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Acute Ethanol Administration Increases Mir-124 Expression via Histone Acetylation in the Brain

Keisuke Mizuo*, Shunichiro Okazaki, Satoshi Watanabe, Hiromasa Inoue and Hiroshi Matsumoto

Department of Legal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

***Corresponding author:** Keisuke Mizuo, Department of Legal Medicine and Molecular Alcohology, Sapporo Medical University School of Medicine, S-1, W-17, Chuo-ku, Sapporo 060-8556, Japan, Tel: +81-11-611-2111; Fax: +81-11-611-3935; E-mail: mizuo@sapmed.ac.jp

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Abstract

Acute ethanol administration causes an enhancement of mesolimbic dopaminergic activity in the brain. The microRNAs miR-124 and miR-132 are highly expressed in the central nervous system and contribute to the regulation of mesolimbic dopaminergic activity. However, the nature of a potential mechanistic association between ethanol and these microRNAs has remained unclear. Recent studies reported that the expression of some microRNAs was regulated by histone acetylation. In this study, we investigated whether ethanol administration can change the expression of miR-124 and miR-132 via histone acetylation in mouse brain. We administered 2 g/kg ethanol (s.c.) to male C57BL / 6J mice. Total RNA was extracted using TRIzol in the whole brain 1 to 12 hr after administration. MicroRNAs were detected by northern blotting. Acetylated histone H3 and histone deacetylases (HDACs) were detected by western blotting analysis and immunohistochemistry. Trichostatin A (TSA) was administered intracerebroventricularly.

Acute ethanol administration increased expression of miR-124 and miR-132 in the mouse whole brain 12 hr after ethanol administration. Acute ethanol administration significantly increased acetylated histone H3 in the nuclear fraction of mouse whole brain, especially the nucleus accumbens and ventral tegmental area. HDAC4 expression was significantly decreased in the nuclear fraction after acute ethanol administration. These findings suggest that acute administration induces histone acetylation by reducing nuclear HDAC4 expression. TSA treatment increased the expression of miR-124 in mouse whole brain. These findings suggest that HDAC4 may directly regulate the expression of miR-124 in the brain. In conclusion, acute ethanol administration induces histone acetylation via reducing nuclear HDAC4 protein, resulting in increased microRNA expression in the brain.

Keywords: Ethanol; microRNA; Histone H3; Brain

Introduction

In the 21st century, it is well-understood that gene expression can be regulated by numerous epigenetic mechanisms, such as the activity of microRNAs, DNA methylation or histone modification [1]. Some microRNAs such as miR-9, miR-124 and miR-132, are highly expressed in the central nervous system [2]. These brain-enriched miRNAs regulate the gene expression during brain development [3,4]. Recently, it has been reported that miR-124 expression in the nucleus accumbens regulates cocaine dependence [5] and miR-132 regulates the differentiation of dopamine neurons [6]. In addition, it is well known that chronic ethanol consumption causes neuronal plasticity [7,8]. These findings suggested to us that miR-124 and miR-132 could play critical roles in the mechanisms underlying ethanol's effects on the brain, as well as its potential to result in abuse and dependence.

Recent reports have also shown that HDAC inhibitors such as trichostatin A modulate the rewarding effects of abused drugs [9-11]. In addition, the expression of some microRNAs can be regulated by histone acetylation [12]. Therefore, we hypothesized that acute ethanol administration might increase the expression of miR-124 and miR-132 via histone acetylation in the brain.

In the present study, we designed to investigate whether acute ethanol administration can change the expression of brain-enriched microRNA miR-124 and miR-132 in mouse brain.

Materials and Method

Animals

These experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Sapporo Medical University school of Medicine and the Ministry of Education, Science, Sports and Culture of Japan and was approved by the Committee on Animal Research, Sapporo Medical University School of Medicine (#09-086).

All the experiments were performed with the objective of minimizing both the number of animals used and any discomfort caused by the experimental protocols. Male C57BL / 6J mice (6–8 weeks old) were obtained from Sankyo Laboratories (Sapporo, Japan). Animals were housed in a room maintained at 22 ± 1°C with a 12 hr light-dark cycle (lights on from 8:00 a.m. to 8:00 p.m.) for the duration of the experiment. Three to nine mice were used as a group in each experiment. Food and water were available *ad libitum*.

