The *Bdnf* Val68 to Met Polymorphism Increases Compulsive Alcohol Drinking in Mice Which Is Reversed by TrkB Activation

Supplemental Information

Supplemental Methods

Generation of Met68BDNF KI Mice

Mice carrying the Valine to Methionine mutated *Bdnf* gene (Met68BDNF) were generated by Taconic (Hudson, NY; previously, Xenogene) on a pure C57BL/6 strain (Figure S2). Sequence comparison between human and mouse *Bdnf* cDNA indicated that Val66 in human cDNA is homologous to Val68 in mouse cDNA (http://weblab.cbi.pku.edu.cn (1)). The mouse genomic BAC clone RP23-393E8 was obtained from Invitrogen (Carlsbad, CA). A 4.4 kb genomic fragment and a 7.7 kb genomic fragment were subcloned from the mouse BAC clone using a recombination-mediated genetic engineering (recombineering) method for 5' and 3' homology arms, respectively. The mouse Met68BDNF mutation was generated in the 3' homology arm by changing the 68th codon for valine (GTC) to methionine (ATG). This gene fragment was then incorporated into a targeting vector containing a neomycin-resistance cassette (Neo) flanked by two LoxP sites (Figure S2A). The target vector was electroporated into B6-3 embryonic stem (ES) cells. Electroporated ES cells were selected and correct gene targeting was confirmed by Southern blotting (Figure S2B). FVB/N blastocysts were injected with correctly targeted ES cells, resulting in chimeric offspring. Male chimeras were bred with female C57BL/6 mice to produce germline-transmitted heterozygote mice (Val/Met), which were then bred with EIIa-Cre mice to excise the Neo cassette. Heterozygote mice (Val/Met) in which the Neo cassette had been successfully removed were then used to set up breeding pairs with C57BL/6 mice.

Heterozygote mice (Val/Met) were therefore used to generate homozygote Met68BDNF (Met/Met) and Val68BDNF (Val/Val) mice.

Animals

Mice were housed under a 12-hr light/dark cycle (lights on at 7:00 AM). Food and water were available ad libitum, and environmental conditions were maintained at a constant temperature (23°C) and humidity (50%). Three-month-old male mice were used in the studies. All procedures involving animals were approved by the University of California San Francisco Institutional Animal Care and Use Committee and were conducted in agreement with the Association for Assessment and Accreditation of Laboratory Animal Care.

Materials

LM22A-4 (N,N,N-tris [2-hydroxyethyl]-1,3,5-benzene tricarboxamide) was developed and synthesized as described in Massa *et al.* (2). Precast NuPAGE Bis-Tris gels, and the Alexa Fluor-488 (catalog #: a-21206) and Alexa Fluor-594 (a-21203) antibodies were purchased from Invitrogen (Carlsbad, CA). Anti-Actin (sc-1616) and anti-BDNF (sc-546) polyclonal antibodies as well as the horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-GFP polyclonal antibody (ab-290) was purchased from Abcam (Cambridge, MA). The anti-NeuN monoclonal antibody (mab-377) was purchased from Millipore (Billerica, MA). Rabbit anti-TrkB antibodies were purchased from Upstate (now EMD Millipore, Billerica, MA). Phosphatase inhibitor cocktails 2 and 3, the anti-GFAP monoclonal antibody (g-3893), saccharin, and quinine were purchased from Sigma-Aldrich (St. Louis, MO). Enhanced chemiluminescence (ECL) reagents were purchased from

Fisher Scientific (Pittsburgh, PA). The bicinchoninic acid protein assay kit was obtained from Pierce Biotechnology (Rockland, IL). The EDTA-free Complete mini protease inhibitor cocktail tablet was purchased from Roche (Indianapolis, IN).

Western Blot Analysis

Mice were killed by cervical dislocation and brains rapidly removed and placed on an ice-cold platform. The vmPFC was dissected and immediately homogenized in ice-cold radio immunoprecipitation assay buffer (containing, in mM: 50 Tris-HCl, 5 EDTA, 120 NaCl, and 1% NP-40, 0.1% deoxycholate, 0.5% SDS, protease and phosphatase inhibitor cocktail inhibitors). Samples were then briefly sonicated using a sonic dismembrator and placed on ice for 30 min. Samples (40 mg) were resolved on NuPAGE TM 4-12% Bis-Tris gradient. After a 2-hr transfer onto nitrocellulose membranes, blots were blocked in 5% milk/TBS-T for 1 hr and probed with anti-BDNF (1:1000), or anti-TrkB (1:1000) or anti-Actin (1:1000) antibodies over night at 4°C. Membranes were then washed and probed with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected using ECL. The optical density of the relevant immunoreactive band was quantified using NIH ImageJ.

Preparation of Solutions

Alcohol solutions were prepared from absolute anhydrous ethyl alcohol (190 proof) diluted to 10% or 20% (v/v) in tap water. The 0.03% saccharin solution was prepared with tap water. Quinine was added to tap water, alcohol and saccharin solutions at concentrations of 0.10, 0.15, 0.20 and 0.30 g/l.

Blood Alcohol Concentration (BAC) Measurements

Trunk blood was collected in heparinized capillary tubes 90 minutes after an intraperitoneal (i.p.) administration of 2.5 g/kg of alcohol (20% v/v in saline). Serum was extracted with 3.4% trichloroacetic acid followed by a 5-minute centrifugation at 420 × *g* and assayed for alcohol content using the NAD^+-NADH enzyme spectrophotometric method (3,4). BACs of serum samples were determined using a standard calibration curve.

Systemic Administration of Drugs

Before the beginning of experiments, mice were habituated to the i.p. administration procedure with a daily injection of saline (0.9% NaCl w/v; Hospira, Lake Forest, IL) for 3 days. On test day, mice received either an i.p. administration of alcohol (20% v/v prepared in 0.9% saline) or saline control (0.9% NaCl). LM22A-4 (prepared in 0.9% saline) was i.p. administered immediately before the beginning of the drinking session at the dose indicated or saline was used for control treatments.

