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Involvement of endocannabinoids in alcohol "binge" drinking: studies of mice with human fatty acid amide hydrolase genetic variation and after CB1 receptor antagonists

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Abstract

Background—The endocannabinoid system has been found to play an important role in modulating alcohol intake. Inhibition or genetic deletion of fatty acid amide hydrolase (FAAH, a key catabolic enzyme for endocannabinoids) leads to increased alcohol consumption and preference in rodent models. A common human single-nucleotide polymorphism (SNP; C385A, rs324420) in the FAAH gene is associated with decreased enzymatic activity of FAAH, resulting in increased anandamide levels in both humans and FAAH C385A knock-in mice.

Methods—As this FAAH SNP has been reported to be associated with altered alcohol abuse, the present study used these genetic knock-in mice containing the human SNP C385A to determine the impact of variant FAAH gene on alcohol "binge" drinking in the drinking-in-the-dark (DID) model.

Results—We found that the $FAAH^{A/A}$ mice had greater alcohol intake and preference than the wild-type $FAAH^{C/C}$ mice, suggesting that increased endocannabinoid signaling in $FAAH^{A/A}$ mice led to increased alcohol "binge" consumption. The specificity on alcohol vulnerability was suggested by the lack of any FAAH genotype difference on sucrose or saccharin intake. Using the "binge" DID model, we confirmed that selective CB1 receptor antagonist AM251 reduced alcohol intake in the wild-type mice.

Conclusions—These data suggest that there is direct and selective involvement of the human FAAH C385A SNP and CB1 receptors in alcohol "binge" drinking.

Keywords

Endocannabinoid system; human FAAH C385A knock-in; CB1 receptor; alcohol drinking

INTRODUCTION

The endocannabinoid system consists of cannabinoids (including anandamide and 2-Arachidonoylglycerol), and cannabinoid receptors (CB1 and CB2). Anandamide-dependent

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signaling has been shown to be regulated by the enzyme involved in its catabolism, fatty acid amide hydrolase (FAAH) [Cravatt et al, 1996, 2001]. Several lines of evidence have recently demonstrated that endocannabinoid is involved in the behavioral effects of alcohol: inhibition or genetic deletion of FAAH, with resultant increases in anandamide levels, increases alcohol consumption and preference [Basavarajappa et al, 2006; Blednov et al, 2007]. CB1 receptor antagonists decrease alcohol intake in Sardinian alcohol-preferring rats [Colombo et al, 1998], attenuate the motivation to consume alcohol in outbred rats [Gallate and McGregor, 1999], and reduce alcohol drinking in mice [Arnone et al, 1997], suggesting that endocannabinoid system is involved in alcohol reinforcement and consumption. In addition, mice lacking CB1 receptors have reduced alcohol drinking or preference [Hungund et al, 2003; Wang et al, 2003; Naassila et al, 2004], and alcohol-induced reward (as assayed in conditioned place preference [Houchi et al, 2005]. A further study demonstrates a critical role of the CB1 receptor in alcohol withdrawal-related symptoms using CB1 knockout mice, though no change in alcohol consumption was found [Racz et al, 2003].

FAAH is the primary catabolic enzyme for anandamide and is a key regulator of anandamide-dependent signaling in the brain. FAAH knock-out mice have been shown to have significantly augmented brain levels of anandamide [Cravatt et al, 2001]. In humans, a common single-nucleotide polymorphism (SNP) (C385A; rs324420) in the FAAH gene has significant effects on the expression and activity of FAAH. This SNP leads to the substitution of an evolutionarily conserved proline at amino acid position 129 with a threonine residue, which in turn renders the FAAH protein more vulnerable to degradation. Accordingly, this polymorphism leads to elevated anandamide levels by reducing steady-state levels of FAAH protein, with resultant increases in anandamide activity [Sipe et al, 2002; Chiang et al, 2004; Dincheva et al, 2015]. The FAAH SNP has been reported to be associated with apparently increased alcohol abuse in some studies [Sipe et al, 2002; Hoenicka et al, 2007; Bühler et al, 2014], but not other studies in alcohol or other drugs [Iwasaki et al, 2007; Tyndale et al, 2007; Proudnikov et al, 2010]. Therefore, the human literature suggests that the FAAH C385A SNP with decreased FAAH activity might alter alcohol consumption. To test this hypothesis directly, we studied FAAH C385A knock-in mice that express the variant A allele of the FAAH polymorphism in place of the C allele, mimicking human variant alleles of FAAH gene [Dincheva et al, 2015]. It has been demonstrated that the FAAH knock-in mouse and human variant allele carriers exhibit several parallel alterations in behavior, including enhanced fear extinction learning, and decreased anxiety-like behaviors [Dincheva et al, 2015]. Using the FAAH knock-in mice, we determined the impact of FAAH genotype on alcohol drinking in the drinking-in-the-dark (DID) model [Rhodes et al, 2005], which allows for limited access (e.g., 4 h/day) to a single alcohol bottle after the beginning of the dark period. It has been well established that C57BL/6J mice become intoxicated and the blood ethanol concentration levels are relatively high during this 4-hour alcohol exposure time in the DID model, and thus the mouse model mimics “binge” drinking to the point of intoxication [Rhodes et al, 2005; Crabbe et al 2011].

