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Inhibition of aberrant cyclin-dependent kinase 5 activity attenuates isoflurane neurotoxicity in the developing brain

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ABSTRACT

Aberrant CDK5 activity is implicated in a number of neurodegenerative disorders. Isoflurane exposure leads to neuronal apoptosis, and subsequent learning and memory defects in the developing brain. The present study was designed to examine whether and how CDK5 activity plays a role in developmental isoflurane neurotoxicity. Rat pups and hippocampal neuronal cultures were exposed to 1.5% isoflurane for 4 h. The protein and mRNA levels of CDK5, p35 and p25 were detected by western blot and QReal-Time PCR. CDK5 activity was evaluated in vitro using Histone H1 as a substrate. Roscovitine (an inhibitor of CDK5) was applied before isoflurane treatment, cleaved Caspase-3, Bcl-2, Bax, MEF2 and phospho-MEF2A-Ser-408 expressions were determined. Dominant-Negative CDK5 was transfected before isoflurane treatment. Neuronal apoptosis was evaluated by Flow cytometry (FCM) and TUNEL-staining. Cognitive functions were assessed by Morris water maze. We found that isoflurane treatment led to an aberrant CDK5 activation due to its activator p25 that was cleaved from p35 by calpain. Inhibition of CDK5 activity with Roscovitine enhanced Bcl-2, and decreased cleaved Caspase-3 and Bax expressions. In addition, isoflurane exposure resulted in a decrease of MEF2 and increase of phospho-MEF2A-Ser-408, which were rescued by Roscovitine or Dominant-Negative CDK5 transfection. Dominant-Negative CDK5 transfection also decreased the percentage of TUNEL-positive cells in isoflurane neurotoxicity. Moreover, Roscovitine remarkably alleviated the learning and memory deficits induced by postnatal isoflurane exposure. These results indicated that aberrant CDK5 activity-dependent MEF2 phosphorylation mediates developmental isoflurane neurotoxicity. Inhibition of CDK5 overactivation contributes to the relief of isoflurane neurotoxicity in the developing brain.

1. Introduction

It is now well established that, both in rodents and primates, inhalational anesthetics (e.g. isoflurane) in isolation are capable of inducing wide neuronal apoptosis in the developing brain (Brambrink et al., 2010; Jiang et al., 2012; Lemkuil et al., 2011). Several investigations have even observed the persistent learning and memory abnormalities during adulthood after the postnatal animal exposure to inhalational anesthetics (Jevtovic-Todorovic et al., 2003; Sanders et al., 2009). Recent studies showed that the neuronal apoptosis was similar in extent and distribution pattern among equipotent anesthetics, such as desflurane, isoflurane and sevoflurane (Istaphanous et al., 2011), indicating no particular

Abbreviations: Calpain, Ca^{2+}-activated protease; CDK5, Cyclin-dependent kinase 5; DIV, Day in vitro; DN CDK5, Dominant-Negative cyclin-dependent kinase 5; FCM, Flow cytometry; LSCM, Laser scanning confocal microscope; MDL 28170, N-benzyloxycarbonylvalylphenylalaninal; MEF2, Myocyte enhancer factor 2; MWM, Morris water maze; PND, Postnatal day; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling; Z-VAD.fmk, N-benzyloxycarbonyl-valine-alanine-aspartate fluoromethylketone.

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advantages related to neurotoxicity of using one particular anesthetic over another. Given that inhalational anesthetics are widely used in clinical settings, especially in neonates and infants, the comprehensive molecular mechanisms underlying the developmental anesthetic neurotoxicity remain to be elucidated.

