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Atp1a2 Contributes Modestly to Alcohol-Related Behaviors

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Abstract

Atp1a2 has been previously studied for anxiety, learning and motor function disorders, and fear. Since *Atp1a2* has been shown to be involved in anxiety and this behavior is a known risk factor for developing alcoholism, we have been investigating *Atp1a2* for its potential role in responses to alcohol. This study utilized *Atp1a2* knockout mice; *Atp1a2* heterozygous mice, with half the amount of protein compared to wild-type mice, were used because *Atp1a2* homozygous null mice die shortly after birth. The alcohol-related behavioral experiments performed were loss of righting reflex (LORR), acute alcohol withdrawal measured by handling-induced convulsions (HIC), drinking in the dark (DID), open-field activity (OFA), and elevated plus-maze (EPM). LORR was a 2-day test that measures acute alcohol sensitivity, and rapid and acute functional tolerance (AFT). HIC was a 3-day test to measure alcohol withdrawal, DID was a 4-day test which measures voluntary alcohol consumption, and OFA and EPM measured anxiety with alcohol exposure. The effect of genotype on alcohol metabolism was also examined. There was a genotype effect on rate of alcohol metabolism, but only in males. There was no effect on alcohol withdrawal severity. The *Atp1a2* heterozygous mice consumed more alcohol than wild-type mice in the DID test, although only in males. In addition, only males were observed to show rapid tolerance in the LORR test while only female heterozygous mice showed a pretreatment effect on AFT. Alcohol exposure had a greater anxiolytic effect in the heterozygous mice compared to wild-type mice, although, again, there were sex effects with only males showing the effect in OFA and only females in the EPM. Although the behavioral results were mixed, there does appear to be a connection between anxiety and alcohol. Overall, the results suggest that *Atp1a2* does contribute to alcohol-related behaviors, although the effect is modest with a clear dependence on sex.

Keywords

ATP1A2; anxiety; alcohol; behavior; heterozygous mouse

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Introduction

Anxiety disorders are the most prevalent psychiatric disorders in the United States, with estimates as high as 28% of the population diagnosed each year (Michael et al., 2007). Anxiety is described as a normal response to stress; however, anxiety can become excessive, producing a recurring negative emotional state with feelings of worry and apprehension, along with cognitive and behavioral changes (Nuss, 2015). Anxiety and alcoholism are often co-morbid, but it has been unclear which condition leads to the other (Buckner & Turner, 2009). There are two hypotheses for this co-morbidity: 1) the hypothesis that increased anxiety results in increased alcohol consumption suggests that the pharmacological effects of alcohol decrease anxiety symptoms, which leads to negative reinforcement and increased risk for developing alcoholism (Quitkin, Rifkin, Kaplan, & Klein, 1972), and 2) the hypothesis that anxiety symptoms are a consequence of the withdrawal syndrome associated with chronic alcohol abuse (George, Nutt, Dwyer, & Linnoila, 1990). Previous research has shown anxiety to consistently correlate with alcohol problems, and generally anxiety appears to precede alcohol use (Buckner & Turner, 2009), but there could be other factors that independently contribute to both conditions, such as genetic predisposition for both, environmental factors, or exposure to prenatal environmental factors (Kushner, Abrams, & Borchardt, 2000).

ATP1A2 is a subunit of the P-type family of Na^+/K^+ -ATPases, which are integral plasma membrane proteins responsible for maintaining the sodium and potassium gradients across cellular membranes with utilization of ATP. This electrochemical gradient fuels central cellular processes, such as the secondary transport of metabolites, provides the basis for electrical excitation in neurons, and drives nutrient and neurotransmitter uptake (Gritz & Radcliffe, 2013). Na^+/K^+ -ATPases are essential in clearing extracellular potassium during neuronal activity and are necessary in the clearance of released glutamate in the synaptic cleft because re-uptake in astrocytes and neurons is driven by the sodium and potassium gradients (Ikeda et al., 2003). ATP1A2 is co-localized with other ion transporters, such as the sodium/calcium ($\text{Na}^+/\text{Ca}^{2+}$) exchanger and the glutamate transporter, which in the central nervous system (CNS) are important in clearance of glutamate and potassium from the extracellular space (Cholet, Pellerin, Magistretti, & Hamel, 2002; James et al., 1999; Rose et al., 2009). In humans, mutations in the *ATP1A2* gene result in familial hemiplegic migraines type II (FHM2), which are a rare autosomal dominant form of migraine with aura (Pietrobon, 2007). FHM attacks are generally longer than the common migraine with aura; however, they share similar symptoms including visual, sensory, and motor symptoms, and aphasia (Pietrobon, 2007).

