**Male mice carrying the BDNF Val68Met polymorphism exhibit alcohol preferences versus social interaction and acute tolerance through malfunction of BDNF in the ventral hippocampus**

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**ABSTRACT**

The brain-derived neurotrophic factor (BDNF) Valine 66 to Methionine human polymorphism results in impaired activity-dependent BDNF release and has been linked to psychiatric disorders including anxiety and alcohol dependence. We previously showed that knock-in mice carrying the mouse Methionine homolog (Met68BDNF) exhibit excessive and compulsive alcohol drinking behaviors as compared to the wild-type Val68BDNF mice. Here, we set out to determine the potential mechanism for the heightened and compulsive alcohol drinking phenotype exhibited by the Met68BDNFmice. We found that male, but not female Met68BDNF mice show social anxiety-like behaviors, and that male Met68BDNF mice show alcohol preferences vs. social interaction. In contrast, alcohol place preference *per se* is similar in Met68BDNF compared with Val68BDNF mice. Alcohol-dependent hyperlocomotion is also reduced in Met68BDNF. We further report that male, but not female, Met68BDNF mice exhibit acute tolerance which is restored in Met68BDNF mice by overexpressing the wild-type Val68BDNF in the ventral hippocampus (vHC). Together, our results suggest that compulsive alcohol drinking in the male Met68BDNF may be due to heighted social anxiety and a lack of alcohol-dependent anxiolysis which may be due to malfunction of BDNF signaling in the vHC.

## Introduction

Brain-derived neurotrophic factor (BDNF) is highly expressed in the CNS and plays an important role in brain development, synaptic plasticity, learning, and memory [1]. BDNF is released both pre and postsynaptically and its release depends on neuronal depolarization [1,2]. A single nucleotide polymorphism (SNP) within the human BDNF gene causes a substitution of valine at position 66 (Val66BDNF) with methionine (Met66BDNF), resulting in a reduction in the activity-dependent release of the neurotrophic factor and thus in the downregulation its downstream signaling [3-5]. The BDNFVal66Met SNP has been linked to multiple neuropsychiatric disorders, including depression and anxiety [6,7], and several reports have suggested that the BDNFVal66Met SNP is also implicated in alcohol use disorder (AUD) [8-13]. In order to evaluate the impact of this BDNF SNP on alcohol related behaviors, we utilized a knock-in strategy in mice to replace the mouse valine homolog at position 68 (Val68BDNF) with methionine (Met68BDNF) and showed that homozygous male Met68BDNF mice consume excessive levels of alcohol in a compulsive manner [14]. We further demonstrated that overexpression of Val68BDNF in the medial prefrontal cortex (mPFC) of Met68BDNF mice converts compulsive, excessive intake to moderate alcohol consumption [14].

AUD is frequently diagnosed in individuals with comorbid neuropsychiatric disorders [15-18]. For example, a global meta-analysis concluded that 27-40% of individuals with major depressive disorder experience an AUD during their lifetime, and 20-40% of people with anxiety disorders also develop an AUD [19]. One potential explanation for this phenomenon is the so-called “self-medication hypothesis”, which posits that individuals consume increasing quantities of alcohol in order to elicit relief for symptoms of unrelated mood disorders [20-22] including social anxiety [23]. Together, these reports show that social anxiety is a risk factor for heavy alcohol use. Heterozygous (Val/Met) and homozygous (Met/Met) genotypes have been linked with social anxiety in humans [13,24]. In analyzing the Pediatric Imaging, Neurocognition, and Genetics (PING) study [25], Li and colleagues recently uncovered a significantly higher self-reported social anxiety score in Met allele carriers than in individuals with homozygote Val

allele [24]. They further showed that social deficits can be recapitulated in a mouse model of the human allele [24]. These data could suggest that Met carriers may consume higher levels of alcohol as a means of reducing anxiety. Here, we examined potential underlying reasons for the heightened alcohol consumption in Met68BDNF mice, including social anxiety and anxiolysis.

## Materials and Methods

### Animals and breeding

Homozygous Val68BDNF (Val/Val) and Met68BDNF (Met/Met) generation (C57Bl6/J background) and characterization is described in Warnault et al. [14]. Female and male Val68BDNF and Met68BDNF mice were separately bred and maintained on site and were tested at the age of 3-5 months. Genotyping was performed as described previously [14]. Juvenile female and male mice (4-6 weeks) were purchased from Jackson Laboratories (Bar Harbor, Main). Mice were housed using a 12-hour light/dark cycle (lights on 7AM–7PM), with ad libitum access to food and water. All procedures were performed in accordance with guidelines from the University of California, San Francisco Institutional Care and Use Committee.

### Materials

AAV1/2-CMV-Val68BDNF-GFP- (AAV-Val68BDNF; ~1 x $10^{12}$ TU/ml) and AAV1/2-CMV-GFP (AAV-GFP; (~1 x $10^{12}$ TU/ml) were produced by the UNC Vector Core (Chapel Hill, NC) and described by Warnault et al. [14]. Ethyl alcohol (190 proof) was purchased from Thermosphere Scientific (Waltham, MA).

### Solution preparation

Alcohol solution for intra peritoneum (i.p.) injection was prepared from absolute anhydrous alcohol (190 proof) diluted to 20% alcohol (v/v) in 0.9% saline solution.

### Behavioral Assays

All behavioral analyses, with the exception of the Loss of Righting Reflex test, were recorded and analyzed using Noldus Ethovision XT software (Wageningen, the Netherlands). Behavioral testing was performed in a dimly lit room (10-15 lux) during the light-cycle, and mice were allowed to habituate to the conditions in the room for at least one hour before testing commenced. All apparatuses were cleaned

first with 70% alcohol and then water between each animal. Test mice were habituated to experimenter handling and i.p. injection of saline for at least three days prior to the start of experiments.

*Three-Chamber Sociability and Social Novelty*

Three-chamber sociability and social novelty tests were adapted from previous studies [26,27]. The three-chamber apparatus (40 x 60 x 25 cm) was divided into three equal zones (40 x 20 x 25 cm) by acrylic walls, connected by small doors (4 x 4 cm). For the sociability test, a novel, wild-type, juvenile (4-8 weeks old), sex-matched mouse (stranger I), was placed in a round wire cage (10 cm diameter) in one of the distal chambers, while an identical, empty cage was placed in the other distal chamber. The experimental mouse was then allowed to freely explore the entire apparatus for 15 minutes, after which point, the mouse was moved into the central chamber and the doors were closed. Cages were then swapped to opposite distal chambers, and a second, more-novel, wild-type, juvenile (4-8 weeks old), sex-matched mouse (stranger II) was placed inside the previously empty cage, in preparation for the social novelty test. Doors were opened, and the experimental mouse was once again allowed to freely explore the entire apparatus. During both tests, the amount of time spent in each chamber was recorded and compared. Preference for a social partner over an empty cage in the sociability test was indicative of normal sociability. Preference for the stranger I mouse over the stranger II mouse in the social novelty task was interpreted as abnormal social behavior.

*Open Field Social Interaction*

On the day before the test, mice were habituated for five-minute habituation session in the open field apparatus (43 x 43 cm). Mice were then placed in the open field with a novel, wild-type, juvenile (4-8 weeks old), sex-matched interaction partner, and their interactions were recorded for five minutes. Cumulative body contact was calculated as time in which the center point of each animal was within 2 cm of the other.

