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Research article

Cyclin-dependent kinase 5/Collapsin response mediator protein 2 pathway may mediate sevoflurane-induced dendritic development abnormalities in rat cortical neurons

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HIGHLIGHTS

- Sevoflurane led to CDK5 activation by increasing p25 expression.
- Sevoflurane increased the expression and cytoplasmic distribution of phospho-CRMP2 (Ser522).
- Sevoflurane suppressed dendritic branching by activating the CDK5/CRMP2 pathway.

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ABSTRACT

Sevoflurane has been reported to induce neurotoxicity and cognitive impairment in the developing brains. However, the underlying molecular mechanisms remain poorly understood. Recent studies have demonstrated aberrant cyclin-dependent kinase 5 (CDK5) activity is implicated in inhaled anesthetic-induced neurotoxicity. CDK5/CRMP2 signaling is involved in the cortical and hippocampal dendritic development. The aim of present study is to investigate whether the CDK5/CRMP2 pathway mediates sevoflurane-induced dendritic development abnormalities. Rat primary cortical neurons were treated with 4% sevoflurane for 6 h, the CDK5 inhibitor roscovitine or the vehicle (0.3% DMSO) was administered 12 h before sevoflurane or carrying gases exposure. Cortical neurons were harvested for further analysis 0 h, 12 h and 24 h after exposure. Sevoflurane exposure for 6 h did not reduce cell viability and slightly increased the expression of cleaved caspase-3. Sevoflurane induced abnormal CDK5 activation by increasing the expression of its activator p25 and promoted the phosphorylation of CRMP2 (Ser522). The increased phospho-CRMP2 (Ser522) was mainly distributed in the cytoplasm of cortical neurons. Sevoflurane significantly reduced the number of primary dendrites and the number of branching points; whereas it did not influence the total dendritic length. Suppression of CDK5 activation with roscovitine attenuated neuronal apoptosis, hyperphosphorylation of CRMP2 (Ser522) and dendritic development abnormalities induced by sevoflurane. Our results indicate that activation of the CDK5/CRMP2 pathway may mediate sevoflurane-induced dendritic development abnormalities in the cortical neurons. The physiological significance of these findings remains to be determined.

Abbreviations: CDK5, Cyclin-dependent kinase 5; CRMP2, Collapsin response mediator protein 2; DMSO, dimethylsulfoxide; DIV, Days in vitro.

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1. Introduction

A long exposure to sevoflurane, an inhalational anesthetic, may induce neurodegeneration in the developing brain and subsequent long-term neurobehavioral abnormalities in rodents and primates [1,2]. Some retrospective studies have also found that children younger than 4 years old exposed to surgery under general anesthesia for more than once have a higher risk of developing disabilities in reading and learning [3,4]. Sevoflurane is widely used in clinical settings, especially in neonates and infants. However, the comprehensive molecular mechanisms underlying the developmental anesthetic neurotoxicity remain to be elucidated.

Cyclin-dependent kinase 5 (CDK5) is a proline-directed serine/threonine kinase and is predominantly activated by binding its own specific partners p35 and p39 in postmitotic neurons [5,6]. Although CDK5 is a cytosolic kinase, the majority of active CDK5-p35 complexes reside in close proximity to the membrane and cytoskeletal elements because p35 have membrane-targeting motifs [7]. Physiological, CDK5-p35 plays a pivotal role in neuronal development by regulating neuronal migration, neurite outgrowth, axon guidance, and synapse formation [8]. Under pathological conditions, p35 can be proteolytically cleaved by Ca^{2+}-activated protease (calpain) to p25 that exhibits higher kinase activity and six-fold longer half-life compared to p35. Because CDK5-p25 complexes lacks the membrane anchoring component, which results in its mislocalization to the cytoplasm and the nucleus to hyperphosphorylate a variety of atypical targets, such as tau, GM130, peroxiredoxins, and collapsin response mediator proteins (CRMP). It is evidenced that CDK5 is hyperactivated and causes neurotoxicity in many neurodegenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD) and ischemic stroke [9,10]. Inhibition of CDK5 overactivation also contributes to diminish isoflurane induced-neurotoxicity in the developing brain [11].

Collapsin response mediator proteins (CRMP1-5) are highly expressed in developing and adult nervous systems and play key roles in axonal guidance [12,13], dendritic spine development [14,15] and synaptic plasticity [16]. CRMP2 binds neuronal cytoskeletal network including tubulin microtubules and actin filaments; whereas phosphorylation of CRMP2 lowers their binding affinity to these cytoskeleton proteins [13,17,18]. In Semaphorin3A (Sema3A) signaling, CRMP2 is phosphorylated by CDK5 at Ser522. This phosphorylation is necessary for the subsequent phosphorylation by glycogen synthase kinase-3 (GSK-3) at Ser518, Thr514, and Thr509 [19]. Recent studies have also identified CDK5/CRMP2 signaling may be involved in the cortical and hippocampal dendritic development [20,21].