Previous research using *Atp1a2* null mutations in the mouse have increased our understanding of its effect on neural activity and whole animal behavior. The homozygous null *Atp1a2* mice are neonatal lethal due to lack of synchronized neuronal firing in the breathing center of the brain (Ikeda et al., 2004; Moseley et al., 2003; Onimaru & Homma, 2007), and the *Atp1a2* heterozygous mice have approximately half the protein compared to wild-type mice (James et al., 1999). A consistent behavioral finding is that the *Atp1a2* heterozygous mice were more anxious than the wild-type mice (Ikeda et al., 2003; Lingrel, Williams, Vorhees, & Moseley, 2007; Moseley et al., 2007). *Atp1a2* heterozygous mice were

hypoactive compared to wild-type mice, based on measurements of total distance traveled and time spent in the corners of the open-field testing box (Moseley et al., 2007), less time spent in and fewer entries into the open arm of the plus maze (Lingrel et al., 2007; Moseley et al., 2007; Segall et al., 2004), and less time spent in the light compartment with fewer transitions between the two compartments in the light/dark box (Ikeda et al., 2003). These behavioral alterations may be the result of impaired reuptake of glutamate and GABA observed in the *Atp1a2* heterozygous mice (Ikeda et al., 2003). The *Atp1a2* heterozygous mice also showed hyperphagia during the light period and suffered from late-onset obesity (Kawakami, Onaka, Iwase, Homma, & Ikeda, 2005). In addition, numerous studies using forward genetic approaches have implicated the broad region around the *Atp1a2* locus in a variety of behavioral and physiological responses, though definitive evidence for the involvement of *Atp1a2* in any of these varied phenotypes is lacking (see Mozhui et al., 2008; Radcliffe et al., 2004).

Since *Atp1a2* heterozygotes show increased anxiety, a known risk factor for developing alcohol-use disorders (AUDs) (Olivier, Vinkers, & Olivier, 2013), we have tested the hypothesis that these mice would show increased drinking behavior compared to their wild-type counterparts. We also sought to determine whether the reduction in ATP1A2 protein seen in the heterozygote mice would affect other alcohol-related behaviors that are not necessarily linked to anxiety, but are thought to be risk factors for the development of AUDs. Specifically, we tested several acute alcohol responses, including sensitivity to the anxiolytic effects of alcohol, sensitivity to withdrawal from alcohol, acute hypnotic sensitivity, and alcohol tolerance. The results were mixed, but indicated modest effects of *Atp1a2*, dependent on sex and the specific behavior being tested.

Materials and methods

Animals

Wild-type and *Atp1a2*^{-/+} breeder mice were obtained from Dr. Jerry Lingrel at the University of Cincinnati (James et al., 1999) and bred in-house in the University of Colorado Anschutz Medical Campus (UCAMC) vivarium, a pathogen-free facility. The *Atp1a2*^{-/+} mice were originally created in the 129/SvJ strain, bred to C57/Black Swiss, and subsequently maintained on a mixed background (James et al., 1999; Moseley et al., 2007). Male and female *Atp1a2* wild-type and heterozygous mice were bred together, which produced only wild-type and heterozygote mice in the same litter. This breeding scheme avoided homozygous null offspring and controlled for any potential maternal effects. Offspring were weaned and sex-separated at 21 days of age. All experiments were conducted with males and females that were group-housed in standard housing containing from 2 to 5 mice per cage (except during DID; see below); the mice were 61 to 102 days old at the time of testing. Separate cohorts of mice were used for each behavioral test, and they were completely naïve to alcohol at the time of testing. The mice were maintained in a constant temperature (22–23 °C), humidity (20–24%), and light (14 h light/10 h dark) environment. The procedures described in this report have been established to ensure the absolute highest level of humane care and use of the animals, and have been reviewed and

approved by the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee.

Alcohol metabolism

Male and female *Atp1a2* heterozygous and wild-type mice were injected intraperitoneally (i.p.) with alcohol (5 g/kg, 16% w/v in normal saline), and blood samples were drawn at 0.5, 1, 1.5, and 2 h after administration. Blood alcohol concentration (BEC) values were determined by spectrophotometry with the use of a reliable enzyme assay (Lundquist, 1959).