*Social-Alcohol Conditioned Place Preference/Aversion (CPP/CPA)*

The social-alcohol CPP/CPA apparatus consisted of two large compartments (20 x 18 x 30 cm) connected by a corridor (20 x 7 x 30 cm). One compartment has lighter colored walls and mesh flooring, while the other contains darker colored walls and grid rod flooring. On the first day of the experiment, mice were subjected to a pre-test, during which they were allowed to freely explore the entire apparatus for 15 minutes, and the time spent in each chamber was recorded. For the next three days, mice were conditioned to associate each of the two chambers, pseudorandomly assigned, with either social interaction paired with an i.p. injection of saline or an i.p. injection of alcohol (2 g/kg). Specifically, during the morning of each conditioning day, each mouse receives an i.p. injection of saline before being placed in their assigned social interaction-paired chamber with a caged, sex-matched, juvenile mouse (6-8 weeks old) for 10 minutes. In the afternoon of each conditioning day, each mouse received an i.p. injection of 20% alcohol (2 g/kg) before being placed in their assigned alcohol-paired chamber for 10 minutes. A post-test was performed on the fifth day of the experiment, in which mice were once again allowed to freely explore the entire apparatus for 15 minutes. The time spent in each chamber during the pre-test and post-test were monitored and quantified.

*Alcohol Conditioned Place Preference*

Alcohol CPP was adapted from Laguesse et al. [28]. Specifically, the apparatus consists of two chambers (17 x 13 x 25 cm) connected by a central door. One chamber consists of lighter colored walls and mesh flooring, while the other consists of darker colored walls and grid rod flooring. The first day of the experiment was considered a pre-test, during which test mice freely explored the entire apparatus for 15 minutes and time spent in each chamber was recorded. The following six experimental days were split into alternating days of saline or alcohol conditioning. On saline conditioning days (2, 4, 6), mice receive an i.p. injection of saline immediately before placement in the saline-paired chamber for five minutes. On alcohol conditioning days (3, 5, 7), mice receive an i.p. injection of alcohol (2 g/kg) before being placed in the alcohol-paired chamber for 5 minutes. Day eight of the experiment consisted of a post-test, during

which test mice freely explore the apparatus for 15 minutes. The time spent in each chamber during the pre-test and post-test were monitored and quantified.

*Open Field Test*

Animal movement and inactivity were recorded for 10 minutes in an opaque, square open field apparatus (43 x 43 cm) 5 minutes after i.p. injection of saline or alcohol (1.25 g/kg). Total distance moved during the test and the time animals spent motionless were monitored and quantified.

*Loss of Righting Reflex*

Loss of Righting Reflex (LORR) was conducted as described previously [29]. Mice received an i.p. injection of a hypnotic dose of alcohol (4.0 g/kg) before being placed in a clean plexiglass cage. The amount of time it took for each animal to lose its' righting reflex (latency) was recorded. At this point, mice were returned to their home cage and placed on their backs. The amount of time it took for each mouse to regain its' righting reflex, e.g., the time when mice could right themselves from their backs three times in one minute (duration), was measured.

*Elevated Plus Maze*

Elevated Plus Maze (EPM) assay was adapted from Walf and Frye [30]. Specifically, mice were injected with either saline or alcohol (1.25 g/kg) and immediately placed on the central platform (5 x 5 cm) of an apparatus elevated 40 cm above the floor, facing one of two closed arms (30 x 5 x 15 cm), which are perpendicular to two open arms (30 x 5 cm). Mice were allowed to freely explore the apparatus and their movement was recorded for 5 minutes. The total amount of time each mouse spent exploring the open arms, and the distal portion of both open arms (outer 15 cm), was monitored and quantified.

**Stereotaxic surgery**

Mice (8-10 weeks old) underwent stereotaxic surgery as described in [14,31] targeting the vHC (Franklin and Paxinos stereotaxic atlas, 3rd edition). Briefly, mice were anesthetized by vaporized isoflurane, and were placed in a digital stereotaxic frame (David Kopf Instruments, Tujunga, CA). Two holes were drilled

above the site of the injection and the injectors (stainless tubing, 33 gauges; Small Parts Incorporated, Logansport, IN) were then slowly lowered into the vHC (AP: -3.0, ML: ±3.0, DV: -3.55, infusion at -3.5 from bregma). The injectors were connected to Hamilton syringes (10 µl; 1701; Harvard Apparatus, Holliston, MA), and infusion was controlled by an automatic pump at a rate of 0.1 µl/min (Harvard Apparatus, Holliston, MA). The injectors remained in place for an additional 10 min to allow the virus to diffuse, and then were gently removed. Mice were allowed to recover in their home cages for at least 3 weeks before further testing to allow for maximal overexpression of BDNF [14].

**Confirmation of viral expression**

Characterization of infection and overexpression was performed as described previously [14,31]. At the end of experiments animals were euthanized by cervical dislocation, and the brains were removed. Brain regions were isolated from a 1-mm-thick coronal section dissected on ice, and a green fluorescence (GFP) signal was visualized using an EVOS FL tabletop fluorescent microscope (ThermoFisher Scientific; Waltham, MA).

**Data analysis**

Graphpad Prism 9 was used for statistical analysis. Data were analyzed using two-way ANOVA, with or without repeated measures, and student's t-test, where appropriate. For two-way ANOVAs, significant main effects or interactions were calculated using Šidák's multiple comparisons test. p-value cutoff for statistical significance was set to 0.05.

## Results

### Aberrant social behavior in male Met68BDNF mice

Met68BDNF mice consume alcohol despite negative consequences [14], however, the underlying cause of this phenotype is unknown. Humans carrying the Met66BDNF allele show increased social anxiety [24], and stress and anxiety including social anxiety are thought to be a major contributor to AUD [19,23]. Thus, to determine if the BDNFVal68Met polymorphism increases susceptibility for social anxiety in mice, we measured sociability and social novelty behaviors in female and male mice using a three-chamber social interaction paradigm [26,27]. First, in the sociability test phase, mice freely explored an apparatus containing, in one distal chamber, a novel, juvenile, sex-matched, C57Bl/6 mouse, identified as "stranger I". The other distal chamber contained an empty cage (Figure 1A. left). Next, in the social novelty test phase, the chambers were swapped and a new social interaction partner, "stranger II", was placed inside the previously empty cage (Figure 1A, right) and interaction time was recorded. We found that male Val68BDNF and Met68BDNF mice spent a similar amount of time interacting with a stranger I mouse in the sociability test (Figure 1B). Female Val68BDNF and Met68BDNF mice also demonstrated statistically similar stranger I investigation times in the sociability test (Figure 1B). Overall, results from the sociability test suggest that the Met68BDNF mutation does not influence social interaction *per se*. Female Val68BDNF and Met68BDNF also spent an equal amount of time interacting with the more novel "stranger II" mouse in the social novelty test (Figure 1C,E). In contrast, male Met68BDNF mice spent significantly less time interacting with the "stranger II" mouse as compared with Val68BDNF mice (Figure 1C-D). These results reveal that male Met68BDNF mice exhibit impairment in the social novelty test, which may indicate social anxiety-like behavior.

To further explore this possibility, female and male Val68BDNF and Met68BDNF mice were subjected to an open field social interaction paradigm. In this assay, test mice were paired with a novel, juvenile, sex-matched, C57Bl/6 partner in an empty open field apparatus (Figure 2A). Male Met68BDNF mice spent significantly less time in physical contact with a novel wild-type mouse than male Val68BDNF

mice (Figure 2B). In contrast, female Val68BDNF and Met68BDNF mice spent equal amounts of time in physical contact with a stranger mouse (Figure 2C). Altogether, male Met68BDNF mice exhibit aberrant social behaviors suggestive of social anxiety.