As introduced above, preclinical investigations have consistently found that the CB1 antagonists decrease alcohol intake and alcohol’s reinforcing and motivational properties [e.g., Arnone et al, 1997; Colombo et al, 2005; Femenia et al, 2010]. In parallel, therefore,

we tested a CB1 antagonist AM251 to confirm the involvement of endocannabinoids in alcohol “binge” DID drinking in wild-type mice.

MATERIALS AND METHODS

Animals

The generation of FAAH C385A SNP mice was described previously (Dincheva et al., 2015). Male $FAAH^{A/A}$ mice and wild-type littermates ($FAAH^{C/C}$) derived from heterozygous $FAAH^{C/A}$ parents were used for all experiments. All animals were kept on a 12/12 light–dark cycle at 22°C with food and water available ad libitum. Mice were genotyped as described previously (Dincheva et al., 2015). At the time the experiments start (at the age of 8-10 weeks), the knock-in $FAAH^{A/A}$, $FAAH^{C/A}$ and wild-type $FAAH^{C/C}$ mice had similar body weight (~27 g).

Both the transgenic male mice and C57BL/6J male mice from the Jackson laboratory (Bar Harbor, ME, USA) (both at the age of 8-10 weeks) were individually housed in ventilated cages fitted with steel lids and filter tops and given ad libitum access to food and water in a stress-minimized facility. Mice were placed on a 12-hour reverse light-dark cycle (lights off at 7:00 am). Animal care and experimental procedures were conducted according to the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources Commission on Life Sciences 1996). The experimental protocols used were approved by the Institutional Animal Care and Use Committee of the Rockefeller University.

Drugs

Ethanol solutions (15% v/v) were prepared from 190 proof absolute ethyl alcohol (Pharmco-AAPER, Brookfield, CT, USA) and diluted in tap water. Sucrose and saccharin were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and diluted in tap water. AM251 was purchased from Tocris Inc. (Minneapolis, MN) and dissolved in 5% Cremophor in physiological saline.

The drinking-in-the-dark (DID) procedure

Mice accessed alcohol drinking in their home cages for 1 week with food available in this one-bottle paradigm with alcohol exposure every day with 1 recording per day (4 hours in the dark cycle). Based on previous publications [Rhodes et al, 2005; Kamdar et al, 2007], the basic paradigm with our modifications was as follows: At the time when the mice started individual housing (1 week before the experiments), the water bottles were replaced with those with sipper tubes to acclimate the mice to the sipper tubes (without ball bearings). Starting at 3 hours after lights off (10:00 am), the water bottles were replaced with 10-ml alcohol pipettes that were cut at both ends and sealed with a rubber stopper and fitted with a stainless steel straight sipper tube. The sipper tubes contained a ball bearing at the end to prevent alcohol leakage. The alcohol pipettes were refilled every day with fresh alcohol solution, and kept for 4 hours and then were replaced with the water bottles. In most of the experiments, alcohol solutions were prepared fresh every 48 hours by mixing alcohol with tap water to reach 15% (v/v) alcohol concentration in tap water. Body weights were recorded every 2 days, and alcohol intake values were recorded at 4 hours every day (to the

nearest 0.1 ml). These data were used to calculate self-administered alcohol dose (i.e., g/kg).