Cyclin-dependent kinase 5 (CDK5) is a member of the family of cyclin-dependent kinases (CDKs) and predominantly active in post-mitotic neurons where its neuron-specific activator p35 and p25 are mainly expressed (Dhavan and Tsai, 2001). Although belonging to the CDK family, CDK5 lacks a role in cell cycle control, but is implicated in an astounding array of neuronal functions, including neuronal survival and death as well as synaptic plasticity (Angelo et al., 2006; Cheung et al., 2006). Physiologically, CDK5 is temporally regulated by p35 (half-life, 20–30 min), leading to an unstable formation of p35/CDK5 complex. Interestingly, under pathological conditions, p35 can be proteolytically cleaved by Ca^{2+}-activated protease (calpain) to the smaller and more stable protein p25 (half life, 2–3 h), resulting in too much CDK5 activity (Kusakawa et al., 2000; Lee et al., 2000). The ensuing inappropriate gain-of-function of CDK5 has been proposed to play a key role in the molecular events linking neurotoxic insults to neuronal apoptosis (Patrick et al., 1999). In addition, p25 has been reported to alter CDK5 localization as generation of p25 resulted in cleavage of the amino-terminal myristoylation site that tethers the p35/CDK5 complex to the intracellular plasma membrane (Asada et al., 2008; Shelton and Johnson, 2004). Moreover, the accumulation of p25/CDK5 complex *per se* causes profound neuronal apoptosis and contributes to the pathogenesis of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease (Chang et al., 2010; Wong et al., 2011).

Myocyte enhancer factor 2 (MEF2, isoforms A–D) family of transcription factors plays key roles in diverse cellular processes including neuronal survival (McKinsey et al., 2002). MEF2s are an endpoint for several critical intracellular signaling pathways that control the molecular machinery of cellular survival and apoptosis (Mao et al., 1999). In addition, inactivation of MEF2 during neuronal apoptosis is associated with hyperphosphorylation of MEF2 (Mao and Wiedmann, 1999). Evidence showed that p25 can mislocalize CDK5 to the nucleus and phosphorylate MEF2 at a distinct serine in its transactivation domain to inhibit MEF2 activity. Specifically, CDK5 directly interacts with MEF2A, and is reported to be responsible for neurotoxicity-induced apoptosis (Gong et al., 2003).

Based on these findings, we postulated that inhalational anesthetics, e.g. isoflurane, may overactivate CDK5, leading to neuronal apoptosis through inhibition of MEF2 in the developing brain. If so, inhibition of CDK5 overactivation may protect developing neurons against isoflurane cytotoxicity. The current study was carried out to test this possibility using rat pups and primary hippocampal neuronal cultures. Characterizing these processes may provide evidence for the possible prevention of developmental anesthetic neurotoxicity.

2. Materials and methods

2.1. Animals and regents

Sprague–Dawley rats were gained from China Academy of Chinese Medical Sciences (Shanghai, China). A total of 128 Sprague–Dawley rats were used in this study, including 16 pregnant rats (embryonic day 18 used for neuronal cultures preparation) and 112 rat pups (postnatal day 7 used for in vivo experiments). All studies performed on animals were approved by the Institutional Animal Care and Use Committee (Shanghai JiaoTong University School of Medicine, Shanghai, China). Animals were housed with food and water available *ad libitum*, and maintained throughout the experiments on a 12-h light–dark cycle. Every effort was made to minimize the suffering and number of animals.

Unless specified, all regents were gained from Abcam (Cambridge, MA, USA), Tocris Bioscience (Ellisville, MO, USA), BD Biosciences (San Jose, CA, USA), Santa Cruz Biotechnology (Santa Cruz, CA, USA), Sigma–Aldrich (St. Louis, MO, USA) and GIBCO Invitrogen (Carlsbad, CA, USA).

2.2. Neuronal cultures and cell transfection

Rat hippocampal neurons were derived from embryonic day 18 Sprague–Dawley rat embryos as described previously (Kaech and Banker, 2007). Briefly, hippocampal tissues were dissected, gently minced and trypsinized (Trypsin 0.05%; 37 °C, 5% CO_2 for 10 min), and the digestion was stopped by DMEM plus 10% heat-inactivated FBS. Cells were plated at the concentration of 3×10^5 per milliliter onto dishes or coverslips which were coated with 100 μg/ml poly-L-lysine. Neurons were maintained in humidified atmosphere of 5% CO_2, 95% O_2 at 37 °C, and fed with Neurobasal media supplemented with 2% B27, 0.5 mM L-glutamine, 10 mM HEPES and 1% penicillin–streptomycin. Half of the medium was replaced with fresh medium every 2–3 days. Dominant-Negative CDK5 (DN CDK5) was prepared as described previously (Tian et al., 2009), and lentiviruses were applied to neuronal cultures on 4 DIV (day in vitro) using the Lipofectamine 2000 reagent (Invitrogen, San Diego, CA, USA). Experiments were performed 72 h after transfection.