Intermittent Access to 0.03% Saccharin Solution

After 1 week of habituation to single housing, mice received intermittent access to saccharin solution (0.03%; w/v). Access to saccharin followed the same weekly intermittent access schedule for alcohol as described in the main text. After 3 weeks, the saccharin solution was adulterated with increasing concentrations of quinine (0.10-0.30 g/l). The concentration of quinine was increased every 2 weeks. Saccharin solution drinking (ml/kg of body weight), total fluid intake (ml/kg of body weight) and the preference ratio (volume of saccharin solution intake/total volume of fluid intake) were recorded after 24 hrs of saccharin access.

Intermittent Access to Quinine Solution

After 1 week of habituation to single housing, mice had intermittent access to 0.10 g/l quinine in tap water solution following the same weekly intermittent access schedule as described for alcohol. After 2 weeks, the concentration of quinine was increased to 0.15 g/l. Quinine solution drinking (ml/kg of body weight), total fluid intake (ml/kg of body weight) and the preference ratio (volume of quinine solution intake/total volume of fluid intake) was recorded after 24 hrs of quinine access.

Testing Compulsive-Like Behavior in the T-Maze

The procedure used here was adapted from Yadin, Friedman, and Bridger (5). The T-maze apparatus consists of two distinctive goal arms, and one start arm. Each arm (including the start arm) measures 29 cm × 5.5 cm × 15 cm, while the central zone measures 5.5 cm × 5.5 cm × 15 cm. To ensure motivation, mice were food-restricted and maintained at 90% of their free-feeding body weight. Mice were exposed ad libitum to chocolate milk solution (0.5 ml) in their home cages to familiarize them with the taste of the solution and avoid neophobia. Animals were then confined once a day for 10 minutes in each of the two goal arms (counterbalanced for order of placement) where 100 µl chocolate milk solution was available over the course of two consecutive days. The following day, individual mice were placed on the start arm with free access to the two goal arms, both of which contained a chocolate milk solution. After drinking the chocolate milk for 5 seconds, each mouse was removed and placed in a holding cage for 10 seconds, after which a new trial was initiated. A maximum of 7 trials per animal were conducted. The latency to reach the reward and the right/left choice made by each mouse was recorded. The

level of compulsive-like behavior was assessed by using the number of goal arm choices made until an alternation occurred. According to this scoring rubric, an animal with very low compulsive-like behavior alternated choice after 2 trials, corresponding to a score of 1.0. In contrast, an animal showing perseverance in the same choice (i.e., a high level of compulsivity) would achieve score of 7.0.

Anxiety Assessment Using the Elevated Plus Maze (EPM) Paradigm

The EPM apparatus consists of two open and two closed arms (30 × 5 cm) with walls 15 cm high and is elevated 40 cm above the ground. The arms extend from a central platform (5 × 5 cm) forming a plus sign. EPM testing took place in a quiet, dimly illuminated room (60 lux at the level of the EPM). Each mouse was tested for 5 min after being placed onto the center platform facing an open arm. Between animals, the EPM apparatus was cleaned with water. The number of open and closed arm entries and the time spent on the various sections of the EPM was scored. Arm entries were scored when an animal put all four paws into the arm. At the end of the test, the number of entries into and the time spent on the open arms are expressed as a percentage of the total number of arm entries and test duration, respectively. The closed and the total number of arm entries were taken as indices of ambulatory activity. Each animal was recorded and behavior later scored by a trained observer blind to genotypes.

Motor Coordination Assessment Using a Rotarod Test

Mice were first trained for two consecutive days to remain on a rotarod (Accurotor; AccuScan Instruments, Columbus, OH) rotating at 10 rpm. The maximum time spend on the rotarod on any given trial was capped 3 minutes. Mice underwent several trials per day until reaching this

criterion. On the third day, mice were initially tested to ensure their ability to stay on the rod at the 3 minutes criterion, after which they were systemically injected with 1.5 g/kg alcohol. Mice were subsequently tested every 15 minutes for 120 minutes. The latency to fall from the rod was recorded in each trial.

Spontaneous Locomotor Activity

Spontaneous locomotor activity was measured in activity-monitoring chambers (43 × 43 cm; Med Associates, St. Albans, VT). Using horizontal photobeams, animal locomotor activity was monitored, including the total distance traveled (cm). Each mouse was recorded for 30 min, after which the animal was removed and the apparatus cleaned between test sessions.

Construction, Preparation, and Infusion of Adeno-Associated Virus (AAV) Overexpressing BDNF into the Mouse vmPFC

The complete *Bdnf* coding DNA sequence was subcloned into the EcoRI restriction enzyme site of the pAAV-IRES-hrGFP vector (Agilent Technologies, Santa Clara, CA). Expression of *Val68BDNF* was driven by the CMV promoter. Empty pAAV-IRES-hrGFP vector was used to construct the control virus. Both constructs contained a fluorescent hrGFP reporter. Adeno-associated virus vector serotype 1/2 (AAV1/2) overexpressing BDNF (AAV-BDNF) and the GFP control virus (AAV-GFP) were produced and purified by the Gene Therapy Center Virus Vector Core Facility at the University of North Carolina.

Surgery. Mice were anesthetized by systemic administration of a mixture of ketamine (120 mg/kg) and xylazine (8 mg/kg). Mice were placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and two holes were drilled above the site of viral injection

(anteroposterior, +2.4 mm from bregma; lateral, ±0.35 mm from bregma). The injectors (stainless tubing, 33 gauges; Small Parts Incorporated, Logansport, IN) were slowly lowered into the vmPFC (dorsoventral, -2.25 mm from bregma). The injectors were connected to Hamilton syringes (10 µl; 1701, Harvard Apparatus, Holliston, MA), and the infusion was controlled by an automatic pump (Harvard Apparatus, Holliston, MA). Mice received 1.0 µl (0.1 µl/min) of AAV-BDNF or AAV-GFP (1 x 10^{12} TU/ml per side). The injectors remained in place for an additional 10 minutes to allow the virus to diffuse, and then were slowly removed. Mice were allowed to recover in their home cages for at least 5 days before further testing.