Experiment I. Genotypic effect on alcohol, sucrose or saccharin drinking in FAAH knock-in mice

After the 4-hour drinking after 4 days of DID, it has been demonstrated that C57BL/6J mice have high alcohol consumption which is correlated with blood alcohol concentrations [Rhodes et al, 2005; Crabbe et al 2011]. The objective of these experiments was to determine whether short access to alcohol for 4 days will lead to stable alcohol intake with potential genotypic difference among $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ mice. Mice of all genotypes were at 8 weeks of age and acclimated for a week prior to testing. During the first day of testing (i.e., session 1), all mice were 9 weeks of age and were given access to 15% alcohol for 4 h. Then they received daily 4-hour access to alcohol for another 3 days. To evaluate alcohol drinking, alcohol intake values were recorded at 4 hours in the dark. To assess further the genotype difference in alcohol preference, mice in $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ genotypes were exposed to the 2-bottle "alcohol (15%) *vs.* water" free choice regimen on day 5 after 4 days of 1-bottle DID experience.

As alcohol is a caloric reinforcer, the specificity of the genotypic difference on alcohol intake was tested on sucrose intake (caloric reinforcer) using the DID paradigm. In these experiments, sucrose exposure procedure was identical to the above alcohol experiments. Mice in $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ genotypes were given access to sucrose (4% or 8%) for 4 hours for 4 days and sucrose intake values were recorded at 4 hours in the dark. To assess further the genotype difference in sucrose preference, mice with $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ genotypes were exposed to the 2-bottle "8% sucrose *vs.* water" free choice regimen on day 5 after 4 days of 1-bottle sucrose (8%) DID experience.

The specificity of the genotype difference on alcohol intake was further tested on saccharin intake (non-caloric reinforcer) using the DID paradigm. In these experiments, the saccharin exposure procedure was identical to the above sucrose experiments. Mice in all three genotypes were given access to saccharin (0.1% or 0.2%) for 4 hours for 4 days. To assess further the genotype difference in saccharin preference, mice with $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ genotypes were exposed to the 2-bottle "0.1% saccharin *vs.* water" free choice regimen on day 5 after 4 days of 1-bottle saccharin (0.1%) DID experience.

Experiment II. Acute administration of AM251 (0.3-1 mg/kg) on alcohol or sucrose drinking in C57BL/6J mice

After 4 days of DID (15% alcohol), the groups assigned as the vehicle-treated mice and AM251-treated mice with matched body weight had similar 15% alcohol intake on day 3 (24 hours before the test day). The AM251 doses chosen were based on literature of rodent alcohol studies [e.g., Femenía et al, 2010]. On the test day, alcohol (15%) was presented 10 min after a single injection of AM251 (0.3, 1 mg/kg) or vehicle, and then alcohol intake values were recorded at 4 hours. Then on day 5, all the 3 groups were tested on 15% alcohol DID for 4 hours for an additional day. In another set of mice, the specificity of the AM251's

effect on alcohol intake at 1 mg/kg dose was tested on sucrose intake (8%), and the sucrose drinking procedure was identical to the above alcohol experiments.

Data analysis

In FAAH knock-in mouse experiments, group differences on alcohol (or sucrose, saccharin) intake during 4-day DID drinking tests were analyzed using 2-way ANOVA for genotype ($FAAH^{A/A}$, $FAAH^{C/A}$, $FAAH^{C/C}$) and time (1, 2, 3, 4 days); group differences on alcohol intake on each day from 1 to 4 days or preference ratio on day 5 were analyzed using 1-way ANOVA for genotype ($FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$). In C57BL/6J wild-type mouse experiments with AM251, group differences on alcohol or sucrose were analyzed using 1-way ANOVA for treatment (vehicle, drug doses). All the ANOVA were followed by Newman-Keuls *post-hoc* tests. The accepted level of significance for all tests was $p < 0.05$. All statistical analyses were performed using *Statistica* (version 5.5, StatSoft Inc, Tulsa, OK).

