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Increased ethanol preference and serotonin 1A receptor-dependent attenuation of ethanol-induced hypothermia in PACAP-deficient mice

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ABSTRACT

Pituitary adenylate cyclase-activating polypeptide (PACAP)-deficient mice display remarkable behavioral changes including increased novelty-seeking behavior and reduced hypothermia induced by either serotonin (5-HT)$_{1A}$ receptor agonists or ethanol. Because 5-HT$_{1A}$ receptors have been implicated in the development of alcohol dependence, we have examined ethanol preference in PACAP-deficient mice using a two-bottle choice and a conditioned place preference test, as well as additive effects of ethanol and 5-HT$_{1A}$ receptor agents on hypothermia. PACAP-deficient mice showed an increased preference towards ethanol compared with wild-type mice. However, they showed no preference for the ethanol compartment after conditioning and neither preference nor aversion to sucrose or quinine. The 5-HT$_{1A}$ receptor agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) restored the attenuated hypothermic response to ethanol in the mutants to similar levels in wild-type mice, with no effect in wild-types. In contrast, the 5-HT$_{1A}$ receptor antagonist WAY-100635 attenuated the ethanol-induced hypothermia in wild-type mice, with no effect in the mutants. These results demonstrate increased ethanol preference in PACAP-deficient mice that may be mediated by 5-HT$_{1A}$ receptor-dependent attenuation of ethanol-induced central inhibition.

Introduction

PACAP is a pleiotropic neuropeptide acting as a neurotransmitter, neuromodulator or neurotrophic factor [1–3]. Previously, we developed mice that lack the PACAP gene (PACAP$^{-/-}$) [4] and demonstrated that these mice exhibit remarkable behavioral changes, including hyperactivity, novelty-seeking behavior, sensory–motor deficits and depression-like behavior [4–6]. These observations suggest a role for altered PACAP-mediated signaling pathways in certain psychiatric disorders. Indeed, an association between single nucleotide polymorphisms in the genes for PACAP and its receptor PAC$_1$ and schizophrenia was identified by case–control comparison [7].

Genetic studies using *Drosophila* mutants revealed altered alcohol sensitivity in mutants with altered cAMP signaling system components such as *rutabaga* (adenylyl cyclase) and *DCO* (protein kinase A catalytic subunit) [8]. *Cheapdate* is also a mutant that has a loss-of-function amnesiac allele which encodes a PACAP-like neuropeptide [9]. Therefore, we addressed whether PACAP-deficiency was associated with altered sensitivity to ethanol, and demonstrated that ethanol-induced hypothermic and hypnotic effects were significantly reduced in PACAP$^{-/-}$ mice [10]. In addition, a considerable body of evidence suggests that dysfunction of central serotonergic neurotransmission is implicated in the pathogenesis and maintenance of alcoholism [11]. Furthermore, the 5-HT$_{1A}$ receptor has been implicated with alcohol consumption and alcohol withdrawal syndrome [12,13]. In PACAP$^{-/-}$ mice, hypothermia induced by the 5-HT$_{1A}$ receptor agonists 8-OH-DPAT or buspirone was significantly decreased [5], and the serotonin metabolite 5-hydroxyindoleacetic acid was slightly decreased in the cortex and striatum [4]. However, it has not yet been addressed whether PACAP-deficiency leads to changes in ethanol preference, and, if this is the case, how the 5-HT$_{1A}$ receptor system is involved in ethanol action in the mutant mice.

To extend these previous studies, we have utilized PACAP-deficient mice to examine ethanol preference using a two-bottle choice test and a conditioned place preference test. In addition, the additive effects of ethanol and 5-HT$_{1A}$ receptor agents on hypothermia were investigated.

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Materials and methods

Animals. All animal experiments were carried out in accordance with protocols approved by the Animal Research Committee of Osaka University, Japan. Generation of PACAP$^{-/-}$ mice by a gene targeting technique has been reported previously [4]. The null mutation was backcrossed onto the genetic background of Crlj:CD1 mice (Charles River, Tokyo, Japan) at least 10 times. All wild-type control and PACAP$^{-/-}$ mice used were obtained from the intercross of animals heterozygous for the mutant PACAP gene, and experiments were conducted with naïve male mice of 2–4 months of age. Mice were housed in a temperature (23 ± 1 °C) and light-controlled room with a 12-h light/12-h dark cycle (lights on from 08:00 to 20:00 h), and allowed free access to water and food, except during behavioral testing.

