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Repression of contexual fear memory induced by isoflurane is accompanied by reduction in histone acetylation and rescued by sodium butyrate

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Editor's key points

- The role of histone acetylation in isoflurane-induced amnesia was studied.
- Repression of contextual fear conditioning by isoflurane correlated with reduced histone acetylation and cFos expression.
- A histone deacetylase inhibitor prevented both the reduction in histone acetylation and the repression of fear conditioning by isoflurane.
- Changes in chromatin modification and gene expression correlate with isoflurane-induced amnesia.

Background. Isoflurane produces amnesia in mice during contextual fear conditioning (CFC) trials. Histone acetylation is a form of chromatin modification involved in the transcriptional regulation underlying memory formation. We investigated whether isoflurane-induced repression of contextual fear memory is related to altered histone acetylation in the hippocampus, and whether it can be rescued by the histone deacetylases inhibitor sodium butyrate (SB).

Methods. Adult C57BL/6 mice were chronically given intraperitoneal injections of SB or vehicle for 28 days. Immediately before CFC training, the mice were exposed to isoflurane or air for 30 min and CFC testing was performed the next day. Hippocampal histone acetylation was analysed 1 h after CFC training. c-Fos, an immediate early gene (IEG) suggested to participate in learning and memory formation, was also investigated at the same timepoint.

Results. Mice exposed to isoflurane showed a reduction in freezing time during the CFC test. These mice also exhibited reduced hippocampal H3K14, H4K5, and H4K12 acetylation 1 h after CFC training, and also decreased c-Fos expression. All of these changes were attenuated in isoflurane-exposed mice that were chronically treated with SB.

Conclusions. Isoflurane suppresses histone acetylation and down-regulates c-Fos gene expression in CA1 of the hippocampus after CFC training. These changes are associated with isoflurane-induced amnesia. The HDAC inhibitor SB prevented repressed contextual fear memory, presumably by promoting histone acetylation and histone acetylation-mediated gene expression in response to CFC training.

Keywords: amnesia; anaesthetics volatile, isoflurane; epigenetics, acetylation; hippocampus; mice; proto-oncogene proteins c-Fos; sodium butyrate

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Animal studies have demonstrated that low inhaled concentrations of isoflurane suppress learning and memory in contextual fear conditioning (CFC).[1–3] The contextual fear memories of animals are gradually represssed when isoflurane inhaled concentration increases from 0% to 0.5% before CFC training. In CFC trials, animals are exposed to a particular neutral context paired with an aversive electric shock. If the context is associated with electric stimuli, fearful responses are observed when animals are placed in that context again. Lesion studies in animals have demonstrated that the hippocampus play a critical role in CFC,[4 5] and CFC has become a common method for studying hippocampus-dependent associative memory.

Alterations in gene expression within seconds or hours after learning are thought to be required for long-term memory formation. There is a wide range of mechanisms underlying regulation of gene expression; modification of chromatin structure has particular significance for gene transcription.[6] Histone acetylation can relax condensed chromatin and result in greater gene transcription.[7–9] Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). Recently, a study of hippocampal memory formation indicated that enhanced histone acetylation due to selective recruitment of HDAC inhibitors can facilitate formation of long-term memory.[10] This result has been confirmed in several studies[11–13] and suggests that histone acetylation-related chromatin modification plays an important role in the transcriptional regulation underlying memory formation.

We hypothesized that gene expression regulation via histone acetylation is involved in isoflurane-induced repression of CFC memory and that HDAC inhibitors could rescue this

effect. To examine this, we investigated the influence of isoflurane on contextual fear memory and hippocampal histone acetylation in response to CFC training. We also determined whether these effects can be attenuated by systemic administration of sodium butyrate (SB), which potently inhibits Class I HDAC, including HDAC1, 2, 3, and 8.[14] In addition, we investigated expression of c-Fos, an immediate early gene (IEG) that has been used as a marker of neuronal activation,[15 16] and assessed its correlation with changes in hippocampal histone acetylation.

Methods

Animals

A total of 164 adult male C57BL/6 mice (3 months old, 20–25 g), obtained from the experimental animal centre of Central South University, were used. Animals were housed in cages and allowed access to food and water ad libitum. Cages were kept in a 12 h light/dark cycle at a room temperature of 24 (1)°C. All procedures were performed with the approval of the animal ethics committee of Xiangya Hospital Central South University and according to local policies. The relevant aspects of the ARRIVE guidelines were adhered to as appropriate.