Drug administration

Ethanol (2 g/kg body weight, 10 ml/kg) was subcutaneously administered. After decapitation under sevoflurane anesthesia, the whole brain was quickly removed 1 to 12 hr after administration and was stored at -80°C until analysis. Trichostatin A (10 nmol/mouse, Kanto chemicals, Tokyo, Japan) was dissolved in saline containing 10% dimethyl sulfoxide (DMSO) and was administered intracerebroventricularly (i.c.v.) 10 min prior to decapitation. Mice were anesthetized with sevoflurane prior to i.c.v. administration. One day previously, a 2 mm double needle (tip: 27 G × 2 mm and base: 22 G × 10 mm) attached to a 25 μl Hamilton microsyringe was inserted unilaterally approximately 1.5 mm from either side of the midline between the anterior roots of the ears.

On the day of the drug injection, the head of the mouse was held against a V-shaped holder and either drug or vehicle was injected into the same opening. The injection volume was 4 μl for each mouse. The control mice have treated with the vehicle in each treatment.

Western blotting

Whole brain was homogenized in ice-cold buffer A containing 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 0.5 mM EGTA, a protease inhibitor cocktail, and 0.32 M sucrose. The homogenate was centrifuged at 1,000 g for 10 min and the pellets were used as the nuclear fraction for Western blotting. An aliquot of tissue sample was diluted with an equal volume of 2× electrophoresis sample buffer containing 2% sodium dodecyl sulfate (SDS) and 10% glycerol with 0.2 M dithiothreitol (DTT).

Proteins (20 μg/lane) were separated by size on 7.5% SDS-polyacrylamide gels, and transferred to nitrocellulose membranes in Tris-glycine buffer containing 25 mM Tris and 192 mM glycine. For immunoblot detection of acetylated histone H3 and HDAC4, the membranes were blocked in Tris-buffered saline (TTBS) containing 0.05% Tween-20 and 5% bovine serum albumin (BSA; SIGMA, St. Louis, MO) for 1 hr at room temperature with agitation. The membranes were incubated with primary antibody diluted in TTBS (1:1000 for acetylated histone H3, HDAC1, 2, 4, 5 and 7, 1:2000 for β-actin as an endogenous control (Cell Signaling Inc., Denvers, MA) containing 5% BSA overnight at 4°C.

The membranes were washed twice for 5 min and twice for 10 min in TTBS, followed by 2 hr incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Cell Signaling Inc.) diluted 1:20,000 in TTBS. Thereafter, the membranes were washed twice for 5 min, and then three times for 10 min in TTBS. The antigen-antibody peroxidase complex was detected using enhanced chemiluminescence (PIERCE, Rockford, IL) according to the manufacturer's instructions and image analysis was performed with the VersaDoc 5000 (Bio-Rad Lab., Hercules, CA). The data were normalized by the levels of β-actin.

Northern blotting

Total RNA in the brain was extracted using TRIzol reagent according to the manufacturer's instructions. The RNA was diluted with an equal volume of formamide and 6× sample buffer. Samples were separated by size gradient on 15% polyacrylamide-8 M urea gels using TBE and transferred to nylon membranes in TBE. After transfer, the membranes were crosslinked by a UV-transilluminator for 5 min.

The membranes were pre-incubated with hybridization buffer for 30 min and incubated with 32 P-labeled probes for miR-9 (5'-UCAUACAGAUAGAUAACCAAAGA-3'), miR124 (5'GGCAUUCACCGCGUGCCUUA3'), miR132 (5'CGACCAUGGCUGUAGACUGUUA-3') or miR-133b (5'- UAGCUGGUUGAAGGGGACCAAA-3') or U6 snRNA as an endogenous control at 37°C overnight. Thereafter, the membranes were washed three times in wash buffer for 10 min, exposed to the imaging plate for 2 to 6 hr, and detected using auto-radiography by FLA-3000 (Fuji Film, Japan). The data were normalized by the levels of U6 snRNA and quantified by Image J.