RESULTS

I. Genotypic differences on 15% alcohol, but not sucrose or saccharin, drinking in FAAH knock-in mice

After exposed to 15% alcohol for 4 days, the $FAAH^{A/A}$ males drank more alcohol than the $FAAH^{C/C}$ ones over the 4-day exposure; daily average alcohol intake is shown in **Figure 1A**. Over 4 days, two-way ANOVA revealed: (1) a significant effect of genotype [$F(2, 64) = 6.22$, $p<0.005$]; and (2) a significant effect of time [$F(3, 64) = 6.49$, $p<0.001$] on alcohol intake. *Post hoc* analysis showed that there was a marginal significance between the $FAAH^{A/A}$ and $FAAH^{C/C}$ mice on day 4 [$p=0.052$]. As the mice showed stable alcohol intake after 3 days of alcohol drinking, consistent with the early report [Rhodes et al, 2005], one-way ANOVA was run on day 4 and showed that the $FAAH^{A/A}$ had significantly more alcohol intake than the $FAAH^{C/C}$ [$F(2,16) = 4.30$, $p<0.05$], When the mice were tested on day 5 for 15% alcohol vs. water choice using 2-bottle 4-hour paradigm, one-way ANOVA showed that the $FAAH^{A/A}$ had significantly more alcohol preference than the $FAAH^{C/C}$ [$F(2, 16) = 3.74$, $p<0.05$] (**Figure 1B**).

During 4 days of sucrose (4% or 8%) drinking, there was no genotype difference over the 4-day exposure. The sucrose intakes in 4% and 8% concentrations from day 1 to day 4 are shown in **Figure 2A and 2B**. To assess further the genotype difference in sucrose preference, mice in $FAAH^{A/A}$, $FAAH^{C/A}$, and $FAAH^{C/C}$ genotypes were exposed to the 2-bottle "8% sucrose *vs.* water" free choice regimen on day 5, and did not show any genotype difference in the sucrose preference (**Figure 2C**).

During 4 days of saccharin (0.1% or 0.2%) drinking, there was no genotype difference over the 4-day exposure. The saccharin intakes in 0.1% and 0.2% concentrations from day 1 to day 4 are shown in **Figure 3A and 3B**. To assess further the genotype difference in saccharin preference, mice in $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ genotypes were exposed to the 2-bottle "0.1% saccharin *vs.* water" free choice regimen on day 5, and did not show any genotype difference in the saccharin preference (**Figure 3C**).

II. Reducing effect of acute administration of AM251 (0.3-1 mg/kg) on 15% alcohol (but not sucrose) drinking in C57BL/6J mice

At 0.3 and 1 mg/kg, AM251 significantly reduced alcohol intake at 4-hour recording time in a dose-dependent manner (**Figure 4**): 1-way ANOVA showed a significant effect on alcohol intake [$F(2, 17) = 8.47$, $p<0.005$]. *Post hoc* analysis showed that: (1) the mice after 0.3 mg/kg AM251 had significantly less alcohol intake than the vehicle control ones [$p<0.05$]; and (2) the mice after 1 mg/kg AM251 had significantly less alcohol intake than the vehicle control ones [$p<0.01$]. There was no difference among the groups 24 hours after the test day (data not shown).

The specificity of the AM251 effect on alcohol intake was tested on sucrose intake (8%), and no effect was found after AM251 at 1 mg/kg on sucrose drinking (**Table 1**).

DISCUSSION

I. Involvement of FAAH in alcohol “binge” drinking in mice

Our first objective in the present study was to investigate the potential of the FAAH SNP C358A genotype variant on altering alcohol “binge” consumption in mice, as some human studies suggest that the FAAH C385A SNP might be associated with increased alcohol vulnerability to alcohol abuse [Sipe et al, 2002; Hoenicka et al, 2007]. As C57BL/6J mice become intoxicated with limited access (e.g., 4 h/day) to a single alcohol bottle after the beginning of the dark period, we purposely used this drinking-in-the-dark (DID) “binge” alcohol drinking model to monitor the drinking behavior. As originally reported [Rhodes et al, 2005], the concentration of alcohol tested in C57BL/6J mice from 10%-30% did not have any effect on total alcohol intake (expressed as gram alcohol /kg body weight), and the highest consumption occurred when the alcohol solution was offered 3 hours at the beginning of the dark cycle and lasted for 4 hours. Based on this basic paradigm, in the present study, the $FAAH^{A/A}$ knock-in mice with C57BL/6J background were offered 15% concentration of alcohol 3 hours after the dark cycle for 4 hours. Using the FAAH C385A SNP mice, we tested whether the FAAH genotypic difference would be associated with vulnerability to alcohol “binge” drinking activity. We found that the $FAAH^{A/A}$ mice displayed higher basal alcohol intake than the wild-type $FAAH^{C/C}$ mice during the 4 days of DID “binge” drinking.

