The BDNF Val68Met polymorphism causes alcohol preference over social interaction and also acute tolerance to the anxiolytic effects of alcohol, a phenotype driven by malfunction of BDNF in the ventral hippocampus in male mice

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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 depression and anxiety. We previously showed that male 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 phenotypes exhibit by the Met68BDNF mice. We found that male but not female Met68BDNF mice show a social anxiety-like behaviors. We further show that male Met68BDNF mice develop a preference for alcohol over social interaction. In contrast, alcohol place preference without having the social interaction as an alternative reward, is similar in male Met68BDNF compared with Val68BDNF mice. Next, we tested whether alcohol is perceived differently in the Met68BDNF and Val68BDNF mice and found that male, but not female Met68BDNF mice are insensitive to the acute anxiolytic action of alcohol. Finally, we show that the acute tolerance to alcohol-dependent anxiolysis is restored by overexpressing wild-type Val68BDNF in the ventral hippocampus (vHC) of the Met68BDNF mice. Together, our results suggest that excessive and compulsive alcohol drinking in the Met68BDNF may be due, in part, 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 results in a substitution of Valine at position 66 (Val66BDNF) with Methionine (Met66BDNF), causing a reduction in the activity-dependent release of the neurotrophic factor and thus to a downregulation its downstream signaling [3-5]. The BDNFVal66Met SNP has been linked to multiple neuopsychiatric disorders, including depression and anxiety [6,7], and several small scale studies 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 [14]. We further demonstrated that the mice drink despite the addition of quinine suggesting that they consume alcohol compulsively [14]. Finally, we reported 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 approximately 20-40% of individuals with major depressive disorder or anxiety also develop 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 from symptoms of mood disorders including social anxiety [20-23]. In fact, 10 percent of people suffering from AUD also endure social anxiety [23]. Furthermore, studies in humans show that those who suffer from AUD are 4.5 times more likely to also exhibit social anxiety [24]. Heterozygous (Val/Met) and homozygous (Met/Met) genotypes have been linked with anxiety and specifically social anxiety in humans [13,25]. For instance, in analyzing the Pediatric Imaging,

Neurocognition, and Genetics (PING) study [26], Li and colleagues recently uncovered a significantly higher self-reported social anxiety score in Met66BDNF allele carriers than in individuals with homozygous Val66BDNF allele [25]. They further showed that social deficits can be recapitulated in the mouse model of the human allele [25].

Here, we examined potential underlying causes for the heightened alcohol consumption in Met68BDNF mice, including social anxiety and alcohol-mediated 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 bred separately and were tested at the age of 8-10 weeks. Genotyping was performed as described previously [14]. Juvenile female and male C57Bl6/J 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 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 intra-peritoneal (i.p.) injection by systemically administrating 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 [27,28]. 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 (4-6 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 (4-6 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. 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 5 minutes in the open field apparatus (43 x 43 cm). Mice were then placed in the open field apparatus with a novel, wild-type, juvenile (4-6 weeks old), sex-matched interaction partner, and their interactions were recorded for 5 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) with one compartment consisting of lighter colored walls and mesh flooring,

while the other one comprising of 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 3 days, mice were pseudorandomly assigned to associate a chamber with either social interaction paired with an i.p. injection of saline or to associate social interaction with an i.p. injection of alcohol (2 g/kg). Specifically, during the morning of each conditioning day, each mouse received an i.p. injection of saline before being placed in their assigned social interaction-paired chamber with a caged, sex-matched, juvenile mouse (4-6 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 recorded and quantified.

Alcohol Conditioned Place Preference

Alcohol CPP was adapted from [29]. 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 is made 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 6 experimental days were split into alternating days of saline or alcohol conditioning. On saline conditioning days (days 2, 4, 6), mice receive an i.p. injection of saline immediately before placement in the saline-paired chamber for 5 minutes. On alcohol conditioning days (days 3, 5, 7), mice received an i.p. injection of alcohol (2 g/kg) before being placed in the alcohol-paired chamber for 5 minutes. Day 8 of the experiment consisted of a post-test, during which test mice freely explored the apparatus for 15 minutes. The time spent in each chamber during the pre-test and post-test were recorded and quantified.

Open Field Test

Animal movement and inactivity were measured 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 recorded and quantified.

Loss of Righting Reflex

Loss of Righting Reflex (LORR) was conducted as described previously [30]. 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 that it took mice to right themselves from their backs three times in one minute (duration), was measured.

Elevated Plus Maze

Elevated Plus Maze (EPM) assay was adapted from [31]. Specifically, mice were injected with either saline or alcohol (1.25 g/kg) and 10 minutes later were 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 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 recorded and quantified.

Stereotaxic surgery

Met68BDNF mice (8-10 weeks old) underwent stereotaxic surgery as described in [14,32] 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 minutes to allow the virus to diffuse and were then 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 Val68BDNF [14].

Confirmation of viral expression

Characterization of infection and overexpression was performed as described previously [14,32]. 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 excessively and compulsively [14], however, the underlying cause of this phenotype is unknown. Humans carrying the Met66BDNF allele show increased social anxiety [25], and stress and anxiety including social anxiety are thought to be major contributors to AUD [19,23]. Thus, to determine if the BDNFVal68Met polymorphism increases susceptibility for social anxiety in mice, we first measured sociability and social novelty behaviors in female and male mice using a three-chamber social interaction paradigm [27,28]. First, in the sociability test phase, mice freely explored an apparatus containing, in one distal chamber, a novel, juvenile, sex-matched, C57Bl/6J 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 female and male Val68BDNF and Met68BDNF mice spent a similar amount of time interacting with a stranger I mouse (Figure 1B) suggesting that sociability *per se* is not affected by the SNP. In the social novelty paradigm in which mice can choose between spending time with the familiar mouse (strange I) or a novel mouse (stranger II), female Val68BDNF and Met68BDNF spent an equal amount of time interacting with "stranger II" mouse (Figure 1C,E). In contrast, male Met68BDNF mice spent significantly less time interacting with the "stranger II" mouse as compared with male Val68BDNF mice (Figure 1C-D). These findings may indicate that Met68BDNF exhibit 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/6J partner in an empty open field apparatus (Figure 2A). Male Met68BDNF mice spent significantly less time in physical contact with a novel juvenile 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). Together, these data unravel a sex-specific, genotype-dependent impairment in social behaviors in Met68BDNF mice suggestive of social anxiety.

