Original article:

Moderate to high ethanol and acetaldehyde administration decreases c-fos protein expression in the hippocampus of Aldh2-knockout and C57BL/6N mice

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Abstract:

Background: Ethanol (EtOH) and acetaldehyde (AcH) have long been associated with many adverse effects in the central nervous system. c-fos has been used as an indirect index of neural activity. ***Method***: Here, we investigated the effects of systemic administration of EtOH and AcH on the expression of c-fos protein in the hippocampus of Aldh2-knockout (Aldh2-KO) mice. The animals received an intraperitoneal injection of saline (control), EtOH (1.0, 2.0 and 4.0 g/kg) or AcH (50, 100 and 200 mg/kg), and the expression of c-fos protein was measured by Western blotting. ***Result:*** We found that EtOH at doses of 2.0 and 4.0 g/kg decreased c-fos in wild-type (WT) mice, whereas EtOH at all three doses tested (1.0-4.0 g/kg) decreased c-fos in Aldh 2-KO mice. Likewise, AcH at doses of 100 and 200 mg/kg had a significant effect in lowering c-fos protein in both WT and Aldh2-KO mice. ***Conclusion:*** Our observations suggest that moderate to high EtOH and AcH can decrease the expression of c-fos protein in the mouse hippocampus. This effect may support, at least in part, the link between c-fos and spatial memory deficits following EtOH and AcH exposure as we have observed in our previous study.

Keyword : Aldh2-KO mice; ethanol; acetaldehyde; c-fos protein; hippocampus

Introduction

Ethanol (EtOH) and acetaldehyde (AcH) have numerous pharmacological and neurobehavioral effects; in fact, some of the effects of EtOH are mediated by its first metabolite AcH. People with deficient low-K_M aldehyde dehydrogenase 2 (ALDH2) activity may accumulate high levels of AcH in their bodies after drinking EtOH[1]. In most previous studies, the effects of AcH have been assessed by the direct administration of AcH or ALDH inhibitors, such as cyanamide or disulfiram (which results in AcH accumulation) in laboratory animals[2-4]. Here, we used Aldh2-knockout (Aldh2-KO) mice as a model of ALDH2-deficient humans to investigate the effects of EtOH and AcH on c-fos protein expression in the hippocampus after intraperitoneal (i.p.) administration of EtOH.

The fos signal has long been used as an indirect index of neural activity. Neurons can respond to extracellular stimulation by modulating the expression of certain immediate early genes, most importantly c-fos, which is known to play an important role in neuronal adaptations and brain plasticity[5,6]. c-fos is widely distributed in the rat brain, particularly in the cortex, hippocampus and cerebellum[7,8], and has been associated with memory[9,10]. EtOH consumption is

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characterized by a wide range of effects that suggest several neuroanatomical targets for this drug. Several works have shown an association between c-fos activation and learning and memory processes in rats. For example, c-fos is essential for spatial memory formation[11-13]. Several studies have demonstrated the effects of EtOH on the expression of c-fos in a large number of neurons in the animal brain[14-17]. Only a very few studies have measured c-fos expression after systemic AcH administration in several areas of the rat brain[14,17].

Our previous study using Aldh2-KO mice suggests that high EtOH and AcH impaired spatial memory performance[18]. Here, we chose to study c-fos expression in the hippocampus because this region is generally considered to play a critical role in the processing of spatial information[19]. Therefore, it is essential to ascertain whether the EtOH- and AcH-induced memory deficit could be related to the expression of c-fos in the hippocampus. Toward this aim, we sought to use Aldh2-KO mice; this genotype has not previously been tested for c-fos expression after systemic EtOH administration and the subsequent changes in AcH levels. We next tested, in the same animals, the effects of AcH on c-fos after direct i.p. administration of AcH to investigate the possibility that AcH may act as a mediator of EtOH consumption. In this study, Western blotting (WB) was used to measure c-fos protein expression in the hippocampus of Aldh2-KO and wild-type (WT) mice 60 min after the i.p. administration of saline, EtOH (1.0, 2.0 and 4.0 g/kg) or AcH (50, 100 and 200 mg/kg).