Immunohistochemistry

At the end of behavioral experiments, mice were deeply anesthetized with Euthasol and perfused with 0.9% NaCl, followed by 4% paraformaldehyde (PFA) in phosphate buffer, pH 7.4. Brains were removed, fixed in PFA for 2 hrs, and transferred to PBS at 4°C. The following day, brains were transferred into 30% sucrose solution and stored for 3 days at 4°C until saturated. Brains were then snap frozen and 50-µm-thick coronal sections were cut on a cryostat (Microm; Thermo Scientific, Wilmington, DE) and collected into 6-well dishes. Free-floating sections containing the injection site in the vmPFC were selected for staining. Sections were blocked with 5% normal donkey serum in PBS for 1 hr and then double labeled with primary antibodies, incubated for 24 hrs at 4°C on an orbital shaker. Sections were labeled for either neuronal marker, NeuN (anti-NeuN antibody, 1:250), or glial marker, GFAP (anti-GFAP antibody, 1:1000) in combination with anti-GFP antibody (1:10,000), diluted in PBS plus 3% BSA, and 0.05% Triton X-100. The sections were then washed 3 times for 5 minutes each in PBS followed by incubation for 4 hrs with the following secondary antibodies: Alexa Fluor 488-labeled donkey anti-rabbit

(1:500) and Alexa Fluor 594-labeled donkey anti-mouse (1:500). After staining, sections were washed 3 times for 5 minutes each in PBS, and mounted with Vectashield mounting medium. Images were acquired using a Zeiss LSM 510 META laser confocal microscope (Zeiss MicroImaging, Jena, Germany).

Data Analysis

Data was analyzed with either unpaired or paired *t*-test, or two-way ANOVAs with or without repeated measures (RM). Significant main effects and interactions of the ANOVAs were further investigated using Fischer LSD *post hoc* analysis or the method of contrasts. Statistical significance was set at $P < 0.05$. Data are presented as mean ± SEM.

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Homo s.   MTILFLTMVISYFGCMKAAPMKEANIRGQGGLAYPGVRTHGTLESVNGPK -50
Mus m.    MTILFLTMVISYFGCMKAAPMKEVNVHGQGNLAYPGVRTHGTLESVNGPR -50
                          66
          AGSRGL--TSLADTFEHVIEELLDEDQKVRPNEENNKDADLYTSRVMLSS -98
          AGSRGLTTTSLADTFEHVIEELLDEDQKVRPNEENHKDADLYTSRVMLSS -100
                          68
          QVPLEPPLLFLLEEYKNYLDAANMSMRVRRHSDPARRGELSVCDSISEWV -148
          QVPLEPPLLFLLEEYKNYLDAANMSMRVRRHSDPARRGELSVCDSISEWV -150

          TAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRG -198
          TAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGYTKEGCRG -200

          IDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR  -247
          IDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGR  -249
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Figure S1. Sequence alignments of *BDNF* from Homo Sapiens and Mus Musculus. Sequence alignments of *BDNF* from Homo sapiens (Homo s.) and Mus musculus (Mus m.) show the high sequence conservation. Alignments were performed using http://weblab.cbi.pku.edu.cn (1).