Sevoflurane has been reported to induce neurotoxicity by disturbing dendritic spine and synapse development in the developing mouse brain [22–24]. However, the molecular mechanism of sevoflurane-induced neurotoxicity is not completely clear. In this study, we postulated that sevoflurane might inhibit dendritic development by overactivating CDK5 and hyperphosphorylating CRMP2 in the developing cortical neurons.

2. Materials and methods

This study was approved by the animal experimentation ethics committee at Sun Yat-sen University and performed in accordance with the Chinese National Health and Medical Research Council (NHMRC) animal ethics guidelines. Cortices were dissected from Sprague–Dawley rat pups at postnatal day 0, and cortical neurons were dissociated with 0.125% trypsin and DNase and then seeded in poly-D-lysine-coated glass coverslips for immunocytochemistry staining at a density of 1×10^4 cells/cm^2 or in 6-well culture plates for Western blotting at a density of 1×10^5 cells/cm^2 and in 96-well plates for CCK-8 cell viability assay at a density of 5000 cells/well. Cultures were maintained in neurobasal A medium (Invitrogen, Carlsbad, CA) containing 2% B27 and 0.5 mM glutamine supplement in an incubator (5% CO_2, 95% air) at 37 °C. The neurons at DIV 3 were pretreated with the CDK5 inhibitor roscovitine (10 μM) or the vehicle (0.3% DMSO) 12 h before sevoflurane or carrying gases exposure.

For the sevoflurane exposure, neurons were placed in an airtight chamber gassed with 4% sevoflurane in the carrying gases. The concentration of sevoflurane in the gases from the outlet of the chamber was measured by a gas analyzer (Datex-Ohmeda, Madison, USA) and reached the target concentrations at 5 min after the onset of gassing. The chamber was sealed after 15-min gassing and kept sealed for 6 h at 37 °C. At the end of 6 h, the concentration of sevoflurane in the chamber was confirmed to be the same as the target concentrations.

Cell viability determined by CCK8 assay was evaluated immediately after the exposure. Briefly, cortical neurons were seeded in 96-well plates with three replicates in each group, and subjected to the various treatments as we described above. At the end of the exposure, 10 μL CCK8 was added to each well and incubated for 1 h at 37 °C. Absorbance at 450 nm was measured with a microplate reader (Thermo Labsystems, Vantaa, Finland). Each experiment was repeated for four times.

For Western blotting studies, the primary cortical neurons in 6-well culture plates were harvested 0 h, 12 h or 24 h after sevoflurane or carrying gase exposure. Western blotting was performed as described previously [25,26]. Briefly, the protein concentrations of samples were determined using the BCA protein assay (Bio-Rad, Herts, UK). Twenty micrograms of each sample were subjected to Western blot analysis. The following antibodies were used with dilution of 1: 1000: anti-cleaved caspase-3 (CST, Danvers, USA), anti-phospho-CRMP2 (Ser522) (Abcam, Cambridge, USA), anti-CRMP2 (CST, Danvers, USA), anti- CDK5 (Abcam, Cambridge, USA), anti-p35/25 (CST, Danvers, USA) and anti-β-actin (CST, Danvers, USA). The band signals of phospho-CRMP2 were normalized to that of CRMP2. Other interesting proteins were normalized to those of β-actin. The results in each group were normalized to that of corresponding control group.

The primary cortical neurons on coverslips were used for double immunofluorescence staining 0 h and 48 h after sevoflurane or carrying gase exposure as we have described previously [25,26]. Briefly, the neurons were fixed in 4% paraformaldehyde, followed by being permeabilized with 0.3% Triton X-100 in Tris-buffered saline and blocked in 5% bovine serum albumin (BSA). The neurons on the coverslips were incubated with the following primary antibodies: rabbit anti-phospho-CRMP2 (Ser522) (1:200) and mouse anti-MAP2a + 2b (1:200; Abcam, Cambridge, USA). The secondary antibodies against the primary antibodies were used and DAPI was used to stain nuclei.

To analyze the dendritic morphology of primary cortical neurons, images of individual neurons at magnification of 400 × were captured randomly with an Olympus BX63 compound microscope (Olympus Corporation, Tokyo, Japan) and analyzed with ImageJ/Fiji software (National Institutes of Health, Bethesda, USA) by two persons blinded to treatments. The experiment was repeated for four times and at least 40 neurons per coverslip were analyzed in each experiment. Dendrites were identified based on selective expression of MAP2a + 2b protein. The number of primary dendrites and number of branching points were averaged from more than 160 neurons from four independent experiments. Total length of dendrites refers to the summed length of all dendrites in a neuron.

Data are presented in mean ± SEM. The Graphpad Prism 6.0 software was used to conduct the statistical analyses. One-way analysis of variance (ANOVA) with Tukey's multiple comparisons were used