Role of Endocannabinoids in Alcohol Consumption and Intoxication: Studies of Mice Lacking Fatty Acid Amide Hydrolase

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Endocannabinoid signaling plays the important role in regulation of ethanol intake. Fatty acid amide hydrolase (FAAH) is a key membrane protein for metabolism of endocannabinoids, including anandamide, and blockade of FAAH increases the level of anandamide in the brain. To determine if FAAH regulates ethanol consumption, we studied mutant mice with deletion of the FAAH gene. Null mutant mice showed higher preference for alcohol and voluntarily consumed more alcohol than wild-type littermates. There was no significant difference in consumption of sweet or bitter solutions. To determine the specificity of FAAH for ethanol intake, we studied additional ethanol-related behaviors. There were no differences between null mutant and wild-type mice in severity of ethanol-induced acute withdrawal, conditioned taste aversion to alcohol, conditioned place preference, or sensitivity to hypnotic effect of ethanol. However, null mutant mice showed shorter duration of loss of righting reflex induced by low doses of ethanol (3.2 and 3.4 g/kg) and faster recovery from motor incoordination induced by ethanol. All three behavioral phenotypes (increased preference for ethanol, decreased sensitivity to ethanol-induced sedation, and faster recovery from ethanol-induced motor incoordination) seen in mutant mice were reproduced in wild-type mice by injection of a specific inhibitor of FAAH activity—URB597. These data suggest that increased endocannabinoid signaling increased ethanol consumption owing to decreased acute ethanol intoxication.
Neuropsychopharmacology (2007) 32, 1570–1582; doi:10.1038/sj.npp.1301274; published online 13 December 2006

Keywords: FAAH; anandamide; mutant mouse; ethanol intake; tolerance; LORR

INTRODUCTION

The endocannabinoid system has been implicated in addictive behavior and in the mechanism of action of several drugs of abuse (see Gardner (2005) for review). This system contains cannabinoid receptors (CB1 and CB2), endogenous cannabinoids, and the molecules involved in the inactivation of endocannabinoids (uptake and enzymatic degradation by fatty acid amide hydrolase (FAAH)) (see Fride (2005), Rodriguez de Fonseca *et al* (2005) for review). Several lines of evidence indicate that the endocannabinoids are involved in the pharmacological and behavioral effects of alcohol (Wang *et al*, 2003; Hungund *et al*, 2003). For example, cannabinoids and alcohol activate the same reward pathways and CB1 receptor antagonists reduce alcohol consumption (see

Mechoulam and Parker (2003) for review). Several endogenous ligands for CB1 receptors have been identified, including anandamide and 2-arachidonoyl-glycerol. These lipid molecules act as retrograde messengers in the central nervous system and are synthesized postsynaptically from lipid precursors (Freund *et al*, 2003). As an endogenous ligand, anandamide may: (1) bind and activate the CB1 receptor *in vitro* (Devane *et al*, 1992; Felder *et al*, 1993), (2) be produced in the brain in response to peripheral pain stimuli (Walker *et al*, 1999), and (3) induce some cannabinoid-like behavioral effects *in vivo*, including hypothermia, analgesia, and motor defects (Crawley *et al*, 1993; Fride and Mechoulam, 1993; Smith *et al*, 1994). Chronic ethanol exposure increases the levels of anandamide in neuroblastoma cells (Basavarajappa and Hungund, 1999a) and in cerebellar granular neurons (Basavarajappa *et al*, 2000). Also, chronic exposure of mice to ethanol resulted in downregulation of CB1 receptors and CB1 receptor agonist-stimulated guanyl-5-*o*-(thio)-triphosphate (GTPγS) binding in synaptic plasma membranes (Basavarajappa and Hungund, 1999b). These researchers hypothesized that changes in endocannabinoid system observed after chronic ethanol intake may play a role in many aspects

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of ethanol action including development of tolerance to ethanol. Naive alcohol-preferring rats show decreased levels of CB1 receptors (Ortiz et al, 2004). Because a CB1 receptor antagonist decreases alcohol intake in both Sardinian alcohol-preferring rats (Colombo et al, 1998) and in C57BL/6 mice (Arnone et al, 1997) and the motivation to consume alcohol in outbred rats (Gallate and McGregor, 1999; Gallate et al, 2004), endocannabinoid signaling may be involved in alcohol reinforcement and consumption. In support of this idea, mice lacking CB1 receptors display reduced alcohol self-administration (Naassila et al, 2004), alcohol-induced place preference (Houchi et al, 2005), and alcohol-induced dopamine release in the nucleus accumbens (Hungund et al, 2003).

FAAH is a key membrane protein for metabolism of anandamide (Cravatt et al, 1996). Cravatt et al (2001) showed that mice lacking this enzyme are severely impaired in their ability to degrade anandamide and when treated with this compound, exhibit a pattern of intense CB1-dependent behavioral responses, including hypomotility, analgesia, catalepsy, and hypothermia. FAAH null-mutant mice show 15-fold augmented brain levels of endogenous anandamide and display reduced pain sensation that is reversed by the CB1 antagonist SR141716A. In human, a missense mutation (385A/385A) of FAAH was associated with problem drug/alcohol use (Sipe et al, 2002).

Taken together, the literature suggests that inhibition of FAAH activity should increase alcohol consumption and might also alter other behavioral actions of alcohol. To directly test this hypothesis, we studied mutant mice with deletion of the FAAH gene as well as a chemical inhibitor of this enzyme.

METHODS

Animals

Null FAAH $(-/-)$ allele mice were created using homologous recombination as described previously and were maintained on original 129/SvJ × C57Bl/6J genetic background (Cravatt et al, 2001). All behavioral analyses were performed on homozygous knockout $(-/-)$ and wild-type $(+/+)$ littermates generated from crosses between heterozygous animals. Mice were group-housed three–five per cage based on sex. Food and water were available ad libitum. The vivarium was maintained on a 12:12 h light: dark cycle with lights on at 0700. The temperature and humidity of the room were controlled. All experiments were performed during the light phase of the light:dark cycle. Mice of both sexes were used for all studies and were 10–16 weeks of age at the time of analysis; within each experiment all mice were of similar age. All experiments were conducted in the isolated behavioral testing rooms in the animal facility to avoid external distractions. All experiments were approved by the Institutional Animal Care and Use Committee.

Alcohol Preference Drinking

The two-bottle choice protocol was carried out as described previously (Blednov et al, 2001). Briefly, mice were allowed to acclimate for 1 week to individual housing. Two drinking tubes were continuously available to each mouse, and tubes were weighed daily. One tube always contained water. Food was available ad libitum, and mice were weighed every 4 days. After 4 days of water consumption (both tubes), mice were offered 3% ethanol (v/v) vs water for 4 days. Tube positions were changed every day to control for position preferences. Quantity of ethanol consumed (g/kg body weight/24 h) was calculated for each mouse and these values were averaged for every concentration of ethanol. Immediately following 3% ethanol, a choice between 6% (v/v) ethanol and water was offered for 4 days, then 9% (v/v) ethanol for 4 days, then 12% (v/v) ethanol for 4 days and finally 15% (v/v) ethanol for 4 days. Throughout the experiment, evaporation/spillage estimates were calculated every day from two bottles placed on an empty cage, one containing water and the other containing the appropriate ethanol solution.

Preference for Nonalcohol Tastants

Wild-type or knockout mice were also tested for saccharin and quinine consumption. One tube always contained water and the other contained the tastant solution. Mice were serially offered saccharin (0.033 and 0.066%) and quinine hemisulfate (0.03 and 0.06 mM), and intakes were calculated. Each concentration was offered for 4 days, with bottle position changed every day. For each tastant, the low concentration was always presented first, followed by the higher concentrations. Between tastants mice had two bottles with water for 2 weeks.

