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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 2h 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.

Graphical Abstract

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Keywords

CREB; HDAC; isoflurane; synapse

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.

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 4h (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 μ M) 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 (Immunoglobulin 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 (P1) 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 μ l (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 6h 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 seconds 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 2h. 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 30s 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.

2.7 Morris water maze test

In the Morris water maze, the hidden platform procedure was performed in a circular tank filled with opaque water as described with minor modifications (Mir et al., 2014), (Zhang et al., 2005). For training, the juvenile mice (PND37) were placed in the tank at four random points and allowed to search and find the platform. During the trials, the mouse was allowed to search for the platform for 60 s. In the event, the mouse didn't find the platform within the 60 s time, the mouse was manually put on the platform for an extra 30 s. Two trials were given every day and the latency for each trial was recorded. A probe trial was performed on day 6 or 7. The mice were allowed to swim in the tank for 60 s without the platform, and performance was assessed on the basis of the time spent in the quadrant in which the hidden platform was originally located (the right quadrant). The investigator was blinded to the experimental groups.

2.8 Statistical analysis

All the data were represented as mean ± standard error mean. Comparisons within the groups were resolved by either Student's *t*-test or one way non-parametric ANOVA. Comparisons only with **p* < 0.05 were considered significant.

2. Results

3.1 Exposure of isoflurane leads to a decrease in loss of neurite outgrowth in primary neurons

To see whether isoflurane has any influence on neurite outgrowth, we overexpressed GFP in primary neuron prior to exposure of isoflurane to visualize dendritic morphology.

Quantitative analysis showed that administration of isoflurane elicits a significant decrease in both total (Fig. 1A, B) and average (Fig. 1A, C) dendritic length. The total and average dendritic length in wild-type mice was 110 and 46 μm respectively, however after isoflurane treatment, the total and average dendritic length were decreased to 40 and 12 μm . Similarly, exposure of isoflurane leads to a significant reduction in synaptic branching (Fig. 1A, D).

3.2 Exposure of isoflurane leads to decrease in both protein and mRNA levels of memory-related genes due to transcriptional inactivation of CREB

To see the influence of isoflurane on genes responsible for cognitive functions in PND7 brain, we measured the expression level of BDNF and C-fos in the cortex by Western blot analysis. We found that the protein levels of both BDNF and C-fos were significantly decreased to the extent of 0.3 fold after isoflurane treatment compared to control mice (Fig. 2A). We have also measured mRNA levels of the *bdnf* and *c-fos* genes by quantitative RT-PCR analysis in primary neurons after exposure to isoflurane. We found that isoflurane causes a significant decrease in mRNA levels of *bdnf* and *c-fos* compared to untreated cells (Fig. 2B, C). Since CREB is one of the major transcription factors to regulate genes such as *bdnf* and *c-fos*, we measured the DNA binding activity of CREB to the *bdnf* and *c-fos* promoters by ChIP assay after treating primary neurons with isoflurane. We found that treatment with isoflurane leads to a significant decrease in CREB binding to the *bdnf* and *c-fos* promoters to the extent of 0.4 fold compared to untreated cells (Fig. 2D). These data suggests that treatment with isoflurane elicits transcriptional impairment of CREB and causes a decrease in expression of its downstream genes such as *bdnf* and *c-fos* both *in-vivo* and *in-vitro*.

3.3 Isoflurane causes an increase in nuclear translocation of HDAC4 in a phosphorylation-dependent manner

Since, HDACs are known to regulate the transcriptional activity of CREB, we treated primary neurons with an HDACs inhibitor to study the influence of isoflurane on transcriptional activation of CREB. Initially, we treated primary neurons with Trichostatin A (TSA), a broad spectrum HDAC inhibitor and sodium butyrate (a specific inhibitor of class I HDAC) prior to treatment with isoflurane. We found that the isoflurane-induced reduction in DNA binding affinity of CREB was rescued in TSA-treated cells but not in cells treated with sodium butyrate (Fig. 3A, B). This result suggests that class II HDACs may influence the isoflurane-induced impairment in CREB activity.

