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Isoflurane-induced inactivation of CREB through histone deacetylase 4 is responsible for cognitive impairment in developing brain

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ABSTRACT

Anesthetics including isoflurane are known to induce neuronal dysfunction in the developing brain, however, the underlying mechanism is mostly unknown. The transcriptional activation of CREB (cyclic AMP response element binding protein) and the alterations in acetylation of histones modulated by several histone deacetylases such as HDAC4 (histone deacetylase 4) are known to contribute to synaptic plasticity in the brain. Here we have shown that administration of isoflurane (1.4%) for 2 h leads to transcriptional inactivation of CREB which results in loss of dendritic outgrowth and decreased expression level of proteins essential for memory and cognitive functions, such as BDNF, and c-fos in the developing brain of mice at postnatal day 7 (PND7). To elucidate the molecular mechanism, we found that exposure to isoflurane leads to an increase in nuclear translocation of HDAC4, which interacts with CREB in the nucleus. This event, in turn, results in a decrease in interaction between an acetyltransferase, CBP, and CREB that ultimately leads to transcriptional inactivation of CREB. As a result, the expression level of BDNF, and c-fos were significantly down-regulated after administration of isoflurane in PND7 brain. Depletion of HDAC4 in PND7 brain rescues the transcriptional activation of CREB along with augmentation in the level of the expression level of BDNF and c-fos. Moreover, administration of lentiviral particles of HDAC4 RNAi in primary neurons rescues neurite outgrowth following isoflurane treatment. Taken together, our study suggests that HDAC4-induced transcriptional inactivation of CREB is responsible for isoflurane-induced cognitive dysfunction in the brain.

1. Introduction

Anesthetics, including isoflurane, are known to induce neuronal dysfunction in the developing brain (postnatal day 5–7, PND5–7) (Fredriksson et al., 2007; Jevtovic-Todorovic et al., 2003; Jevtovic-Todorovic et al., 2001; Loepke et al., 2009; Zou et al., 2011). During this process, isoflurane alters synaptic plasticity with changes that persist for at least 4 weeks post-exposure; however, unlike other anesthetics, isoflurane does not induce neuronal apoptosis (Briner et al., 2010). Neonatal exposure to anesthetics results in neurocognitive and behavioral abnormalities during adolescence and adulthood (Jevtovic-Todorovic et al., 2003; Satomoto et al., 2009); although the mechanism by which this toxicity occurs is not clear.

Chromatin remodeling, especially through histone tail acetylation, which alters the compact chromatin structure and changes the accessibility of DNA to regulatory proteins, is emerging as a fundamental mechanism for regulating gene expression (Borrelli et al., 2008; Riccio, 2010). Notably, it was demonstrated that the histone deacetylases (HDACs) are one of the major regulators of histone acetylation and synaptic plasticity (Morris et al., 2010; Peixoto and Abel, 2013; Riccio, 2010). Within the Class I HDACs, HDAC 1, 2, and 8 are found primarily in the nucleus, whereas HDAC3 is found in both the nucleus and the cytoplasm and is also membrane-associated. Class II HDACs (HDAC4, 5, 6, 7 9, and 10) are able to shuttle in and out of the nucleus, depending on the signals (de Ruijter et al., 2003; Longworth and Laimins, 2006). However, only HDAC4, 5 and 9 are present in the brain. The export of HDAC4 to the cytoplasm depends on its interaction with a 14-3-3 chaperone protein (Ellis et al., 2003; Grozinger and Schreiber, 2000) and is due to its phosphorylation at Ser-246, Ser-467 and Ser-632 by CaMK4 (Calcium/Calmodulin-dependent protein kinase 4)(Backs et al., 2006).

Neurotrophic factors, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), act by turning on genetic programs, especially those associated with cyclic AMP response element-binding (CREB)(Guan et al., 2009; Lonze and Ginty, 2002; Nott et al., 2008). CREB modulated transcription of genes by recruiting another transcriptional co-activator called CBP (CREB binding protein) that contains the intrinsic HAT domain (Cardinaux et al., 2000; Vo and Goodman, 2001) that has been thought to be important in the actions of numerous growth factors including the neurotrophins.

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In the present study, we have shown that down-regulation of CREB mediates the anesthetic actions of isoflurane. Exposure to isoflurane facilitates translocation of HDAC4 into the nucleus, where HDAC4 prevents transcriptional activation of CREB through a direct protein-protein interaction, which results in a decrease in neurite outgrowth and cognitive impairment in mice.

2. Materials and methods

Animal care and animal experiments were approved by the Institutional Animal care and Use Committee of Augusta University (AU, Augusta, USA) and were performed according to the Guide for the Care and Use of Laboratory Animals approved by the National Institutes of Health (NIH), USA.