Ethanol-Induced Acute Withdrawal

Mice were scored for handling-induced convulsion (HIC) severity 30 min before and immediately before i.p. ethanol administration. The two predrug baseline scores (PRE) were averaged. A dose of 4 g/kg of ethanol in saline was injected i.p. and the HIC score was tested every hour until the HIC level reached base-line. Acute withdrawal was quantified as the area under the curve but above PRE level (Crabbe et al, 1991). Briefly, each mouse is picked up gently by the tail and, if necessary, gently rotated 180°, and the HIC is scored as follows: 5, tonic–clonic convulsion when lifted; 4, tonic convulsion when lifted; 3, tonic–clonic convulsion after a gentle spin; 2, no convulsion when lifted, but tonic convulsion elicited by a gentle spin; 1, facial grimace only after a gentle spin; 0, no convulsion.

Conditioned Place Preference

The conditioned place preference protocol was carried out as described previously (Blednov et al, 2003b). Four identical acrylic boxes (30 × 15 × 15 cm³) were separately enclosed in ventilated, light, and sound-attenuating chambers (Med Associates, St Albans, VT). Each box has two compartments separated by wall with a door. The two compartments each have a different type of floor (either bars—[GRID−] or small round holes—[GRID +]). Infrared light sources and photodetectors were mounted opposite each other at 2.5-cm intervals along the length of each box, 2.2 cm above the floor. Occlusion of the infrared light beams was used to measure general activity and

location of the animal (left or right) within the box. Total activity counts and location of the animal (left or right compartment) within the box were recorded by computer. The floors and the inside of the boxes were wiped with water and the litter paper beneath the floors was changed between animals. The main principles of conditioned place preference procedure have been described earlier (Cunningham *et al*, 1993). Ethanol was used at dose 2.0 g/kg (i.p.).

Conditioned Taste Aversion

Subjects were adapted to a water-restriction schedule (2 h of water per day) over a 7-day period. At 48-h intervals over the next 10 days (days 1, 3, 5, 7, 9, and 11), all mice received 1-h access to a solution of saccharin (0.15% (w/v) sodium saccharin in tap water). Immediately after 1-h access to tastant, mice received injections of saline or ethanol (2.5 g/kg) (days 1, 3, 5, 7, and 9). All mice also received 30-min access to tap water 5 h after each saccharin access period followed by injection of ethanol to prevent dehydration (days 1, 3, 5, 7, and 9). On intervening days, mice had 2 h continuous access to water at standard time at the morning (days 2, 4, 6, 8, and 10).

Loss of Righting Reflex

Sensitivity to ethanol was determined using the standard duration of loss of righting reflex (LORR) (sleep time) assay. Ethanol was diluted in 0.9% saline (20% v/v) and administered in doses adjusted by injected volumes. Mice were injected with ethanol and when they became ataxic, they were placed in the supine position in V-shaped plastic troughs until they were able to right themselves three times within 30 s. Sleep time was defined as the time from being placed in the supine position until they regained their righting reflex. During all sleep time assays, room temperature was 22°C. Mice that failed to lose the righting reflex (misplaced injections) or had a sleep time greater than two standard deviations from the group mean were excluded from the analysis.

Initial Sensitivity to LORR

Mice were given ethanol and 3 min later they were tested for an LORR (see above) greater than 1 min. The 95% confidence limits were determined using the 'up and down' method (see below) with an ethanol log dose interval of 0.0138, which corresponds to approximately a 0.1 g/kg ethanol dose difference at doses tested. The up and down method was used as described by Dixon and Massey (1969). Mice were injected with an initial dose and tested, and the results from each animal determined the dose that the next animal would receive. If the mouse did not display an LORR greater than 1 min, then the next dose of drug administered would be increased (by a 0.0138 log interval of the dose). If the mouse showed an LORR for more than 1 min, then the next dose of drug administered would be decreased (by a 0.0138 log interval of the dose). Each mouse was used for only one dose. The consequence of events (successful performance of task—1 or not successful performance of task—0) was used for statistical analyses. The ED50 values were determined by the following equation: 95%

$$CI \ (\text{confidential interval}) = \text{dosing increment} \times \sqrt{2/n} \times 1.96,$$

where n is the last n trials and 1.96 reflects the 0.05 α level (Dixon and Massey, 1969). For each trial with a given drug, six subsequent mice ($n = 6$) were used.

Rotarod

Mice were trained on a fixed speed rotarod (Economex; Columbus Instruments; speed of rod, 5.0 r.p.m.), and training was complete when mice were able to remain on the rotarod for 60 s. Every 10 min after injection of ethanol (2 g/kg i.p.), each mouse was placed back on the rotarod and latency to fall was measured until mouse was able to stay on the rotarod for 60 s.

Drug Injection

All alcohol (Aaper Alcohol and Chemical, Shelbyville, KY) solutions were made in saline (20% (v/v)) and injected i.p. with a volume of 0.1 ml/10 g of body weight. Inhibitor of FAAH—URB597 (Cayman Chemical, Ann Arbor, MI; 0.5 mg/kg i.p.) was prepared as a suspension of 0.5 mg of URB597 in 10 ml of saline with 4–5 drops of Tween-80 and injected to wild-type FAAH (+/+) and FAAH (−/−) knockout male mice in volume 0.1 ml/10 g of body weight 2 h before administration of ethanol in LORR and rotarod experiments. Published data suggest that this injection procedure provides complete and relatively long-lasting inhibition of FAAH. For example, Fegley *et al* (2005) showed that systemic administration of URB597 (0.3 mg/kg i.p.) to rats produced a time-dependent inhibition of [^{3}H]anandamide hydrolysis in brain membranes. Enzyme inhibition reached a maximum value within 15 min of drug administration and persisted for at least 16 h. This effect was associated with a parallel increase in brain anandamide.

In two-bottle choice paradigm, URB597 (0.5 mg/kg i.p.) was injected to FAAH (+/+) and null mutant FAAH (−/−) male mice 30 min before lights off. This time was chosen because the major fluid intake occurs during the dark phase (Brown *et al*, 2004) and brain anandamide is elevated for at least 16 h after injection of URB597 (Fegley *et al*, 2005). Control mice of both genotypes received i.p. injection of saline with 4–5 drops of Tween-80. Two bottles—one with water and another one with one of concentrations of ethanol—were placed into the cages immediately after light off and ethanol and water intakes were measured for 24 h as described above. Each concentration of ethanol was presented for 2 days. Tube positions were changed daily to control for position preferences. Bottles were weighed daily.

Ethanol Metabolism

Animals were given a single dose of ethanol (3.8 g/kg i.p.) and blood samples were taken from the retro-orbital sinus in 30, 60, 120, 180, and 240 min after injection. Blood alcohol concentration (BEC) values, expressed as milligram ethanol per ml blood were determined spectrophotometrically by an enzyme assay (Lundquist, 1959).

Statistical Analysis

Data are reported as the mean ± SEM value. The statistics software program GraphPad Prizm (Jandel Scientific, Costa

Madre, CA) was used throughout. To evaluate differences between groups, analysis of variance (ANOVA) (two-way ANOVA with Bonferroni *post hoc* analysis) and Student's *t*-test was carried out.

RESULTS

Ethanol Consumption

In a two-bottle test in which mice could drink either water or an ascending series of ethanol concentrations (3, 6, 9, 12, and 15%), mutant mice lacking FAAH displayed increased preference for ethanol ($F_{(1,90)} = 9.6$; $p < 0.01$, main effect of genotype and $F_{(4,90)} = 10.4$; $p < 0.0001$, main effect of concentration for females; $F_{(1,90)} = 28$; $p < 0.0001$, main effect of genotype and $F_{(4,90)} = 15$; $p < 0.0001$, main effect of concentration for males). No genotype × concentration interaction was found (Figure 1a and b). The amount of ethanol consumed depended on genotype ($F_{(1,90)} = 4.1$; $p < 0.05$ and $F_{(1,90)} = 31$; $p < 0.0001$ for female and male mice, respectively), concentration ($F_{(4,90)} = 6.5$; $p < 0.001$ and $F_{(4,90)} = 2.9$; $p < 0.05$ for female and male mice respectively) but there was no genotype × concentration interaction (Figure 1c and d). There were no differences between wild-type and null-mutant female mice in total intake of fluid (water + ethanol) (Figure 1e). However, total fluid intake was slightly increased in null-mutant male mice ($F_{(1,90)} = 6.7$; $p < 0.05$, main effect of genotype) (Figure 1f). There was no main effect of concentration or genotype × concentration interaction on total intake.