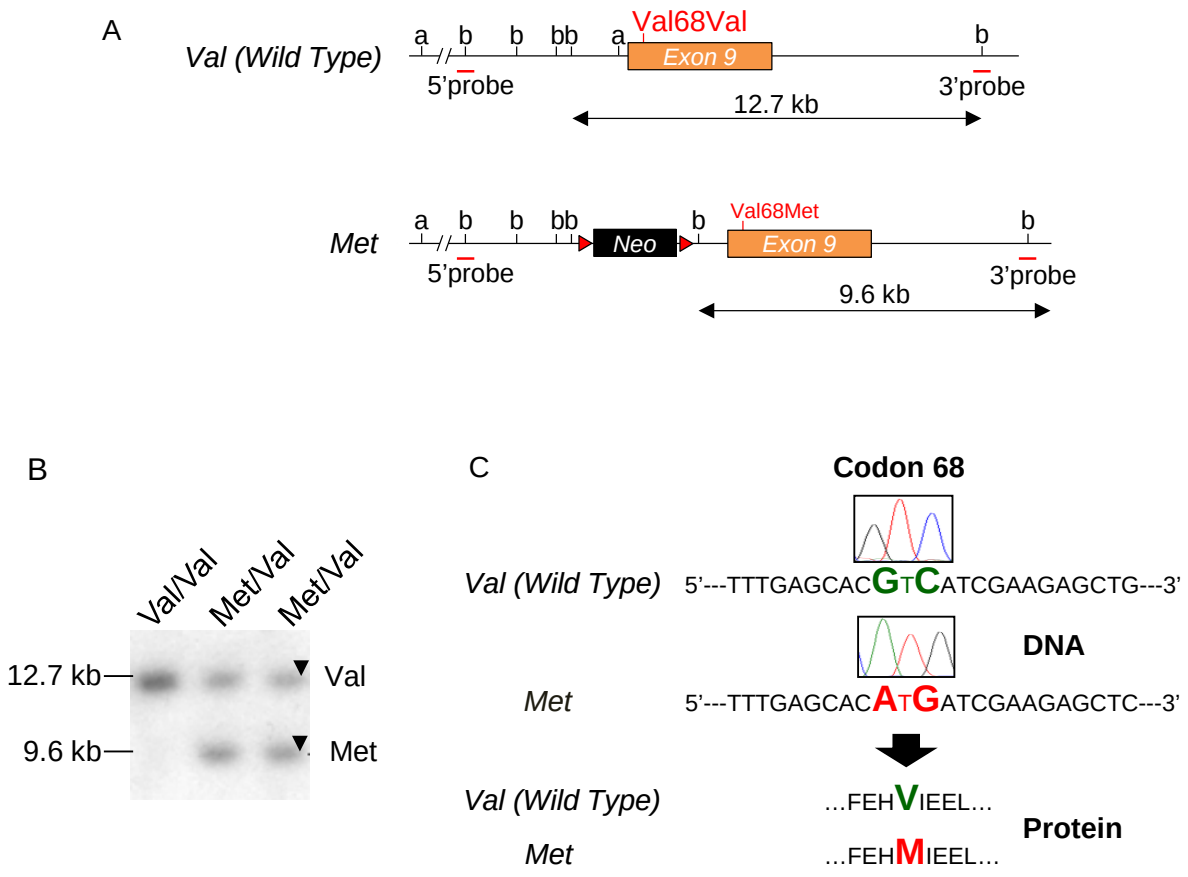

Figure S2. Generation and validation of the Met68BDNF mice. (**A**) Schematic diagram of the strategy used to introduce the Val68Met polymorphism into the mouse *Bdnf* gene. To obtain the amino acid substitution of Methionine (Met) in the place of Valine (Val), two point mutations at codon 68 were engineered: GTC à ATG. A neomycin resistance cassette (Neo) flanked by loxP sites (depicted as triangles) was also introduced with the Met68 allele. Restriction sites were as follows: a, Afl II and b, Bgl II. (**B**) Southern blots of representative recombined embryonic stem cell clones for Met68BDNF variant. The Bgl II restriction enzyme digestion and 3' probe indicated in (**B**) were used to detect the homologous replacement of the *Bdnf* gene. Image depicts the wild type allele (12.7 kb) and the knock-in allele (9.6 kb). (**C**) The upper panel shows the DNA sequencing results validating the nucleotide base pair mutations in the *Bdnf* gene. The lower panel confirms the Val/Met substitution in the protein at the codon 68.

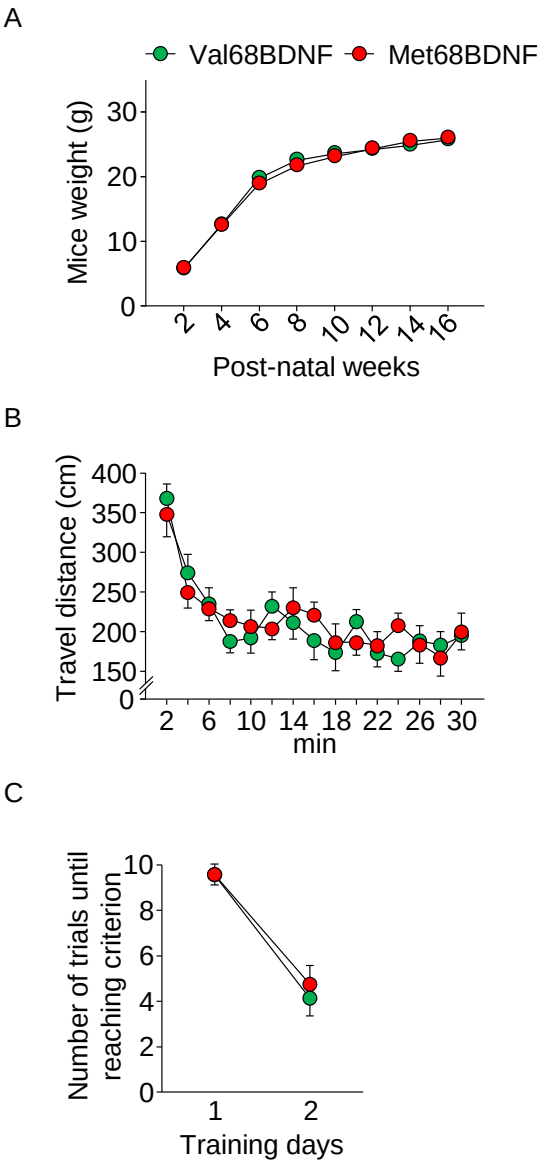

Figure S3. Met68BDNF polymorphism does not hinder gross development or basal locomotion, and sensorimotor coordination. (**A**) Growth curves of Met68BDNF and Val68BDNF mice during the first sixteen post-natal weeks. Mice were weighed every two weeks on the same day and at the same time. Met68BDNF polymorphism does not affect growth (two-way RM-ANOVA, $F_{(1,27)} = 0.27$, $P = 0.60$). (**B**) Basal locomotor activity was recorded for 30 minutes. No difference between genotypes was found (two-way RM-ANOVA, $F_{(1,29)} = 0.13$, $P = 0.72$). (**C**) Mice were trained for 2 consecutive days to perform the rotarod task. Data represent the number of trials necessary to reach the criterion of 180 seconds on the rod without falling during the 2 consecutive training days. There was no difference between genotypes in the acquisition of the task (two-way RM-ANOVA, $F_{(1,26)} = 0.30$, $P = 0.58$). Results are expressed as mean ± SEM. (**A**) $n = 15$ per genotype, (**B**) $n = 14$-17 per genotype and (**C**) $n = 11$-17 per genotype.