**Male Met68BDNF mice exhibit social aversion and alcohol preference in a social/alcohol conditioned place preference/aversion test**

Previous research demonstrated that individuals carrying at least one Met66BDNF allele report higher levels of social anxiety [24]. We therefore examined the possibility that mice carrying the mutation will prefer alcohol vs. social interaction. To test this possibility, we conducted a social-alcohol conditioned place preference/conditioned place aversion (CPP/CPA) paradigm, in which mice were conditioned to associate one distinct chamber with social interaction with a novel partner each morning for three days, and a second, distinct chamber with acute alcohol administration each afternoon for three consecutive days (Figure 3A). We then compared the time each mouse spent freely exploring each chamber following conditioning (post-test) with the amount of time mice spent exploring the same chambers prior to conditioning (pre-test). We found that male Val68BDNF mice did not exhibit a stronger preference for either social interaction or alcohol, as evidenced by no significant change in the time they spent exploring the social-paired and alcohol-paired chambers in the post-test, compared with during the pre-test (Figure 3B). In contrast, male Met68BDNF mice, a significant preference lower preference for social interaction chamber and a higher preference for the alcohol-paired chamber (Figure 3B).

Next, in order to determine whether male Met68BDNF mice prefer the alcohol chamber over social interaction because they find it more rewarding, we next used an alcohol CPP test (Figure 4A). In the post-test, male Val68BDNF and Met68BDNF mice spent significantly more time in the alcohol-paired chamber following conditioning than they did in the pre-test (Figure 4B). Mice from both genotypes also spent less time in the saline-paired chamber in the post-test, compared to the pre-test (Figure 4B). These results demonstrate that Met68BDNF mice experience the rewarding effects of alcohol to a similar degree as

Val68BDNF mice, suggesting that Met68BDNF mice may prefer the alcohol-associated chamber because of social anxiety.

**Male Met68BDNF mice are resistant to alcohol-induced locomotor hypoactivity and to the sedative effects of alcohol**

Alcohol tolerance has been associated with increased propensity to develop AUD [32,33]. We therefore examined the possibility that the polymorphism results in alteration in mice sensitivity to the acute actions of alcohol. First, we examined mouse locomotion in response to systemic administration of alcohol in the open field test. Specifically, male Val68BDNF and Met68BDNF received saline or a dose of 1.25 g/kg of alcohol and were placed in an open field chamber five minutes later. The distance the mice moved or the time they spent motionless were examined for ten minutes. We found that the Val68Met polymorphism did not affect general locomotion, as similar movement and motionless times were recorded in male Val68BDNF and Met68BDNF mice that received a systemic dose of saline (Figure 5A-B). In contrast, male Met68BDNF mice covered a significantly greater distance compared to male Val68BDNF mice following an acute systemic administration of alcohol (Figure 5A). Furthermore, male Met68BDNF mice spent significantly less time motionless compared with male Val68BDNF mice immediately after acute alcohol administration (Figure 5B).

We further tested whether male Met68BDNF mice would exhibit an abnormal response to a sedative dose of alcohol using a loss of righting reflex (LORR) paradigm. Specifically, we measured how long it took for mice to lose their righting reflex after receiving a systemic administration of a hypnotic dose (4g/kg) of alcohol, and how long the loss of righting reflex lasted in each animal. We found that it took significantly longer for male Met68BDNF mice to lose their righting reflex, compared to male Val68BDNF mice (Figure 5C). Furthermore, following LORR onset, male Met68BDNF mice also exhibited a much quicker recovery from the sedative effects of alcohol, as indicated by a group mean LORR duration less than half as long as the mean duration for male Val68BDNF mice (Figure 5D).

Together, these results suggest that male Met68BDNF exhibit a broad resistance to the acute effects of alcohol.

**Male Met68BDNF mice exhibit acute tolerance to the anxiolytic action of alcohol**

Next, we determined whether the Val68Met BDNF polymorphism alters the anxiolytic properties of alcohol in female and male mice. To do so we used an elevated plus maze paradigm (EPM) in which mice received a systemic dose of alcohol (1.25 g/kg) or saline and were then placed in the center of an elevated plus maze apparatus, which is composed of two enclosed "safe" arms and two open "unsafe" arms, which do not have barriers on their perimeters (Figure 6A). Alcohol administration significantly increased the time female and male Val68BDNF mice spent in the open arms, and specifically in the distal portion of the open arms compared with mice receiving saline, suggesting an anxiolytic response to alcohol (Figure 6B-D). The Val68Met BDNF polymorphism did not affect the anxiolytic actions of alcohol in female mice, as female Met68BDNF mice injected with alcohol (1.25 g/kg) also spent more time in the open arms and their distal regions, compared to female Met68BDNF mice injected with saline (Figure 6B-D). In contrast, we found that male Met68BDNF mice exhibit a significant resistance to the anxiolytic effects of alcohol, as the administration of alcohol did not increase mice open arm or distal open arm exploration times (Figure 6B-D). Overall, these data demonstrate a sex-specific, genotype-dependent impairment in the acute actions of alcohol including alcohol-mediated anxiolysis

**Overexpression of Val68BDNF in the ventral hippocampus of male Met68BDNF mice restores the anxiolytic effects of alcohol**

Finally, we set to counteract the behavioral deficits in male Met68BDNF by restoring normal BDNF signaling via overexpression of the wild-type Val68BDNF. The ventral hippocampus (vHC) plays a central role in anxiety-like behaviors in rodents [34-39]. Therefore, we hypothesized that BDNF gates anxiety-like behaviors and promote alcohol-dependent anxiolysis and that malfunction of BDNF signaling

manifests in behaviors such as resistance to the anxiolytic actions of alcohol. To test this possibility, the vHC of Met68BDNF mice were infected with adeno-associated (AAV) virus expressing either wild-type Val68BDNF or a GFP control (Figure 7A). Three weeks later, mice received systemic administration of saline or alcohol (1.25 g/kg) immediately prior to placement on the EPM apparatus. Similar to results reported above (Figure 6), male Met68BDNF infected with AAV-GFP in the vHC were resilient to the anxiolytic effects of alcohol and spent a similar amount of time in the open arms, and their distal portions, following alcohol or saline administration (Figure 7B-D). Overexpression of wildtype Val68BDNF in the vHC of the Met68BDNF mice, however, was sufficient to rescue alcohol-mediated anxiolysis (Figure 7B-D). Specifically, overexpression of wild-type BDNF in the vHC of Met68BDNF mice led to a significant increase in time mice spent in the open arms and distal open arms following alcohol administration, to a level similar to what was detected in both female and male Val68BDNF mice (Figure 7B-D). Together, these data imply that deficits in BDNF signaling in vHC circuitry in carriers of the Met68BDNF allele contribute to impaired alcohol-mediated anxiolysis.

## Discussion

In this study we provide evidence to suggest that a substitution of the amino acid Valine to Methionine in BDNF produces numerous sex specific phenotypes that are associated with AUD. Our data further suggest that proper BDNF signaling is required for several neurobehavioral responses to acute alcohol exposure, and that the vHC is a key node in mediating the anxiolytic effects of alcohol. Specifically, we show that male, but not female, mice carrying the Met68BDNF allele exhibit social anxiety phenotypes, and prefer alcohol to social interaction. We further report that Met68BDNF are resistant to the hypolocomotive, sedative and anxiolytic properties of alcohol. Finally, we show that overexpression of the wild-type Val68BDNF in the vHC of the Met68BDNF mice restore normal anxiolytic responses to alcohol.