Immunohistochemistry

Mice were deeply anesthetized with sodium pentobarbital and perfused transcardially with 4% pafaformaldehyde (pH 7.4). The brains were then quickly removed and thick coronal slices of the forebrain were initially dissected using a brain block. The coronal slices were post-fixed in 4% paraformaldehyde for 2 hr, and subsequently permeated with 20% sucrose for 24 hr, then 30% sucrose for 24 hr, and then frozen in embedding compound with liquid nitrogen.

Frozen sections (8 μm thickness) were cut using a cryostat and thaw-mounted onto silan-coated glass slides. The brain sections were blocked with 10% normal goat serum in 0.01 M PBS for 1 hr at room temperature. Primary antibody (1:100 for acetylated histone H3, Cell Signaling Tec.) was diluted in 0.01 M PBS containing 10% NGS and incubated overnight at 4°C. The sections were then rinsed and incubated with the appropriate HRP-conjugated secondary antibody for 2 hr at room temperature. The sections were rinsed and incubated with reacting solution for 5 min.

The slides were then coverslipped with mounting medium. Immunolabeling was detected and photographed with a BZ-9000 camera (Keyence Inc., Tokyo, Japan). The density of acetylated histone H3 labeling was measured with a computer-assisted imaging analysis system (Image J program, developed at the National Institutes of Health available at http://rsbweb.nih.gov/ij). A rectangle was positioned over the nucleus accumbens or ventral tegmental area of control mice. The number of positive cells was calculated. The same box was then dragged to the corresponding position on the nucleus accumbens or ventral tegmental area of ethanol treated mice, and the number of positive cells was again calculated.

Statistical analysis

Data represent the mean ± S.E.M. Comparisons between groups were performed using Student's t-test or ANOVA with Bonferroni/Dunnett's tests using PRISM (GraphPad Software Inc., La Jolla, CA).

Results

Figure 1 shows the expression of miR-124 (A) and miR-132 (B) in the mouse brain before ethanol administration. One hour after ethanol administration, miR-124 and miR-132 were significantly increased in the brain and remained high for 12 hr after administration (Figure 2A and B). On the other hand, the microRNAs miR-9 and miR-133b were not detected in mouse brain (Figure 1C and D).

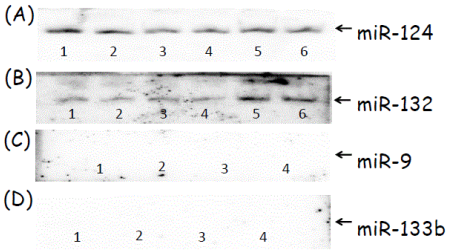

Figure 1: Detection of microRNAs by northern blot analysis. Representative northern blot of (**A**) miR-124 and (**B**) miR-132 in mouse whole brain before ethanol administration. We were unable to detect either (**C**) miR-9 or (**D**) miR-133b in whole mouse brain prior to ethanol administration. (A) Lane 1, 2, 5: Saline; Lane 3, 4, 6: Ethanol; (B) Lane 1, 3, 4: Saline; Lane 2, 5, 6: Ethanol; (C) Lane 1, 2: Saline; Lane 3, 4: Ethanol; (D) Lane 1, 2: Saline; Lane 3, 4: Ethanol.