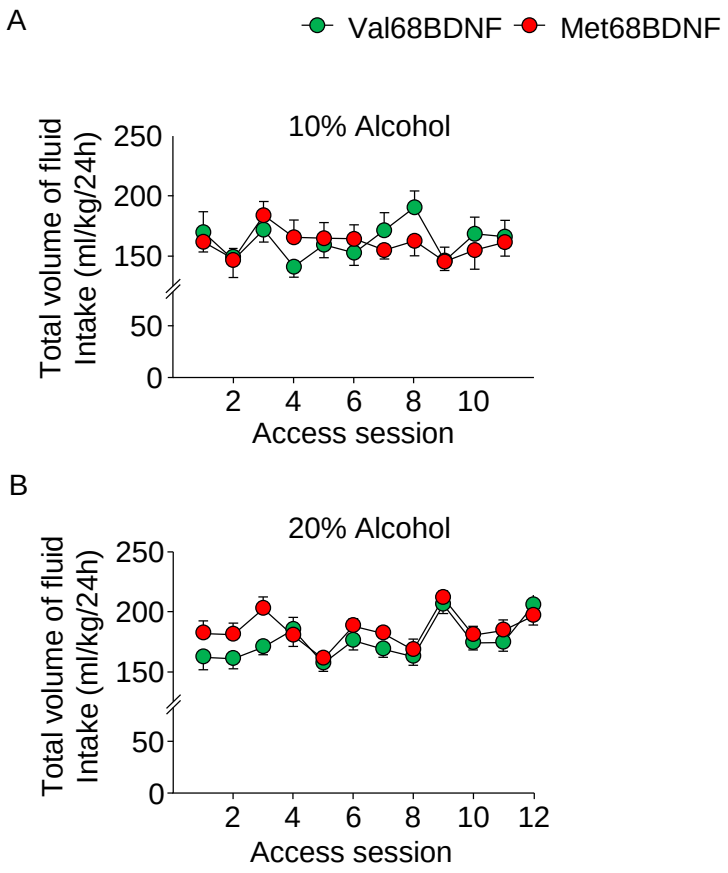

Figure S4. Total volume of fluid consumed during the intermittent alcohol drinking procedure. Total volume of fluid consumed by Met68BDNF and Val68BDNF mice during intermittent access to 10% alcohol (**A**), and intermittent access to 20% alcohol (**B**) procedures. No genotype differences were found: two-way RM-ANOVA, $F_{(1,36)} = 0.05$, $P = 0.82$ (**A**); and $F_{(1,35)} = 0.01$, $P = 0.92$ (**B**). Results are expressed as mean ± SEM. $n = 19$ (**A**) and 18-19 (**B**) per genotype.