We report that male Met68BDNF mice exhibit aberrant social interaction behaviors, indicative of a social anxiety-like phenotype. Previous reports linked social anxiety with the Met66BDNF allele in human populations [13,24], and Li et al. further showed that a different mouse model of the human BDNFVal66Met SNP, also suggests a link between impaired BDNF signaling and social deficits [24]. It is important to note however, that the BDNFVal66Met mouse model [40], in which the endogenous mouse BDNF gene was replaced with a human sequence, also includes a long carboxy-terminal Histidine repeats tag that may affect the interpretation of the data. Indeed, these two mouse models display divergent behavioral phenotypes [41], including heightened baseline anxiety-like behavior in the mouse model from Chen et al. [40], which we did not observe.

We found that male Met68BDNF also exhibited aversion to a social-paired chamber and a preference for an alcohol-paired chamber in a social-alcohol CPP/CPA test. However, alcohol-place preference was normal in BDNFMet68 mice suggesting that social deficits and not increased alcohol reward are likely to be the cause of the mice preferring the chamber that was associated with alcohol and not the chamber that was associated with social interaction. Ten percent of subjects suffering from AUD also endure social anxiety [42], and those who suffer from AUD are 4.5 times more likely to also exhibit

social anxiety [23,42-44]. In addition, social anxiety disorders are strongly correlated with increased risk for heavy alcohol use [42,43,45,46]. Furthermore, social stress during adolescence increases alcohol intake in male and female C57Bl/6 mice [47], and predicts alcohol intake in humans [48]. Together, these reports show that social anxiety is a risk factor for heavy alcohol use. We previously showed that male Met68BDNF mice consume alcohol both excessively and compulsively [14]. Several human studies report higher average alcohol consumption in Met66BDNF allele carriers [12,13]. It would be of great interest to determine whether human carriers of the Met66BDNF alleles exhibit social anxiety as well as AUD.

We observed that the Met68BDNF mice are resistant to the acute effects of alcohol suggesting that the mutation leads to the development of acute alcohol tolerance. It is unlikely, that the acute tolerance to the sedative, hypolocomotive and anxiolytic actions of alcohol is due to enhanced alcohol metabolism for two reasons: First, blood alcohol concentration was the same in Val68BDNF and Met68BDNF mice 90 minutes after receiving a dose of 2.5g/kg of alcohol [14], and second, since overexpression of wild-type Val68BDNF in the vHC of Met68BDNF mice was sufficient to rescue alcohol anxiolysis. Classic studies by Schuckit and colleagues showed that male children of fathers with AUDs demonstrate heightened acute alcohol tolerance, which coincides with an increased risk for problem drinking [32,49,50]. Animal studies further support the hypothesis that alcohol resistance leads to elevated alcohol consumption. For instance, rats selectively bred to prefer alcohol exhibit acute tolerance to alcohol, especially compared with rats bred to be alcohol-averse [51,52]. In addition, murine knockout of genes encoding neuropeptide Y (NPY) and Epidermal Growth Factor Receptor Pathway Substrate 8 (Eps8) each result in decreased sensitivity to the behavioral effects of alcohol and increased alcohol consumption [53,54]. Nevertheless, not much is known about the mechanisms underlying acute tolerance to alcohol's actions [55]. Thus, our findings are of importance as we discovered malfunction of a gene within a specific brain region plays a critical role in various phenotypes associated with acute tolerance.

Interestingly, we observed sex differences between the male and female Met66BDNF mice as disruption in BDNF function does not seem to affect social behavior in female animals, as well as their acute response to alcohol. In line with our findings, human data indicates that male, but not female, Met66BDNF allele-carriers experience higher incidences of major depressive disorder [56]. In addition, sex differences in social behavior have been widely described [57-62], and are likely to be caused by differences in neuronal activity, receptor density and hormonal signaling. However, further studies are needed to determine whether BDNF in female mice plays a role in other behaviors, including gating alcohol use [63], which is mediated in part via corticostriatal circuitries [31].

We establish that BDNF in vHC neurons, plays a critical role in mediating alcohol-induced anxiolysis. BDNF acts both in an autocrine and a paracrine manner. Specifically, BDNF is released in an activity dependent manner postsynaptically, and more commonly through axonal terminals [1,2]. Thus, it is plausible that BDNF synthesized in the vHC is released postsynaptically and activates TrkB receptors in dendrites of the same neurons or presynaptically by targeting other neurons within the vHP. Alternatively, BDNF produced in vHC neurons and released in target regions may influence behaviors via signaling in those brain regions. For example, the vHC extends neuronal projections to, and receives them from, the basolateral amygdala (BLA) and the mPFC, and connections between these three brain regions are linked with anxiety and specifically with social anxiety-like behaviors in rodents [35,64-67]. Furthermore, vHC neurons projecting to the lateral septum (LS) were shown to suppress anxiety-like behavior [66] whereas vHC to mPFC circuits promote anxiety [38,66]. In contrast, vHC neurons that project to the nucleus accumbens (NAc) [68] or mPFC [69] were shown to drive social reward memory. In rats, alcohol dependence has been shown to specifically increase synaptic excitability in the vHC [70], and inactivation of a projection from the ventral subiculum of the hippocampus to the NAc shell decreases context-induced alcohol relapse [71]. Moreover, we previously found that overexpressing wild-type BDNF in the mPFC of Met68BDNF mice is sufficient to reverse compulsive alcohol consumption in adult mice [14] which raises the possibility that overexpression of wild-type BDNF in the mPFC mimics the

endogenous BDNF in vHC to mPFC circuit. Future studies are required to map vHC BDNF neurons and their target regions and to determine whether BDNF in these circuits plays a role in other alcohol-related phenotypes including social anxiety phenotypes, alcohol preferences vs. social interaction. And compulsive alcohol drinking.

Li and colleagues showed that the social deficits in Met66BDNF carrier mice are caused, at least in part, by reduced function in a medial orbitofrontal cortical-basolateral amygdalar (mOFC-BLA) circuit during development, and that these deficits can only be rescued when wild-type BDNF is overexpressed during a peri-adolescent developmental window [24].

In summary, in this study we demonstrate that male Met68BDNF mice exhibit anxiety-like phenotypes and are resistant to numerous behavioral effects of alcohol. Our data also bring forward the importance of BDNF-expressing neurons in the vHC in the anxiolytic actions of alcohol. Finally, this study suggests that BDNF-mimetics [72] should be evaluated for the treatment of AUDs and related disorders in human carriers of the Met66BDNF allele.

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**Author Contributions:** JJM contributed to experimental design, data acquisition, data analysis, and manuscript preparation. SAS contributed to experimental design and data acquisition. ZWH contributed to data acquisition. DR conceived the study. contributed to experimental design and to manuscript preparation.

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## Figure Legends

**Figure 1. Male Met68BDNF mice exhibit social anxiety-like behaviors**

(**A**) Three-chamber sociability and social novelty paradigms. In the 15-minute sociability test, one distal chamber contained an empty cage and the other contained a juvenile, wild-type mouse (Stranger I). In the 15-minute social novelty test, Stranger I was relocated to the opposite chamber and a novel mouse (Stranger II), was placed in the other. (**B**) Female and male Val68BDNF (green and blue) and female and male Met68BDNF mice (orange and red) spend about the same amount of time interacting with the stranger I mouse during the sociability test. Two-way ANOVA, main effect of sex, $F(1, 54) = 61.83$, $p < 0.0001$. (**C**) Male Met68BDNF mice spend significantly less time interacting with a stranger II mouse in the social novelty test than male as compared to Val68BDNF mice ($p < 0.001$). Female Val68BDNF and Met68BDNF mice spend a similar amount of time interacting with a stranger II mouse in the social novelty test. Two-way ANOVA, interaction effect between sex and genotype, $F(1, 53) = 15.58$, $p < 0.001$, main effect of sex, $F(1, 53) = 6.701$, $p < 0.05$, main effect of genotype, $F(1, 53) = 5.735$, $p < 0.05$. (**D-E**) Heatmaps of mean mouse position for male and female Val68BDNF and Met68BDNF and cohorts during the social novelty test shown in (**C**). Stranger I mice are shown on the left of each heatmap, and stranger II are shown on the right side. All data are represented as mean ± SEM. *** $p < 0.001$. Female Val68BDNF: n = 15, all other experimental groups: n=14.