Consistent with the early report [Rhodes et al, 2005], the alcohol intake on day 1 was essentially lower than those on days 2–4, and the intakes on days 3–4 were reasonably stable. After the 4-hour DID on day 4, the $FAAH^{A/A}$ mice had higher alcohol consumption than the wild-type $FAAH^{C/C}$ mice. In parallel, the $FAAH^{A/A}$ mice displayed greater alcohol preference than the wild-type $FAAH^{C/C}$ mice in the test on day 5 when the alcohol and water choice were available. The specificity on alcohol was suggested by the lack of any FAAH genotype difference in sucrose or saccharin intake. It has been demonstrated that this $FAAH^{A/A}$ polymorphism leads to elevated anandamide levels, by reducing steady-state levels of FAAH protein in $FAAH^{A/A}$ variants in mouse brain [Dincheva et al, 2015]. Therefore, our new results on alcohol “binge” drinking behavior suggest that the $FAAH^{A/A}$-induced increase in endocannabinoid activity may play a role in modulating alcohol drinking

in mice. Our finding shows that $FAAH^{A/A}$ knock-in mice displayed elevated alcohol consumption, consistent with some human studies showing that the FAAH C358A polymorphism is associated with increased vulnerability to alcohol abuse [e.g., Sipe et al, 2002; Hoenicka et al, 2007]. In another human study, however, the CC genotype has been reported to be associated with high risky alcohol consumption [Bühler et al, 2014]. Our data are also consistent with earlier studies of FAAH knockout mice showing that enhanced endocannabinoid activity in the FAAH deficient mice have higher preference for alcohol and more voluntary alcohol consumption [Basavarajappa et al, 2006; Blednov et al, 2007]. Together, our results show for the first time that altered endocannabinoid activity due to impaired FAAH function in $FAAH^{A/A}$ knock-in mice leads to increased alcohol consumption.

II. Involvement of CB1 receptors in alcohol “binge” drinking in mice

Previous pharmacological studies have demonstrated a role of brain endocannabinoid system in the neural circuitry regulating alcohol consumption and motivation to consume alcohol in different rodent models: CB1 receptor agonists and antagonists stimulate and suppress, respectively, alcohol intake, alcohol self-administration, and alcohol’s motivational properties [Colombo et al, 2005]. Specifically, it has been demonstrated that selective CB1 receptor antagonist AM251 decreased alcohol consumption in fawn-hooded rats [Femenia et al, 2010]. In the present study, we confirmed the result in mice that selective blockade of CB1 receptor after acute pretreatment with AM251 attenuated alcohol drinking in a dose-dependent manner. In rodents, both the cannabinoid receptors and the opioid receptors have long been considered key receptors related to increased vulnerability to develop alcohol dependence and relapse, and their interactions may provide the mechanisms through which cannabinoids are able to modulate the alcohol drinking [Manzanares et al, 1999; Zhou and Kreek, 2014]. Indeed, it has been found that the AM251 pretreatment reversed endogenous opioid alterations after chronic alcohol exposure [Femenia et al, 2010]. Together, our results confirm previous studies in rats that selective blockade of CB1 receptors decreases alcohol consumption, and extend the finding into mice.

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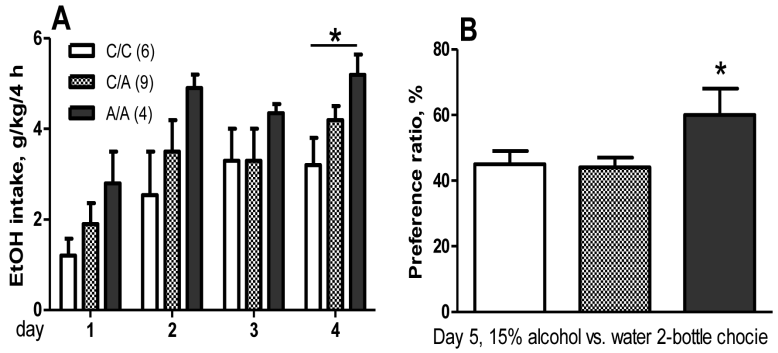

Figure 1.