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

Social anxiety is a risk factor for AUD [23,24], and previous research demonstrated that humans carrying at least one Met66BDNF allele report higher levels of social anxiety [25]. Since male Met68BDNF mice exhibit a social anxiety phenotypes, we examined the possibility that mice carrying the mutation 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 3 days, and a second, distinct chamber with acute alcohol administration each afternoon for 3 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 exhibited a significantly lower preference for the social interaction chamber and a higher preference for the alcohol-paired chamber (Figure 3B), indicative of a simultaneous social aversion and alcohol preference.

Next, in order to determine whether male Met68BDNF mice prefer the alcohol chamber over social interaction because they find alcohol more rewarding, we used an alcohol CPP paradigm (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 prefer the alcohol-associated chamber in the social-alcohol CPP/CPA test because of social anxiety.

Male Met68BDNF mice are resistant to alcohol-induced hypolocomotion and sedation

Alcohol tolerance has been associated with increased propensity to develop AUD [33-35]. We therefore examined the possibility that the BDNFVal68/Met polymorphism alters male mice sensitivity to the acute actions of alcohol. We therefore tested whether male Met68BDNF mice exhibit an abnormal response to an acute sedative dose of alcohol as compared to male Val68BDNF mice. Specifically, we utilized a loss of righting reflex (LORR) paradigm and measured how long it took mice to lose their righting reflex after receiving a systemic administration of a hypnotic dose (4g/kg) of alcohol, and how long the LORR lasted in each animal. We found that it took male Met68BDNF mice significantly longer to lose their righting reflex as compared to male Val68BDNF mice (Figure 5A). 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 being less than half as long as the mean duration for male Val68BDNF mice (Figure 5B). Together, these results suggest that male Met68BDNF exhibit tolerance to the acute effects of a high dose of alcohol .

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

As the data above indicate, male Met68BDNF mice exhibit tolerance to the acute actions of alcohol (Figure 5). We next examined whether acute alcohol tolerance is more generalized and also whether alcohol is perceived differently in the two genotypes. We therefore, evaluated the acute anxiolytic properties of alcohol in male Met68BDNF mice as compared to male Val68/BDNF. We also determined whether female Met68BDNF also exhibit acute tolerance phenotype compared to female Val68BDNF 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 10 minutes later were placed in the center of an elevated plus maze apparatus, which is composed of two enclosed arms and two open arms that do not have barriers on their perimeters (Figure 6A). Administration of alcohol (1.25 g/kg) significantly increased the time Val68BDNF male mice spent in the open arms (Figure 6B-E), and specifically in the distal portion of the open arms compared with mice receiving saline, suggesting an anxiolytic response to alcohol (Figure 6B-E). In contrast, we found that male Met68BDNF mice exhibit a significant resistance to the anxiolytic effects of alcohol, as acute systemic administration of 1.25 g/kg of alcohol did not alter Met68BDNF mice' exploration time in the open arm or distal open arm as compared to male Val68BDNF mice (Figure 6B-E). The reduction in exploration time in the male Met68BDNF mice was not due to changes in locomotion in response to alcohol administration (Supplementary Figure 1). In contrast, acute systemic administration of alcohol (1.25 g/kg) was anxiolytic in both female Val68BDNF and Met68BDNF mice as both genotypes spent more time in the open arms and their distal regions, compared to female Val68BDNF and Met68BDNF mice injected with saline (Figure 6B-E). Overall, these data demonstrate a sex-specific, genotype-dependent impairment alcohol-mediated anxiolysis. Our data further confirm that male mice carrying the Met68BDNF allele exhibit tolerance to the acute actions of alcohol.

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

The ventral hippocampus (vHC) plays a role in anxiety-like behaviors in rodents [36-41]. Interestingly the vHP has also been associated with anxiolysis [42]. We therefore hypothesized that BDNF in the vHC plays a role in dampening anxiety-like behaviors and in promoting alcohol-dependent anxiolysis. We further hypothesized that malfunction of BDNF signaling in the vHC manifests in behaviors such as resistance to the anxiolytic actions of alcohol. To test this possibility, the vHC of male Met68BDNF mice was infected with adeno-associated (AAV) virus expressing either wild-type Val68BDNF or a GFP control (Figure 7A). Three weeks later a timepoint in which viral infection is

maximal, mice received systemic administration of saline or alcohol (1.25 g/kg) 10 minutes prior to placement on the EPM apparatus. Similar to results reported in 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 saline or alcohol administration (Figure 7B-D). In contrast, male Met68BDNF mice infected with Val68BDNF in the vHC spent significantly more time 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 6B-D), a rescue that was not due to alterations in locomotion (Supplementary Figure 2). Together, these data imply that deficits in BDNF/TrkB signaling in vHC circuitry in carriers of the Met68BDNF allele contribute to impaired alcohol-mediated anxiolysis and to the development of acute tolerance which were restored by overexpression of wildtype BDNF in the vHP.