**Figure 2. Male Met68BDNF mice exhibit aberrant social interaction**

(**A**) Open. Field social interaction paradigm. Male and female Val68BDNF and Met68BDNF mice were placed in an empty open field apparatus with a novel mouse for 10 minutes and body contacts were recorded. (**B**) Male Met68BDNF (red) mice spent significantly less time in contact with a novel interaction partner than male Val68BDNF mice (blue) ($p < 0.01$). Two-tailed Student's t test, t=2.947, df=17. (**C**) There is no significant difference in total body contacts with a novel interaction partner between female Val68BDNF and Met68BDNF mice (green and orange). Two-tailed Student's t test, t=0.5858, df=26. All

data are represented as mean ± SEM; ** $p < 0.01$. (B): Male Val68BDNF n = 10, Male Met68BDNF: n = 9, (C): Female Val68BDNF and Met68BDNF n = 14.

**Figure 3. Male Met68BDNF mice demonstrate social aversion and alcohol preference in a social-alcohol place conditioning test**

(**A**) Outline of the social-alcohol CPP/CPA test. On day 1 mice explored the entire apparatus for 15 minutes. In the morning of days 2-4 conditioning days, mice received an i.p. injection of saline before a 10 minute social interaction period in the social assigned chamber. In the afternoon of days 2-4 mice received an i.p. injection of 2 g/kg of alcohol prior to being placed in the alcohol-paired chamber for 10 minutes. On day 5, mice were allowed to freely explore the entire apparatus for 15 minutes. (**B**) Male Met68BDNF mice (red) spend significantly less time in the social-paired chamber in the post-test than they do in the pre-test ($p < 0.0001$), and significantly more time in the alcohol-paired chamber in the post-test compared to the pre-test ($p < 0.01$). Male Val68BDNF mice (blue) do not exhibit any place preference or aversion in the social-alcohol CPP/CPA test. Two-way RM ANOVA, interaction effect of conditioning and genotype, $F (3, 40) = 15.17$, $p < 0.0001$. All data are represented as mean ± SEM; ** $p < 0.01$, **** $p < 0.0001$. Val68BDNF: n = 12, Met68BDNF: n = 10.

**Figure 4. Male Met68BDNF mice do not exhibit heightened alcohol conditioned place preference**

(**A**) Alcohol place preference paradigm. Mice underwent a pre-test with free exploration of both chambers. On alternating conditioning days, mice were placed in a saline- or alcohol-paired chamber immediately after receiving an i.p. injection of saline or 2 g/kg of alcohol. During the post test, mice were once again allowed to freely explore the entire apparatus. (**B**) Male Val68BDNF (blue) and Met68BDNF (red) mice exhibit a significantly higher preference or CPP score for the alcohol-paired chamber, compared with the saline-paired chamber ($p < 0.05$, both groups). Two-way ANOVA, main effect of conditioning, F (1, 24)

= 14.94, $p < 0.001$. All data are represented as mean ± SEM; * $p < 0.05$. Val68BDNF: n = 9 (2 were removed due to health issues), Met68BDNF: n = 17.

**Figure 5. Male Met68BDNF mice are resistant to alcohol-induced locomotor impairments and the sedative effects of alcohol**

Impairment of locomotion after systemic administration of alcohol (1.25 g/kg) was measured in an open field test. (**A**) Bar graph depicting locomotion immediately after the administration of saline or alcohol to male Val68BDNF mice (blue) and Met68BDNF mice (red). Met68BDNF mice move a significantly greater distance than Val68BDNF mice in the open field test ($p < 0.05$). Two-way ANOVA, interaction effect between genotype and alcohol treatment, $F(1, 27) = 8.500$, $p < 0.01$. (**B**) Male Met68BDNF mice (red) also spend significantly less time motionless than Val68BDNF counterparts (blue) in the open field test following alcohol treatment ($p < 0.01$). Two-way ANOVA, interaction effect between genotype and alcohol treatment, $F(1, 27) = 9.611$, $p < 0.01$; main effect of genotype, $F(1, 27) = 6.328$, $p<0.05$.

(**C**) Mice received 4 g/kg of alcohol and the time it took for sedation to set in, and the duration of the sedation were recorded. The latency between alcohol injection (4 g/kg) and the point at which mice do not right themselves after being placed on their backs is significantly greater in male Met68BDNF mice (red) than it is in male Val68BDNF mice ($p < 0.05$). Two-tailed Student's t test, $t = 2.439$, df = 13. (**D**) The total duration of LORR for male Met68BDNF mice (red) is significantly shorter than it is for mice Val68BDNF mice (blue) ($p < 0.01$). Two-tailed Student's t test, $t = 3.736$, df = 13. Data are represented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$. Val68BDNF + alcohol (A-B): n = 7, Val68BDNF (C-D): n = 7, all other experimental groups: n = 8.

**Figure 6. Male Met68BDNF exhibit acute tolerance to the anxiolytic action of alcohol**

(A) Immediately following i.p. injection of saline or alcohol (1.25 g/kg), mice were placed in the center of the elevated plus maze apparatus and allowed to explore freely. The time mice spent in the open arms

and in the distal portion of the open arms was measured. (**B**) Female Met68BDNF mice (orange) and Val68BDNF mice (green) spend significantly more time in the open arm of an elevated plus maze following i.p. injection of alcohol (1.25 g/kg). Male Val68BDNF mice (blue) also spend significantly more time in the open arm following alcohol administration, whereas male Met68BDNF mice (red) do not spend significantly more time in the open arm following alcohol administration ($p > 0.05$). Two-way ANOVA, main effect of alcohol treatment, $F (1, 75) = 64.07$, $p < 0.0001$. (**C**) Female Met68BDNF and Val68BDNF mice also spend significantly more time in the distal portion open arm following i.p. injection of alcohol (1.25 g/kg). Male Val68BDNF mice likewise spend significantly more time in the distal open arm. Male Met68BDNF mice do not spend significantly more time in the distal open arm following alcohol administration ($p > 0.05$), compared with following saline treatment. Two-way ANOVA, interaction effect between genotype and treatment, $F (3, 75) = 3.290$, $p < 0.05$; main effect of treatment, $F (1, 75) = 44.25$, $p < 0.0001$. (**D**) Heatmaps representing mean relative position of animals in each group. Open arms are represented horizontally, and closed arms extend vertically from the center. Data are represented as mean ± SEM; * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. Male Val68BDNF + saline: n = 15, male Val68BDNF + alcohol: n = 14, male Met68BDNF + saline: n = 13, male Met68BDNF + alcohol: n = 14, female Val68BDNF + saline: n = 6, female Val68BDNF + alcohol: n = 7, female Met68BDNF + saline: n = 7, female Met68BDNF + alcohol: n = 7.