Loss of righting reflex

The loss of righting reflex assay (LORR) was conducted as a 2-day test to determine rapid tolerance, acute functional tolerance (AFT), and alcohol sensitivity (Radcliffe, Floyd, & Lee, 2006). On day 1, mice were injected (i.p.) with either 0.9% normal saline (0.01 mL/g) or alcohol (5 g/kg, 16% w/v in normal saline), a pretreatment dose which has been shown to elicit rapid tolerance in the LORR assay without being overtly toxic (Radcliffe et al., 2006). To avoid the potential effects of associative learning that might contribute to tolerance, no behavioral testing was done on day 1; mice were injected and placed back in the home cage with littermates. Twenty-four hours following this single administration, all groups were tested for duration of LORR following a single acute dose of alcohol (i.p.; 3 g/kg, 16% w/v in normal saline). LORR was tested using the modified procedure of Ponomarev & Crabbe, 2002, which allows for a more accurate estimate of AFT. Immediately after injection, the mouse was placed into a small Plexiglas® cylinder fitted with square end caps. The cylinder was rotated 90° every 2–3 sec until the time at which the mouse became and remained supine for at least 5 sec; this was defined as the loss of righting reflex. A 20-μL retro-orbital blood sample was drawn at this point for determination of BEC at loss of righting (initial sensitivity; BEC1). The animals were tested for recovery of LORR every 3–6 min thereafter. A second blood sample (BEC2) was drawn when the animal was able to right itself within a 5-sec period after being placed in a supine position or could not be placed on its back after 8 successive 90° turns of the cylinder. Duration of LORR (“sleep time”) was defined as the time between the loss and regaining of the righting reflex. An increase in BEC2 from BEC1 was interpreted as development of AFT, which was quantitatively expressed as the difference between BEC2 and BEC1. Rapid tolerance was defined as a decrease in the duration of LORR in the alcohol pretreatment group compared to the saline pretreatment group. In contrast to AFT, which is tolerance that develops within an acute alcohol administration session, rapid tolerance is observed 1 day after a single administration of alcohol in naïve mice and is thought to be the initial stage of chronic tolerance development (for further discussion, see Radcliffe et al., 2006).

Handling-induced convulsions

Alcohol withdrawal was measured by handling-induced convulsions (HIC) scores following an acute dose of alcohol (Metten & Crabbe, 1994). Mice were picked up by the tail, observed for 10 sec, and convulsions were scored. If no convulsion occurred, the mice were rotated in a 180° arc for 5 sec and observed for HICs. HICs were measured on a scale of 0–7, with 0 being no seizure activity at all and 7 being full spontaneous tonic-clonic seizures observed in the home cage. Baseline HIC scores were determined, and mice were given a 4-

g/kg intraperitoneal injection of alcohol (16% w/v in saline) once a day for 3 consecutive days. The HICs were scored hourly for 12 consecutive hours and at 24 h starting immediately after the first injection and after each injection thereafter. Each test was digitally recorded to confirm the original observations.

Drinking in the dark

“Drinking in the dark” (DID), which is considered to be a model of binge drinking, was used to test alcohol consumption (Rhodes, Best, Belknap, Finn, & Crabbe, 2005). Mice were switched to a reverse light/dark schedule 2 weeks prior to testing (lights-off at 12:00 PM and lights-on at 10:00 PM). One week before testing began, the mice were individually housed with ball-bearing sipper water bottles. Mice were weighed 1 h before lights-out on testing days. Three hours after lights out, the water bottles were replaced with a 25-mL glass tube with a ball-bearing sipper containing 20% alcohol (v/v in tap water). Fluid amounts were measured at time zero and again at 2 h. The alcohol was removed at the 2-h time point and replaced with water. The procedure was repeated for 3 days. On day 4, the alcohol bottles were available for 4 h and the fluid levels were measured at time zero, 2 h, and 4 h. After the last reading, mice were sacrificed by CO₂ anesthesia followed by decapitation, and two 20-μL blood samples from trunk blood were collected to measure BEC. Intake was expressed as g/kg alcohol per 2-h or 4-h time period.

Open-field activity

Locomotor activity and position preference were measured in the open field as a measure of anxiety (modified from Crabbe, Johnson, Gray, Kosobud, & Young, 1982). Mice were injected (i.p.) with either 0.9% normal saline (0.01 mL/g) or alcohol (1 or 2 g/kg, 16% w/v in normal saline). Immediately after injection, the mouse was placed into the center of a large white Plexiglas® open box (44 cm × 44 cm × 20 cm) with the room lights on. Locomotor activity and position preference were measured automatically over a 20-min time period using digital recording equipment (EthoVision XT; Noldus Information Technology, Leesburg, VA).