Discussion

In this study we provide evidence to suggest that male carriers of the Met68BDNF allele exhibit social anxiety, alcohol preference over social interaction and acute tolerance to the sedative and anxiolytic actions of alcohol. Finally, we show that overexpression of the wild-type Val68BDNF in the vHC of the Met68BDNF mice restores normal anxiolytic responses to alcohol suggesting that proper BDNF signaling in the vHC is required for alcohol-dependent anxiolysis.

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 [43], and those who suffer from AUD are 4.5 times more likely to also exhibit social anxiety [23,24,43,44]. In addition, social anxiety disorders are strongly correlated with increased risk for heavy alcohol use [43-46]. 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]. Thus, it is plausible the mice and humans carrying the BDNF SNP consume alcohol compulsively in part to alleviate social anxiety. Therefore, it would be of great interest to determine whether social anxiety and AUD are correlated in human carriers of the Met66BDNF allele.

We observed that male Met68BDNF mice are resistant to the acute sedative and anxiolytic actions of alcohol suggesting that this mutation leads to the development of acute alcohol tolerance. It is unlikely, that the acute tolerance to 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-dependent anxiolysis. Acute tolerance is a well-known characteristic of AUD [35] and classic longitudinal studies by Schuckit and colleagues showed that heightened acute alcohol tolerance, coincides with an increased risk for AUD [33,34]. Not much is known about the mechanisms underlying alcohol tolerance [35]. Thus, our findings are of importance as we discovered malfunction of a gene plays a critical role in both acute tolerance and excessive compulsive alcohol consumption [14].

Interestingly, we observed sex differences between the male and female Met68BDNF mice. Specifically, disruption in BDNF function does not seem to affect social behavior and acute response to alcoho in female Met66BDNF animals. In line with our findings, human data indicates that male, but not female Met66BDNF allele human carriers experience higher incidences of major depressive disorder [49]. In addition, sex differences in social behavior have been widely described [50-55]. However, further studies are needed to determine whether BDNF in female mice plays a role in other behaviors, including gating alcohol use, which is mediated in part via corticostriatal circuitries in male mice [14,56-58].

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 [37,42,59-61]. Furthermore, vHC neurons projecting to the lateral septum (LS) were shown to suppress anxiety-like behavior [42] whereas vHC to mPFC circuits promote anxiety [40,42]. In contrast, vHC neurons that

project to the nucleus accumbens (NAc) [62] or mPFC [63] were shown to drive social reward memory. In rats, alcohol dependence has been shown to specifically increase synaptic excitability in the vHC [64], and inactivation of a projection from the ventral subiculum of the hippocampus to the NAc shell decreases context-induced alcohol relapse [65]. 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.

We found that mouse male carriers of the mouse Met68BDNF allele exhibit social anxiety-like phenotypes and Li et al. showed that mouse male carriers of the human Met66BDNF allele also show social anxiety phenotypes [25]. The authors further showed that the social deficits in the male Met66BDNF carrier mice are caused by reduced function of BDNF in a medial orbitofrontal cortical mOFC) to BLA circuit during development, and that these deficits can only be rescued when wild-type Val66BDNF is overexpressed during a peri-adolescent developmental window of Met66BDNF mice but not during adulthood [25]. Although we did not specifically examine whether social anxiety can be rescued in the adult Met68BDNF mice, we were able to rescue compulsive alcohol drinking phenotype by overexpressing Val68BDNF in the mPFC of adult Met68BDNF mice [14], and here we show that alcohol-dependent anxiolysis can be restored by overexpressing Val68BDNF in the vHP of adult Met68BDNF mice. These data suggest that malfunction of BDNF during development does not play a role in all tested behaviors. Another important difference between the two mouse lines is that we did not observe a baseline anxiety differences in mice carrying the Val68BDNF and Met68BDNF alleles ([14] and data herein), whereas mice generated by Li and colleagues carrying the human Met66BDNF allele, show heighten basal anxiety levels [66]. Furthermore, Vandenberg et al. compared the behaviors of Met66BDNF and

Met68BDNF mice as well as their wild-type counterparts in a battery of cognitive paradigms and found numerous additional differences [67]. It is important to note that our knockin mouse line and Li and colleagues knockin mouse line are markedly different. First, Chen et al. replaced the mouse *BDNF* gene with the human *BDNF* sequence and introduced a single mutation to produce the Met66BDNF allele [66] whereas we introduced two mutations to replace a Valine 68 with Methionine in the mouse gene to create the Met68BDNF allele [14]. Another important difference is that Chen et. al included a Histidine tail at the C-terminal of the protein [66], whereas our mouse line did not contain additional amino acids [14]. This difference is of importance as the addition of a Histidine tag can produce none-physiological changes to the function of the endogenous protein. First, the addition of multiple Histidines was shown to increase protein stability [68], and to also change the confirmation and activity of recombinant proteins [69,70]. Thus, differences in Met66BDNF and Met68BDNF half-life and/or confirmation could play a role in the behavior of the mouse. More studies are required to clarify why mice carrying the val66/68MetBDNF alleles show such differences in neurobehavioral phenotypes.

In summary, in this study we demonstrate that male Met68BDNF mice exhibit social anxiety-like phenotypes and are resistant to the acute 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 [71] should be evaluated for the treatment of AUDs and related disorders in human carriers of the Met66BDNF allele.

Funding and Disclosure: This research was supported by the National Institute of Alcohol Abuse and Alcoholism R37AA01684 (DR) and F32AA028422 (JJM). None of the authors have a conflict of interest.

Acknowledgments: The authors thanks JiHwan Yu for his help. Some of the figures were made in part using BioRender.