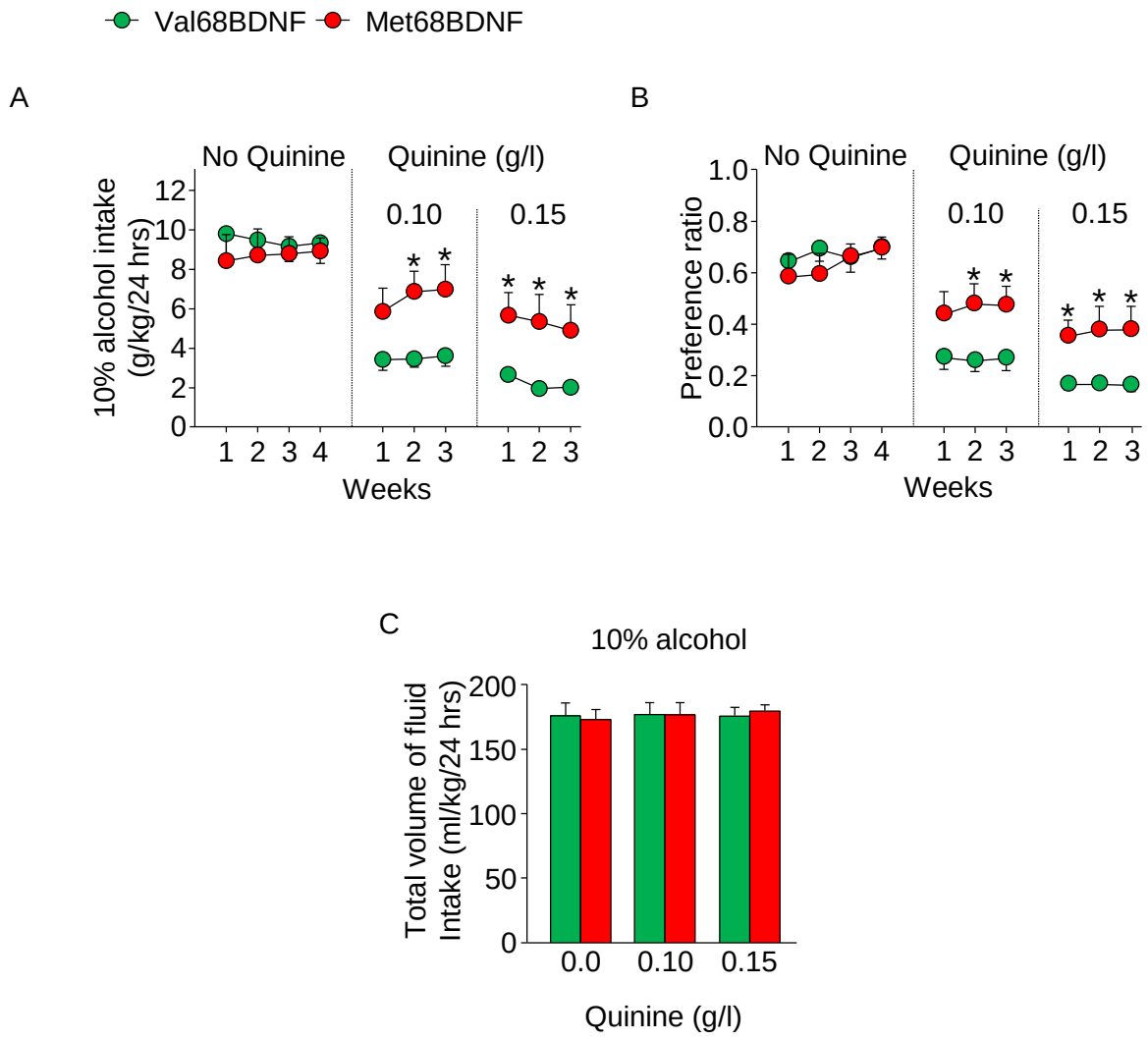

Figure S5. Weekly time course of 10% alcohol drinking before and after quinine adulteration and total fluid intake of Met68BDNF and Val68BDNF. After 4 weeks of intermittent access to 10% alcohol, quinine was added to the alcohol solution in increasing concentrations (0.0-0.15 g/l; each concentration was maintained for 3 weeks before being increased). Data shown are the weekly averages of intake (**A**; g/kg/24 hrs) and preference (**B**) for 10% alcohol and total fluid intake (**C**). Two-way RM-ANOVA showed a main effect of genotype on the level of intake (**A**; $F_{(1,20)} = 14.7$, $P = 0.001$) and preference for alcohol (**B**; $F_{(1,20)} = 11.0$, $P < 0.01$), and a main effect of quinine concentration on intake level (**A**; $F_{(3,60)} = 20.6$, $P < 0.001$) and preference for alcohol + quinine (**B**; $F_{(3,60)} = 13.7$, $P < 0.001$). (**C**) Total fluid intake during intermittent access of 10% alcohol solution, in presence or absence of quinine (0.10-0.15 g/l) Results are expressed as mean ± SEM; *$P < 0.05$ compared to Val68BDNF mice the same week, method of contrasts; n = 9-11 per genotype.

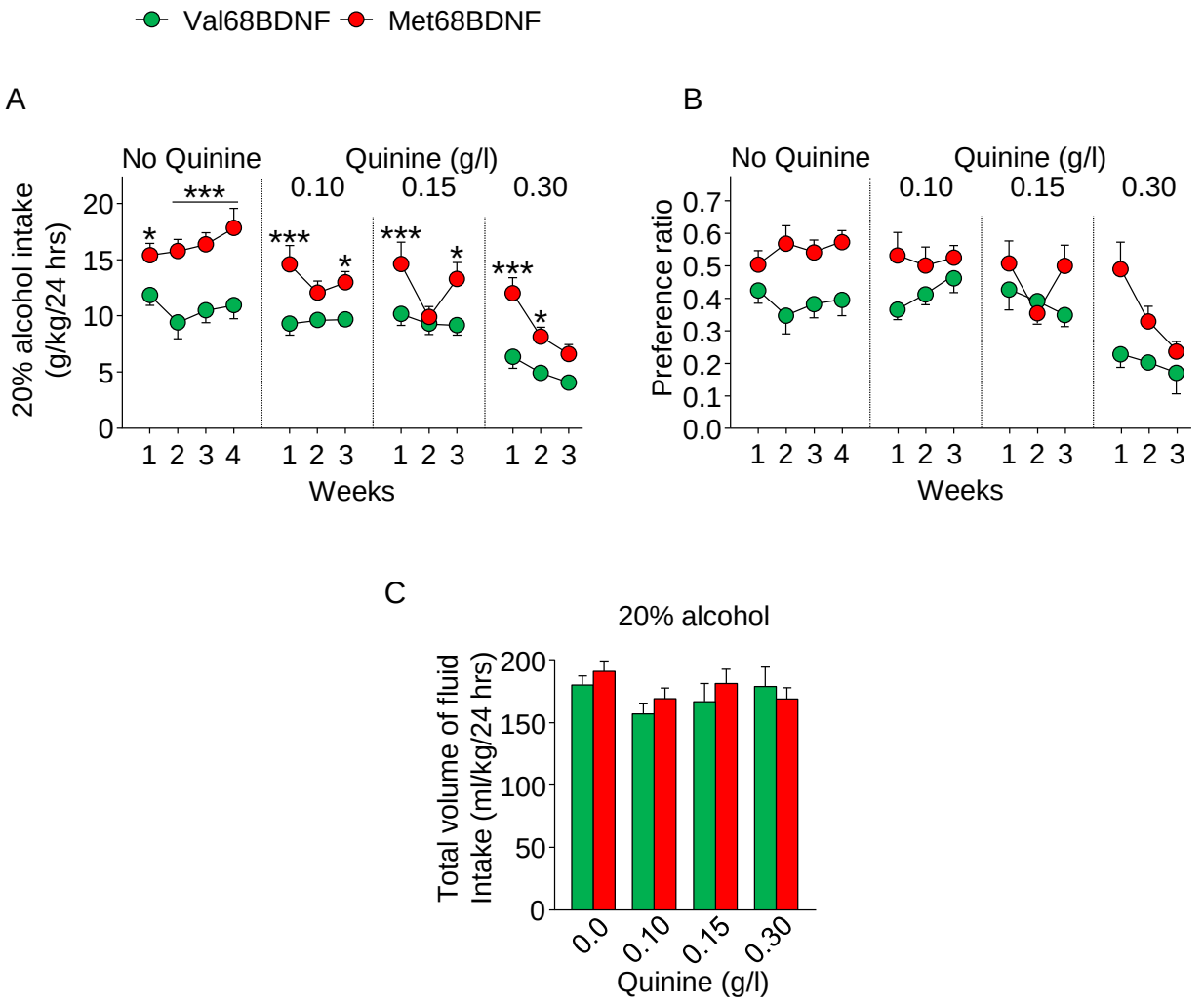

Figure S6. Weekly time course of 20% alcohol drinking before and after quinine adulteration and total fluid intake of Met68BDNF and Val68BDNF. After 4 weeks of intermittent access to 20% alcohol, quinine was added to the alcohol solution (0. 0-0.30 g/l, concentrations were increased every 3 weeks). Data shown are the weekly averages of 20% alcohol intake (**A**; g/kg/24 hrs) and preference (**B**) and total fluid intake (**C**). (**A**) Two-way RM-ANOVA, significant main effect of the genotype [$F_{(1,20)} = 18.35$, $P < 0.001$] and a significant interaction genotype x session [$F_{(12,240)} = 1.9$, $P < 0.05$]. (**B**) Two-way RM-ANOVA, with only a significant main effect of the genotype [$F_{(1,20)} = 11.44$, $P < 0.01$] as compared to the Val68BDNF mice before and after quinine adulteration of the 20% alcohol solution. (**C**) Total fluid intake during intermittent access of 20% alcohol solution, in presence or absence of quinine (0.10-0.15 g/l). Results are expressed as mean ± SEM; **P* < 0.05 and ****P* < 0.001 compared to Val68BDNF mice the same week, LSD post hoc test; *n* = 9-11 per genotype.