Elevated plus-maze

An elevated plus-maze was used to measure anxiety using the modified procedure of Stinchcomb, Bowers, & Wehner, 1989. The elevated plus-maze consists of two open arms (30 cm × 5 cm), a center platform (5 cm × 5 cm), and two closed arms with non-transparent walls (30 cm × 5 cm × 14 cm) elevated 37 cm off the ground. Mice were injected (i.p.) with either 0.9% normal saline (0.01 mL/g) or alcohol (0.5 or 1 g/kg, 16% w/v in normal saline). Fifteen minutes after injection, the mouse was placed into the central platform of the maze with its head facing a closed arm. The frequency of entry into the open and closed arms as well as the amount of time spent in the open and closed arms was measured by digital recording over a 15-min time period (EthoVision XT; Noldus Information Technology, Leesburg, VA).

Statistical methods

Data were analyzed using SPSS Statistics 23 (IBM). All data are expressed as the mean $\pm$ SEM. Statistical significance was determined by three-way ANOVA, two-way ANOVA, logistical regressions, and Student's *t* tests for two-group comparisons. Behavioral tests with three sources of variation (LORR with sex, genotype, and pre-treatment; OFA and EPM with sex, genotype, and dose) were analyzed using three-way ANOVA, and tests with two sources of variation (DID and metabolism with sex and genotype) were first analyzed with two-way ANOVA. If there were no differences between sexes, the groups were collapsed and analyzed by Student's *t* tests. HIC scores and alcohol clearance in the LORR study were analyzed using logistical regression. Significance for all tests was set at a *p* value below 0.05.

Results

Alcohol metabolism

Alcohol metabolism was analyzed by three-way repeated-measures ANOVA (time $\times$ sex $\times$ genotype; Fig. 1). As expected, there was a significant main effect of time [$F(3,21) = 36.3$, $p < 0.001$]. There also was a significant main effect of sex [$F(1,7) = 15.1$, $p < 0.01$] and a significant genotype $\times$ sex interaction [$F(1,7) = 8.3$, $p < 0.05$]. Because of the interaction, a two-way repeated-measures ANOVA was conducted to determine whether there was a genotype effect within each sex. For both males and females, there was not a significant main effect of genotype nor of time $\times$ genotype interaction. There was a significant genotype $\times$ sex interaction [$F(1,7) = 5.8$, $p < 0.05$] for rate of alcohol metabolism as measured by the slope of time vs. BEC, but no main effect of genotype ($p > 0.1$). Simple-effects analysis, however, indicated that the rate of metabolism was faster in male heterozygous mice compared to wild-type mice [$t(4) = 3.2$, $p < 0.05$]. Note that the sample size for each group was small ($n = 3$), and therefore the results should be interpreted with caution.

Loss of righting reflex

Acute sensitivity and AFT in male and female wild-type mice and *Atp1a2* heterozygous mice were examined using the LORR assay (Fig. 2). Three-way ANOVA indicated a significant effect of sex [$F(1,158) = 21.8$, $p < 0.001$] and a significant sex $\times$ genotype $\times$ pretreatment interaction [$F(1,158) = 8.4$, $p < 0.01$] (Fig. 2a). Simple-effects analysis showed a genotype effect in alcohol-pretreated males [$t(39) = 2.7$, $p < 0.05$] and also a pretreatment effect (rapid tolerance) in wild-type males [$t(40) = 2.6$, $p < 0.05$], but no significant effects were seen in females.

The blood ethanol concentration at the regaining of the righting reflex (BEC2) is shown in Fig. 2b. Close inspection of Figs. 2a and 2b reveal a generally inverse relationship between BEC2 and sleeptime, which is what would be expected barring any major effects of genotype or pretreatment on alcohol metabolism. As with sleeptime, there was a significant sex $\times$ genotype $\times$ pretreatment interaction [$F(1,158) = 5.2$, $p < 0.05$]. In addition, simple-effects analysis indicated a significant difference between saline and alcohol pretreatment for the wild-type males [$t(40) = 3.0$, $p < 0.01$] and also between wild-type and heterozygous males in the alcohol pretreatment group [$t(39) = 2.4$, $p < 0.05$]. Both of these effects appear

to be driven by the development of rapid tolerance in the wild-type males (increased BEC2 in the alcohol pretreatment group), but not in the heterozygous males. Unlike sleeptime, BEC2 showed a significant effect of pretreatment [$F(1,158) = 9.4, p < 0.01$] with no significant effect of sex.