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. YE contributed to data analysis and manuscript preparation. DR conceived the study. contributed to experimental design and 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*. *Sociability test* (left). The distal chamber contained an empty cage, and the other chamber contained a sex match juvenile C57Bl/6J mouse (Stranger I). Female (green) and male (blue) Val68BDNF, and female (orange) and male (red) Met68BDNF mice were allowed to interact with the Stranger I mouse for 15 minutes, and the time a mouse spent with Stranger I mouse was recorded and quantified. *Social novelty test* (right). Stranger I was relocated to the distal chamber, and a sex matched novel juvenile C57Bl/6J mouse (Stranger II), was placed in the other chamber. Female (green) and male (blue) Val68BDNF and female (orange) and male (red) Met68BDNF mice were allowed to interact with stranger I or Stranger II for 15 minutes and the time a mouse spent with stranger I or stranger II was recorded and quantified. (**B**) Female and male Val68BDNF and Met68BDNF mice spent 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 spent significantly less time interacting with a stranger II mouse in the social novelty test as compared to male Val68BDNF mice ($p < 0.001$). Female Val68BDNF and Met68BDNF mice spent a similar amount of time interacting with a stranger II mouse. 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 mice 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. *** $p < 0.001$, ns = none-significance. 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 (blue) and female (green) Val68BDNF and male (red) and female (orange) Met68BDNF mice were placed in an open field apparatus with a novel sex matched juvenile C57Bl/6J mouse for 10 minutes and body contacts were recorded. (**B**) Male Met68BDNF mice spent significantly less time in contact with a novel interaction partner than male Val68BDNF mice ($p < 0.01$). Two-tailed Student's 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. 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 place preference/aversion 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 and the time spend in the social and alcohol chambers were recorded and quantified. (**B**) Male Met68BDNF mice (red) spent significantly less time in the social-paired chamber in the post-test than they did 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 place preference or aversion. **C**) Representative heatmaps of mouse position for Val68BDNF and Met68BDNF cohorts during the social-alcohol CPP/CPA shown in (**B**). The social chamber is depicted on the left and the alcohol chamber is depicted on the right for each heatmap. The upper portion of the heatmap (above the horizontal dotted line) represents the connecting hallway between chambers. 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 Val68BDNF and Met68BDNF mice exhibit alcohol place preference

Mice underwent an alcohol place preference paradigm in which they were first able to freely explore both chambers (pre-test). On alternating conditioning days, mice were placed in a saline- or alcohol-paired chamber after receiving an i.p. injection of saline or 2 g/kg of alcohol. During the post-test day, mice were once again allowed to freely explore the entire apparatus and time spend in each of the chambers were recorded and quantified. (**B**) Male Val68BDNF (blue) and Met68BDNF (red) mice exhibit a significantly higher preference 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 the sedative effects of alcohol

(**A**) 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 was significantly greater in male Met68BDNF mice (red) than male Val68BDNF mice (blue) ($p < 0.05$). Two-tailed Student's t test, $t = 2.439$, $df = 13$. (**B**) The total duration of LORR for male Met68BDNF mice (red) was significantly shorter than it was 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: n = 7, Met68BDNF: n = 8.

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

(**A**) Ten minutes following i.p. injection of saline or alcohol (1.25 g/kg), mice were placed in the center of the elevated plus maze and allowed to freely explore the apparatus. The time mice spent in the open arms (**B**) and in the distal portion of the open arms (**C**) was measured. (**B-C**) Following an i.p. administration of alcohol (hatched bars), female (green) and male (blue) Val68BDNF mice and female Met68BDNF mice (orange) spent significantly more time in the open arm (**B**) and the distal portion of the open arm (**C**) than upon receiving an injection of saline (open bars). Male Met68BDNF mice (red) exploration time in the open arm (**B**) and the distal portion of the open arm (**C**) was unaltered following a systemic administration of alcohol (hatched bars) or saline (empty bars). (**B**) Two-way ANOVA, main effect of alcohol treatment, $F(1, 75) = 64.07$, $p < 0.0001$. (**C**) 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 signal in the vHC of a Met68BDNF mouse that received AAV-Val68BDNF in the vHC. (**B-C**). Male Met68BDNF that received AAV-GFP (green) in the vHC spend similar time in the open arm (**B**) or distal open arm (**C**) following i.p. injection of alcohol (1.25 g/kg) (hatched bars) as compared to male Met68BDNF mice receiving saline (open bars). Met68BDNF mice that received AAV-Val68BDNF (purple) in the vHC spent significantly

more time in the open arm (**B**) or distal open arm (**C**) following a systemic administration of alcohol (hatched bars) compared to the administration of saline (open bars). (**B**) 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**) 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.

Supplemental Figure 1. Alcohol does not alter locomotion in the elevated plus maze test in Val68BDNF and Met68BDNF mice

(**A**) Male Met68BDNF (red) mice travel the same distance on the elevated plus maze as male Val68BDNF (blue) mice following i.p. injection of saline (empty bars) or 1.25 g/kg of alcohol (hashed bars). Female Val68BDNF (green) and Met68BDNF (orange) mice also travel the same distance i.p. injection of saline (empty bars) or 1.25 g/kg of alcohol (hatched bars). Two-way ANOVA, main effect of alcohol treatment, $F(3, 75) = 13.34$, $p < 0.0001$; main effect of sex $(1,75) = 10.93$, $p < 0.01$. Data are represented as mean ± SEM. 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.

Supplemental Figure 2. BDNF overexpression in the vHC does not impact alcohol-dependent locomotion in the elevated plus maze

(**A**) Mal Met68BDNF mice that received AAV-Val68BDNF in the vHC (purple) move the same distance as Male Met68BDNF mice that received AAV-GFP in the vHC (green) following i.p. injection of either saline (empty bars) or 1.25 g/kg of alcohol (hatched bars). Two-way ANOVA, main effect of alcohol

treatment, $F(1, 38) = 10.64$, $p < 0.01$. Data represented as mean ± SEM. Male Met68BDNF + AAV-GFP: n = 11, Male Met68BDNF + AAV-Val68BDNF: n = 10.