Methods

Animals

Aldh2-KO mice were generated as previously reported[20]. These mice were maintained and backcrossed with the C57BL/6J strain for more than 10 generations. They have the same genetic background, except for Aldh2. Two eight-week-old male/female pairs were obtained from the Department of Environmental Health at the University of Occupational and Environmental Health in Japan and were bred at the Kagawa University animal facility. Breeding pairs from this strain were used to generate the experimental groups. WT mice have the same genetic background as C57BL/6J mice and were bred in our animal facility. All experiments were conducted with male mice. Each was 10-12 weeks in age and weighed 24-28 g. All animals were housed in controlled temperature (21 ± 3˚C),

humidity (50-70%) and light (12-h light-dark cycle) conditions.

Experimental groups

Aldh2-KO and WT male mice were divided into eight experimental groups (n=5 per group): (a) saline (10 ml/kg, 0.9% NaCl), (b) EtOH (1.0 g/kg), (c) EtOH (2.0 g/kg), (d) EtOH (4.0 g/kg), (e) 4-methylpyrazole (4-MP) + AcH (50 mg/kg), (f) 4-MP + AcH (100mg/kg), (g) 4-MP + AcH (200 mg/kg), (h) 4-MP alone. 4-MP (an alcohol dehydrogenase inhibitor) was administered before the AcH to block the reversible conversion of AcH to EtOH. The EtOH (20%, w/v) and AcH (4%, w/v) were dissolved in 0.9% saline, and all injections were administered i.p. in a volume of 10 ml/kg. 4-MP (82 mg/kg) i.p. was given an hour before the injection of AcH. After mice received an i.p. injection of saline, EtOH or AcH, brain tissue was collected 60 min later. A high dose of EtOH (4 g/kg) was given according to the previous work[21].

Brain sample

Mice were killed by cervical dislocation and then decapitated. Brains were quickly removed and were washed in cold saline for twice. The hippocampus from one half-brain was removed and immediately transferred to a 2.5 tube containing 0.4 ml of Radioimmuno precipitation assay buffer (RIPA) lysis buffer (SantaCruz) for protein. The tissue samples were subsequently homogenized with Polytron (PT 2500E, Switzerland), and an additional 0.4 ml of buffer was added with 10 μl of each phenylmethylsulfonyl fluoride (PMSF), Na orthovanadate and phosphatase inhibitor for a total sample volume of 0.8 ml. After centrifugation at 12,000g at 4 °C for 15 min, the supernatant was used for protein concentration. The protein content of the supernatant was determined by Bradford (Bio-Rad, Hercules, CA) using bovine serum albumin (Sigma Chemical, St. Louis, MO) as the standard. The total protein concentration was measured at 590 nm using a microplate reader (SH-9000Lab, Corona Electric).

Western blot analyses

Samples were run on 10% SDS PAGE with molecular weight markers (Bio-Rad), then transferred electrophoretically to PVDF membranes. The blots were blocked at room temperature with 5% (w/v) non-fat dried milk in Tris-buffered saline (pH 7.4) containing 0.1% tween-20. Membranes were subsequently incubated at 4°C overnight with primary antibodies against c-fos (1:1000, sc-52, SantaCruz) and β-actin (1:2000, Cat# 013-24553, Wako Pure Chemical Industries Ltd, Japan). The immunoblots were incubated with a horseradish peroxidase-

Mostofa Jamal, Ayako Ito, Naoko Tanaka, Kiyoshi Ameno, Takanori Miki, Hiroshi Kinoshita

conjugated anti-rabbit IgG (GE Healthcare Japan, Tokyo) for 1 h, and then signals were visualized using Luminata Crescendo Western HRP Substrate (Millipore). The band intensities were evaluated by an LAS-1000plus lumino-imaging analyzer (Fujix). The relative expression of protein levels was normalized to the β-actin level in each sample.

Statistics

All values were expressed as the mean ± standard error of the mean (SEM); values of *P*<0.05 were considered significant. The data were analyzed with StatPlus 2009 (version 5.8) software using a two-way analysis of variance (ANOVA). Post-hoc tests were performed using the Tukey–Kramer test.