SB administration and isoflurane delivery

SB was dissolved in saline as vehicle and chronic treatment was administered as 1.2 g kg^{-1} once-daily intraperitoneal injections for 28 days (Fig. 1A). The control group animals received saline injections. All injections were given between 7:00 and 9:00 p.m.

For isoflurane delivery, mice were kept in a gas-proof box (40×35×25 cm, with a gas inlet and outlet) for 30 min. The box was filled with the target concentration of isoflurane (0.4 vol% isoflurane+30% O_2, 1 litre min^{-1}) through the inlet, and the outlet gas concentration was monitored by Capnomac Ultima anaesthesia monitor (Daetex-Ohmeda/GE Healthcare, Wauwatosa, WI, USA). After exposure to isoflurane, each animal was quickly transferred into the CFC training chamber which was filled with the same concentration of isoflurane. For control group animals, the gas-proof case and training chamber were filled with oxygen-enriched air (30% O_2, 1 litre min^{-1}).

Behavioural procedure

Animals were handled for 4 days, and on the day of experiments, they were transported to the laboratory at least 2 h before isoflurane delivery and CFC training. For CFC, the transparent Plexiglas training chamber (40×30×26 cm) was placed into a soundproof box (75×60×45 cm) with a camera fixed on the top. The video of each animal during CFC was captured by ANY-maze software (Stoelting Co, Wood Dale, IL, USA.). The floor of training chamber had 28 iron bars which could provide electric shock. Mice were kept in the training chamber about 5 min for CFC training. In the first 2 s of the 5th minute, mice received an electric footshock of 1.0 mA for 2 s. 'Freezing' behaviour was measured with ANY-maze software. A freezing score of <30 is considered to enter the freezing state and >40 was defined to be off freezing state. The quantification of freezing time during CFC training started at the 2nd minute and was terminated before the footshock was given. For assessment of contextual fear memory, freezing time at 24 h post-training was measured for three consecutive minutes in the training chamber.

Isoflurane is an inhaled anaesthetic that supplies several essential elements of anaesthesia: hypnosis, immobility, and amnesia. Previous studies[1–3] have shown that the concentrations of isoflurane required to suppress contextual fear memory are 0.3–0.5 vol%. We used a 0.4 vol% isoflurane for our memory-repression model in view of the above-described studies and our preliminary experiments because this concentration induces sufficient amnesia with minimal immobility (data not shown). Mice exposed to 0.4 vol% isoflurane for 30 min showed a reduction in freezing during the CFC test, whereas freezing time of each group during CFC training was similar, regardless of whether or not the mice were exposed to isoflurane. In addition, when a single 1.0 mA footshock was given, all mice, including the groups exposed to isoflurane for 30 min, showed visible nociceptive reflexive behaviours, including screaming, jumping, and withdrawal (data not shown).

To investigate whether exposure to isoflurane itself has an influence on freezing behaviour in response to novel context, mice exposed to isoflurane or air for 30 min underwent the same training procedure as above, but did not receive a footshock during a mock training session.

To ensure the objectivity of the study, the researchers who were in charge of CFC test, immunohistochemistry and western-blot analysis which were described in following sections did not know the treatment and training program of each group until unblinding.

Tissue preparation and immunohistochemistry

At 1 h after CFC training, mice were killed by rapid cervical dislocation. Isolated brains were fixed using 4% paraformaldehyde for 24 h at 4°C. After cryoprotection in 30% sucrose–PBS for 2 days at 4°C, brains were embedded in 30% sucrose and stored at 4°C. Tissue was cryo-sectioned at 50 μm thick and the hippocampus processed for immunohistochemistry. Tissue sections were incubated successively with primary antibody (12 h, 4°C), serum (10 min, room temperature), and biotin-conjugated secondary antibodies (30 min, 37°C). The primary antibodies used were: anti-acetyl H3K9, #9671, 1:200, Cell Signaling Technology, Danvers, MA, USA; anti-acetyl H3K14, #4318, 1:200, Cell Signaling Technology; anti-acetyl H4K5, #9672, 1:1600, Cell Signaling Technology; anti-acetyl H4K12, SC-34266, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA; anti-c-Fos, #2250, 1:200, Cell Signaling Technology. The tissue sections were then incubated with streptavidin–peroxidase complex (Maixin, Fuzhou, China) for 10 min. Immunoreactivity was seen by incubating the sections with 3, 3′-diaminobenzidine (DAB, Maixin) for 2–10 min. Sections for c-Fos analysis were counterstained with haematoxylin. Finally, sections were dehydrated, washed, and fixed onto gelatin-coated slides (China National Medicines, Shanghai, China). The hippocampal CA1, CA3, and DG regions of all