Figure 1

Male Met68BDNF mice exhibit social novelty anxiety-like behavior

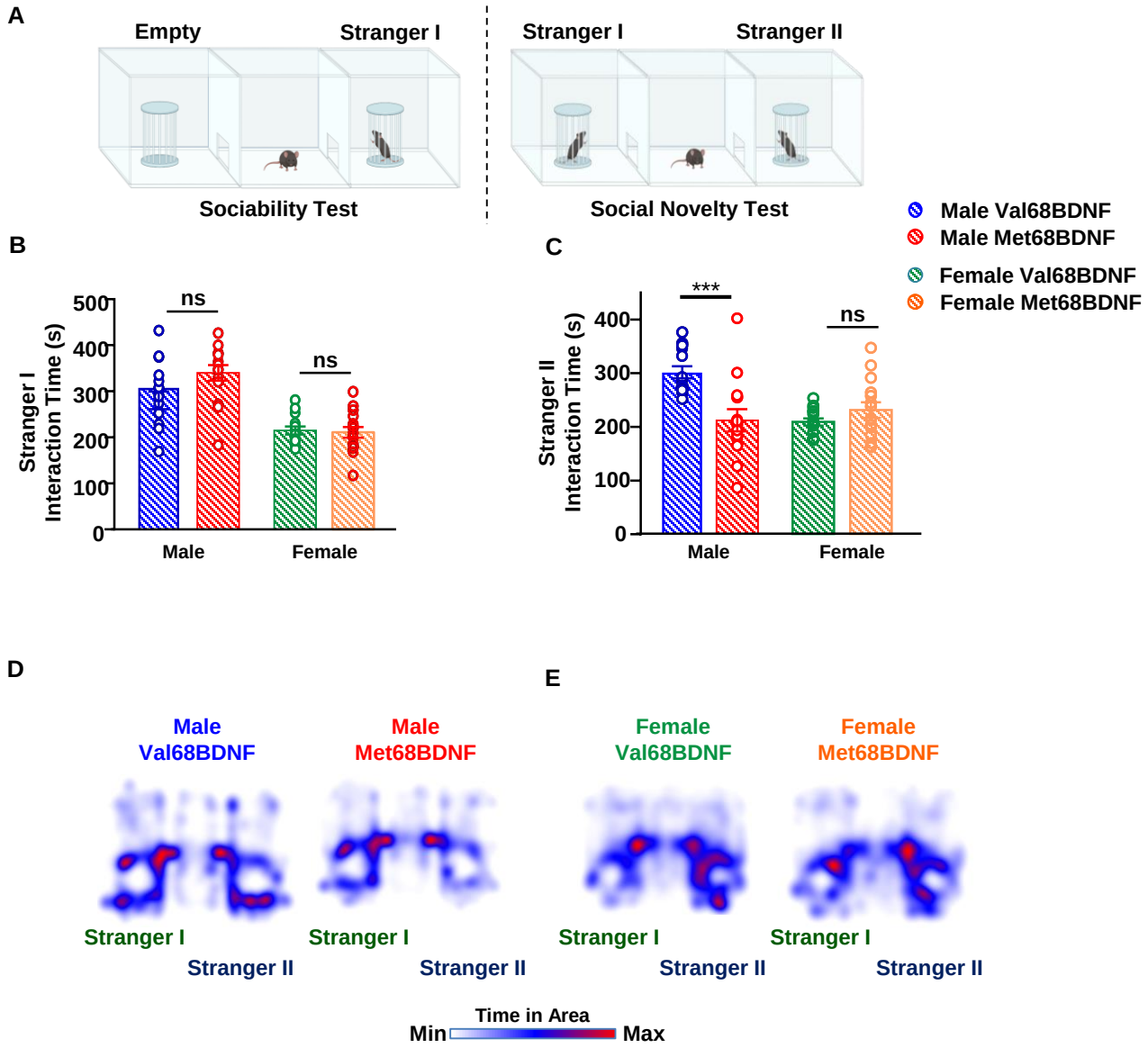
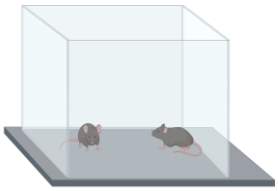


Figure 2

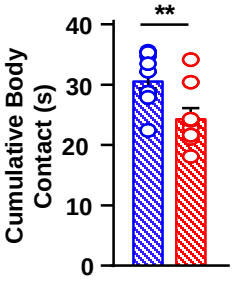
Male Met68BDNF mice exhibit social interaction anxiety-like behavior

A



Open Field Social Interaction

B



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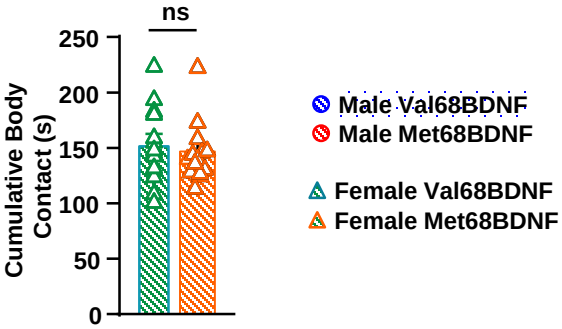