Ethical clearance: All animal experiments were approved by the Kagawa University Animal InvestigationCommittee.
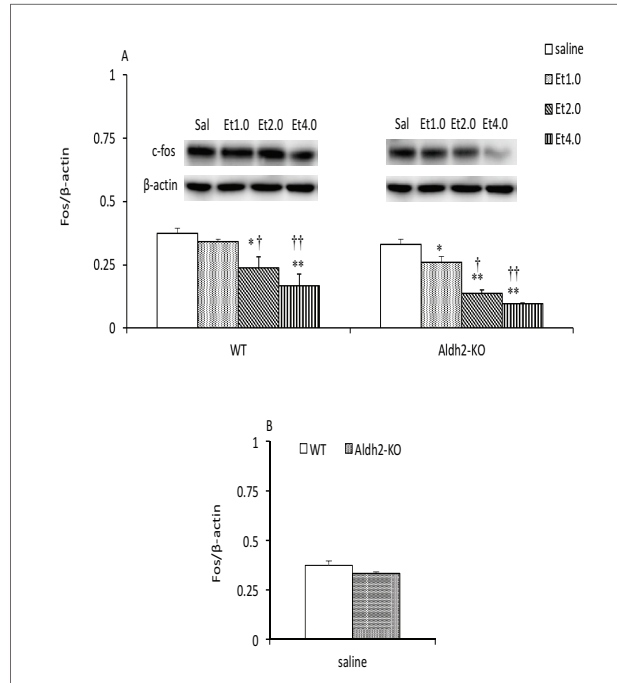
Results

Figure 1: c-fos protein after exposure to EtOH (1.0, 2.0 and 4.0 g/kg) in the hippocampus of WT mice and Aldh2-KO mice (A) and the genotype differences on c-fos (B). Data represent mean ± SE (*n* = 5). *P<0.05 and **P<0.01 versus saline; †P<0.05 and ††P<0.01 versus EtOH (1.0 g/kg). Sal; saline, Et1.0; ethanol 1.0 g/kg; Et2.0, ethanol 2.0 g/kg; Et4.0, ethanol 4.0 g/kg.

Fig. 1 shows the effects of different doses of EtOH on c-fos protein expression in the hippocampus of WT and Aldh2-KO mice (A). The results of the ANOVA revealed that there was a significant main effect of EtOH treatment (df 3,32; *F*=29.209; *p*<0.001), but

there was no significant interaction between treatment × animal (df 3,32; *F*=0.414; *p*=0.744). For WT mice, further analysis with Tukey-Kramer post hoc tests show that treatment with 2.0 (*P*=0.005) and 4.0 g/kg (*P*<0.001) of EtOH resulted in a significant decrease in the level of c-fos when compared with saline. There was a significant difference in c-fos between the 1.0 g/kg EtOH and 2.0 g/kg EtOH (*P*=0.041) groups, and between the 1.0 g/kg EtOH and 4.0 g/kg EtOH (*P* < 0.001) groups. For Aldh2-KO mice, the post hoc analysis with the Tukey–Kramer test showed that treatment with 1.0 (*P*=0.049), 2.0 (*P*<0.001) and 4.0 g/kg (*P*<0.001] of EtOH resulted in a significant decrease in the level of c-fos when compared with the saline group. Significant differences were found between the 1.0 g/kg EtOH and 2.0 g/kg EtOH (*P*<0.013) groups, and between the 1.0 g/kg EtOH and 4.0 g/kg EtOH (*P*<0.001) groups. There was no significant difference in c-fos expression in the saline group between WT and Aldh2-KO mice (*P*=0.055) (B).
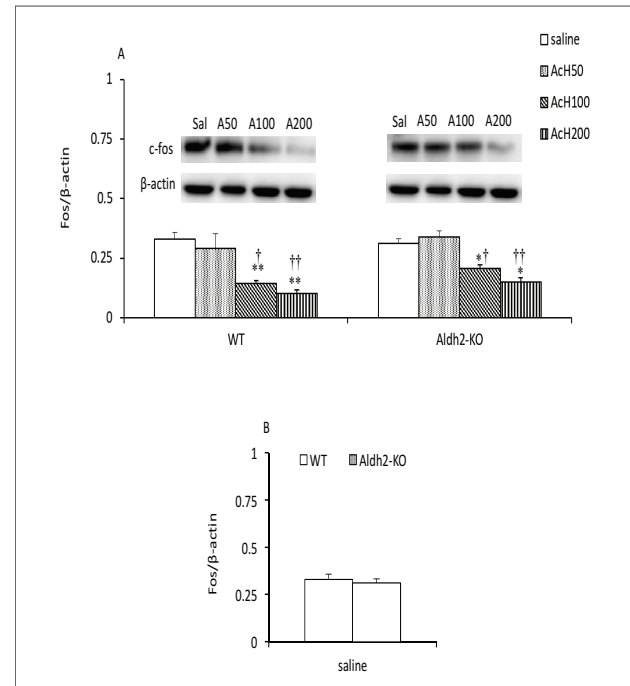

Figure 2: c-fos protein after exposure to AcH (50, 100 and 200 mg/kg) in the hippocampus of WT and Aldh2-KO mice (A) and the genotype differences on c-fos (B). Data represent mean ± SE (*n* = 5). *P<0.05 and **P<0.01 versus saline; †P<0.05 and ††P<0.01 versus AcH (50 mg/kg). Sal; saline, AcH50; AcH 50 mg/kg; AcH100, AcH 100 mg/kg; AcH200, AcH 200 mg/kg.