Preference for Nonalcohol Tastants

Mice lacking FAAH did not differ from wild-type mice in preference for saccharin (male mice: $F_{(1,36)} = 0.12$, $p > 0.05$ and $F_{(1,36)} = 4.8$, $p < 0.05$, main effect of genotype and concentration, respectively; female mice: $F_{(1,36)} = 1.3$, $p > 0.05$ and $F_{(1,36)} = 2.6$, $p > 0.05$, main effect of genotype and concentration, respectively) or for quinine solutions (male mice: $F_{(1,36)} = 0.04$, $p > 0.05$ and $F_{(1,36)} = 11$, $p < 0.01$, main effect of genotype and concentration, respectively; female mice: $F_{(1,36)} = 0.13$, $p > 0.05$ and $F_{(1,36)} = 1.3$, $p > 0.05$, main effect of genotype and concentration, respectively) (Table 1). There were also no significant differences in total fluid intake (g/kg/day) between wild type and null-mutant mice for saccharin (male mice: $F_{(1,36)} = 3.0$, $p > 0.05$ and $F_{(1,36)} = 3.2$, $p > 0.05$, main effect of genotype and concentration respectively; female mice: $F_{(1,36)} = 0.8$, $p > 0.05$ and $F_{(1,36)} = 1.2$, $p > 0.05$, main effect of genotype and concentration, respectively) as well as for quinine (male mice: $F_{(1,36)} = 0.12$, $p > 0.05$ and $F_{(1,36)} = 0.05$, $p < 0.01$, main effect of genotype and concentration, respectively; female mice: $F_{(1,36)} = 2.1$, $p > 0.05$ and $F_{(1,36)} = 0.01$, $p > 0.05$, main effect of genotype and concentration, respectively).

Depressant Effects of Ethanol

The duration of LORR (sleep time) produced by ethanol was decreased in both null allele male and female mice compared with wild-type mice ($F_{(1,45)} = 4.6$; $p < 0.05$—

dependent on genotype; $F_{(2,45)} = 13$; $p < 0.0001$—dependent on dose, for female mice and $F_{(1,40)} = 10$; $p < 0.01$—dependent on genotype; $F_{(2,40)} = 32$; $p < 0.0001$—dependence on dose, for male mice) (Figure 2a and b). Only male mice show significant genotype × dose interaction ($F_{(2,40)} = 4.1$; $p < 0.05$). *Post hoc* analysis revealed differences between wild-type and null mutant male mice for effect of ethanol at doses 3.2 g/kg ($p < 0.05$) and 3.4 g/kg ($p < 0.01$). Although the interaction of genotype and dose was not significant for female mice, separate *t*-test analysis revealed that FAAH ($-/-$) null mutant female mice exhibited shorter durations of loss of the righting reflex following the 3.2 g/kg ($p < 0.01$) and 3.4 g/kg ($p < 0.05$) ethanol doses.

Genotypic differences in duration of ethanol-induced LORR can be due to differences in sensitivity, acute tolerance, or ethanol clearance. Because acute tolerance may be initiated immediately following acute ethanol administration, genotypic differences in duration might reflect both initial sensitivity and acute tolerance mechanisms. Thus, we determined the median effective dose (ED_{50}) required to produce LORR to ascertain whether FAAH null mutant mice were initially less sensitive to ethanol. The doses of ethanol tested ranged from 1.8 to 2.8 g/kg, and 5–6 ethanol doses were tested in each sex and genotype group. The genotypes did not differ in initial sensitivity to ethanol-induced LORR ($ED_{50} \pm 95\%$ confidence interval: males ($n = 6$ per genotype): 2.1 ± 0.1 and 2.0 ± 0.1 g/kg for wild-type and knockout mice, respectively; females ($n = 6$ per genotype): 2.5 ± 0.1 and 2.4 ± 0.1 g/kg for wild-type and knockout mice, respectively).

Motor-Incoordination Effect of Ethanol

Acute administration of ethanol (2 g/kg) produces incoordination and null mutant mice recover from this motor-incoordination faster than wild-type mice ($F_{(1,126)} = 12$; $p < 0.001$—dependent on genotype; $F_{(8,126)} = 77$; $p < 0.0001$—dependent on time, for female mice and $F_{(1,238)} = 47$; $p < 0.0001$—dependent on genotype; $F_{(16,238)} = 53$; $p < 0.0001$—dependent on dose, for male mice) (Figure 4a and b). There was a significant genotype × time interaction ($F_{(8,126)} = 2.9$; $p < 0.01$ for female mice and $F_{(8,238)} = 2.7$; $p < 0.001$ for male mice). However, no differences were found between wild-type and null-mutant mice in acute motor-incoordination produced by several doses of ethanol (range 1.0–2.0 g/kg; measurement 10 min after injection) (Figure 3c and d). This suggests that the mutation mainly increases tolerance development without a change in initial sensitivity, consistent with the results presented above for the LORR tests.

Conditioned Taste Aversion

Although there was no difference in consumption of saccharin on trial 0 (before conditioning) between wild-type and FAAH null-mutant mice (77 ± 5 and 86 ± 2 g/kg body weight, for females and 58 ± 3 and 59 ± 4 g/kg body weight, for males, respectively), to attempt to correct for initial fluctuations in tastant intake and facilitate presentation of the data, intake was calculated as a percentage of the trial 0 consumption for each subject by dividing the amount of saccharin solution consumed on subsequent condition-
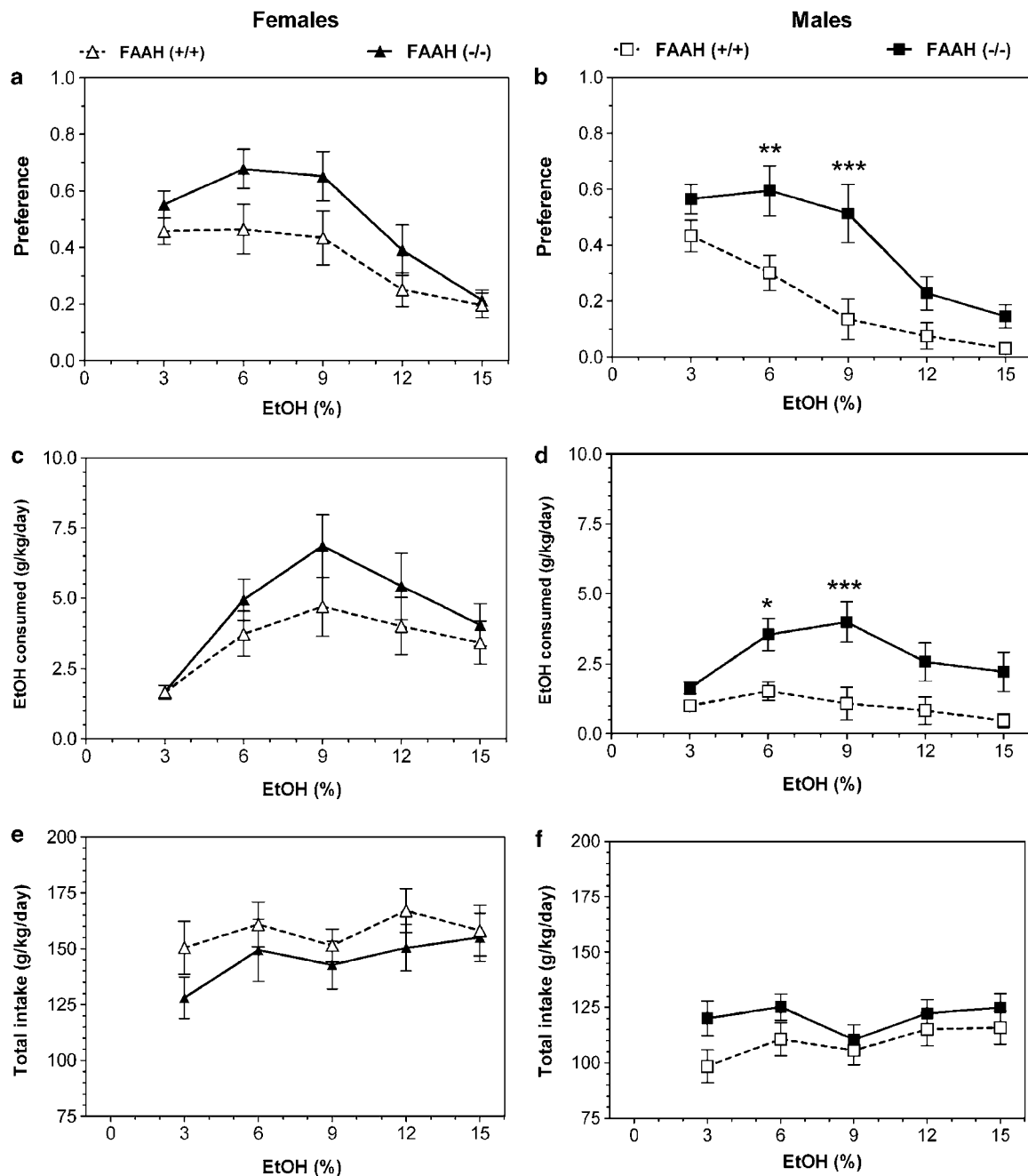
Figure 1 Increased ethanol preference and consumption in FAAH knockout (−/−) mice. (a) Males—preference for ethanol. (b) Females—preference for ethanol. (c) Males—amount of ethanol consumed. (d) Females—amount of ethanol consumed. (e) Males—total fluid intake. (f) Females—total fluid intake. $n = 10$ for all groups. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$—significant differences relative to wild-type mice for same concentration of ethanol (Bonferroni post-test).