Genotypic difference in alcohol (15%) intake in 4-day drinking-in-the-dark (DID) model (**A**) and alcohol preference (15% alcohol vs. water) on day 5 (**B**) in FAAH knock-in mice. (**A**) On the test days, alcohol at 15% concentration was presented 3 hours after the beginning of dark cycle, and then alcohol intake values were recorded at 4 hour time point for 4 consecutive days in the $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ mice; and (**B**) the mice were tested on day 5 for 15% alcohol vs. water choice using 2-bottle 4-hour paradigm after the 4-day DID drinking test. Genotypic difference: *$p<0.05$ vs. $FAAH^{C/C}$ mice. The values presented in the figure are mean ± SEM.

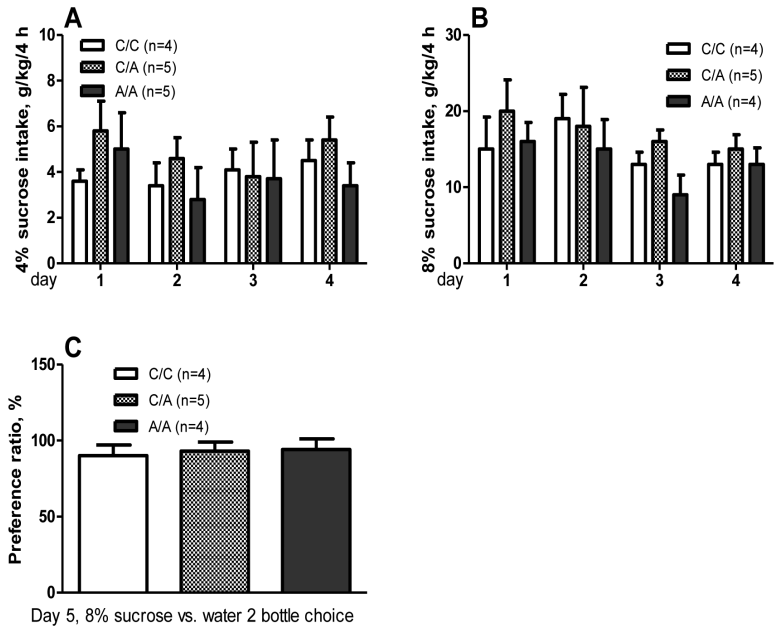

Figure 2.

No genotype differences in 4% (**A**) or 8% (**B**) sucrose intake in 4-day drinking-in-the-dark (DID) model, or sucrose preference (8% sucrose vs. water) on day 5 (**C**) in FAAH knock-in mice. (**A** and **B**) On the test days, sucrose was presented 3 hours after the beginning of dark cycle, and then their intake values were recorded at 4 hours for 4 consecutive days in the $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ mice; and (**C**) the mice were tested on day 5 for 8% sucrose vs. water choice using 2-bottle paradigm, and then their intake values were recorded at 4 hours. The values presented in the figure are mean ± SEM.