2.3. Isoflurane exposure

Neuronal cultures and rat pups were placed in a tightly sealed plastic chamber at 37 °C as described previously (Wang et al., 2013). 1.5% isoflurane was exposed for 4 h. For neuronal cultures, isoflurane was flushed in a gas mixture of 95% O_2 and 5% CO_2. For in vivo study, 7 PND (postnatal day) rat pups were treated with isoflurane in a gas mixture of 30% O_2 and 70% N_2. Control experiments were performed in the same manner, except no isoflurane was added when flushing the chamber. The gas mixture was delivered at a flow rate of 2 L/min. The concentrations of isoflurane, O_2 and CO_2 in the chamber were continuously monitored using a Datex Capnomac Ultima gas analyzer (Datex Ohmeda, USA). After isoflurane exposure, rat pups were euthanized by overdose pentobarbital (100 mg/kg i.p.) for Western blot, others were transferred to their cages for behavior study at 28 PND.

2.4. Western blot

The hippocampal tissues and neuronal cultures were harvested and subjected to lysis buffer. The protein concentrations were determined by Bradford Protein Assay (Bio-Rad, Hercules, California, USA). 30 μg lysates were loaded and separated by 10%–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene fluoride (PVDF) membrane which was incubated with appropriate primary antibodies followed by secondary antibodies (Luo et al., 2008). Bands were processed with ECL detection reagents, and developed on Kodak radiography film (Fuji, Japan). The bands were digitally scanned and analyzed using Image J software (Available at: http://rsb.info.nih.gov/ij/).

2.5. Immunocytochemistry

Hippocampal neurons were fixed with 4% paraformaldehyde in PBS for 15 min. The neurons were permeabilized in buffered 0.3% Triton X-100 for 15 min and then blocked with 10% goat serum for 1 h. Neurons were incubated with the anti-CDK5 (1:100) for 48 h at 4 °C. Secondary antibody to CDK5 was Goat Anti-Mouse coupled to TRITC (Red) incubated at 37 °C for 1 h. Nuclei were stained with Hoechst 33342 (0.25 μg/ml). Neuronal DNA damage was quantified by TUNEL-staining according to the manufacturer's protocol. Briefly, neuron-bearing coverslips were fixed, permeabilized with 0.01% Triton X-100 in phosphate buffered saline (PBS) containing 1% sodium citrate, and then incubated (30 min, 37 °C) with the TUNEL reaction mixture. The coverslips were washed, incubated (1 h, 37 °C) with Rhodamine (TRITC)-Streptavidin (Jackson, West Grove, PA, USA), and mounted on glass slides. Twenty fields were evaluated per culture. Three separate cultures were included in each experimental group. Immunofluorescence was captured by Laser scanning confocal microscope (Zeiss, Germany).

2.6. Flow cytometry analysis

After treatment with isoflurane, neuronal cultures were double labeled with Annexin V-FITC and propidium iodide (PI) using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Jose, CA, USA) for flow cytometry (FCM) analysis. Briefly, the cells were washed with PBS and then detached with 0.25% trypsin-EDTA. Cells were resuspended in 1 ml Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). 2×10^5 cells were stained with Annexin V-FITC (5 μl) and PI (0.5 μg/ml) in 100 μl of binding buffer at 4 °C, according to the protocol of manufacturer. Samples were incubated in the dark at room temperature for 15 min. Subsequently, 400 μl of binding buffer was added, and samples were analyzed using a tri-laser FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.7. CDK5 kinase activity assay

CDK5 kinase assay was performed as described previously (Zhao et al., 2009). Briefly, hippocampus tissues and hippocampal cultures were prepared in