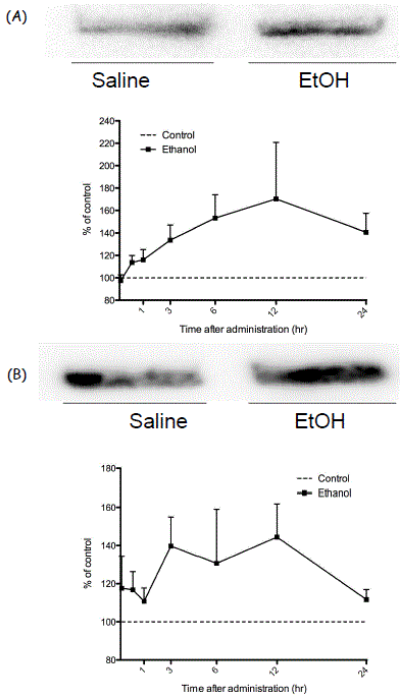

Figure 2: Time-course of the expression of (A) miR-124 and (B) miR-132 in mouse whole brain after acute ethanol administration. (Upper panel) Representative northern blot of (A) miR-124 and (B) miR-132 in mouse whole brain over a 12-hr period following acute ethanol administration. (Lower panel) A significant increase in the expression of both (A) miR-124 and (B) miR-132 in ethanol-treated (2 g/kg, s.c.) mice as compared to saline-treated (control) mice (ANOVA: $F_{1,80} = 4.63$, $p < 0.05$; ethanol vs. control: $F_{1,70} = 8.41$, $p < 0.01$). Values are expressed as a percentage of the average value of the control group. Each point represents the mean ± SEM of 5–9 mice.

Figure 3 shows the changes in acetylated histone H3 in mouse whole brain over a 12-hr period after ethanol administration, reflecting a significant increase compared to vehicle controls. In contrast, HDAC4 was significantly decreased in mouse whole brain 12 hr after administration (Figure 4), indicating that acute ethanol administration decreased HDAC4 activity.

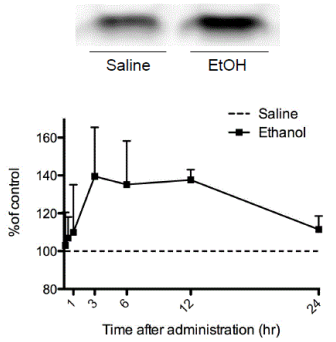

Figure 3: Time-course of the expression of acetylated histone H3 after acute ethanol administration in mouse whole brain. (Upper panel) Representative western blot of acetylated histone H3 in mouse whole brain over a 12-hr period following acute ethanol administration. (Lower panel) A significant increase in the acetylation of histone H3 in ethanol-treated (2 g/kg, s.c.) mice compared to saline-treated (control) mice (ANOVA: $F_{1,65} = 7.48$, $p < 0.01$). Values are expressed as a percentage of the average value of the control group. Each point represents the mean ± SEM of 3–8 mice.

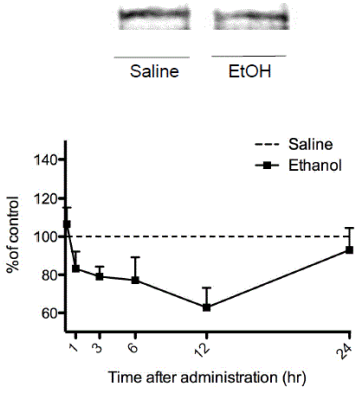

Figure 4: Time-course of the expression of histone deacetylase (HDAC) 4 in mouse whole brain following acute ethanol administration. (Upper panel) Representative western blot of HDAC4 in mouse whole brain over a 12-hr period following acute ethanol administration. (Lower panel) A significant reduction in the expression of HDAC4 in ethanol-treated (2 g/kg, s.c.) mice as compared to saline-treated (control) mice (ANOVA: $F_{1,50} = 5.05$, $p < 0.05$). Values are expressed as a percentage of the average value of the control group. Each point represents the mean ± SEM of 3–8 mice.

Immunohistochemical study showed that acute ethanol administration increased acetylated histone H3-like immunoreactivity in the nucleus accumbens (Figure 5) and ventral tegmental area (Figure 6). As shown in higher magnification images, acetylated histone H3 positive cells were dramatically increased in mice administered ethanol (Figure 5B and 6B). Furthermore, using quantitative analysis, acetylated histone H3 positive cells were significantly increased in the nucleus accumbens and ventral tegmental area after ethanol administration compared to vehicle controls (*p<0.05, *p<0.05, student's t-test, Figure 5C and 6C, respectively).