We were interested in understanding which HDAC II is responsible for impairment of CREB. Since class II HDACs (HDAC4, 5, 7 and 9) functions of cells by altering its cytosolic and nuclear pool we were interested to see whether isoflurane has any influence on the cellular localization of HDAC4, 5 and 9. We excluded measuring HDAC7 because it does

not present in the brain. We prepared both cytosolic and nuclear fraction from both untreated and isoflurane-treated mouse brain. We found that only the protein level of HDAC4 was significantly increased in the nuclear fraction of isoflurane-treated brain samples; however, the nuclear level of HDAC5 and 9 remained unaltered (Fig. 3C). To further confirm the nuclear accumulation of HDAC4, primary neurons were treated with isoflurane and cells were monitored by confocal microscopy. We found that nuclear level of HDAC4 was increased 4 fold after isoflurane treatment compared to control cells (Fig. 3D). We also found that the phosphorylated form of HDAC4 is exclusively cytosolic; however de-phosphorylation of HDAC4 leads to its nuclear accumulation. We monitored phosphorylation of HDAC4 in both isoflurane-treated and untreated lysates from a primary neuron and found that treatment with isoflurane causes a significant decrease in the phosphorylation level of HDAC4 at Ser 632 and Ser 246 residues (Fig. 3E). Quantitative analysis suggests that the phosphorylation level of HDAC4 was decreased to 0.35 fold compared to control cells. Taken together, our result suggests that isoflurane-induced dephosphorylation of HDAC4 facilitates its nuclear transport.

3.4 HDAC4 interacts with CREB in the nucleus following isoflurane treatment

We were interested in understanding the underlying mechanism through which HDAC4 impairs the transcriptional activity of CREB in the nucleus. To see whether the nuclear accumulation of HDAC4 alters the level of histone acetylation, we measured histones H3 and H4 acetylation by the co-immunoprecipitation (IP) assay. Briefly, acetylated proteins were pulled down by acetylated lysine antibody and H3 and H4 were detected by Western blot analysis. We found that the acetylated level of H3/H4 remains unaltered in the isoflurane-treated brain compared to control brain samples (Fig. 4A). This result suggests that acetylation of histones is not responsible for impairment of transcriptional activity of CREB.

To monitor whether augmentation in the nuclear level of HDAC4 has any direct influence on CREB, we monitored the interaction between HDAC4 and CREB in PND7 brain. We found that administration of isoflurane significantly increased the interaction between CREB and HDAC4 (Fig. 4B), and quantitative analysis suggests that the binding was increased more than 3 fold following isoflurane treatment. Confocal microscopic analysis of primary neurons treated with isoflurane also results in co-localization of HDAC4 and CREB inside the nucleus (Fig. 4C). To further confirm the interaction between HDAC4 and CREB, we incubated these cells with isoflurane in a dose-dependent manner (0.5, 1.0, and 1.5%). Cell lysates were subjected to Co-IP measurement to determine the HDAC4-CREB interaction. We found that the interaction was increased significantly as the dose of isoflurane was increased (Fig. 4D). Since the transcriptional activity of CREB depends on its phosphorylation at Ser133 residue as well as its interaction with CBP, a histone acetyltransferase, we also monitored S133 phosphorylation of CREB and the CBP-CREB interaction in isoflurane-treated cells. We found that the interaction between CBP and CREB was decreased significantly compared to control cells (Fig. 4E); however, the phosphorylation level of CREB remained unaltered (Fig. 4F).

3.5 Depletion of HDAC4 rescues transcriptional activation of CREB and protein level of BDNF following isoflurane exposure

Based on our findings, we hypothesized that depletion of HDAC4 may rescue the transcriptional inactivation of CREB. Thus, we depleted HDAC4 in PND7 brain after administration of lentivirus particles of HDAC4 RNAi prior to exposure of isoflurane. We confirmed the depletion of HDAC4 in the cortex by Western blot analysis using lysates from the cortex (Fig. 5A). We also measured the CBP-CREB interaction by the CO-IP method. Interestingly, we found that depletion of HDAC4 rescues the interaction between CBP and CREB (Fig. 5B). To see whether depletion of HDAC4 rescues DNA binding activity of CREB, we treated primary neurons with isoflurane after administration of lentiviral RNAi particles of HDAC4. We found that depletion of HDAC4 causes an increase in the binding of CREB to the *bdnf* promoter by 2.2 fold compared to control RNAi-treated cells (Fig. 5C). We also analyzed protein levels of C-fos and BDNF in cortical lysates after depletion of HDAC4 in the brain. Consistent with our ChIP analysis, we found that the protein levels BDNF and C-fos were increased more than 2.5 fold after depletion of HDAC4 (Fig. 5D). These data suggests that nuclear accumulation of HDAC4 prevents the formation of an active transcriptional complex of CREB in the nucleus and subsequently decrease the synthesis of downstream genes of CREB, which are important for neurite outgrowth and cognitive function in the brain.