2.1. Biochemical studies

Primary neuron cultures were obtained from cerebral cortices of embryonic day 17–18 C57BL/6 mice as described (Mir et al., 2014; Sen and Snyder, 2011). Primary neurons (days *in vitro*, 4–7) were treated with isoflurane (1.4%) for 4 h (Head et al., 2009; Lemkuil et al., 2011). In the presence or absence of treatment with isoflurane, cells were lysed with lysis buffer and the supernatant was used for Western blotting using anti-CREB, anti-CREB phosphor (S133), anti-HDAC4, anti-BDNF, anti-c-fos, anti-HDAC4 phosphor (S632), anti-HDAC4 phosphor (S246) and anti-actin antibodies. Antibodies were obtained either from Cell Signaling Technology or Santa Cruz Biotechnology. For *in vivo* studies, cortex was isolated from both isoflurane-treated and untreated mice, and the lysates were used for Western blot hybridization. For confocal microscopy, serial coronal sections (12 μM) were prepared using a cryostat microtome, and sections were processed as described (Farook et al., 2013; Mir et al., 2014). Total RNA was isolated (SV RNA Isolation kit; Promega, Madison, WI) and reverse transcription RT-PCR was performed as described (Farook et al., 2013). For chromatin immunoprecipitation (ChIP) assays, we used a chromatin immunoprecipitation assay kit purchased from Millipore following the instructions from the supplier. Briefly, primary neurons were stimulated with or without isoflurane. After sonication, lysates containing soluble chromatin were incubated overnight with an anti-CREB antibody or with normal rabbit IgG (Immunoglobin G). DNA–protein immunocomplexes were precipitated with protein A-agarose beads, washed, and eluted. The eluates were used as templates in PCR using the primers as described (Kapoor et al., 2013; Mir et al., 2014).

2.2. Isoflurane treatment in mice

Mice were anesthetized as described (Xie et al., 2008; Zhang et al., 2010). C57BL/6 mice (postnatal day 7; PND7) were randomly assigned to either an anesthesia or control group. Mice randomized to the anesthesia group received either 1.4% isoflurane in 100% oxygen for 2 h in an anesthetizing chamber, whereas the control group received 100% oxygen at an identical flow rate for 2 h in an identical chamber. Anesthesia was terminated by discontinuing isoflurane and placing animals in a chamber containing 100% oxygen until 20 min after return of the righting reflex. They were then returned to individual home cages until sacrificed by decapitation 6 h after isoflurane anesthesia. The brain was removed rapidly, and the prefrontal cortex was dissected out and frozen in liquid nitrogen for subsequent processing for biochemical studies.

2.3. Intra-cortical injection

New born pups (*P*1) were cryo-anesthetized by placing them at 0 °C for 3 min before injection as described (Kim et al., 2013; McLean et al., 2014; Passini and Wolfe, 2001). Then 1.2 μl (10^6–10^7 pfu/ml) of GFP (Green Fluorescent Protein) conjugated lentiviral particles of either HDAC4 RNAi or control RNAi ($n = 9$) were slowly injected into the cortex of each hemisphere located anteroposterior −2.0 mm, lateral +1.5 mm and vertical −1.5 mm to a depth of 1 mm. All injections were performed with a 32 gauge Hamilton syringe. After injections, pups were placed on a warming pad until they regained normal color and resumed movement. Six days after viral infection, depletion of HDAC4 in coronal tissues was detected by Western blot with the anti-HDAC4 antibody. The efficiency of transduction of lentiviral particles in the postnatal brain was monitored by confocal microscopy using an antibody specific to GFP and MAP2, a neuronal marker. On day 7, mice were exposed to isoflurane treatment. After 6 h mice were sacrificed and cortex was used for biochemical assays. This method is highly reproducible, persistent and widespread for neuronal transduction.

2.4. Loss and return of righting reflex

Anesthetic sensitivity was assessed behaviorally using the loss of righting reflex (LORR). A mouse unable to turn itself prone to all four feet within 10 s was considered to have lost its righting reflex and entered a hypnotic state. All mice were observed until they had regained the righting reflex (RORR) at which point the mouse was able to right itself two consecutive times within 1 min of each other. We analyzed LORR and RORR of mice following the procedure as described with modifications after isoflurane exposure (Hu et al., 2012). To acclimatize animals to the testing environment, mice were placed in 200-ml cylindrical open circuit chambers for 2 h daily for the 4 days before anesthetic testing. Mice were exposed to a concentration of 1.5% isoflurane in 100% oxygen for 2 h. Chambers were rotated 180° to assess LORR of each mouse. Body temperature was maintained at 36.0° ± 0.2 °C. The anesthetic gas was discontinued and time to RORR was recorded.

2.5. Neurite outgrowth

The dendritic length assay was performed as described (Sen and Snyder, 2011), with modifications. Briefly, primary neurons were transfected with GFP, and images of neuronal morphologies were captured based on immunoreactivities against GFP, using the 510 META confocal laser-scanning microscope (LSM) system (Zeiss, LA, USA) after treatment with isoflurane. Dendrites and axons were identified by standard morphological criteria. Because the majority of neurons in our cortical culture preparation possessed only one clearly classifiable axon and one or more dendrites, neurons with nonpyramidal morphological features (such as multiple axons or no classifiable processes) were excluded from analyses. The average and total length were determined manually using Neuron J1.0.0 plug-in software for ImageJ (National Institutes of Health). Representative images were acquired using the 510 META confocal LSM with a 40× objective. All analyses were performed by an observer blinded to the identity of the transfected constructs.

2.6. Rotarod

To examine motor coordination and learning in the juvenile mice (PND37), a rotor-rod test was performed as described (Mir et al., 2014; Tsuji et al., 2013). The mice were strictly selected with regard to body weight and eye-opening to reduce data variations. Six trials were continuously performed at 4–40 rpm (rotations per minute). After a 30 s interval, the time that each mouse stayed on the rod and number of falls was measured (maximum time 120 s for the constant mode and 300 s for the accelerating mode). Trials were performed on three successive days to evaluate motor learning. The investigator was blinded to the experimental groups.