ing trials by the amount of saccharin solution consumed on trial 0 (before conditioning). Ethanol–saccharin pairings produced reduction in saccharin intake across trials compared with saline–saccharin pairings, indicating the development of CTA in both genotypes of female mice ($F_{(1,65)} = 310$; $p < 0.0001$—effect of treatment and $F_{(4,65)} = 3.2$; $p < 0.05$—dependence on trial, for wild-type mice and $F_{(1,65)} = 224$; $p < 0.0001$—effect of treatment and $F_{(4,65)} = 3.2$; $p < 0.05$—dependence on trial, for knockout mice) as well as in male mice ($F_{(1,65)} = 141$; $p < 0.0001$

—effect of treatment, for wild-type mice and $F_{(1,69)} = 140$; $p < 0.0001$—effect of treatment, for knockout mice) (Figure 4a and b). However, there were no differences in development of CTA between wild-type and FAAH null-mutant mice of both sexes.

Place Conditioning

Following control saline injections, male mice spent substantially less time on the bar floor than on the floor

with round holes $(F(1,38) = 84; p < 0.0001$, main effect of floor) (Figure 5b). *Post hoc* analysis show the significant reduction of time spent on the bar floor for male mice of both genotypes $(p < 0.001$ for both genotypes). No main effect of genotype was found $(F(1,40) = 0.01; p > 0.05)$. Female mice also spent less time on the bar floor than on the floor with round holes $(F(1,36) = 24; p < 0.0001$, main effect of floor) (Figure 5a). However, *post hoc* analysis show a significant reduction only for female knockout mice $(p < 0.001)$. No main effect of genotype was found $(F(1,38) = 0.01; p > 0.05)$. Taking into account this original preference for one type of floor, we calculated place conditioning only for the group of mice injected with ethanol paired with their less favorite type of floor (the bar floor, GRID−). The percent of time spent on the bar floor

by saline- and ethanol-injected male mice of each genotype is shown in Figure 5d. Male mice of both genotypes spent more time on the bar floor when it was paired with ethanol than when paired with saline, reflecting development of conditioned place preference $(F(1,26) = 12; p < 0.01$, main effect of treatment). However, there was no difference in development of place conditioning between the genotypes $(F(1,36) = 3.2; p > 0.05$, main effect of genotype). In contrast, female mice show effects of genotype $(F(1,36) = 15; p < 0.001)$ but no effect of treatment $(F(1,26 = 0.2); p > 0.05)$ (Figure 5c).

Ethanol Acute Withdrawal Severity

A single 4 g/kg ethanol dose suppressed basal HIC in both the knockout and wild-type mice of both sexes for about 5 h, followed by increased HIC (Figure 6a and b). FAAH null-mutant and wild-type mice of both sexes did not differ in levels of basal HIC. Animals of both genotypes and sexes demonstrated signs of withdrawal (HIC scores higher than the basal level). However, there were no differences in area under the curves for HIC and above the basal level during withdrawal either for females $(1.5 \pm 0.7$ and 1.3 ± 0.6 for wild-type and knockout, respectively) or for males $(2.1 \pm 0.6$ and 2.2 ± 0.3 for wild type and knockout, respectively) (Figure 6c and d).

Ethanol Metabolism

There were no differences in metabolism of ethanol between wild-type and knockout mice of both sexes. Thus, ethanol clearance expressed in mg of ethanol per dl of blood in 1 h was: 59 ± 3 and 63 ± 4 for wild-type and knockout female mice, respectively, and 48 ± 6 and 50 ± 4 for wild-type and knockout male mice, respectively.

URB597 Administration *In Vivo*

To determine whether the differences in ethanol behavioral phenotypes of the null-mutant mice are the result of deletion of FAAH or changes resulting from loss of the

Table I Saccharin and Quinine Preference in Mice Lacking FAAH

	Male		Female	
	Wild type	**FAAH (−/−)**	**Wild type**	**FAAH (−/−)**
Saccharin 0.033%				
Preference	0.67 ± 0.09	0.74 ± 0.05	0.85 ± 0.05	0.80 ± 0.06
Total intake (g/kg)	132.7 ± 10.1	150.2 ± 10.4	222.8 ± 15.1	239.7 ± 34.0
Saccharin 0.066%				
Preference	0.88 ± 0.08	0.85 ± 0.06	0.93 ± 0.03	0.89 ± 0.06
Total intake (g/kg)	149.5 ± 13.1	183.8 ± 21.4	247.2 ± 21.4	291.1 ± 54.8
Quinine 0.03 mM				
Preference	0.20 ± 0.06	0.22 ± 0.04	0.44 ± 0.08	0.39 ± 0.07
Total intake (g/kg)	111.6 ± 6.9	110.3 ± 9.1	145.2 ± 11.1	132.6 ± 8.8
Quinine 0.06 mM				
Preference	0.09 ± 0.04	0.06 ± 0.02	0.33 ± 0.09	0.32 ± 0.08
Total intake (g/kg)	114.5 ± 6.4	110.8 ± 5.7	146.2 ± 11.6	129.6 ± 7.8

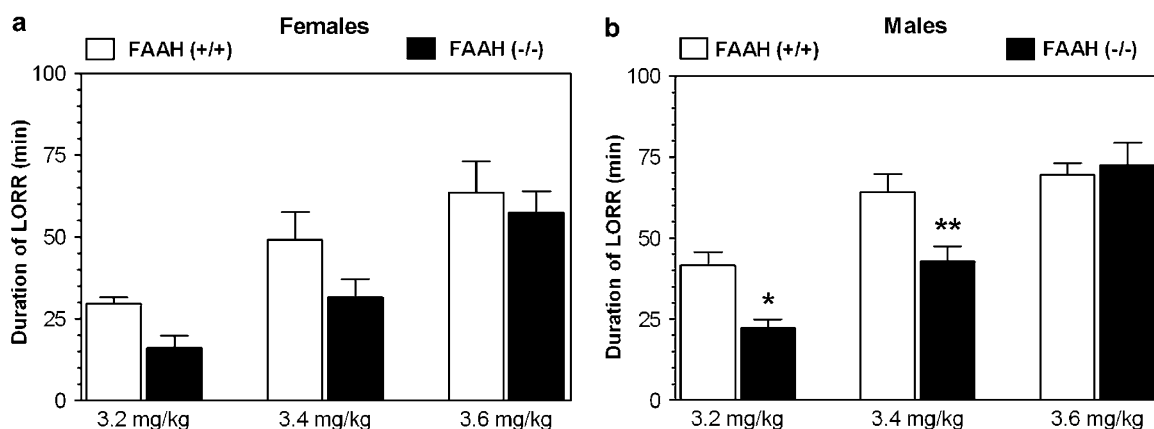Values represent mean+SEM group sizes were $n = 10$ per sex and genotype.