AFT showed no significant main effects of sex or genotype, but there was a significant sex $\times$ genotype $\times$ pretreatment interaction [$F(1,158) = 4.5, p < 0.05$] (Fig. 2c). Although AFT was increased in wild-type males that received an alcohol pretreatment, which is consistent with their decreased sleeptime, the effect was not significant. Instead, only females showed significant simple effects – there was a pretreatment effect in the heterozygous females [$t(41) = 2.9, p < 0.01$] and a genotype effect among females in the alcohol pretreatment group [$t(39) = 2.5, p < 0.05$].

Alcohol withdrawal

Handling-induced convulsions were studied to determine whether *Atp1a2* expression affected alcohol withdrawal. The seizure scores for all of the mice tested were very low; i.e., nearly half the mice scored 0's throughout the entire 3-day test and no single mouse ever scored above a 1, which is a facial grimace when spun. The peak mean HIC scores across all groups ranged between 0.01 and 0.25, and there were no significant main effects or interactions in withdrawal severity across any of the three days. Since there was no difference between sexes, they were combined and reanalyzed. Again, there was no difference in withdrawal due to genotype (data not shown).

Drinking in the dark

Atp1a2 heterozygous mice have previously been shown to have increased anxiety (Ikeda et al., 2003; Moseley et al., 2007), and because anxiety is a risk factor for alcoholism this led to the hypothesis that the heterozygous mice would voluntarily consume more alcohol. Overall, females of both genotypes consumed more alcohol on day 4 than the males, which resulted in a significant sex effect [$F(1,86) = 28.3, p < 0.001$], but no significant main effect of genotype (Fig. 3a). During the first 2 h on day 4, *Atp1a2* heterozygous male mice drank more than their wild-type controls [$t(45) = 2.2, p < 0.05$]. Alcohol consumption on day 4 was significantly correlated between the first and last 2 h [$r^2 = 0.3, p < 0.01$] (Fig. 3b), and BEC was significantly correlated to total alcohol consumed on day 4 [$r^2 = 0.2, p < 0.001$]. There was a significant interaction between genotype and sex for BEC [$F(1,86) = 4.2, p < 0.05$]; however, there were no sex or genotype effects.

Open-field activity

The total distance traveled, position preference, and rearings were measured to determine the anxiolytic effects of alcohol. There was no main effect of genotype on total distance, but there was a dose effect [$F(2,109) = 23.9, p < 0.001$], which was expected, and there was an interaction of sex $\times$ genotype $\times$ dose [$F(2,109) = 4.5, p < 0.05$] (Fig. 4). The 1-g/kg dose caused an increase in the distance traveled in the male *Atp1a2* heterozygous mice compared to wild-type mice [$t(18) = 1.7, p < 0.05$]. Significant dose effects were observed for time spent in the border (dose-dependent increase) [$F(2,109) = 13.6, p < 0.001$], time spent in the center (dose-dependent decrease) [$F(2,109) = 13.3, p < 0.001$], and the amount of time spent

in the corners (dose-dependent increase) [$F(2,109) = 7.1, p < 0.001$], as well as the number of rearings (dose-dependent decrease) [$F(2,109) = 277.0, p \ll 0.001$], but there were no other significant main effects or interactions for these measures. Time spent in the corners and on the borders tended to increase, while time in the center tended to concomitantly decrease in a dose-dependent fashion without regard to sex or genotype (data not shown). The number of rearings decreased dramatically as a function of dose, from an average of approximately 115 with saline to fewer than 10 at the highest dose, again without regard to sex or genotype.

