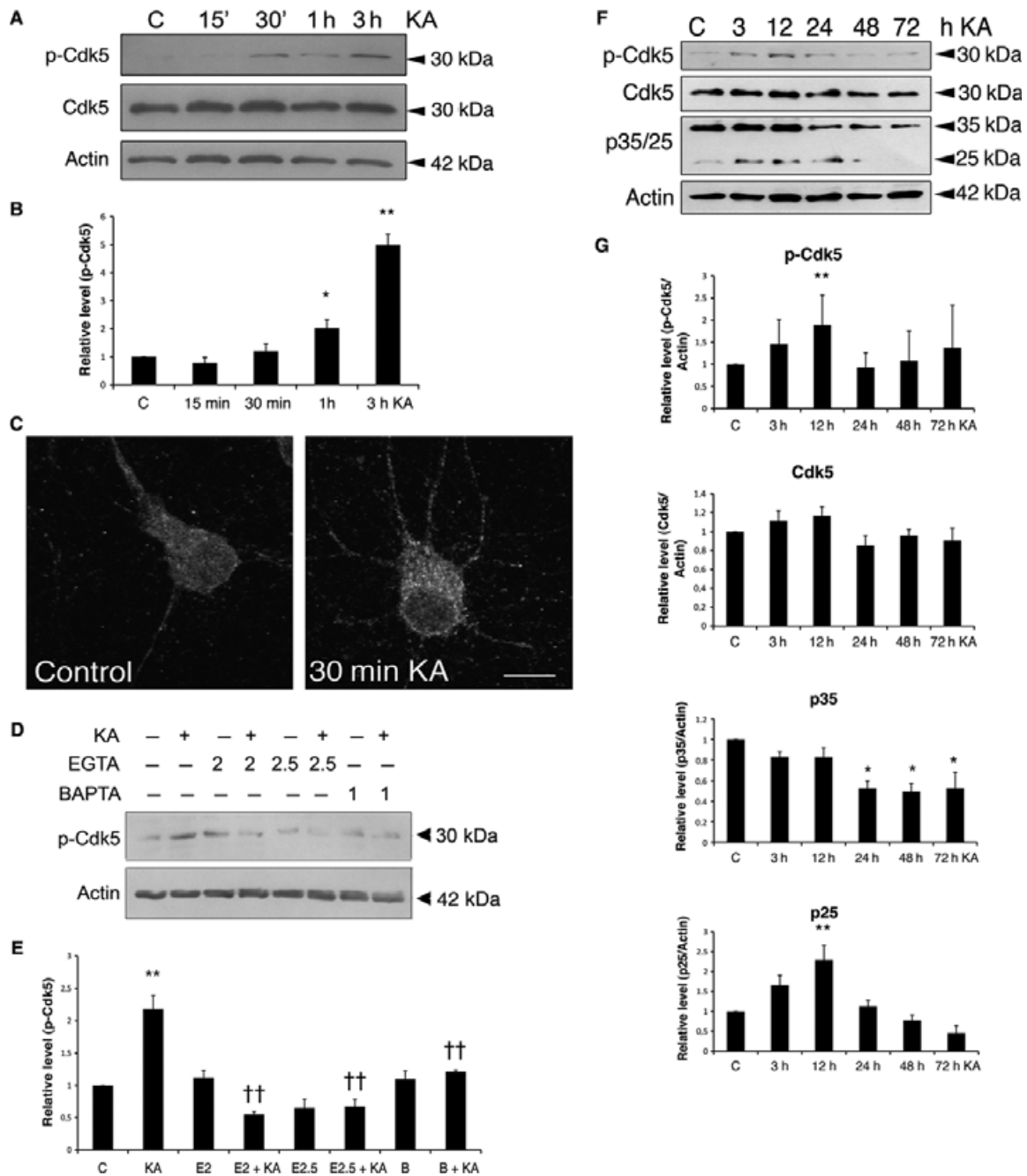


FIG. 4. KA treatment induces Cdk5 phosphorylation in hippocampal neurons in culture and *in vivo*. Hippocampal neurons were treated with 100  $\mu$ M KA for different times and analyzed further as indicated. Administration of KA *in vivo* was performed as in Fig. 1. (A and B) Immunoblots. Phosphorylation of Cdk5 (p-Cdk5) was induced by KA stimulation with no changes in total Cdk5 levels. Values are means + SEM,  $n = 4$ . \*\* $P < 0.01$  and \* $P < 0.05$  for KA vs. control (C). (C) Immunostaining of cultured neurons. Phosphorylated Cdk5 is localized particularly within neurites at 30 min after KA stimulation. Scale bar, 10  $\mu$ m. (D and E) Immunoblots. Pre-treatment with 2 mM EGTA (E2) or 2.5 mM EGTA (E2.5) or 1 mM BAPTA-AM (B) reduced the increase in p-Cdk5 induced by KA. Values are means + SEM,  $n = 4$ . \*\* $P < 0.01$  for KA vs. C and †† $P < 0.01$  for EGTA+KA and BAPTA-AM+KA treatments vs. KA. (F) *In vivo* experiments. KA was administered for 12 h. Note the increase in the levels of p25 and p-Cdk5 in the hippocampus by KA.  $\beta$ -actin was used as control (C). (G) Values are means + SEM,  $n = 4$ . \*\* $P < 0.01$  for 12 h KA vs. C in the case of p-Cdk5 and p25, and \* $P < 0.05$  for 24 h KA vs. C in the case of p35.

Recently, Cdk5 was also shown to regulate the  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor subunit GluR2-mediated synaptic activity via phosphorylation of D-catenin (Poore *et al.*, 2010).

In this work, we have studied the involvement of Cdk5 in KA-induced excitotoxicity in neurons, and particularly analyzed the synaptic proteins, GluR6/7 and PSD95, that are important for proper

glutamatergic neurotransmission in the mossy fibers in the hippocampus. We show here that KA stimulation induced a phosphorylation of Cdk5 and an increased cleavage of the regulatory protein p35 in cultured hippocampal neurons. Similar changes were also observed in hippocampus *in vivo* with the largest effects observed at 12 h after KA administration. As studied *in vitro*, the cleavage of p35 and the increase in p-Cdk5 depended on the increase in intracellular