Figure 2 Reduced depressant effect of ethanol in FAAH knockout (−/−) mice. (a) Females—duration of LORR. (b) Males—duration of LORR. $n = 6$–9 for males and $n = 6$–12 for females. *$p < 0.05$; **$p < 0.01$—significant differences relative to wild-type mice for same dose of ethanol (Bonferroni post-test).

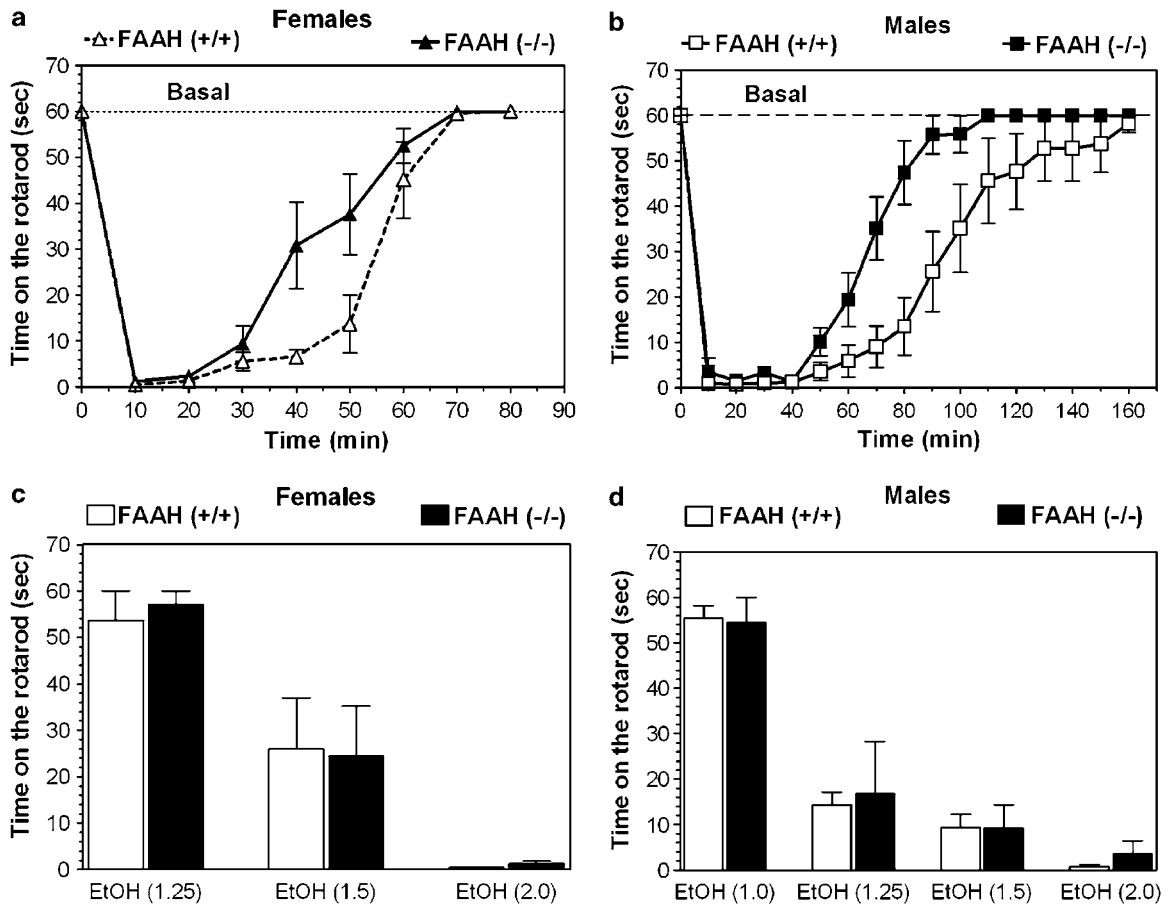**Figure 3** Faster recovery from motor incoordination effects of ethanol in FAAH knockout (−/−) mice. (a) Females. Time on the rotarod. Recovery from motor incoordination effect of ethanol (2 g/kg). (b) Males. Time on the rotarod. Recovery from motor incoordination effect of ethanol (2 g/kg). (c) Females. Acute sensitivity to motor incoordination effects of ethanol. (d) Males. Acute sensitivity to motor incoordination effects of ethanol. $n = 8$ for all groups for a and b. $n = 5–8$ for all groups for (c) and (d).
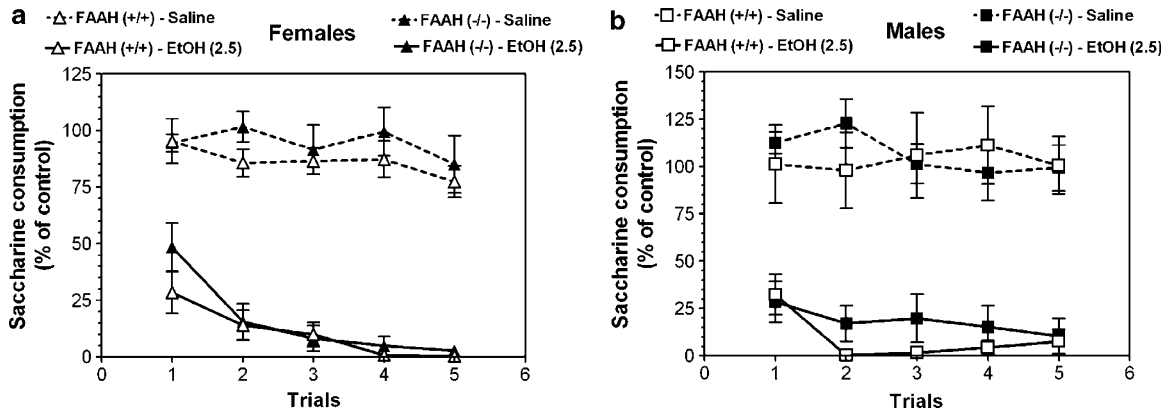
Figure 4 No differences in ethanol induced conditioned taste aversion between wild type and FAAH knockout (−/−) mice. (a) Females—changes in saccharin consumption with trial. (b) Males—changes in saccharin consumption with trial. $n = 5–6$ for saline injection for all genotypes. $n = 9–10$ for groups with ethanol injection.

enzyme, we studied an inhibitor of FAAH activity—URB597—on several ethanol behaviors.

As shown in Figure 7a and c, injection of URB597 (0.5 mg/kg) significantly increased preference for ethanol ($F(1,70) = 18$; $p < 0.001$ main effect of treatment) as well as the amount of ethanol consumed ($F(1,70) = 13$; $p < 0.001$