Figure 3

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

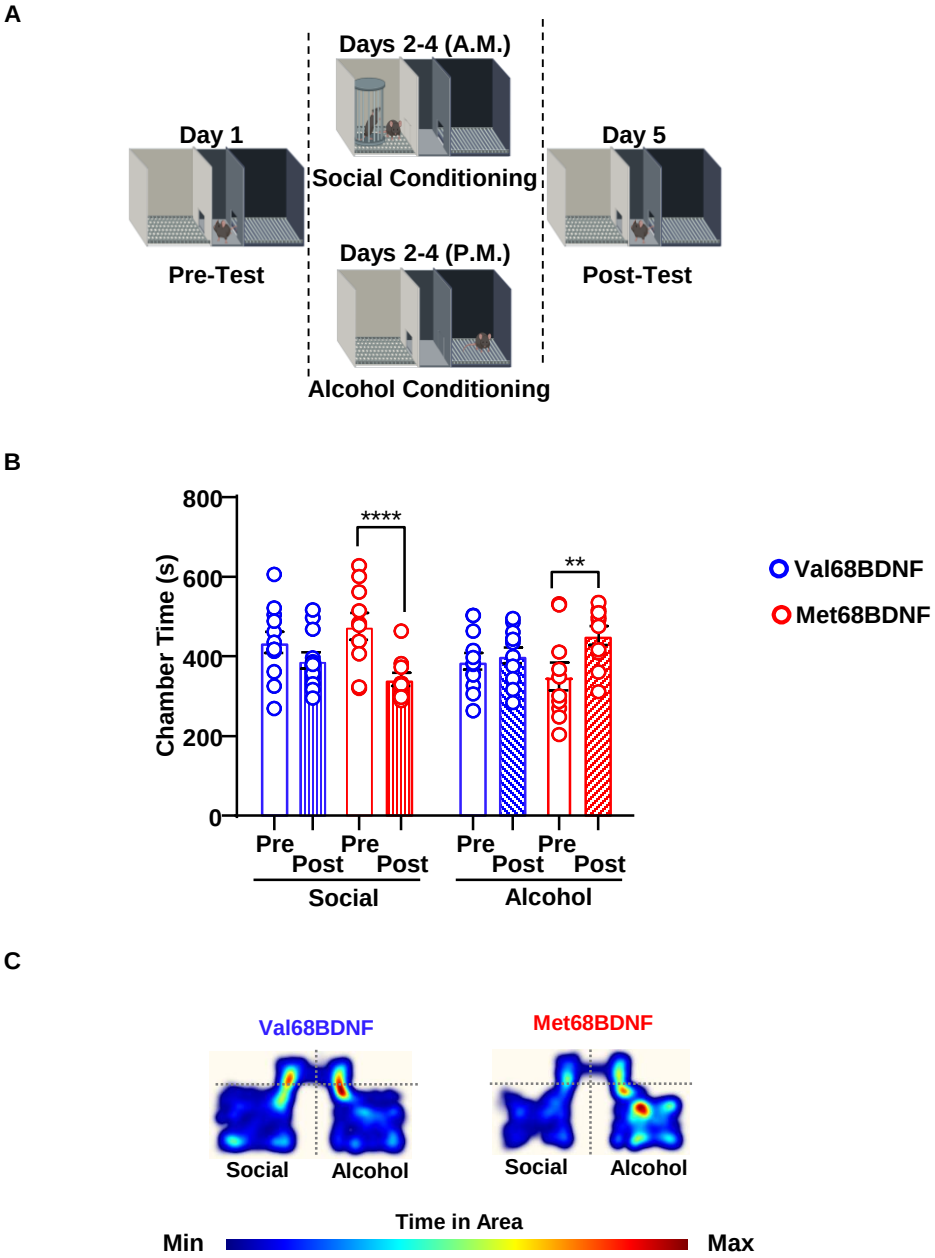


Figure 4

Male Val68BDNF and Met68BDNF mice exhibit alcohol place preference

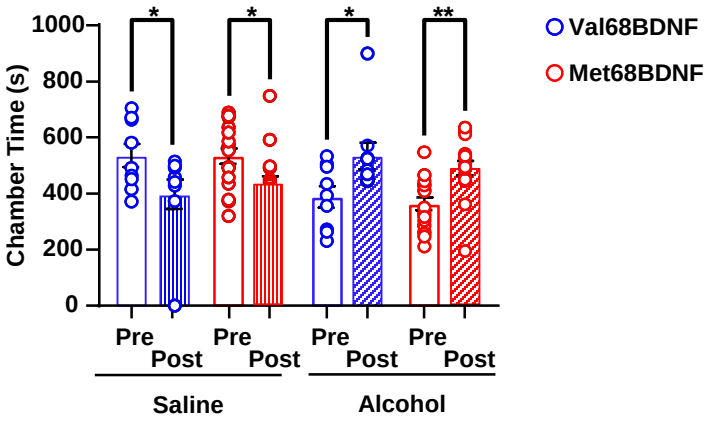


Figure 5