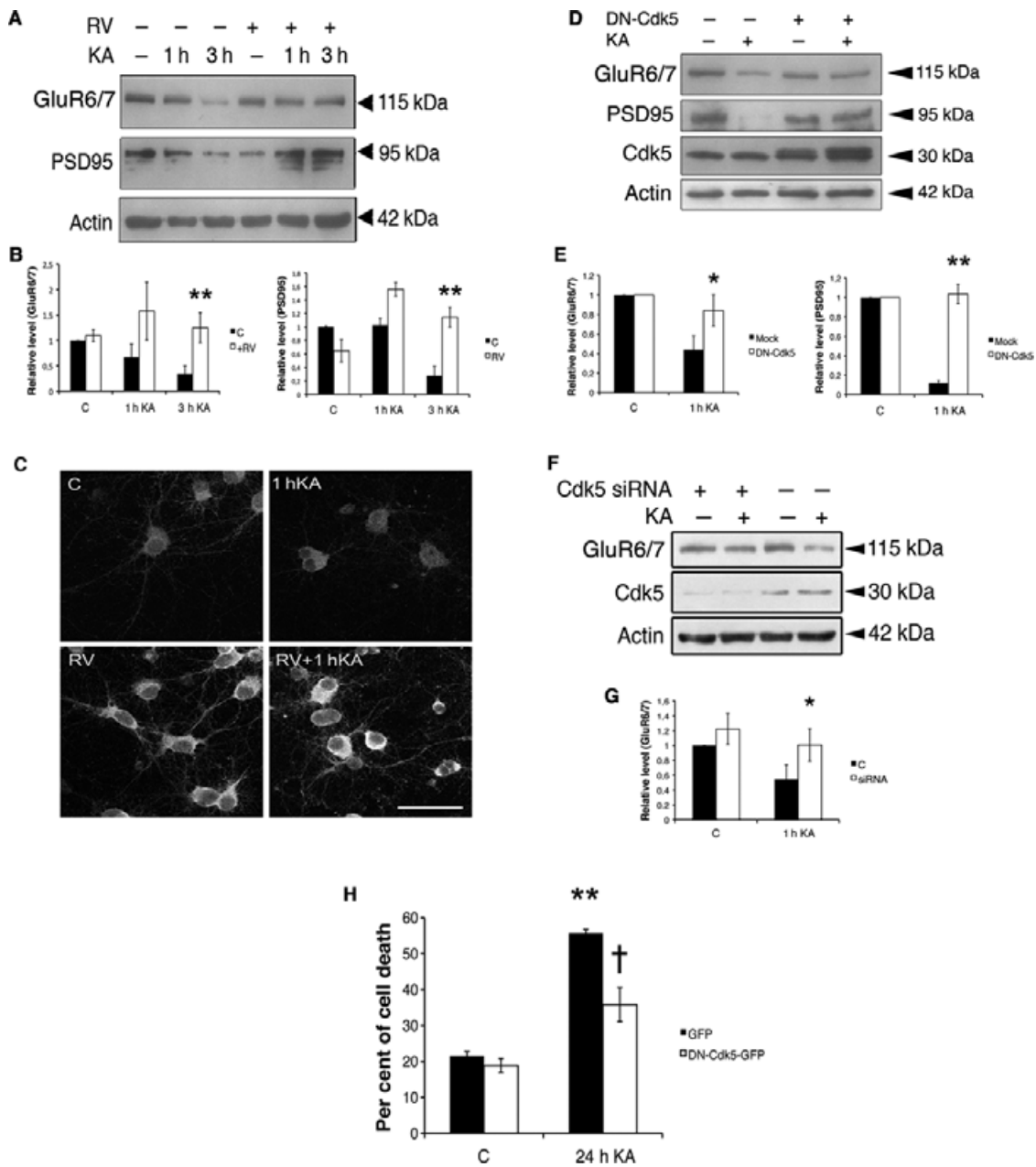


FIG. 5. Inhibition of Cdk5 counteracts KA-induced downregulation of PSD95 and GluR6 in cultured hippocampal neurons. Hippocampal neurons were treated with 100  $\mu$ M KA alone or in the presence of the cyclin-dependent kinase inhibitor roscovitine (RV). In some experiments, neurons were transfected with siRNA against Cdk5 or with the dominant-negative (DN) Cdk5 construct prior to KA treatments. (A and B) Immunoblots. Addition of RV counteracts the downregulation of GluR6/7 and PSD95 induced by KA. Values are means  $\pm$  SEM,  $n = 3$ .  $^{*}P < 0.01$  for RV+KA vs. KA at 3 h. (C) Confocal images of hippocampal neurons were acquired as described in Materials and methods. Note the increased GluR6/7 immunostaining by roscovitine in KA-treated neurons. Scale bar, 50  $\mu$ m. (D and E) Immunoblots. Expression of DN-Cdk5-hemagglutinin reduced the decrease in GluR6/7 and PSD95 induced by KA at 3 h. Values are means  $\pm$  SEM,  $n = 3$ .  $^{*}P < 0.05$  or  $^{**}P < 0.01$  for DN-Cdk5+KA vs. KA in the case of both GluR6/7 and PSD95. (F and G) Immunoblots. Cdk5-siRNA reduced Cdk5 levels in neurons at 5 days of transfection and reduced the decrease in GluR6/7 induced by KA. Values are means  $\pm$  SEM,  $n = 3$ .  $^{*}P < 0.05$  for siRNA+KA vs. KA. (H) Neurons transfected with DN-Cdk5-EGFP or with EGFP as controls (C) were stimulated with 100  $\mu$ M KA for 24 h. Nuclei were labeled with Hoechst blue and the number of transfected cells with condensed or fragmented chromatin was counted as an index of degenerating cells. Values are means  $\pm$  SEM,  $n = 3$ .  $^{*}P < 0.01$  for KA vs. C and  $^{\dagger}P < 0.05$  for DN-Cdk5+KA vs. KA.