main effect of treatment) in wild-type male mice. By contrast, the drug had no effect in FAAH (−/−) knockout male mice, indicating that its action on ethanol intake is probably due to inhibition of FAAH activity (Figure 7b and d). Consistent with data obtained with naïve mice, null-mutant male mice treated with saline showed significantly
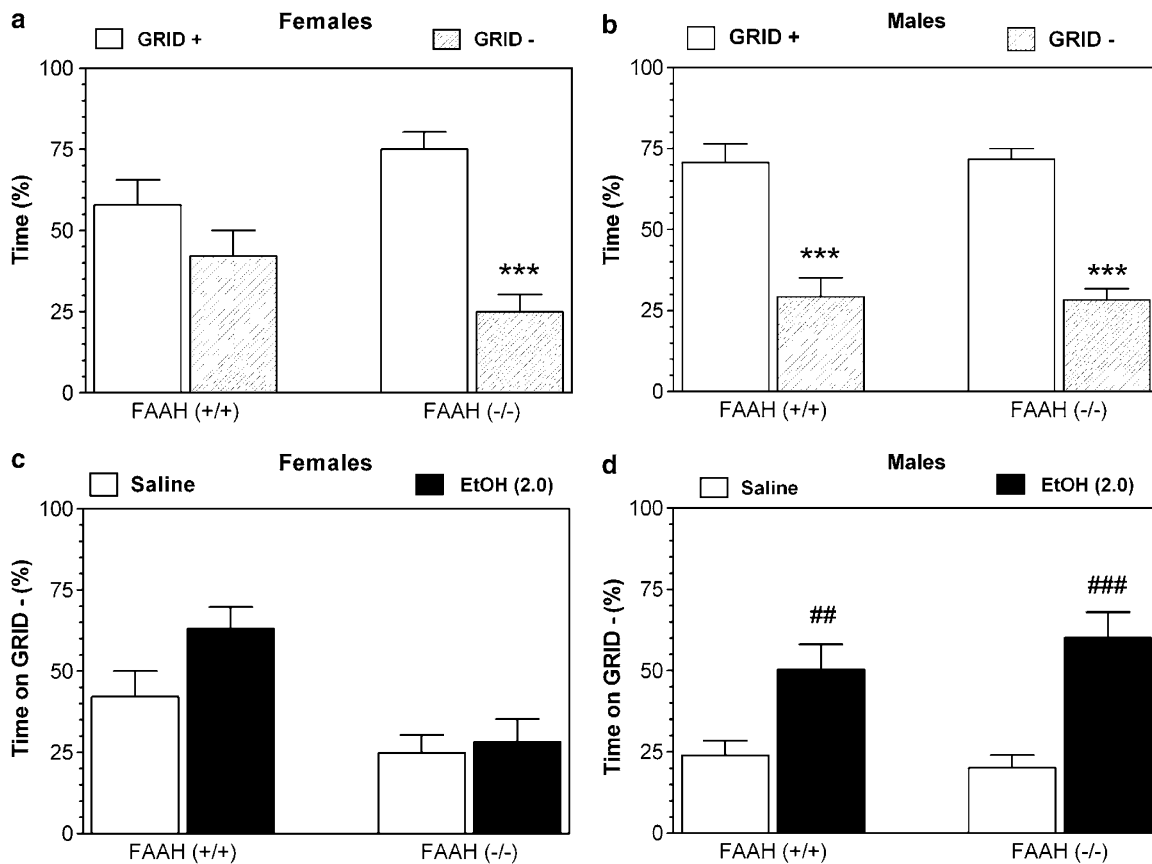
Figure 5 Lack of FAAH does not change the ethanol-induced conditioned place preference. (a) Females—percent of time spent on different floors during 30-min test session in control saline-treated groups. (b) Males—percent of time spent on different floors during 30-min test session in control saline-treated groups. (c) Females—percent of time spent on the bars by saline-treated groups and GRID—conditioned subgroups (ethanol injection was paired with bars). (d) Males—percent of time spent on the bars by saline-treated groups and GRID—conditioned subgroups (ethanol injection was paired with bars). ***$p < 0.001$—significant differences between time spent on (GRID +) and (GRID−) for saline-treated groups of mice of correspondent genotype (Bonferroni post-test). ##$p < 0.01$; ###$p < 0.001$—significant differences in time spent on [GRID−] between saline- and ethanol-treated groups of mice of correspondent genotype (Bonferroni posttest). $n = 10$ for saline-injected groups of each genotype and sex, $n = 11$ for ethanol-injected groups of each genotype and sex.

higher ethanol intake ($F(1,60) = 4.3$; $p < 0.05$ main effect of genotype) and preference for ethanol ($F(1,60) = 4.7$; $p < 0.05$ main effect of genotype) than wild-type mice from saline-treated group. There were no differences in ethanol intake between drug-treated wild-type mice and saline-treated null-mutant mice, consistent with the idea that the drug has the same effect as the mutation.

In wild-type female mice injection of URB597 (0.5 mg/kg) significantly increased the amount of ethanol consumed ($F(1,70) = 8.9$; $p < 0.001$ main effect of treatment) and there was a trend toward increased preference for ethanol ($F(1,70) = 3.2$; $p = 0.07$ main effect of treatment) (Figure 8a and c). By contrast, the drug had no effect on preference and amount of ethanol consumed in FAAH ($-/-$) knockout female mice (Figure 8b and d). Consistent with data obtained with naïve mice, null-mutant female mice treated with saline showed significantly higher ethanol intake ($F(1,70) = 6.3$; $p < 0.05$ main effect of genotype) and a trend for difference in preference for ethanol ($F(1,60) = 3.0$; $p = 0.08$ main effect of genotype) than wild-type female mice from saline-treated group. There were no differences in parameters of ethanol intake between

drug-treated wild-type female mice and saline-treated null female mice.

The effect of URB597 on ethanol LORR in wild-type male mice was dependent on treatment ($F(1,27) = 9.7$; $p < 0.01$) and dose of ethanol ($F(1,27) = 96$; $p < 0.001$) with significant treatment × dose interaction ($F(1,27) = 12.3$; $p < 0.01$) (Figure 9a). *Post hoc* analysis showed that injection of URB597 attenuated the hypnotic effect only at the lowest dose (3.2 g/kg) of ethanol tested. Wild-type male mice injected with URB597 recovered from motor incoordination effect of ethanol faster than saline-treated group ($F(1,221) = 28.9$; $p < 0.001$ main effect of treatment) (Figure 9b).

DISCUSSION

Taken together, these results show that deletion of FAAH significantly increases the preference for alcohol and amount of alcohol consumed by mice. Our data are in agreement with the results of recent pharmacological studies suggesting a role of brain endocannabinoid system
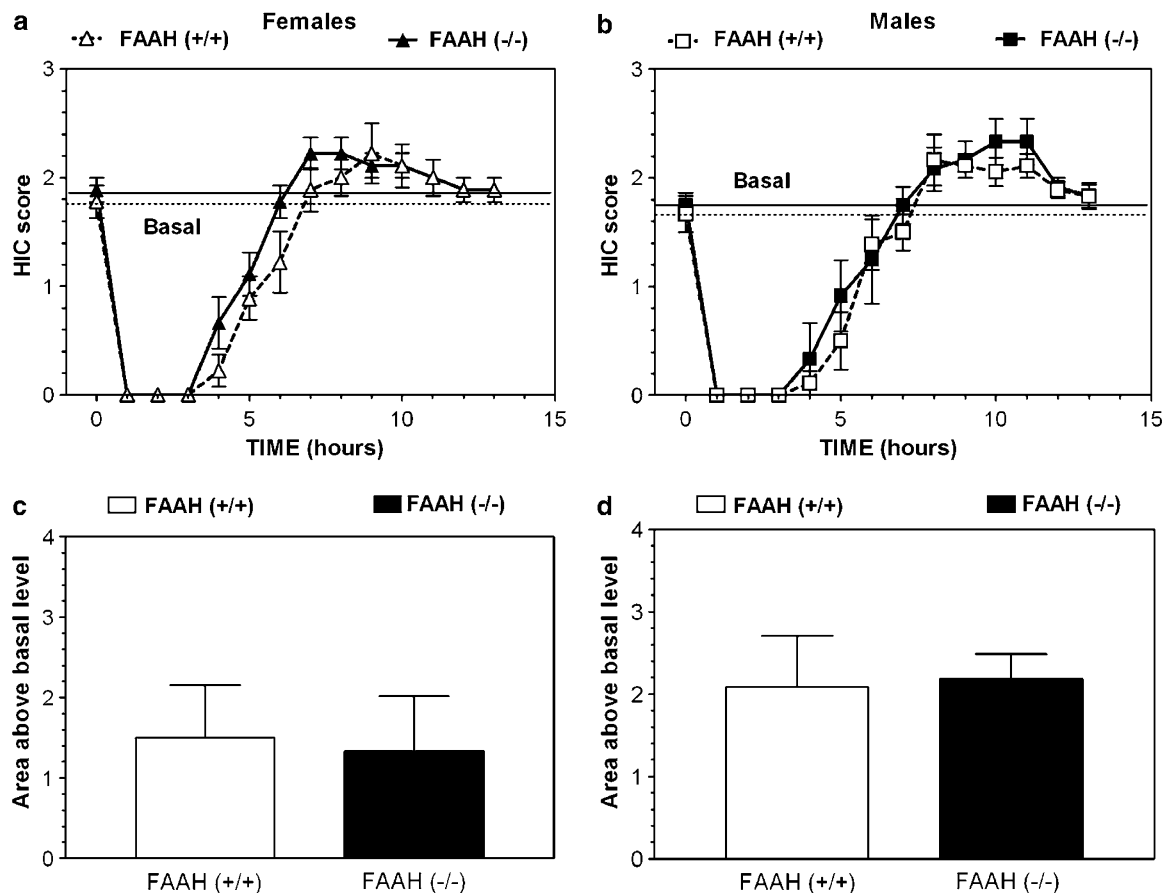
Figure 6 Severity of acute ethanol withdrawal was not changed in FAAH (−/−) knockout mice. (a) Females—HIC score. (b) Males—HIC score. (c) Females—area under the HIC score and above the correspondent basal level. (d) Males—area under the HIC score and above the correspondent basal level. $n = 7$–9 for male mice and $n = 9$ for female mice of both genotypes.