To monitor whether inhibition of HDAC4 has any influence on the anesthetic effect after exposure of isoflurane, PND7 mice were treated with TSA, prior to exposure to anesthetics followed by LORR and RORR tests. We found that treatment with TSA does not make any significant difference between times to LORR/RORR after isoflurane exposure (Fig. 5E).

To further monitor the influence of isoflurane on mRNA levels of memory genes such as *bdnf* and *c-fos*, we treated primary neurons with isoflurane in a dose-dependent manner (0, 0.5, 0.8, 1.0, and 1.5%) prior to administration of either control RNAi or HDAC4 RNAi lentiviral particles. We found that isoflurane leads to a dose-dependent decrease in mRNA levels of both *bdnf* and *c-fos* in cells having control RNAi; however, the mRNA levels of these genes remained almost unaltered in cells depleted of HDAC4.

3.6 Depletion of HDAC4 rescues dendritic outgrowth in primary neurons following isoflurane exposure

To monitor whether depletion of HDAC4 can rescue isoflurane-induced reduction in dendritic outgrowth, we administered lentiviral particles of HDAC4 RNAi in primary cortical neuron overexpressing GFP protein to visualize the dendritic morphology under confocal microscopy (Fig. 6A, B). Then total and average dendritic lengths were then measured along with synaptic branching. We found that depletion of HDAC4 prevents the loss of both total and average dendritic lengths (Fig. 6C, D). Moreover, the number of synaptic branches was increased in cells lacking HDAC4 after isoflurane exposure compared to control RNAi-treated cells (Fig. 6E).

3.7 Depletion of HDAC4 rescues cognitive impairment following isoflurane exposure

To administer lentiviral particles of HDAC4 into the cortex of P1 mice, the mice were cryo-anesthetized and virus particles were delivered using a 32 gauge Hamilton syringe. Brain sections of PND7 mice were analyzed under a confocal microscope to determine the efficacy of lentiviral transduction. We found that GFP-positive cells were co-localized with MAP2 a marker for mature neurons (Fig. 7A, B) which suggests that lentiviral transduction is mostly neuronal. We also found that the protein level of HDAC4 was decreased significantly, to the extent of 80%, which was evidenced by Western blot hybridization (Fig. 7C, D); however, the protein level of actin remains unaltered in both control RNAi and HDAC4 RNAi depleted mice. These data substantiate the specificity of HDAC4 RNAi in the cortex.

To see whether cryo-anesthetizing before administration of lentivirus particles has any influence on anesthetic depth induced by isoflurane, we performed the righting reflex test in both cryo-anesthetized and non-cryoanesthesia groups. We did not find any significant difference in time to LORR or RORR between these two groups of mice (Fig. 7E), indicating that cryo-anesthetizing has no influence on isoflurane-induced anesthetic depth. In another control, both HDAC4 RNAi and control RNAi-treated mice were subjected to LORR and RORR along with PBS to see whether administration of lentiviral particles has any effect on anesthetic depth. Again, we did not find any difference in time for either LORR or RORR among these groups of mice (Fig. 7F).

To characterize the influence of HDAC4 on isoflurane-induced neurological outcomes, rotarod and Morris water maze (MWM) tests were performed after depletion of HDAC4 in the cortex at PND37 old (juvenile) mice. In rotarod tests, we found that the number of falls per min (Fig. 7G) increased, and the latency to first fall (Fig. 7H) were decreased in mice exposed to isoflurane. However, depletion of HDAC4 causes a significance improvement in rotarod tests, which was evidenced by a decrease in a number of falls per min and an increase in latency to first fall (Fig. 7G, H). Consistent with rotarod data, we found that treatment with isoflurane causes a decrease in the latency to find the platform (Fig. 7I) and the time spent in the right quadrant (Fig. 7J). However, depletion of HDAC4 improves the memory function which was evidenced by an increase in the latency to find the platform and the time spent in the right quadrant (Fig. 7I, J). These data suggest that depletion of HDAC4 prevents isoflurane-induced cognitive impairment in the developing brain.