calcium brought about by KA as this was blocked by BAPTA-AM and EGTA. Inhibition of the calcium-dependent protease calpain also reduced the p35 cleavage induced by KA. This indicates that KA can activate calpain at an early stage, which links the calcium increase to subsequent downstream cellular events in excitotoxicity. Our study with KA confirms previous data showing a cleavage of p35 after activation of GluRs using NMDA,  $\alpha$ -amino-3-hydroxy-5-

methylisoxazole-4-propionic acid and glutamic acid as agonists (Kerokoski *et al.*, 2004). However, the links between p35 and the deregulation of Cdk5 and alterations in synaptic proteins and neuronal excitotoxicity induced by KA have not been documented before.

It has been shown that KARs participate in the excitatory postsynaptic currents occurring between the mossy fibers and CA3



pyramidal cells (Castillo *et al.*, 1997). In the pre-synaptic compartment, KARs affect the neurotransmitter release through activation of GluR6/GluR7 (Lerma, 2006). In addition, extrasynaptic KARs, in CA1 pyramidal cells, may increase neuronal excitability through a metabotropic action (Melyan *et al.*, 2002). In *GluR6* gene-deleted mice, the mossy fiber excitatory post-synaptic currents and the pre-synaptic actions of KARs are missing (Mulle *et al.*, 1998; Contractor *et al.*, 2001). Recently, KARs were shown to control the efficacy of spike transmission between pre-synaptic granule cells and CA3 pyramidal cells by a combination of pre- and post-synaptic actions (Sachidhanandam *et al.*, 2009). Polymorphisms in the *GluR6* gene have been associated with a number of mental disorders, e.g. schizophrenia and bipolar disorders (Beneyto *et al.*, 2007; Blackwood *et al.*, 2007). As shown in *GluR6* gene-deleted mice, activation of GluR6 is central for epileptiform seizures induced by KA and for cell death in the hippocampal CA3 area (Mulle *et al.*, 1998).

In this work, we have identified Cdk5 as an important downstream target for the action of KA in hippocampal neurons. Blocking of the Cdk5 pathway by roscovitine or using the DN-Cdk5 construct restored the levels of GluR6/7 and PSD95 in hippocampal neurons stimulated by KA. Roscovitine is an inhibitor of the cyclin-dependent kinase family of proteins including Cdk5, and it has been shown to be neuroprotective in animal models for focal ischemia and in different tauopathies (Wen *et al.*, 2007; Menn *et al.*, 2010). The structurally related compound olomoucine also reduces ischemia-induced reactive astrogliosis (Zhu *et al.*, 2007). However, both roscovitine and olomoucine may also have unwanted effects that may preclude their direct application in neurological disorders. It was recently reported that the Cdk5/p25 inhibitory peptide, which targets only the deregulated Cdk5/p25 complex (Zheng *et al.*, 2002), showed promising effects in nerve cell cultures (Kanungo *et al.*, 2009). However, more studies on this peptide are needed to elucidate its effects *in vivo* as a potentially beneficial agent in neurodegeneration (Kanungo *et al.*, 2009).

Taken together, the present findings show an essential function of Cdk5 in the regulation of synaptic integrity and GluR6/7 and PSD5 levels in excitotoxicity induced by KA in hippocampal neurons. Cdk5 has different protein target proteins and affects various signaling events in neurons. The mechanisms through which Cdk5 influences synaptic proteins and excitotoxicity in neurons remain to be studied further.

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## Abbreviations

BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid acetoxymethyl ester; CA, cornu ammonis; Cdk5, cyclin-dependent kinase-5; DN, dominant-negative; EGFP, enhanced green fluorescent protein; EGTA, glycol-bis(2-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid; GluR, glutamate receptor; GRIK, glutamate receptor ionotropic kainate; KA, kainic acid; KAR, kainate receptor; NMDA, *N*-methyl-D-aspartate; p-Cdk5, phospho-cyclin-dependent-kinase-5; PSD95, post-synaptic density protein 95; siRNA, silencing RNA.

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