in the neural circuitry regulating alcohol consumption and motivation to consume alcohol in different rodent models of excessive alcohol consumption. These results show that cannabinoid receptor agonists and antagonists stimulate and suppress, respectively, alcohol intake, alcohol self-administration, and alcohol's motivational properties (see Colombo et al (2005) and, Maldonado et al (2006) for review). Hansson et al (2006) demonstrated a decrease of FAAH expression and activity in prefrontal cortex of alcohol-preferring AA rats compare with nonpreferring ANA rats. They also showed that elevated endocannabinoid transmission in brain of AA animals was accompanied by a compensatory downregulation of CB1 signaling. This is consistent with the observation that high-drinking C57BL/6 mice have lower levels of CB1-binding sites, but higher receptor affinity and coupling than low-drinking DBA/2 mice (Hungund and Basavarajappa, 2000). Our results are also consistent with a recent study of FAAH null mutant mice (Basavarajappa et al, 2006), although their changes in ethanol intake and preference were sex-dependent, whereas we found similar results from both sexes. These inconsistencies may be due to differences in the genetic background of the mutant mice used in the two studies. The wild-type mice used by Basavarajappa et al (2006) displayed much higher alcohol consumption than our mice and this may have limited the ability of the mutation to

further increase drinking. Because female mice have higher alcohol consumption than male mice, such a 'ceiling effect' may be more pronounced for females, which is consistent with the published data.

There are several possible behavioral mechanisms for increased ethanol intake in the two-bottle choice paradigm. For example, mice can increase consumption of ethanol because of decreased aversive properties of alcohol as well as of increase (or decrease) in ethanol reward (for review see Chester and Cunningham, 2002). However, our studies of conditioned taste aversion and conditioned place preference in these mutant mice indicate that changes in these properties of ethanol do not account for the decreased alcohol consumption. Alcohol withdrawal severity is also inversely correlated with alcohol consumption in mice (Metten et al, 1998) raising the possibility that increased alcohol withdrawal could account for the differences in alcohol consumption. However, FAAH mutants did not differ from wild-type mice in severity of acute ethanol-induced withdrawal.

Some studies have shown a negative correlation between hypnotic (loss of righting reflex) effects of ethanol and voluntary ethanol consumption (Hodge et al, 1999; Thiele et al, 1998, 2000; Spanagel et al, 2002), but this is not always the case (Boehm et al, 2003, 2004; Blednov et al, 2003a, b). Our results showed that reduction of the duration of
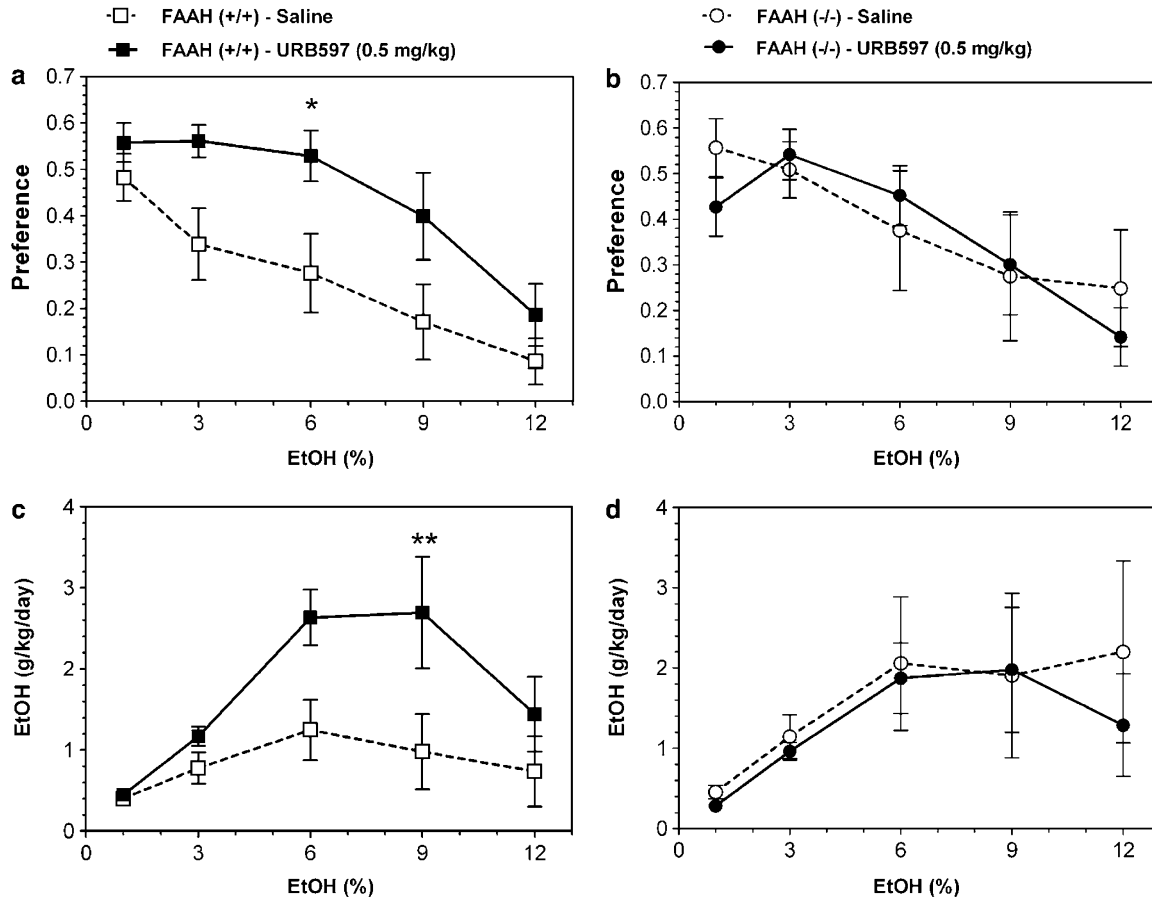
Figure 7 Inhibitor of FAAH—URB597 (0.5 mg/kg) increased voluntary ethanol preference and intake in wild-type but not in FAAH knockout (−/−) male mice. (a) Preference in wild-type mice. (b) Preference in FAAH knockout (−/−) mice. (c) Consumption of ethanol (g/kg/day) in wild-type mice. (d) Consumption of ethanol (g/kg/day) in FAAH knockout (−/−) mice. *$p < 0.05$; **$p < 0.01$—significant differences between saline- and URB597-treated groups of mice for same dose of ethanol (Bonferroni post-test). $n = 6$ for each group of knockout mice and $n = 8$ for each group of wild-type mice.