Fig. 2 shows the effects of different doses of AcH on c-fos protein expression in the hippocampus of

WT and Aldh2-KO mice (A). An ANOVA revealed a significant main effect of AcH treatment (df 3,32; F=23.683; P<0.001), but there was no significant interaction between treatment × animal (df 3,32; F=0.833; p=0.486). For WT mice, the post hoc analysis with the Tukey–Kramer test showed that treatment with 100 (P<0.001) and 200 mg/kg (P<0.001) of AcH resulted in a significant decrease in the level of c-fos when compared with the administration of saline. Significant differences were found between the 50 mg/kg AcH and 100 mg/kg AcH (P=0.007) groups, and between the 50 mg/kg AcH and 200 mg/kg AcH (P<0.001) groups. The 4-MP alone group had no effects on c-fos in WT mice (data not shown). For Aldh2-KO mice, the post hoc analysis with the Tukey–Kramer test showed that treatment with 100 (P=0.04) and 200 mg/kg (P=0.002) of AcH resulted in a significant decrease in the level of c-fos when compared with the administration of saline. Significant differences were found between the 50 mg/kg AcH and 100 mg/kg AcH (P=0.013) groups, and between the 50 mg/kg AcH and 200 mg/kg AcH (P<0.001) groups. The 4-MP alone group had no effects on c-fos in Aldh2-KO mice (data not shown). There was no significant difference in c-fos expression in the saline group between WT and Aldh2-KO mice (P=085) (B).

Discussion

This study was undertaken to delineate, for the first time, the effect of acute treatment with different doses of EtOH and AcH on c-fos protein in the hippocampus of Aldh2-KO and WT mice. The main results we report here are the following: 1) 2.0 and 4.0 g/kg of EtOH decreased c-fos in WT mice, whereas EtOH at all three doses tested deceased c-fos in Aldh2-KO mice, indicating that both EtOH and AcH mediate this effect; and 2) AcH at doses of 100 and 200 mg/kg decreased c-fos protein in both WT and Aldh2-KO mice, suggesting that AcH itself exerts this effect. These findings are the first to suggest that EtOH and/or AcH can decrease the expression of c-fos protein in the hippocampus of mice.

A series of studies has demonstrated the effects of EtOH on the expression of c-fos in a large number of neurons in the rat brain. Their results demonstrated that EtOH-induced changes in c-fos are variable in rat brains, increasing in many cases (3, 14-16), but decrease[17] or having no significant effect in others[22,23]. Here, we chose mice to give an i.p. injection of different doses of EtOH (1.0, 2.0 and 4.0 g/kg) so that we could observe the effects of EtOH and subsequent changes in AcH levels on the expression of c-fos in

the hippocampus. The high AcH concentration in the EtOH groups of Aldh2-KO mice is probably due to the constant rate of EtOH oxidation over a wide range of EtOH concentrations (24). We, selected C57BL/6 mice instead of rats because the two respond differently to EtOH administration[25]. C57BL/6 mice have a greater availability of hepatic enzyme alcohol dehydrogenase (ADH) than other inbred strains of mice[26]. The difference in ADH activity among the mouse strains may suggest a difference in the extent or mechanism of damage caused by EtOH.