Elevated plus-maze

Time spent in the open and closed arms, as well as the number of entries into the open and closed arms, was measured in the elevated plus-maze. The number of total entries over 15 min is shown in Fig. 5a. There were no significant main effects or interactions. There also were no significant effects on the percent of entries into the open arms (Fig. 5b). There was, however, a significant genotype $\times$ dose interaction on percent time in the open arms during the 15 min of the test [$F(2,97) = 3.7, p < 0.05$] (Fig. 5c). Simple-effects analysis indicated a significant difference between wild-type and heterozygote mice only in females and only at the 1.0-g/kg dose [$t(17) = 2.4, p < 0.05$]. We also examined the first 5 min of the test before the animals started to habituate to the apparatus (Figs. 5d–5f). Similar to the entire 15-min test, there were no significant main effects or interactions on total arm entries (Fig. 5d). For percent of entries into the open arms during the first 5 min, there were no significant differences between sex or genotype, but there was a significant overall dose effect [$F(2,97) = 4.1, p < 0.05$] (Fig. 5e). In contrast, there were significant main effects of genotype [$F(1,97) = 4.9, p < 0.05$], dose [$F(1,97) = 4.0, p < 0.05$], and a significant genotype $\times$ dose interaction [$F(2,97) = 3.4, p < 0.05$] for percent time in the open arms during the first 5 min (Fig. 5f). Alcohol appeared to increase percent time in the open arms in the heterozygous males and females compared to the wild-type mice at the 0.5 and 1.0 doses, but there was a significant difference only in the females at the 1.0-g/kg dose [$t(17) = 2.5, p < 0.05$]. It is worth noting that the percent time spent in the open arms was decreased in the heterozygote mice compared to wild-type mice for both males and females at the 0-g/kg dose, but this effect did not reach statistical significance.

Discussion

A genetic link to AUDs has been well established, yet, with the exception of the major alcohol metabolizing enzymes, the underlying molecular basis of this link remains mostly a mystery (Edenberg & Foroud, 2013). This is for a variety of reasons, including small effect size of individual genes and clinical heterogeneity. Model organism studies have been useful in identifying candidate genes that potentially influence the human condition, often with the use of transgenic technologies (Crabbe, Phillips, Harris, Arends, & Koob, 2006). The goal of this study was to determine whether *Atp1a2* influenced the behavioral effects of alcohol by examining a battery of relatively simple behavioral tests using the *Atp1a2* heterozygous mouse. This was not a typical null mutant study as the homozygous nulls die shortly after birth; it was more like an *Atp1a2* dosage study because the heterozygote mice have half the amount of enzyme which has been shown to be the cause of fairly robust phenotypic effects,

most notable increased anxiety (Ikeda et al., 2003; Lingrel et al., 2007; Moseley et al., 2007). Overall, the results indicate a modest role for *Atp1a2* in alcohol-related behaviors, although we were unable to replicate the increased anxiety phenotype that has been observed by other investigators. Moreover, the metabolism study did detect significant interactions involving genotype, and therefore it is not possible to rule out metabolic effects on the few behaviors for which genotype effects were observed.

Perhaps the most widely used test of acute alcohol sensitivity is the loss of righting reflex or “sleeptime”. We observed a significant main effect of sex on sleeptime, which has been widely reported in the literature (DeFries, Wilson, Erwin, & Petersen, 1989). In addition, the *Atp1a2* mice were more sensitive than the wild-type mice, as indicated by a longer sleeptime, but only in males that had been pretreated with alcohol, which has become part of our standard sleeptime test to examine for possible effects on rapid (1 day) tolerance. Indeed, the pretreatment effect was due to the wild-type males developing a significant rapid tolerance, but not the heterozygotes. It is not possible to know to what extent these sleeptime results were due to metabolic effects since there was a genotype effect on rate of clearance in males and a significant three-way interaction on metabolism. In addition, we do not know whether there was a metabolism effect 24 h after a pretreatment, similar to the way in which the LORR tests were conducted, since that was not specifically tested. It should be noted, however, that BEC2 and sleeptime showed a very similar, and as expected, inverse relationship, especially in males, which argues against a major effect of metabolism. For AFT, the non-significant increase in alcohol-pretreated wild-type males is consistent with their decrease in sleeptime. The results in alcohol-pretreated heterozygous females were also consistent, but opposite to the males – a non-significant decrease in sleeptime with a significant increase in AFT. It may be possible that these sex effects are dose-dependent; i.e., different pretreatment or test doses may have elicited similar patterns of responding from males and females. These issues could potentially be resolved by expanding both the pretreatment and test doses for the LORR experiment, but that was beyond the scope of the current study.

Atp1a2 mutations in humans have been shown to cause seizures (Deprez et al., 2008; Jurkat-Rott et al., 2003), and seizures are a side effect of alcohol withdrawal. In addition, we have observed a highly significant genetic correlation between audiogenic seizure susceptibility and *Atp1a2* mRNA abundance in the BXD recombinant inbred strains (unpublished). For these reasons, we expected to see an increase in alcohol withdrawal severity in the *Atp1a2* heterozygous mice. As expected, seizures were not detected in naïve mice (baseline score on day 1) of either genotype, and seizure scores after withdrawal were very low. However, there was no significant difference between sexes or genotype. This may have been because this was a brief, acute time period of alcohol treatment and potential neuronal adaptations involving *Atp1a2* may not have been fully or even partially manifested at this point. It would be interesting to determine if the *Atp1a2* mice show a difference following a more prolonged exposure to alcohol.