ethanol LORR by the mutation was most clear for low doses of ethanol. Because ethanol LORR is mediated by multiple molecular mechanisms, it is likely that distinct molecular mechanisms are responsible for hypnotic effect of different doses of ethanol. For example, at least four quantitative trait loci on different mouse chromosomes and 23 candidate genes have been identified, which are largely responsible for the heritable component of hypnotic sensitivity to ethanol in selected ILS and ISS strains of mice (Markel et al, 1997; Bennett and Johnson, 1998; Ehringer et al, 2001; MacLaren et al, 2006). Furthermore, genotypic differences in duration of ethanol-induced LORR can reflect differences in sensitivity, acute tolerance or ethanol clearance (Radcliffe et al, 2005). Because there were no differences in either initial sensitivity to LORR or in ethanol clearance, the faster recovery of mice lacking FAAH from motor incoordination may be due to development of ethanol tolerance. This is consistent with data from Basavarajappa and Hungund (2005) showing a role of the endocannabinoid system in the development of alcohol tolerance. The faster recovery from ethanol-induced acute intoxication combined with higher alcohol consumption may be relevant to the observation that individuals who are family history positive for alcoholism are innately less sensitive to alcohol that family-history-negative individuals and are at

increased risk for alcoholism (Schuckit, 1994; Schuckit et al, 2005; Schuckit and Smith, 2006). Our data show that deletion of FAAH elevates voluntary alcohol consumption, consistent with human studies showing that a polymorphism in the FAAH gene (Sipe et al, 2002) reduces FAAH protein levels and activity (Chiang et al, 2004) and is linked with increased vulnerability to drug use and alcoholism. Any comparison of mouse models to human alcoholism is inherently speculative, and it is important to recognize that the levels of alcohol consumption achieved in our study do not produce intoxication. However, it also important to note that the tests (discussed above) that show genetic correlations with our continuous voluntary access model are tests using relatively high doses of ethanol (eg LORR and withdrawal severity). Thus, there may be common mechanisms that regulate both voluntary intake of low amounts of alcohol and responses to much larger doses.

One of the problems in interpretation of results obtained with knockout mice is whether compensatory changes in expression of other genes occur as a result of deletion of particular gene (Ponomarev et al, 2006). Although this issue has not yet to be directly explored with FAAH knockout mice, it should be noted that all three behavioral differences between wild-type and null-mutant mice have been
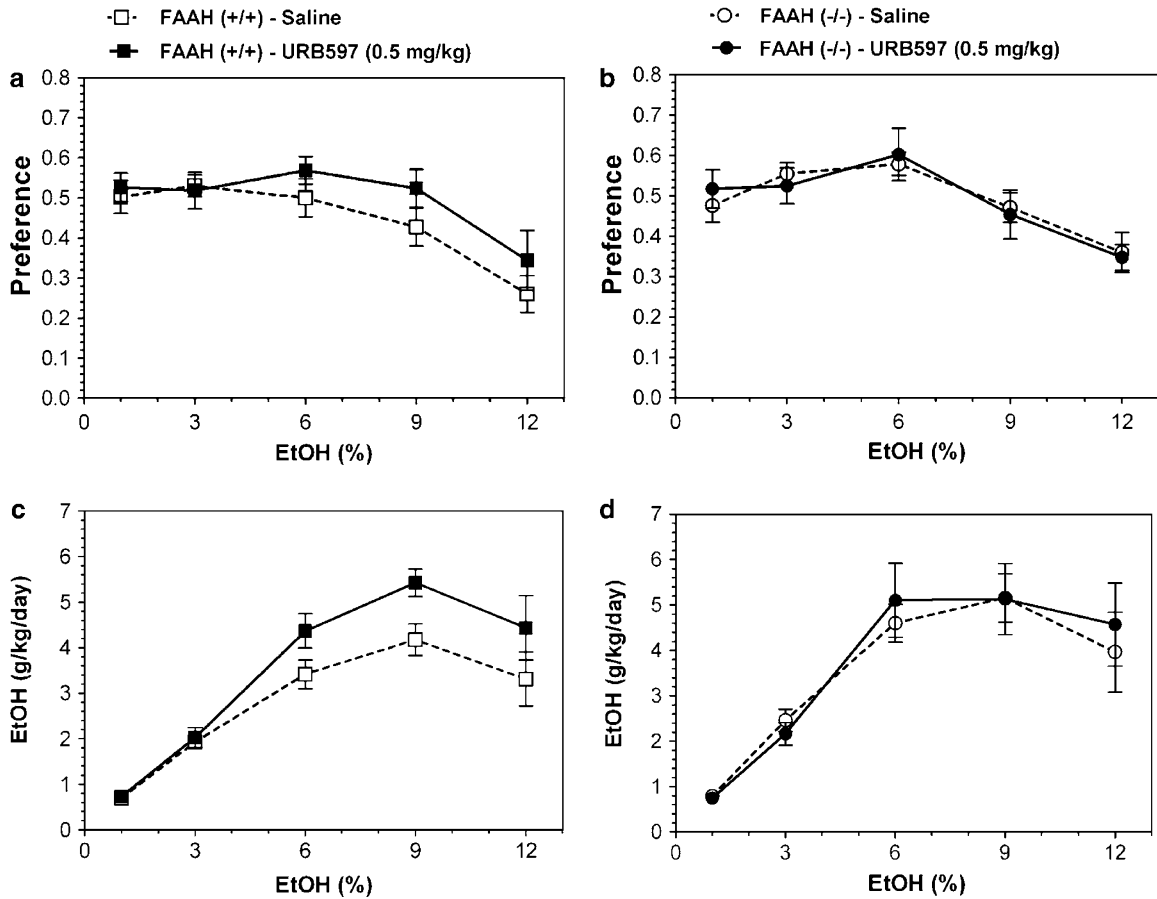
Figure 8 Inhibitor of FAAH—URB597 (0.5 mg/kg) increased voluntary ethanol intake in wild-type but not in FAAH knockout (−/−) female mice. (a) Preference in wild type mice. (b) Preference in FAAH knockout (−/−) mice. (c) Consumption of ethanol (g/kg/day) in wild-type mice. (d) Consumption of ethanol (g/kg/day) in FAAH knockout (−/−) mice. $n = 8$ for each group of knockout mice and wild-type mice.
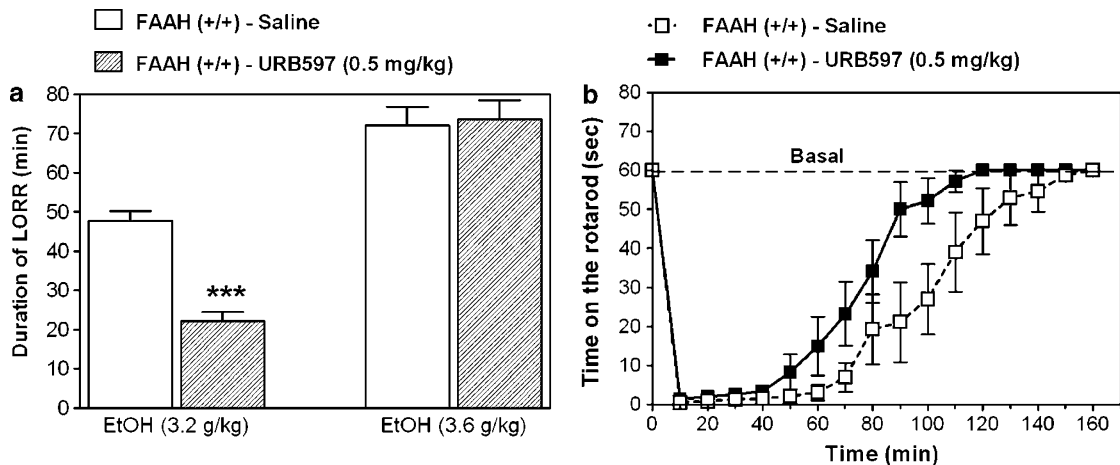

Figure 9 Inhibitor of FAAH—URB597 (0.5 mg/kg) reduced depressant and motor incoordination effects of ethanol in wild-type male mice. (a) Duration of LORR. (b) Time on the rotarod. Recovery from ethanol (2 g/kg) motor incoordination. $n = 7$–8 for saline- and ethanol-treated groups. ***$p < 0.001$ —significant differences saline- and URB597-treated groups for similar dose of ethanol (Bonferroni post-test).

reproduced in wild-type mice after administration of the selective FAAH inhibitor URB597. Furthermore, this drug had no effect in FAAH (−/−) knockout mice, indicating its actions are probably due to inhibition of FAAH activity. Although we cannot completely rule out effects on other systems, these findings argue against a role for compensa-

tory changes and suggest a direct role for FAAH in alcohol actions.

In summary, these results show that impaired FAAH function leads to increased alcohol intake and point to FAAH as a potential genetic and therapeutic target for excessive alcohol consumption.

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