3. Discussion

In the present study, we have demonstrated a novel mechanism whereby exposure of a volatile anesthetic such as isoflurane causes impairment of the transcriptional activation of CREB through an HDAC4-dependent manner. Exposure of isoflurane augments interaction between HDAC4 and CREB, which causes a decrease in the DNA binding activity of CREB due to a decrease in interaction between the CREB and CBP proteins (Fig. 7K). We have also provided evidence in support of the fact that depletion of HDAC4 rescue neurite outgrowth and transcriptional activation of CREB in mice.

Several lines of evidence have suggested that decrease in the level of BDNF is one of the major factors for anesthetic-induced impairment in synaptic plasticity and cognitive function

in the brain. It was shown that clinically used general anesthetics administered at the peak of brain development usually 7 days of postnatal age in rats induce neuroapoptosis damage in the developing brain via BDNF modulated apoptotic cascade (Lu et al., 2006). In the thalamus, anesthesia causes a decrease in the BDNF protein level and the activated Akt levels, without any effect on P75^{ntr} (p75 neurotrophin receptor) and ceramide levels resulting in activation of caspase-9 and caspase-3 activities, leading to apoptotic neurodegeneration (Head et al., 2009; Lemkuil et al., 2011). Here we have elucidated the underlying mechanism where inactivation of CREB is responsible for the loss of BDNF in the developing brain.

Several studies have convincingly demonstrated that HDACs contribute to manipulating memory traces that form the basis of robust, persistent memories in the brain. HDACs also are known to augment the consolidation of memory related to object recognition in mice (Graff and Tsai, 2011; Lee and Silva, 2009; Zovkic et al., 2013). Furthermore, it was also shown that HDAC1, 2, and 3 plays a major role in memory formation. However, the role of HDAC4 in memory formation and cognitive functions are not largely understood. Here we have shown that isoflurane specifically induces HDAC4 in the brain and in primary neuron culture, which results in the loss of genes responsible for cognitive functions.

Thus, the question was how HDAC4 influences memory formation and cognitive functions in the developing brain. One possible mechanism is that HDACs may affect histone acetylation status in the chromatin (Nott et al., 2008; Riccio, 2010). Earlier it was shown that acetylation of H3 leads to structural changes in chromatin that in turn influence memory formation, although the acetylation level of H4 remains unchanged. Further studies have suggested that an increase in acetylation of histones near the promoter region of genes that are responsible for learning and memory (e.g., CREB) facilitates gene expression programs (Bredy et al., 2007; Lubin et al., 2008; Sen, 2014; Sen and Snyder, 2011). Strikingly, in our study, we have shown that treatment with isoflurane has no influence on the status of histone acetylation. Thus, we can exclude the possibility that alteration in the level of histone acetylation is responsible for impairment of the transcriptional activation of CREB. The other alternative explanation for the isoflurane-induced decrease in CREB activity is that HDAC4 directly manipulate its activity. In fact, we found that HDAC4 interacts directly with CREB in the nucleus after administration of isoflurane both *in vivo* and *in-vitro*. We have provided evidence that the interaction between HDAC4 and CREB prevents CREB from binding with its coactivator CBP inside cells. It is possible that HDAC4 and CBP compete for the same binding site of CREB. Our hypothesis was substantiated by the data where we have shown that depletion of HDAC4 augments the interaction between CREB and CBP as well as increases the expression level of *bdnf* and *c-fos*. Moreover, our data shows that interaction between HDAC4 and CREB does not alter the phosphorylation level of CREB but causes a decrease in CBP-CREB interaction. Thus, we conclude that HDAC4 regulates CREB's function independent of its phosphorylation status.