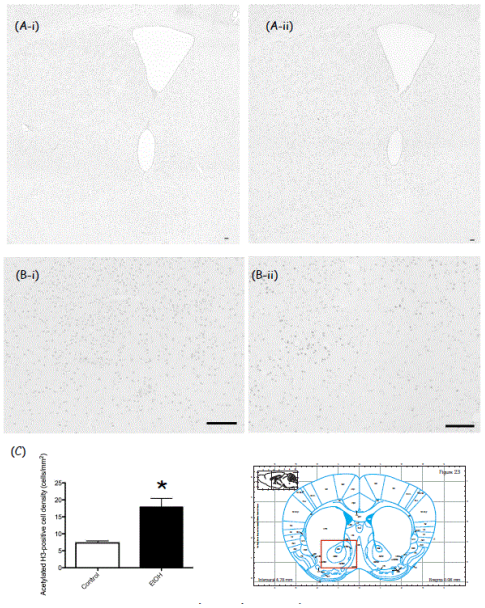

Figure 5: Increase in acetylated histone H3 in the brains of mice administered ethanol in nucleus accumbens. (**A**) Low magnification images around the nucleus accumbens in ethanol treated (EtOH) and saline-treated (control) mice (A-i: EtOH; A-ii: control). (**B**) High magnification images showed that acute ethanol administration clearly increased the expression of acetylated histone H3 as compared to control mice (B-i: EtOH; B-ii: control). (**C**) Quantification of acetylated histone H3 positive cells in nucleus accumbens. Acute ethanol administration significantly increased the density of acetylated histone H3 positive cells as compared to control mice (*p<0.05, student's t-test). Each column represents the mean ± SEM of three independent samples. Mice were perfused with phosphate buffered saline (PBS) followed by freshly-prepared 4% paraformaldehyde in PBS 12 hr after ethanol administration. Scale bar = 50 μm.

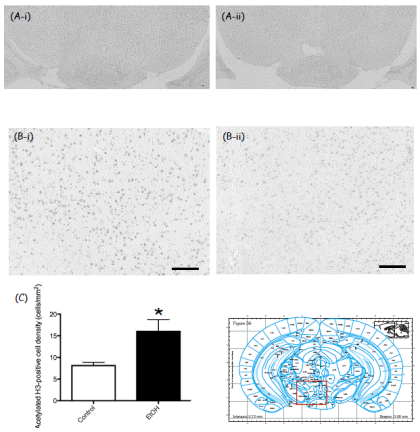

Figure 6: Increase in acetylated histone H3 in the brains of mice administered ethanol in ventral tegmental area. (**A**) Low magnification images around the ventral tegmental area in ethanol treated (EtOH) and saline-treated (control) mice (A-i: EtOH; A-ii: control). (**B**) High magnification images showed that acute ethanol administration clearly increased the expression of acetylated histone H3 as compared to control mice (B-i: EtOH; B-ii: control). (**C**) Quantification of acetylated histone H3 positive cells in ventral tegmental area. Acute ethanol administration significantly increased the density of acetylated histone H3 positive cells as compared to control mice (*p < 0.05, student's t-test). Each column represents the mean ± SEM of three independent samples. Mice were perfused with phosphate buffered saline (PBS) followed by freshly-prepared 4% paraformaldehyde in PBS 12 hr after ethanol administration. Scale bar = 50 μm.

Administration of TSA (10 nmol/mouse, i.c.v.) significantly increased the expression of miR-124 in mouse whole brain at the 10-min assessment timepoint (*p<0.05, student's t-test, Figure 7).

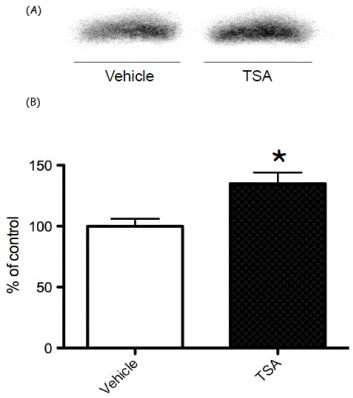

Figure 7: Acute administration of TSA increased the expression of miR-124 in the brain. (**A**) Representative northern blot of miR-124. (**B**) A significant increase in the expression of miR-124 in the whole brain 10 min after i.c.v. administration of TSA as compared to controls (*p < 0.05, student's t-test). Each column represents the mean ± SEM of 6–7 mice.