Two-bottle choice test. The two-bottle choice test was carried out as described previously [14,15] with slight modifications. Mice were habituated to drinking from two bottles containing water over 2 days and were then given free access to water and a solution of ethanol in water. The concentration of ethanol was increased every 3 days, ranging from 0% to 10% (v/v). The positions of the bottles were changed every day to control for position preferences. The same procedures were used to test for sweet (sucrose) and bitter (quinine) taste preferences.

Conditioned place preference test. The conditioned place preference test was carried out as described previously [16,17] with slight modifications. The apparatus consisted of a box divided into two compartments of equal size (20 × 20 cm). One compartment had black walls and a floor covered with wire mesh. The other compartment had white walls and a smooth textured floor that was odorized with a few drops of 2% (v/v) acetic acid.

For the pre-conditioning phase (Day 1–3), each mouse was placed in the white compartment and allowed to freely explore both compartments for 10 min. This was repeated twice a day with a 6 h interval. The exploration was videotaped and the amount of time spent in each compartment was determined. For the conditioning phase (Days 4–7), half of the animals from each genotype received ethanol (1 g/kg, intraperitoneally), and each mouse was confined to the white compartment for 15 min. After a 6-h washout period, they were administered with the vehicle solutions (saline) and confined to the black compartment for 15 min. For the post-conditioning phase (Day 8), each mouse was placed in the white compartment and then allowed to freely explore both compartments for 10 min. The conditioned score represented the difference in the amount of time spent in the white compartment between the post- and pre-conditioning sessions.

Measurement of rectal temperature. Rectal temperature was recorded with a Physitemp Bat 12 digital thermometer (Physitemp Instruments Inc., Clifton, USA) prior to and following an injection of ethanol (2.5 g/kg, intraperitoneally). 8-OH-DPAT (0.05 mg/kg, subcutaneously) or WAY-100635 (0.1 mg/kg, subcutaneously) was given 10 min before the ethanol injection.

Reverse transcription (RT)-polymerase chain reaction (PCR) analysis. Semi-quantitative RT-PCR was performed as described previously [18]. Primer sequences [19], and the number of PCR cycles were as follows: 5-HT$_{1A}$ receptor: 5′-ACCATCTACTCCACTTTC GGCG-3′ (sense), 5′-TTCACTGTCTTCCTCTCACGGG-3′ (antisense), 29 cycles; 5-HT$_{1B}$ receptor: 5′-AGGAGCAGGGTATTCAGTGCG-3′ (sense), 5′-TGTCCAGCGTCCAGTGACCG-3′ (antisense), 29 cycles; β-actin: 5′-GATGGTGGGTATGGGTCAGAAGGA-3′ (sense), 5′-GCTC ATTGCCGATAGTGATGACCT-3′ (antisense), 24 cycles. The β-actin housekeeping gene was simultaneously reverse-transcribed and amplified as an internal reference. PCR amplification consisted of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 1 min. PCR products were analyzed with a fluorescent image analyzer (FluorImager 595; Molecular Dynamics, Japan). The numbers of cycles were optimized for each primer pair to obtain linearity between the amount of cDNA and PCR product.

Results

Increased ethanol preference in PACAP$^{-/-}$ mice

In the two-bottle choice test, PACAP$^{-/-}$ mice displayed a significantly increased preference to ethanol (Fig. 1B, C). PACAP$^{-/-}$ mice consumed more than two times the amount of 3% (v/v) or 10% (v/v) ethanol solution compared with water, while wild-type mice consumed similar amounts of either concentration of ethanol solution and water. The total liquid consumption did not differ between the two genotype groups indicating normal drinking behavior in PACAP$^{-/-}$ mice (Fig. 1A). PACAP$^{-/-}$ mice showed neither preference nor aversion to the non-alcoholic tastants, sweet (1.7% (w/v) sucrose) and bitter (0.1 mM quinine) (Fig. 1D, E).

Ethanol-induced conditioned place preference test in PACAP$^{-/-}$ mice

PACAP$^{-/-}$ mice showed increased place preference to the white compartment on day 2 during a pre-conditioning period compared with wild-type mice. However, PACAP$^{-/-}$ mice showed no preference to the ethanol compartment in the post-conditioning phase on day 8 (Fig. 2).