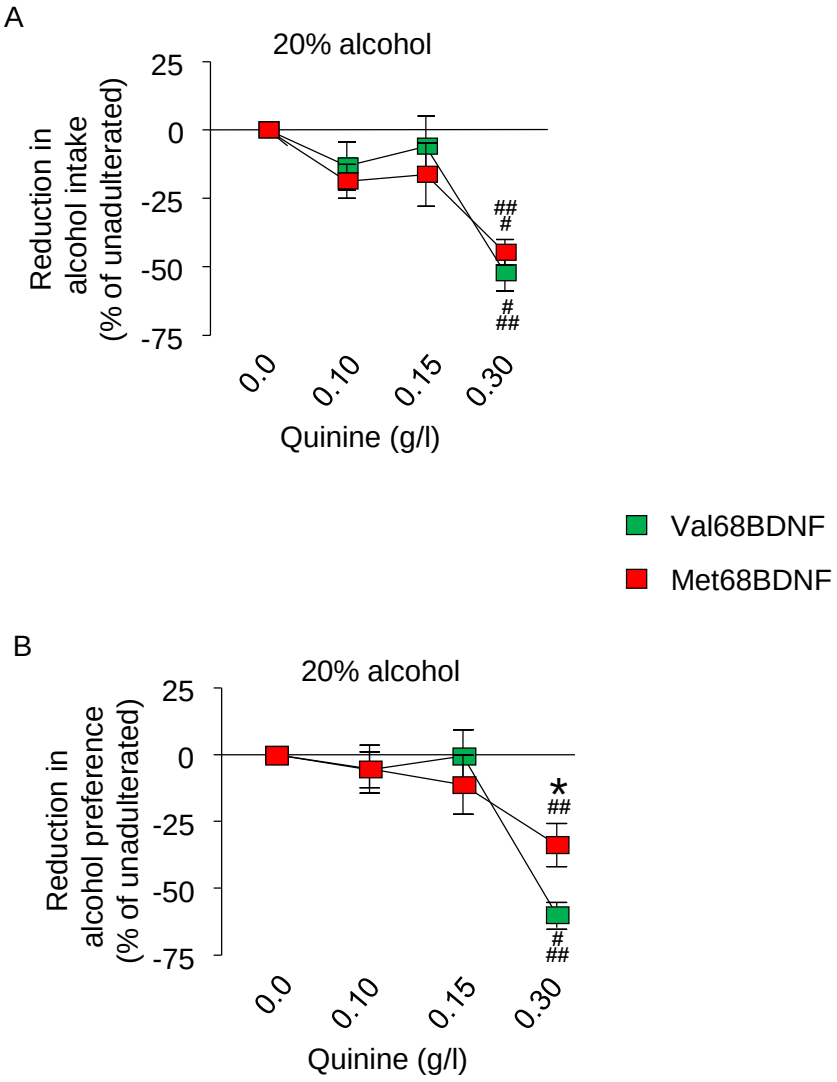

Figure S7. Reduction in intake and preference of a 20% alcohol solution in the presence or absence of quinine. Quinine concentration (0.0-0.30 g/l) was gradually increased to the 20% alcohol solution every 3 weeks. Intake of (**A**) and preference for (**B**) a 20% alcohol solution. Results are expressed as mean ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to 0.0 g/l quinine in the same genotype; $^{*}P < 0.05$ Met68BDNF mice compared to Val68BDNF at the same dose of quinine, method of contrasts; n = 9-11 per genotype.

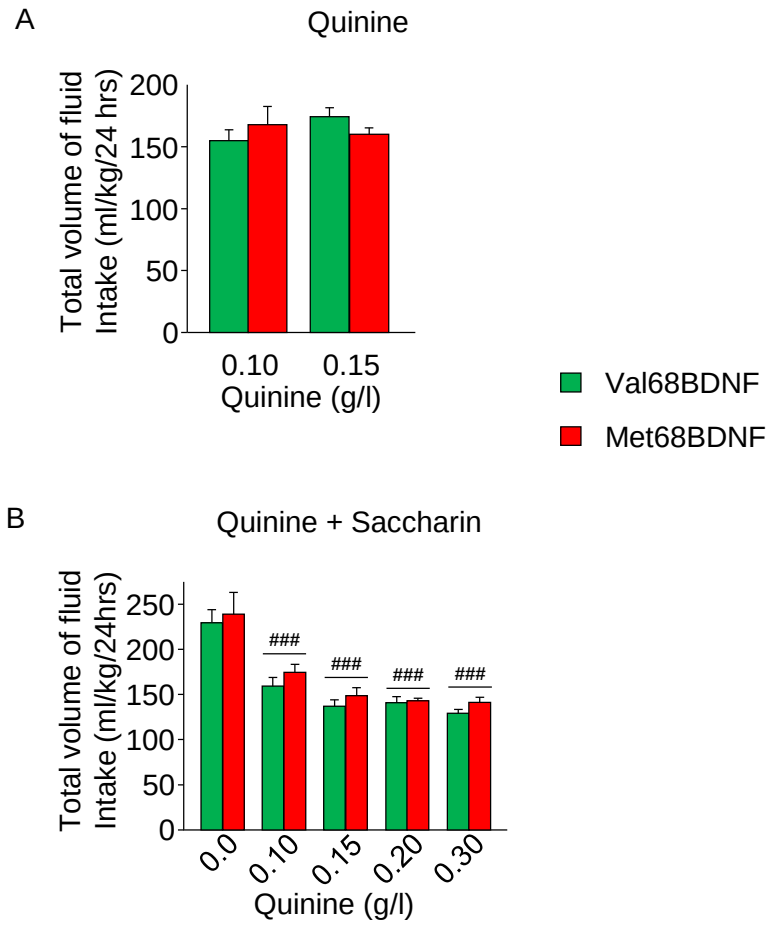

Figure S8. Total volume of fluid consumed during the quinine solution or quinine-adulterated saccharin solution drinking procedure. Total body fluid of quinine (**A**-**B**) in the absence or presence (**B**) of saccharin. (**A**) Two-way RM-ANOVA; $F_{(1,12)} = 0.19$, $P = 0.67$. (**B**) Two-way RM-ANOVA; $F_{(1,16)} = 0.05$, $P = 0.82$. Quinine-adulteration reduced total fluid intake as compared to drinking only saccharin ($F_{(4.48)} = 16.2$, $P < 0.001$). Results are expressed as mean ± SEM, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to saccharin drinking mice; (**A**) $n = 7$ per genotype and (**B**) $n = 7$ per genotype.