Discussion

In the present study, we show that acute ethanol administration increased the expression of miR-124 and miR-132 in mouse whole brain. The microRNAs miR-124 and miR-132, widely expressed in the brain, play essential roles in neuronal development and plasticity [13,14]. In particular, miR-124 has been reported to specifically regulate neurite outgrowth, neuronal differentiation and synaptic plasticity [15,16]. Therefore, our results suggest the possibility that acute ethanol administration may increase synaptic plasticity in the brain via the expression of miR-124. It has been reported that miR-132 regulates the dopamine neuron differentiation and dopamine signaling [6,17]. In addition, it is well known that single or repeated ethanol treatment enhances extracellular dopamine release [18-20]. Our findings suggest that the changes in miR-132 following acute ethanol administration may affect mesolimbic dopaminergic system.

We observed that the levels of expression of miR-124 and miR-132 were kept high for 12 hr after administration. On the other hand, we observed that ethanol was no longer detected in blood 3 hr after administration (data not shown). Thus, it appears that the modulation of miRNAs by ethanol is long-lasting and occurs independently of the blood ethanol concentration.

Although miR-9 and miR-133b were not detected in the mouse brain either before or after ethanol administration, it has been reported that miR-9 is a key regulator of ethanol tolerance [21]. Our findings suggest that a single ethanol administration may not be sufficient to cause tolerance to ethanol. It has also been reported that miR-133b expression is specifically expressed in midbrain dopamine neurons [22,23]. The fact that we did not detect either miR-9 or miR-133b suggests that they are expressed at lower levels than miR-124 or miR-132 in whole mouse brain. Thus, these miRNAs may be present and functionally active in the brain, but nevertheless cannot be detected by northern blotting. To better understand the association of these miRNA in the actions of ethanol, further studies will be needed.

Scott et al. previously reported that some miRNAs were increased by treatment with an HDAC inhibitor, LAQ824 [12]. In the present study, we delineated how ethanol induces expression of miR-124 and miR-132 in the brain via HDAC inhibition. First, we investigated whether ethanol directly affects histone acetylation in the mouse brain. We observed that acute ethanol administration significantly increased the expression of acetylated histone H3 in the brain nuclear fraction. Our results are consistent with those of Choudhury and Shukla, who reported that ethanol loading increased histone H3 acetylation in rat hepatocytes [24]. Similarly, Pandy et al. reported that chronic ethanol treatment modulates histone acetylation in the amygdala region [25]. Therefore, our results support the interpretation that acute ethanol administration facilitates histone H3 acetylation in the brain. Furthermore, HDAC4 in the nuclear fraction was significantly decreased after acute administration of ethanol, whereas the expression of other HDAC subtypes (HDAC1, 2, 5, 7) was not affected by actute ethanol administration. Therefore, these findings suggest that HDAC4 may be a key modulator in ethanol-induced histone acetylation in the brain.

We also found that acute ethanol administration increased acetylated histone H3 in the ventral tegmental area and nucleus accumbens. Several studies have shown that the mesolimbic dopamine system, which projects to the nucleus accumbens from the ventral tegmental area, can be a critical site for the initiation of ethanol dependence [26-28]. To clarify whether ethanol-induced histone acetylation modulates the expression of miR-124, we investigated the levels of miR-124 after i.c.v. administration of trichostatin A, an HDAC inhibitor [29]. TSA treatment significantly increased the expression of miR-124 in mouse brain. This finding suggests that HDAC inhibition rapidly increased the expression of miR-124. Taken together, our findings provide evidence that ethanol increases brain miRNA expression by reducing HDAC activity.

In conclusion, acute ethanol administration increases histone acetylation via the reduction of HDAC activity, which in turn results in increased expression of miR-124 and miR-132. Interestingly, Wang et al. proposed that acetylation of histone H3 by HDAC4 is an important molecular mechanism underlying reinforcement provided by abused drugs such as cocaine [30], and Hobora et al. showed that mRNAs of HDACs is changed in patients with mood disorders [31]. These findings of the present study suggest that ethanol-induced miR-124 and miR-132 expression may be involved in the development of ethanol tolerance and/or in the priming of ethanol dependence.

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