Male Met68BDNF mice are resistant to the sedative effects of alcohol

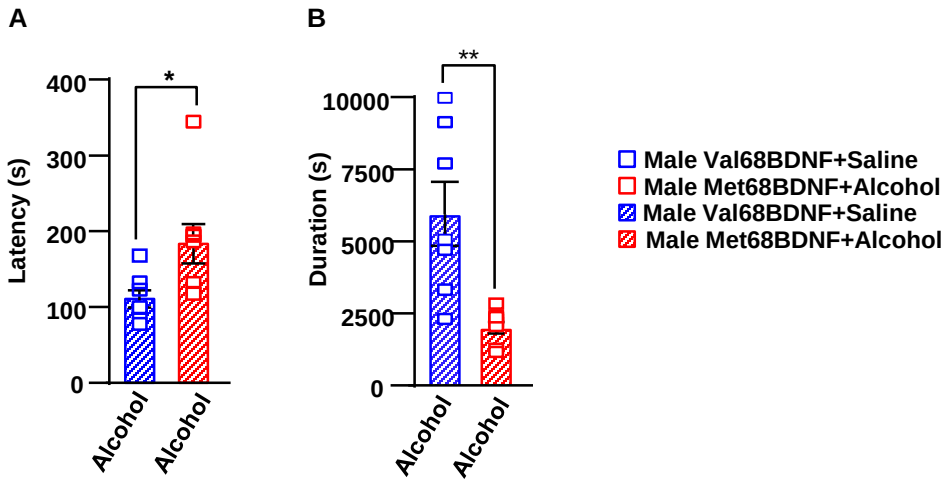


Figure 6

Male Met68BDNF exhibit acute tolerance to the anxiolytic action of alcohol

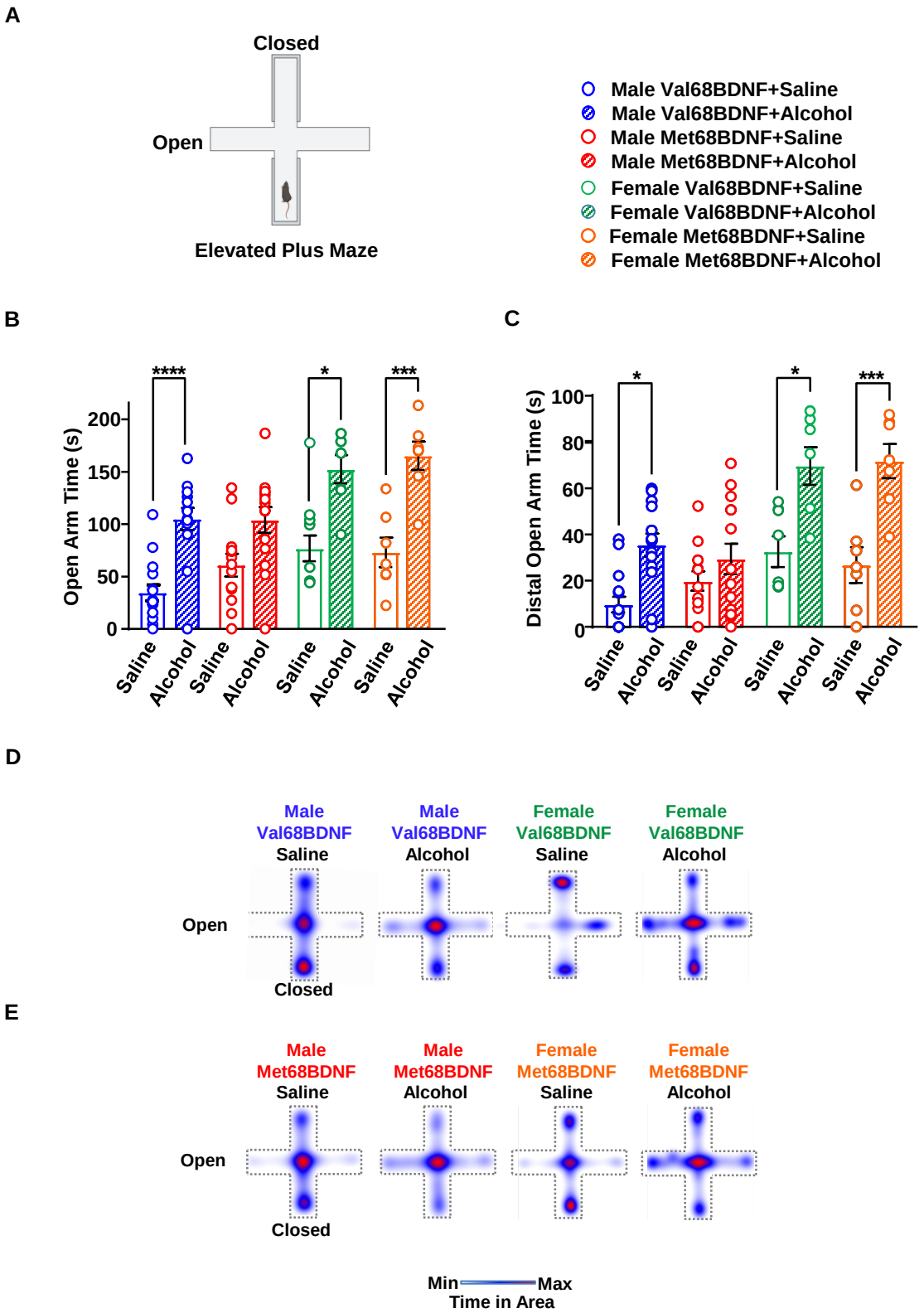
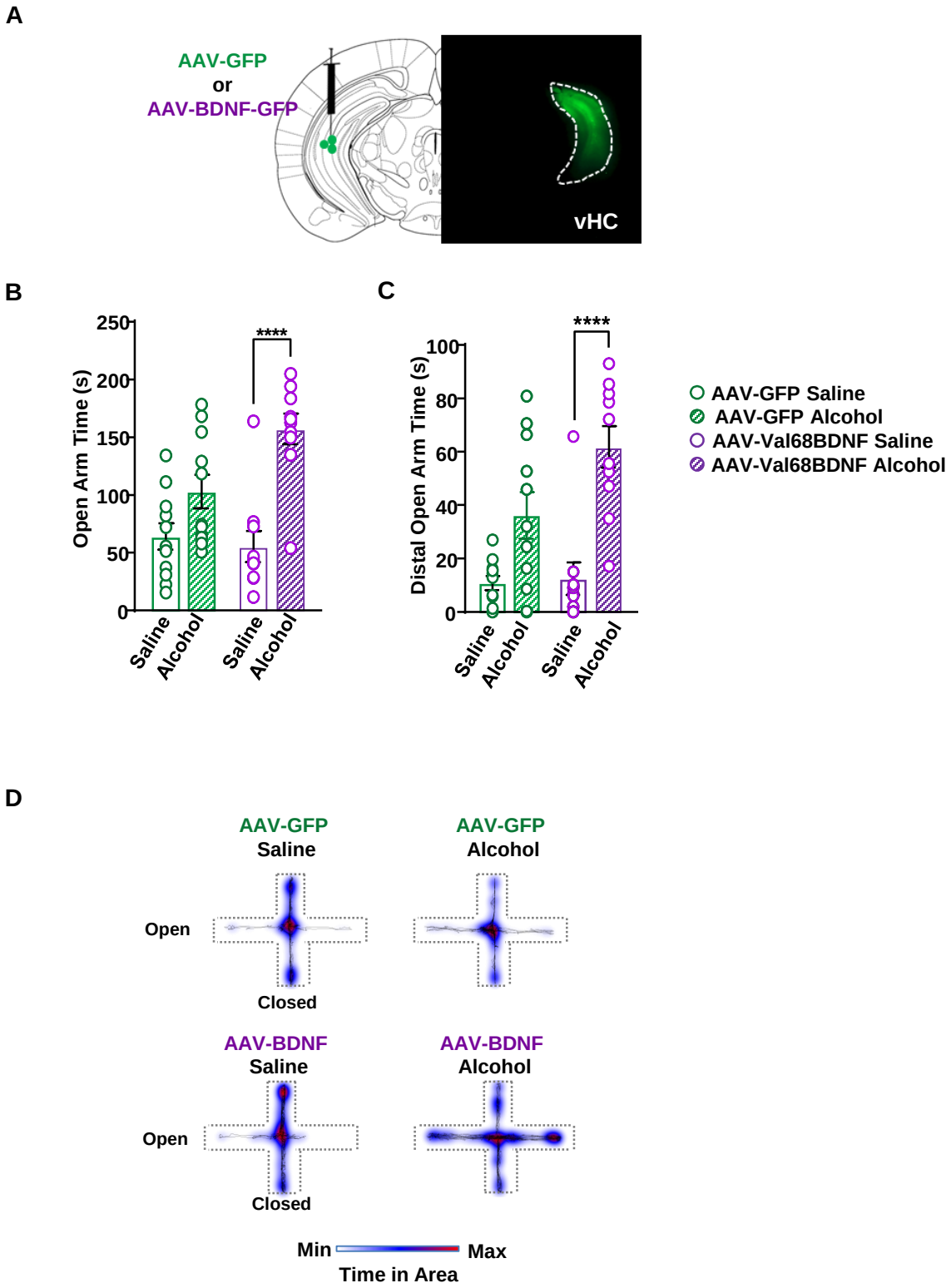