Restoration of attenuated ethanol-induced hypothermia in PACAP$^{-/-}$ mice by activation of 5-HT$_{1A}$ receptors

In accordance with our previous results, ethanol-induced hypothermia at a much reduced level in PACAP$^{-/-}$ mice compared with wild-type mice ($P < 0.001$, two-way ANOVA with repeated measures; Fig. 3A). The 5-HT$_{1A}$ receptor agonist 8-OH-DPAT restored this attenuated hypothermic response to ethanol in the mutants (Fig. 3C; $P < 0.001$, two-way ANOVA with repeated measures) to similar levels in wild-type mice (Fig. 3A), with no effect in wild-types (Fig. 3B). In contrast, the 5-HT$_{1A}$ receptor antagonist WAY-100635 attenuated the ethanol-induced hypothermia in wild-type mice (Fig. 3B; $P < 0.001$, two-way ANOVA with repeated measures), with no effect in the mutants (Fig. 3C).

It has been demonstrated that pre-synaptic 5-HT$_{1A}$ receptors mediate the hypothermic response [20], and the receptor density correlates with hypothermic response to the 5-HT$_{1A}$ receptor agonist [21], therefore, we carried out RT-PCR analysis using β-actin mRNA as an internal control to quantify the expression of the receptor mRNA levels. There was no significant difference in the expression of 5-HT$_{1A}$ or 5-HT$_{1B}$ receptor mRNA in the brainstem and hippocampus between wild-type and PACAP$^{-/-}$ mice (Fig. 3D).

Discussion

In the present study, we have observed an increased ethanol preference in PACAP$^{-/-}$ mice. Since previous studies have shown a correlation between voluntary ethanol consumption and taste factors in rodents [22], we examined the preference to sweet and bitter flavored solutions in the mutant mice. However, we observed neither a preference nor aversion to the two tastants. In addition, total liquid consumption did not differ significantly between PACAP$^{-/-}$ and wild-type mice. The results suggest that the increased ethanol preference in PACAP$^{-/-}$ mice may not be ascribable to altered taste preference but may be due to the changes in the pharmacological effects of ethanol on the mice.

In the conditioned place preference test [16,17], a well-established model of ethanol reward, PACAP$^{-/-}$ mice exhibited in-

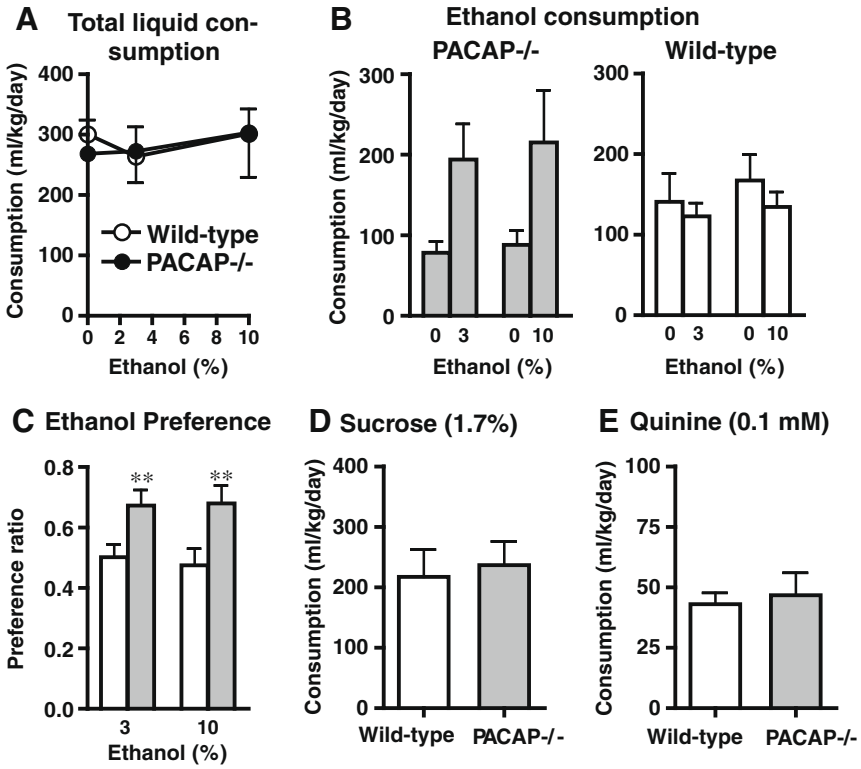

Fig. 1. Ethanol preference in PACAP$^{-/-}$ mice. (A) Total liquid consumption, (B) ethanol consumption, (C) ethanol preference ratios, (D, E) preference for non-alcohol tastants, sweet (1.7% (w/v) sucrose, D) and bitter (0.1 mM quinine, E) were examined in PACAP$^{-/-}$ (filled circles and bars) and wild-type (open circles and bars) mice. Preference ratios were determined as volume of ethanol consumed per total volume of liquid consumed. Values are the mean ± SEM (n = 11–12 per group, A–C; n = 5 per group, D and E). $^{**}P < 0.01$ compared with wild-type mice.