Here we have shown that depletion of HDAC4 in cortex affects spatial memory following exposure of isoflurane to the neonatal brain. Previously, it was demonstrated that spatial memory which is generally evaluated by the Morris Water Maze (MWM) test, is attributed to both hippocampus and prefrontal cortex (PFC) (D'Hooge and De Deyn, 2001; Euston et

al., 2012). The interaction between PFC and hippocampus support the assimilation of new memories into presenting a network of knowledge (Preston and Eichenbaum, 2013). Moreover, after using a different version of the MWM test, recent studies have shown that mPFC plays a significant role in retaining spatial information in the hippocampus for efficient navigation (Jo et al., 2007). Based on this literature, we can explain how manipulation of the HDAC4/CREB signaling pathway in cortex affects spatial memory after exposure to isoflurane in the neonatal brain.

Collectively, these results provide a novel mechanism where HDAC4 impairs transcriptional activation of CREB without altering the acetylation status of histones. Abolishment of HDAC4 stimulates dendritic outgrowth by increasing the BDNF level through transcriptional activation of CREB. Thus, our study provides a mechanistic framework on which HDAC4 inhibitor can be tested as a therapeutic approach to prevent neurotoxicity upon exposure to anesthetics.

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Highlights

- Isoflurane leads to an accumulation of HDAC4 in the nucleus.
- Nuclear HDAC4 interacts with CREB.
- The interaction between CREB and CBP was abolished.
- Transcriptional activity of CREB was downregulated and induce cognitive dysfunction in the developing brain

Figure 1. Treatment with isoflurane decreases neurite outgrowth

(A) Primary neurons were overexpressed with GFP and treated with isoflurane. Neurons were imaged by confocal microscopy to monitor dendritic morphology. (B–C) Total (B) and average (C) dendritic length were measured after isoflurane treatment. (D) Branch point/cell analysis in neurons after isoflurane treatment. * $p < 0.05$, $n = 9$, one-way ANOVA, mean $\pm$ Standard Error Mean.

Figure 2. Treatment with isoflurane affects transcriptional activation of CREB

(A) Mice were exposed to isoflurane, and protein levels of bdnf and c-fos were measured by Western blot analysis. The protein levels were quantitated using image J software. (B–C) Real-time RT-PCR analysis was performed to measure the mRNA level of bdnf (B) and c-fos (C) in primary neurons treated with isoflurane. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (D) ChIP assay was performed using lysates isolated from isoflurane-treated brain (cortex) lysates. The images are shown as inverted of the original images. Quantitative measurement of the intensity of PCR product after ChIP analysis. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean.

Figure 3. Decrease in phosphorylation of HDAC4 is responsible for its nuclear localization after isoflurane treatment

(A) Primary neurons were treated with isoflurane with or without treatment of TSA (100 nM) or sodium butyrate (20 mM). CREB binding to the *bdnf* promoter was monitored by ChIP assay. (B) Quantitative analysis of CREB binding to the *bdnf* promoter. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (C) Nuclear and cytosolic fractions were isolated after treatment with isoflurane in mice. The protein level of HDAC4, 5 and 9 was monitored in each fraction by western blot hybridization. Quantitative analysis of protein level of HDAC4, 5 and 9 in both nuclear and cytosolic fraction after isoflurane treatment. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (D) Confocal microscopic analysis to monitor HDAC4 in primary neurons after treatment with isoflurane. (E) The phosphorylation level of HDAC4 at its Ser632 and Ser246 residues were monitored by Western blot analysis in lysates isolated from primary neuron culture treated with or without isoflurane. Quantitation of phosphorylation of HDAC4 level after isoflurane treatment. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean.

Figure 4. HDAC4 interacts with CREB

(A) After isoflurane treatment, protein lysates were used for immunoprecipitation (IP) using anti-acetyl lysine antibody and protein level of H3 and H4 were measured by Western blot analysis. (B) Mice were treated with isoflurane and cortical lysates were precipitated with the anti-HDAC4 antibody. The protein level of CREB was monitored by Western blot hybridization. Quantitative analysis of protein intensity after coIP to analyze HDAC4 interaction with CREB. $*p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (C) Confocal microscopic analysis of HDAC4 and CREB after isoflurane treatment in primary neurons. Arrowhead indicates co-localization of HDAC4 and CREB. (D) Mice were treated with isoflurane in a dose-dependent manner and interaction between HDAC4 and CREB was measured by co-IP studies. (E) Mice were treated with isoflurane and cortical lysates were precipitated with the anti-CBP antibody. The protein level of CREB was monitored by Western blot hybridization. Quantitative analysis of protein intensity after coIP to analyze HDAC4 interaction with CREB. $*p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (F) Phosphorylation of CREB at residue S133 was monitored by Western blot analysis. $*p < 0.05$, $n = 6$, one-way ANOVA, mean $\pm$ Standard Error Mean.