Anxiety is frequently co-morbid with AUDs, and it is believed alcohol is used to manage distress associated with the disease (Buckner & Turner, 2009). Previous findings that indicated that the *Atp1a2* mice exhibit increased anxiety thus led to the hypothesis that they

would drink more alcohol (Ikeda et al., 2003; Lingrel et al., 2007; Moseley et al., 2007; Segall et al., 2004). Male *Atp1a2* mice did in fact consume more alcohol, although it was only during the first 2 h on day 4. Females of both genotypes consumed more alcohol than the males; however, there was no effect of genotype in the females. As with alcohol withdrawal, a more prolonged and perhaps heroic exposure, such as the chronic intermittent ethanol exposure procedure (Becker, 2013), may be required to more fully express an effect on drinking. Overall, the mice drank small amounts of alcohol and showed similarly low BEC levels. We cannot say for certain that these modest values were due to genetic background, but we note that the 129S1/SvImJ strain, while not the exact substrain that was used to generate the *Atp1a2* KO, previously has been shown to consume the lowest amount of alcohol in the DID test among a large number of inbred strains at less than 2 g/kg/4 h, with only the DBA/2J showing slightly lower consumption (Rhodes et al., 2007). Average consumption for all groups during the 4-h day-4 test in the current study was nearly 4 g/kg. This value that is substantially higher than the 129S1/SvImJ may be due to the presence of C57BL/6 alleles.

Open-field activity and the elevated plus-maze were used to investigate potential interactions between alcohol and genotype on anxiety. Previous OFA behavioral testing with alcohol has shown that the 1-g/kg dose significantly increased exploratory activity and the 2-g/kg dose initially increased activity with a reduction of activity approximately 10 min after injection, due to the sedative effects of alcohol (Crabbe et al., 1982; Palmer, McKinnon, Bergstrom, & Phillips, 2002; Stinchcomb et al., 1989). We also observed a dose effect on total distance, but in our case, increasing dose generally caused a decrease in locomotor activity with the difference possibly due to a genetic background effect. There was very little effect of genotype on open-field activity, except for an increase in total distance traveled at just a single alcohol dose in the *Atp1a2* heterozygous males. This may indicate that the heterozygote mice were more sensitive to the anxiolytic effects of alcohol; indeed, a similar result was observed for males at the 1-g/kg dose in the elevated plus-maze, although it was not significant. Females, however, did show a significant effect of 1 g/kg alcohol in the elevated plus-maze, but no effect in the open field.

Clearly, the interaction between sex, genotype, and alcohol treatment on measures of anxiety is complex. Interestingly, we did not observe the significant genotype effect that has been previously published with open-field activity and elevated plus-maze (Ikeda et al., 2003; Moseley et al., 2007); i.e., in those studies, the *Atp1a2* heterozygous mice consistently displayed a high-anxiety phenotype. This lack of an anxiety phenotype could be due to several factors. First, all our mice were weighed and injected. This handling could have affected their behavior, whereas the mice used by Ikeda et al. (2003) were completely naïve. The mice used in the study by Moseley et al. (2007) were previously tested in the elevated zero-maze prior to testing in the open field, whereas independent cohorts were used in all of our studies. It is also possible that the genetic background had changed sufficiently to alter the anxiety phenotype, as has been well documented for other phenotypes in genetically modified mice (see Bailey, Rustay, & Crawley, 2006). Nonetheless, in both the open-field test and the elevated plus-maze, the *Atp1a2* heterozygous mice were slightly more anxious in the absence of alcohol compared to the wild-type mice in our studies, but this difference was not statistically significant. Interestingly, the main finding of the elevated plus-maze

study was that the *Atp1a2* heterozygous mice were more sensitive to the anxiolytic effects of low-dose alcohol, i.e., they showed *less* anxiety than the wild-type mice, but only with alcohol on board. This can be easily seen with the percent time spent in the open arms during the first 5 min of the test, which shows a significant main effect of genotype. This appears to be driven by the increased values in both male and female alcohol-treated *Atp1a2*^{-/+} mice.