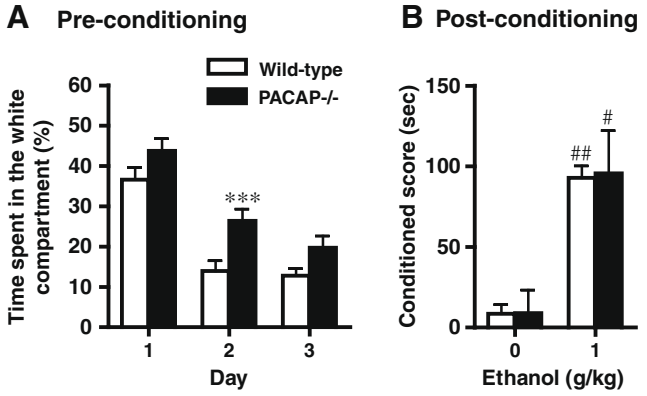

Fig. 2. Ethanol-induced conditioned place preference test in PACAP$^{-/-}$ mice. Pre-conditioning (A) and post-conditioning (B) time spent in the white (ethanol-paired) compartment were examined in PACAP$^{-/-}$ (filled bars) and wild-type (open bars) mice. Values are the mean ± SEM (n = 6 per group). $^{***}P < 0.001$ compared with wild-type mice; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with 0 g/kg ethanol in the same genotype.

creased place preference to the white compartment on day 2 during a pre-conditioning period. This result may be related to impaired habituation to a novel environment in PACAP$^{-/-}$ mice, which we previously demonstrated in an open-field test [4]. However, in the post-conditioning phase on day 8 after conditioning for 4 days, PACAP$^{-/-}$ mice exhibited conditioning to ethanol similar to that in wild-type mice indicating that the effect of the ethanol reward was not different between the two groups. This may be in accordance with a previous observation that PACAP$^{-/-}$ mice showed normal methamphetamine-induced behavioral sensitization compared with wild-type mice [23].

Dysfunction of central serotonergic neurotransmission has been implicated in the pathogenesis and maintenance of alcoholism associated with negative mood states and excessive alcohol intake, which may be mediated in part by reduced alcohol-induced sedation [11]. Indeed, PACAP$^{-/-}$ mice have been shown to have a reduced hypnotic response to a higher dose of ethanol (4.0 g/kg body weight) as well as an attenuated hypothermic response to ethanol (2.5 g/kg body weight) [10] or the 5-HT$_{1A}$ receptor agonists 8-OH-DPAT (0.1–0.5 mg/kg body weight) and buspirone (10 mg/kg body weight) [5]. Therefore, we further examined whether the 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT, could restore the attenuated ethanol-induced hypothermia in PACAP$^{-/-}$ mice. Interestingly, 8-OH-DPAT at a relatively low dose (0.05 mg/kg body weight) restored the attenuated hypothermic response to ethanol in mutants to similar levels in wild-type mice, with no effect in wild-types. This was also true vice versa, where the 5-HT$_{1A}$ receptor antagonist WAY-100635 (0.1 mg/kg body weight) attenuated the ethanol-induced hypothermia in wild-type mice, with no effect in the mutants.

As the hypothermic response to the 5-HT$_{1A}$ receptor agonist 8-OH-DPAT is known to be mediated by 5-HT$_{1A}$ autoreceptors [20], impaired 5-HT$_{1A}$ autoreceptor function and therefore increased serotonergic neurotransmission would be expected in PACAP$^{-/-}$ mice. However, these mice had slightly decreased 5-hydroxyindoleacetic acid, a 5-HT metabolite, in the cortex and striatum compared with wild-type mice [4], suggesting low brain 5-HT turnover rate rather than increased serotonergic neurotransmission. In addition, as the 5-HT$_{1A}$ receptor density has been shown to correlate with hypothermic response to the 5-HT$_{1A}$ receptor agonist [21], we examined the 5-HT$_{1A}$ receptor mRNA levels in the mutant mice. However, no significant difference in the mRNA levels was observed in the brainstem and hippocampus between wild-type