**Figure 7. Overexpressing BDNF in the vHC of male Met68BDNF rescues the anxiolytic effect of alcohol**

(**A**) Confirmation of Val68BDNF overexpression. Image (10x) depicts GFP expression in the vHC of a Met68BDNF mouse that received AAV-Val68BDNF in the vHC. (**B**). Male Met68BDNF that received AAV-GFP in the vHC do not spend significantly more time in the open arm following i.p. injection of alcohol (1.25 g/kg), while Met68BDNF mice that received AAV-Val68BDNF in the vHC spend significantly more time in the open arm ($p < 0.0001$). Two-way ANOVA, interaction effect between virus

and alcohol treatment, $F(1, 38) = 5.644$, $p < 0.05$; main effect of alcohol treatment, $F(1, 38) = 28.34$, $p < 0.0001$. (**C**) Met68BDNF mice that received AAV-Val68BDNF (purple) in the vHC also spend significantly more time in the distal portion of the open arms following alcohol (1.25 g/kg) administration, compared with saline. Met68BDNF mice that received AAV-GFP (green) in the vHC, however, did not spend significantly more time in the distal open arms following alcohol injections. Two-way ANOVA, main effect of alcohol treatment, $F(1, 38) = 31.08$, $p < 0.0001$; main effect of virus, $F(1, 38) = 4.126$, $p < 0.05$. (**D**) Heatmaps of group mean position on the elevated plus maze. Open arms are horizontal, and closed arms extend vertically from the center. Data represented as mean ± SEM; **** $p < 0.0001$. Male Met68BDNF + AAV-GFP: n = 11, Male Met68BDNF + AAV-Val68BDNF: n = 10.

**Figure 1**

**Male Met68BDNF mice exhibit social novelty anxiety-like behavior**

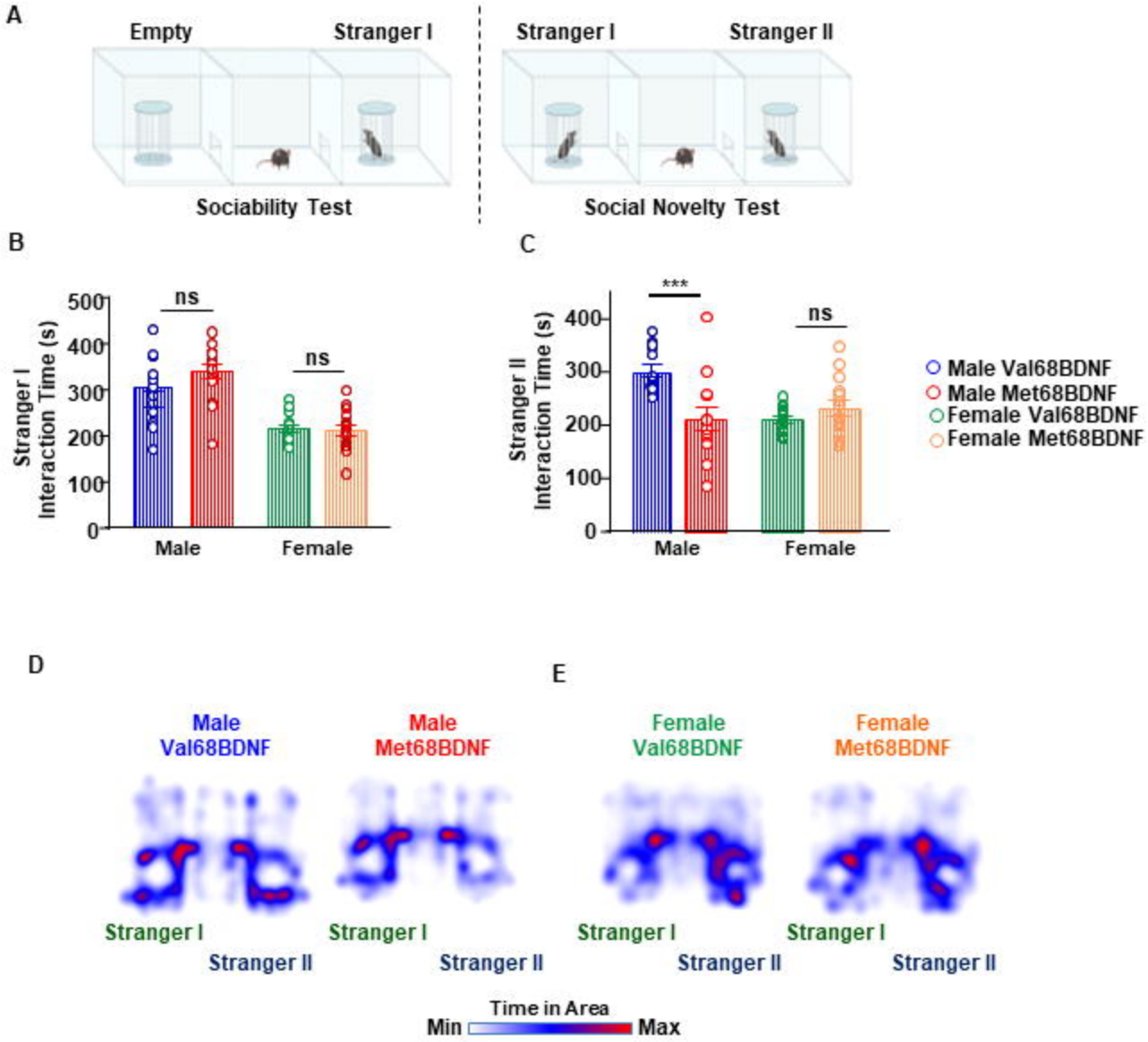
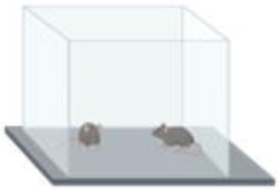


**Figure 2**

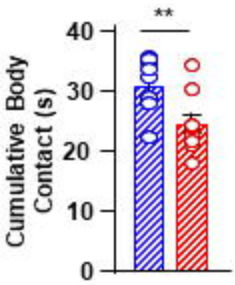
## Male Met68BDNF mice exhibit social interaction anxiety-like behavior