Glutamate transporters are coupled to Na⁺/K⁺-ATPases (Rose et al., 2009), and the clearance of extracellular potassium and released glutamate is dependent on *Atp1a2* (Ikeda et al., 2003). It has been hypothesized that mutations in *Atp1a2* that are known to cause FHM2 produce an increase in extracellular glutamate along with a decrease in the removal of extracellular potassium (Koenderink et al., 2005; Montagna, 2004; Pietrobon, 2007). It is possible that the *Atp1a2* heterozygous mice also have reduced glutamate clearance which may be the driving factor for the increased anxiety seen in the heterozygous *Atp1a2* mice that has been observed by others and to a lesser extent in our studies (Ikeda et al., 2003). It thus may be that the modest increases in the alcohol-mediated anxiolytic effects that we observed in the heterozygous *Atp1a2* mice may be the result of an interaction between the reduced activity of *Atp1a2* (due to half the amount of the enzyme in the heterozygotes) and the known alcohol-mediated reduction in glutamatergic signaling (Valenzuela, 1997). This hypothesis, however, is purely speculative and needs to be confirmed.

In summary, the data show that the loss of one copy of *Atp1a2* has modest effects on alcohol-mediated behaviors. The sleeptime study hints that the *Atp1a2* heterozygotes may have altered neuroadaptive responses to alcohol, although only in males. Longer-term chronic alcohol treatments may be required for more pronounced effects on tolerance as well as on withdrawal. There was a consistent but small effect of an increased anxiolytic response to acute alcohol in the *Atp1a2* heterozygote mice, although it was dependent on sex and the specific behavioral test. This may in some way be related to complex effects on glutamate release and uptake, both of which influence extracellular glutamate. In any case, it is possible that this is why the *Atp1a2* mice drank more, although, again, it was sex-dependent – only male *Atp1a2* mice showed an increase in drinking behavior. Overall, there were complex interactions between genotype, sex, and alcohol treatment, suggesting that *Atp1a2* makes a modest contribution to alcohol-related behaviors, though it should be noted that at least some of the observed genotype effects might have been due to metabolic differences. Further investigation into these effects should probably focus on chronic alcohol treatment and potential neuroadaptive responses mediated by *Atp1a2*.

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Highlights

- The objective was to determine whether decreased *Atp1a2* contributes to variation in alcohol-related behaviors.
- Decreased *Atp1a2* did not affect alcohol metabolism or alcohol withdrawal.
- *Atp1a2* heterozygous mice were less anxious with alcohol exposure.
- A connection between anxiety and alcohol exists despite mixed behavioral results.

Fig. 1. Alcohol metabolism in the Atp1a2 mice

Shown are the least-squares linear regression for each genotype and sex from which the slope was derived. Significant main effects of time ($p < 0.001$), sex ($p < 0.01$), and sex $\times$ genotype interaction ($p < 0.05$) for BEC. Significant genotype $\times$ sex interaction ($p < 0.05$) for slope. $n = 3$ per sex and genotype.

Fig. 2. LORR and AFT in the *Atp1a2* mice

a) Duration of LORR (“sleep time”); b) BEC at the regaining of the righting reflex (BEC2); c) acute functional tolerance (AFT). $n = 20$ per sex and genotype; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; SE = Saline pre-treatment, EE = Ethanol pre-treatment (5 g/kg).

Fig. 3. Drinking in the Dark in the Atp1a2 mice

a) Alcohol consumption during hours 0–2 and hours 2–4 of the test on day 4; b) correlation between day 4 hours 0–2 and hours 2–4 in individual mice ($r^2 = 0.3$, $p < 0.01$); c) correlation between total consumption (hours 0–4) and BEC at the end of the 4-h drinking session on day 4 individual mice ($r^2 = 0.2$, $p < 0.001$). $n = 18$ – 29 per sex and genotype; * $p < 0.05$, ** $p < 0.001$.

Fig. 4. Open-field activity in the Atp1a2 mice

Total distance traveled measured over 20 min in the open-field arena. $n = 10$ per group, * $p < 0.05$, ** $p < 0.001$.

Fig. 5. Elevated plus-maze in the Atp1a2 mice

a) Total entries during the entire 15-min test; b) percent entries into the open arms during the entire 15-min test; c) percent time spent in the open arms during the entire 15-min test; d) total entries during the first 5 min of the test; e) percent entries into the open arms during the first 5 min of the test; f) percent time spent in the open arms during the first 5 min of the test. n = 6–10 per genotype and sex; * $p < 0.05$.