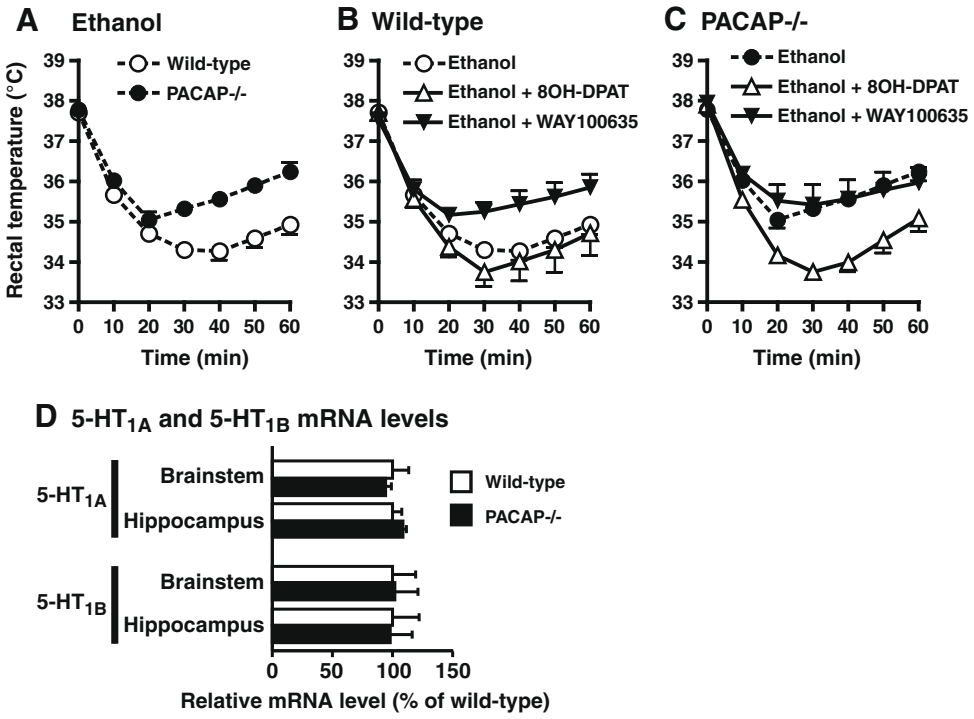

Fig. 3. Ethanol and 5-HT_{1A}-agonist-induced hypothermia and 5-HT_{1A} mRNA levels. (A) $PACAP^{-/-}$ (filled circles) and wild-type (open circles) mice were injected with ethanol (2.5 g/kg body weight). (B and C) $PACAP^{-/-}$ (B) and wild-type (C) mice were injected with ethanol (2.5 g/kg body weight) either alone (open circles) or in combination with 8-OH-DPAT (0.05 mg/kg body weight; open triangles) or WAY-100635 (0.1 mg/kg body weight; inverted filled triangles). Rectal temperature was measured at the indicated times. Values are the mean ± SEM (n = 5–7 per group). (D) 5-HT_{1A} and 5-HT_{1B} mRNA levels were analyzed by RT-PCR in the brainstem and hippocampus of wild-type (open bars) and $PACAP^{-/-}$ (closed bars) mice. Values are the mean ± SEM (n = 4–7 per group).

and $PACAP^{-/-}$ mice. We further carried out RT-PCR and microarray analyses, but to date, we have failed to confirm changes in the expression of genes that are probably responsible for altered hypothermic response seen in $PACAP^{-/-}$ mice (data not shown), although these data do not exclude the possibility of altered functions of 5-HT_{1A} autoreceptors in $PACAP^{-/-}$ mice.

In conclusion, the present results suggest that the increased ethanol preference in $PACAP^{-/-}$ mice may be related to diminished ethanol-induced central nervous system effects, in which an impaired 5-HT_{1A} receptor function is likely be implicated. In the present study, we could not address the direct relationship between the increased ethanol preference and 5-HT_{1A} receptor signaling pathway in $PACAP^{-/-}$ mice. This issue needs to be examined in a future study. Brain derived neurotrophic factor heterozygous knock-out mice have been shown to display increased ethanol intake and heightened aggressiveness as well as decreased 5-HT_{1A} receptor function at the level of receptor–G protein interaction [15]. Interestingly, $PACAP^{-/-}$ mice also exhibit remarkable behavioral changes (see Introduction). The present results, taken together with previous data, suggest that PACAP-signaling pathways are involved in ethanol consumption and preference that implicate altered 5-HT_{1A} receptor function.

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