Our results revealed a significant decrease in c-fos protein after treatment with 2.0 and 4.0 g/kg of EtOH in WT mice, while in Aldh2-KO mice, this effect was seen at all three doses of EtOH (1.0, 2.0 and 4.0 g/kg) indicating a role for AcH in EtOH's mechanism of action (Fig. 1). The finding of a decrease in c-fos after low-moderate-high EtOH exposure in Aldh2-KO mice has led to the suggestion that EtOH can depress c-fos in the hippocampus which may depend on the metabolism of EtOH to AcH. In support of this possibility is a study showing that administration of a low to moderate dose of alcohol (0.5 and 1.5 g/kg) led to the suppression of c-fos only in the hippocampus[17]. EtOH at a dose of 1.5 g/kg may also produce strong suppression of hippocampal memory and block both basal and experience-dependent c-fos mRNA induction in the hippocampus[27]. EtOH-induced suppression of hippocampal c-fos and memory is likely mediated by central GABAergic, glutamatergic and dopaminergic systems (28). Although we did not find any changes of c-fos in WT mice at a low dose of EtOH (1.0 g/kg). We chose this dose as the lowest one used in our study since a low dose (1.0 g/kg) of EtOH does not have consistent memory effects in WT mice[18]. Notably, the pattern of decrease in c-fos with EtOH in this study was similar at a dose (2.0 g/kg) that is able to affect spatial memory performance in both WT and Aldh2-KO mice[18]. Moreover, EtOH-induced effects on c-fos are brain-region specific[17] suggest the possibility that the reduction in c-fos reflects a non-specific EtOH-related cellular toxicity. Taken together, the present results suggest that EtOH-induced suppression of hippocampus-dependent memory could be through downregulation of c-fos expression in the hippocampus.

Previously, we measured brain AcH concentrations in mice following direct administration of AcH in Aldh2-KO and WT mice[24]. Their results showed that AcH levels are more than two-fold higher in the brains of Aldh2-KO mice compared to those in WT mice probably due to ALDH2 deficiency. The enzyme

ALDH2 is likely to responsible for the majority of AcH oxidation. Although AcH concentration was not measured in the current study, we expect that the data would be similar to those we reported in our previous study. AcH levels used in this experiment were much greater than those achieved naturally, and such high levels of AcH in the blood may therefore enter the brain.

Thus far, very few studies have measured c-fos expression after systemic AcH administration in several areas of the rat brain, most of which have a substantial dopamine innervation[14,29]. They have used lower (0.032 g/kg) and higher (0.1 to 0.5 g/kg) doses of AcH. The pattern of results found by those studies was different from our results: increase[14] and no changes[29]. In another study, AcH does cause a significant increase in c-fos mRNA in the paraventricular nucleus of the rat hypothalamus, where ALDH inhibitor cyanamide with EtOH was administered to obtain a high concentration of AcH[3]. However, no study has previously examined c-fos expression in the hippocampus of mice after systemic administration of AcH. Our results revealed a significant decrease in c-fos after treatment with 100 and 200 mg/kg of AcH in both genotypes of mice, whereas a lower dose (50 mg/kg) had no effects in either genotype of mice (Fig. 2). The reason for these discrepancies in c-fos expression between previous studies and ours is not clear, but it might be due to the different strains and brain regions used. The activity of c-fos is modulated by interactions with a number of kinases. For example, the activation of c-fos is regulated by MAPK pathways[30]. Protein kinase C (PKC) also stimulates the expression of the c-fos gene[31] Our results suggest that EtOH and AcH can inhibit c-fos expression by affecting MAPK and PKC pathways. The mechanism underlying this effect needs to be clarified.

Our results reflect the same pattern of results found in behavioral studies demonstrating that the systemic injection of EtOH and AcH induces sedative as well as depressive and memory-impairing effects[4,18,32]. c-fos expression in the hippocampal dentate gyrus is associated with major depression[33]. Moreover, c-fos expression in the cortex and the dorsal hippocampus is necessary for spatial memory formation[11,13]. The inhibition of c-fos in the brain resulted in a significant increase in the number of reference and working memory errors[11]. It is possible to argue that the acute administration of EtOH and AcH, which in turn results in decreased c-fos expression in the hippocampus, could contribute to these spatial memory deficits.

Conclusion

We can conclude from the present study combined with previous studies that exposure of mice to a moderate to high dose of EtOH and AcH can cause the expression of c-fos to decrease in the hippocampus. Although, c-fos induction could reflect the functional activity of the neurons, our data provide evidence for the first time of a likely inhibition of c-fos by EtOH and/or AcH in the mouse hippocampus; additional work is required to confirm this conjecture by using other immediate early gene markers to evaluate the effects EtOH and AcH on this brain area.

Conflict of interest disclosure

The authors have no conflict of interest to declare

Individual Contribution of the Authors:

Conceptual work: Jamal M

Data collection: Jamal M, Ito A, Tanaka N, Ameno K, Miki T, Kinoshita H

Manuscript writing: Jamal M

Editing of final manuscript: Jamal M, Ito A, Tanaka N, Ameno K, Miki T, Kinoshita H

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