A



Open Field Social Interaction

B




C

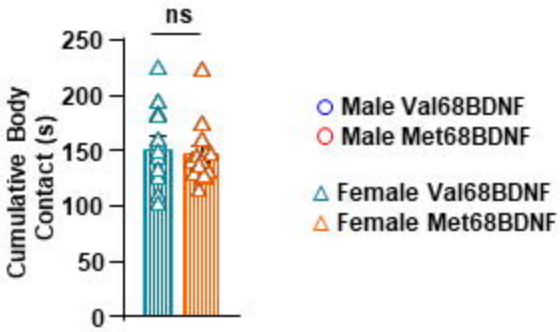


Figure 3

**Male Met68BDNF mice demonstrate social aversion and alcohol preference in a social-alcohol place preference test**

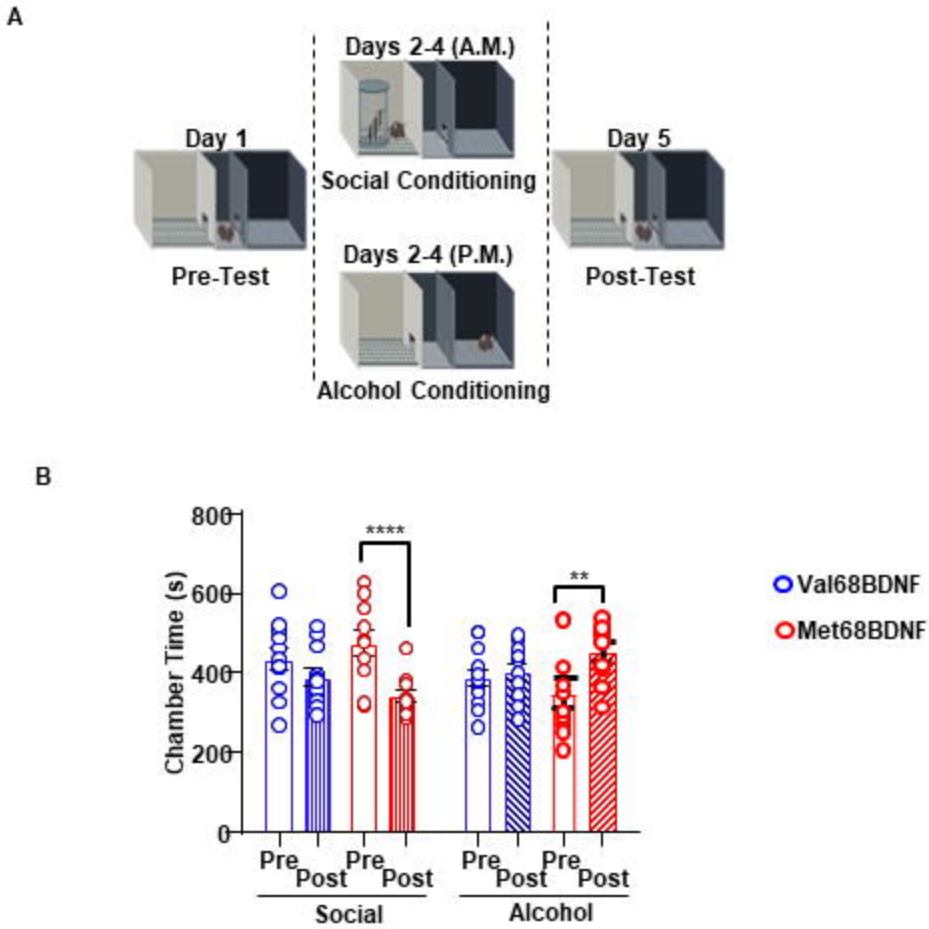


Figure 4

**Male Met68BDNF mice do not exhibit heightened alcohol conditioned place preference**

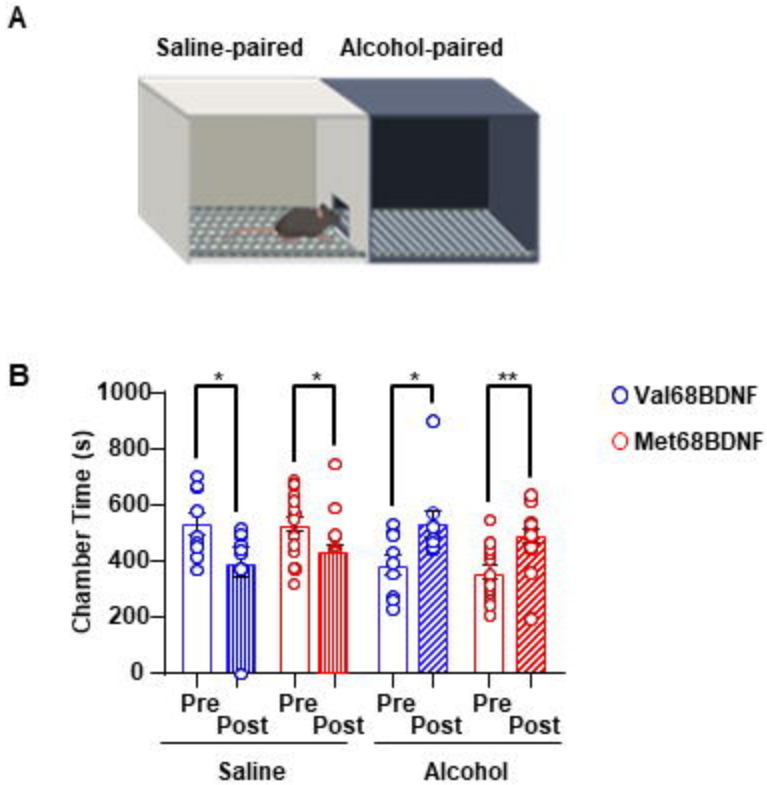