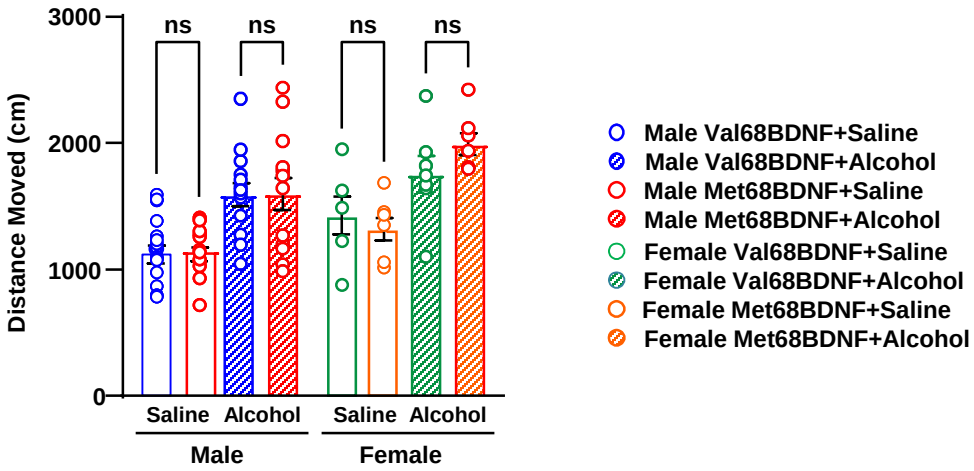
Figure 7

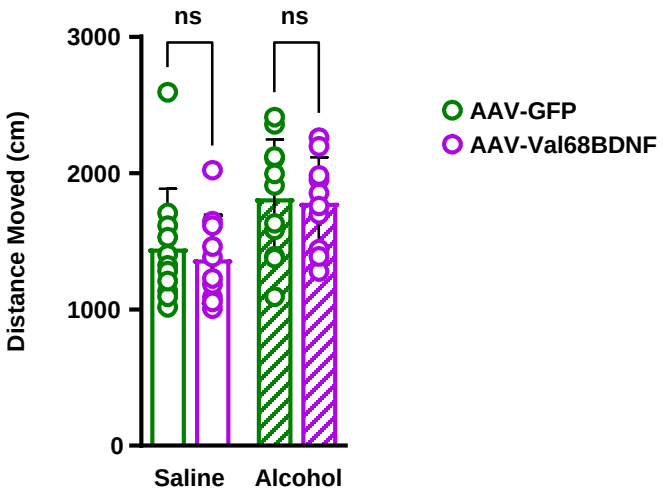
Overexpressing Val68BDNF in the vHC of male Met68BDNF mice rescues the anxiolytic effect of alcohol



Supplemental Figure 1

Alcohol does not alter locomotion in the elevated plus maze test in Val68BDNF and Met68BDNF mice



Supplemental Figure 2

BDNF overexpression in the vHC does not impact alcohol-dependent locomotion in the elevated plus maze test