Figure 5. Depletion of HDAC4 rescues transcriptional activity of CREB following exposure of isoflurane

(A) Depletion of HDAC4 after administration of HDAC4 RNAi in the cortex was confirmed by Western blot hybridization in both treated and untreated brain lysates. Quantitative analysis of HDAC4 level after administration of HDAC4 RNAi in the cortex. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (B) Co-IP analysis to measure binding between CREB and CBP in cortical lysates isolated from either HDAC4 RNAi or control RNA-treated brain lysates. Quantitative analysis of the intensity of CREB protein in cortex depletion of HDAC4 following isoflurane treatment. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (C) ChIP assay to monitor CREB binding to bdnf promoter in cortical lysates isolated from either HDAC4 RNAi or control RNA treated brain lysates. Quantitative analysis of CREB binding to bdnf promoter after depletion of HDAC4 following isoflurane treatment. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean. (D) Western blot analysis to measure protein level of BDNF, c-fos, and Arc in cortical lysates isolated from either HDAC4 RNAi or control RNA treated brain lysates. (E) Time to measure LORR and RORR after exposure of isoflurane with or without TSA treatment. (F) Cells were treated with isoflurane in a dose-dependent manner prior to depletion of either HDAC4 in cells. mRNA level of both bdnf and c-fos were measured by quantitative RT-PCR analysis. * $p < 0.05$, $n = 5$, one-way ANOVA, mean $\pm$ Standard Error Mean.

Figure 6. Depletion of HDAC4 rescues neurite outgrowth following exposure of isoflurane (A–B) confocal microscopic images of primary neurons after depletion of HDAC4 prior to treatment with isoflurane. (C–D) Measurement of total (C) and average (D) dendritic length after depletion of HDCA4 in primary neurons following treatment with isoflurane. (E) Branch point per cell was measured in primary neurons overexpressing either HDAC4 RNAi or control RNAi following treatment with isoflurane. * $p < 0.01$, $n = 9$, one-way ANOVA, mean $\pm$ Standard Error Mean.

Figure 7. Depletion of HDAC4 rescues cognitive impairment after isoflurane exposure in mice (A) Cartoon of a brain slice including the location of administration of lentiviral particles in the cortex. (B) Confocal microscopic analysis of the cortex of PND6 brain where lentiviral particles of either control RNAi or HDAC4 RNAi were administered through intracortical injection. Lentiviral particles were stained with GFP and neuronal cells were stained with MAP2. (C–D) Western blot analysis (C) and quantitative analysis of HDAC4 level (D) of HDAC4 after administration of lentiviral particles of either HDAC4 or control RNAi in mice. * $p < 0.05$, $n = 7$, one-way ANOVA, mean $\pm$ Standard Error Mean. (E) Time to LORR and RORR of cryo-anesthetized mice after exposure of isoflurane at PND7. (F) Time to LORR and RORR after exposure of isoflurane following administration of either PBS, control RNAi or HDAC4 RNAi. (G–H) Motor coordination was measured in both control and HDAC4 RNAi mice after treatment with isoflurane. A number of falls/min (G) and latency to first fall (H) were measured in both groups of mice. (I–J) Latency to find the platform (I) and time spent in the right quadrant (J) in MWM test were measured for both groups of mice. * $p < 0.05$, $n = 10–12$, two-way ANOVA, mean $\pm$ Standard Error Mean. (K) A model representing how isoflurane leads to cognitive dysfunction by impairment of CREB. In control cells, HDAC4 is mostly localized to the cytosol. After treatment with isoflurane, HDAC4 translocates to the nucleus and interacts with CREB, which results in impairment of

transcriptional activation of CREB. As a result, mRNA levels of genes such as bdnf and cfos were decreased which ultimately leads to cognitive dysfunction in the developing brain.

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