**Figure 5**

**Male Met68BDNF mice are resistant to alcohol-induced locomotor impairments and the hypnotic effects of alcohol**

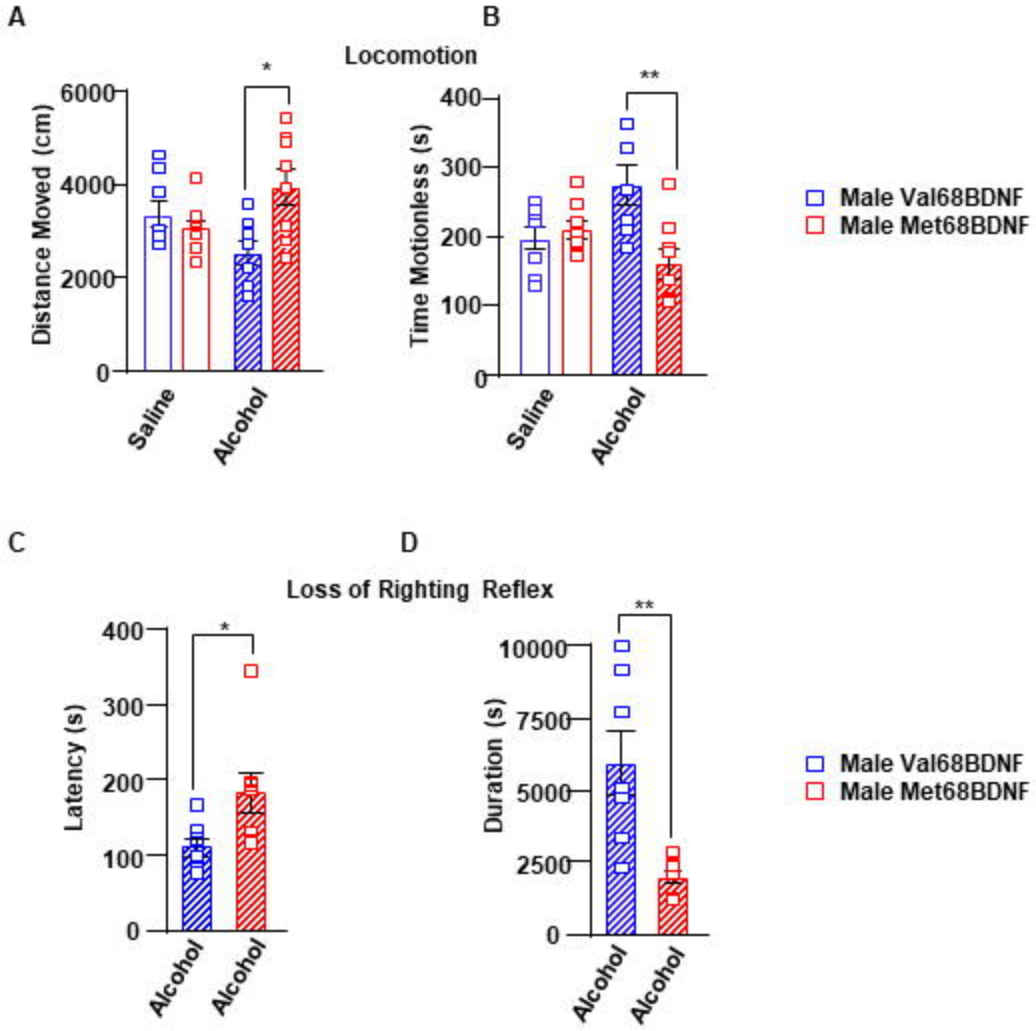


**Figure 6**

## Male Met68BDNF exhibit acute tolerance to the anxiolytic action of alcohol

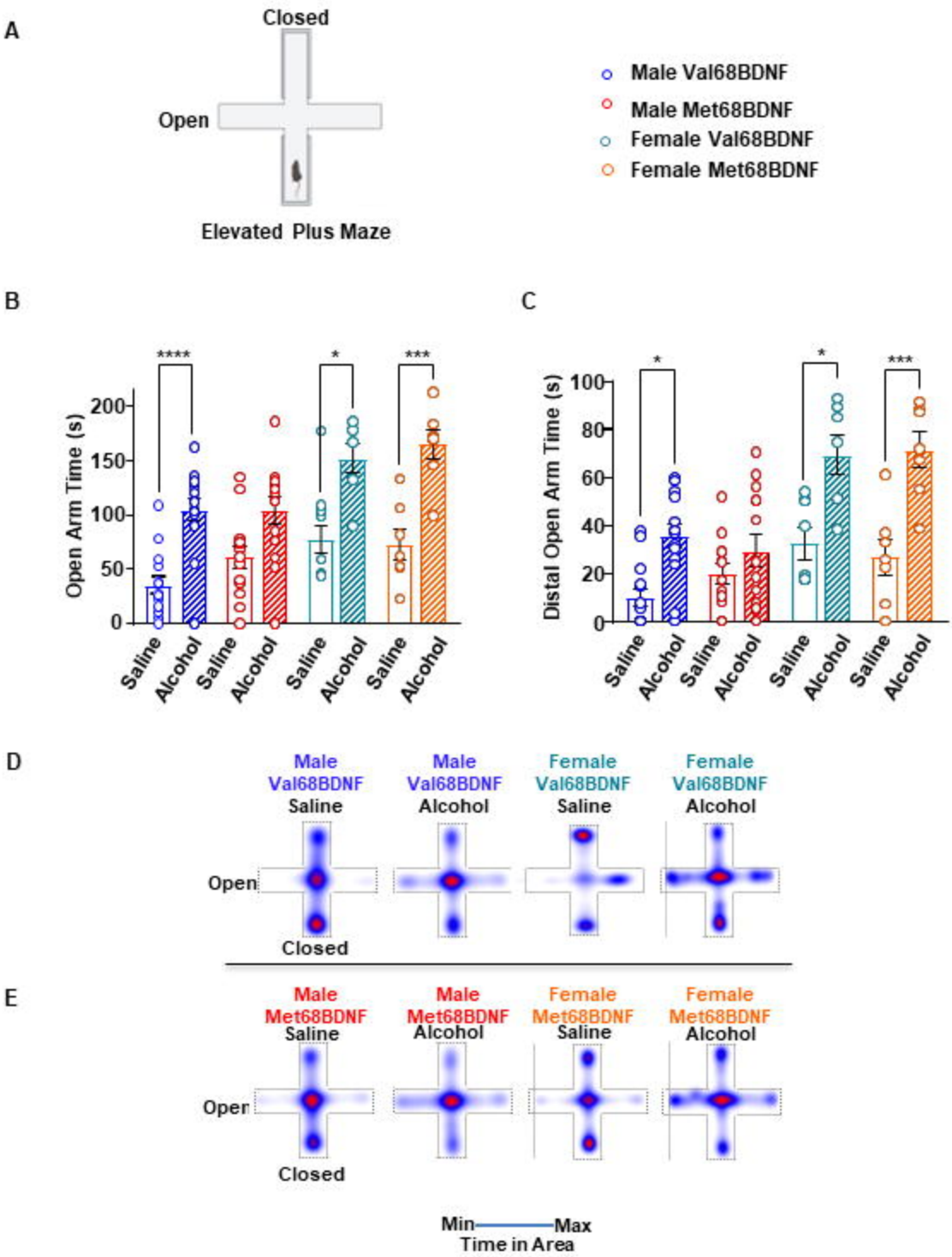
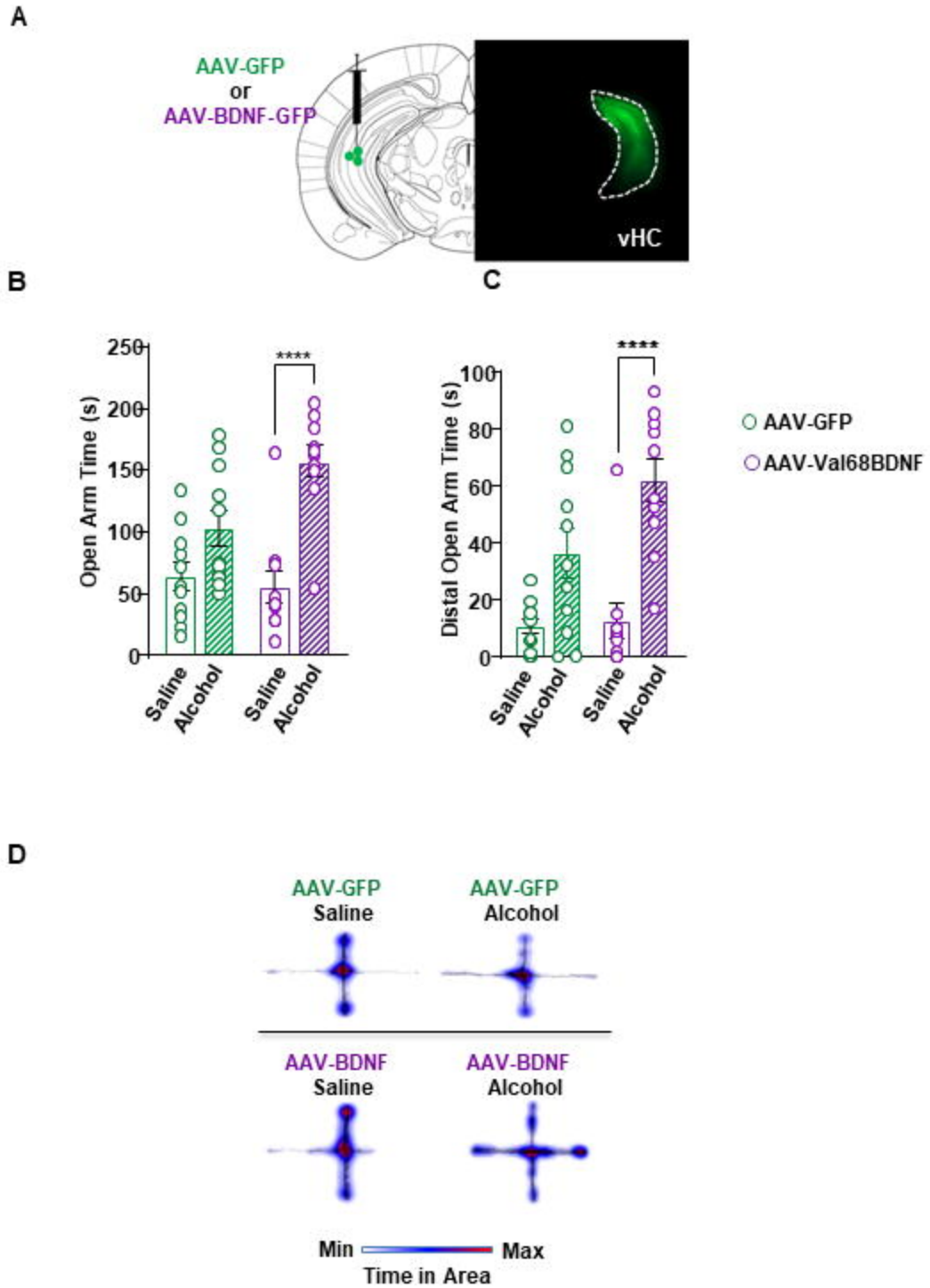


Figure 7

**Overexpressing Val68BDNF in the vHC of male Met68BDNF mice rescues the anxiolytic effect of alcohol**