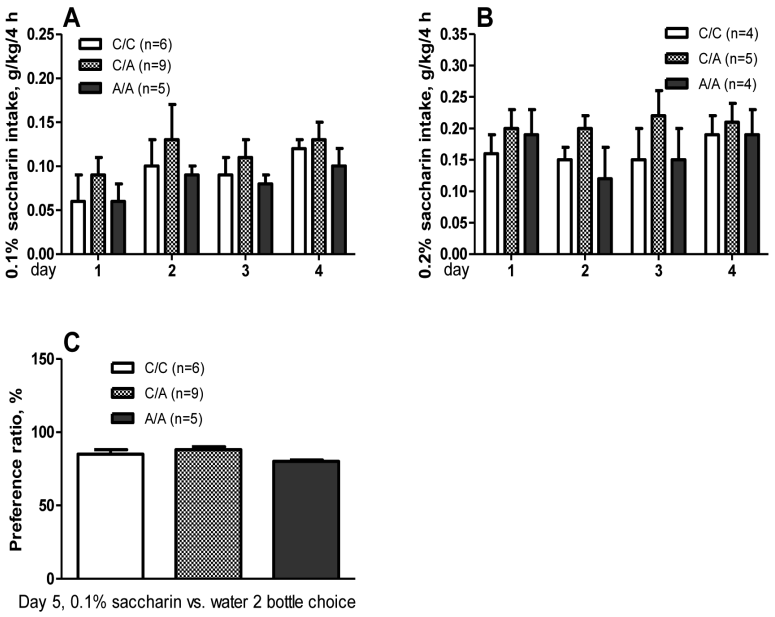

Figure 3.

No genotype differences in 0.1% (**A**) or 0.2% (**B**) saccharin intake in 4-day drinking-in-the-dark (DID) model, or saccharin preference (0.1% saccharin vs. water) on day 5 (**C**) in FAAH knock-in mice. (**A** and **B**) On the test days, saccharin was presented 3 hours after the beginning of dark cycle, and then their intake values were recorded at 4 hours for 4 consecutive days in the $FAAH^{A/A}$, $FAAH^{C/A}$ and $FAAH^{C/C}$ mice; and (**C**) the mice were tested on day 5 for 0.1% saccharin vs. water choice using 2-bottle paradigm, and then their intake values were recorded at 4 hours. The values presented in the figure are mean ± SEM.

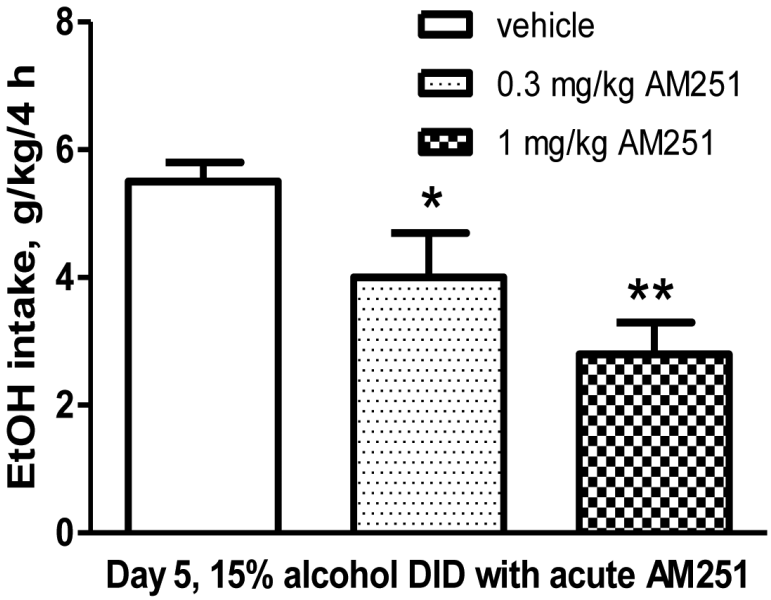

Figure 4.

Effect of acute AM251 administration at 0.3 or 1 mg/kg on alcohol (15%) intake on day 5 after 4-day drinking-in-the-dark (DID) model in C57BL/6J mice. The mice were assigned to one of 3 treatment groups: (1) vehicle as control; (2) 0.3 mg/kg AM251; and (3) 1 mg/kg AM251. AM251 effect: *$p<0.05$, **$p<0.01$ vs. vehicle. The values presented in the figure are mean ± SEM.

Table 1

Effect of acute AM251 administration at 1 mg/kg on sucrose (8%) intake on day 5 after 4-day drinking-in-the-dark (DID) model in C57BL/6J mice. The groups assigned as the vehicle-treated or AM251-treated mice had similar sucrose intake 24 hours before the test day (data not shown). On the test day, 8% sucrose was presented 10 min after a single i.p. injection of AM251 or vehicle, and then sucrose intake values were recorded at 4 hours. The mice were assigned to one of 2 treatment groups: (1) vehicle as control; and (2) 1 mg/kg AM251. The values presented in the Table are mean ± SEM.

	Vehicle N = 7	**1 mg/kg AM251 N = 8**
8% sucrose, g/kg/4h	19 ± 3	18 ± 2