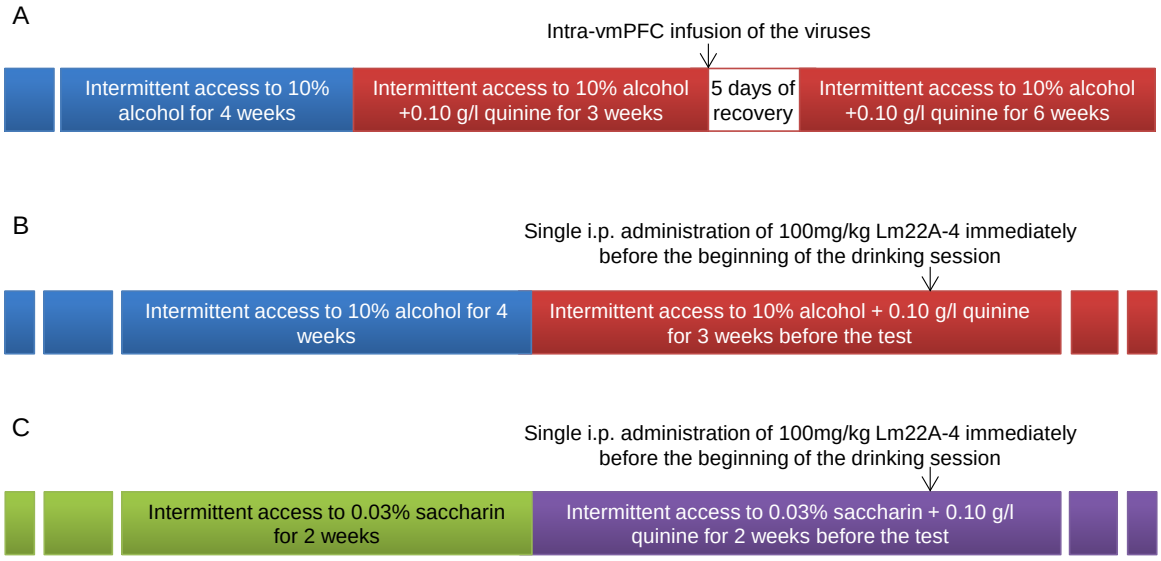

Figure S9. Timeline of the experience shown in Figures 6C-D and 7. (**A**) Timeline of the experiment shown in Figure 6C-D. Met68BDNF mice had intermittent access to 10% alcohol during 24 hrs access starting at 12:00 PM (on Mondays, Wednesdays, and Fridays) for 4 weeks followed by 3 weeks of intermittent access to a 10% alcohol containing 0.10 g/l quinine (alcohol+quinine) solution. AAV-BDNF or the AAV-GFP control was then bilaterally infused into the vmPFC. After 5 days of recovery, access to the alcohol+quinine solution was resumed and recorded over 6 weeks. (**B**) Timeline of the experiment shown in Figure 7A-B. Met68BDNF and Val68BDNF mice had intermittent access to 10% alcohol for 24 hrs starting at 12:00 PM (Mondays, Wednesdays, and Fridays) for 4 weeks. After this period, quinine (0.10 g/l) was added to the alcohol solution. (**C**) Timeline of the experiment shown in Figure 7C. Mice had intermittent access to 0.03% saccharin for 24 hrs starting at 12:00 PM (Mondays, Wednesdays, and Fridays) for 2 weeks, and then the saccharin solution was adulterated with quinine (0.10 g/l).

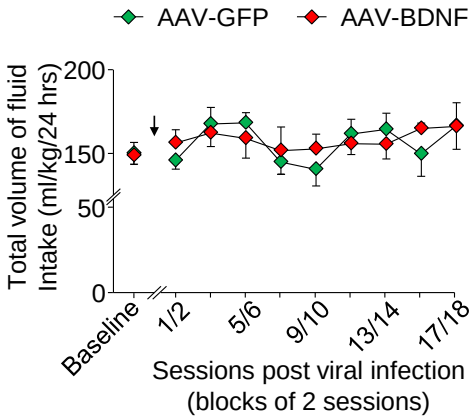

Figure S10. Total volume of fluid consumed during the alcohol drinking procedure in Met68BDNF mice overexpressing Val68BDNF in the vmPFC. Total volume of fluid intake by Met68BDNF mice during the access to 10% alcohol + quinine solution. Arrow indicates the time point of the intra-vmPFC infusion of the viruses. No effect of the overexpression of Val68BDNF in the vmPFC was found: two-way RM-ANOVA, $F_{(1,19)} = 0.05$, $P = 0.82$. Results are expressed as mean ± SEM. $n = 10$-11 per group.

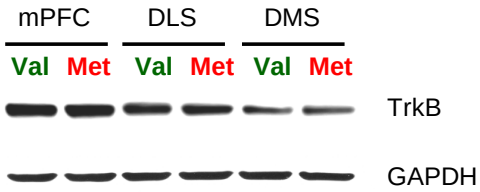

Figure S11. Levels of TrkB in the mPFC, DLS and DMS of Met68BDNF and Val68BDNF mice. TrkB levels in the mPFC (**A**), DLS (**B**) and DMS (**C**) of Met68BDNF and Val68BDNF were measured by western blot analysis. $n = 2$ per genotype.

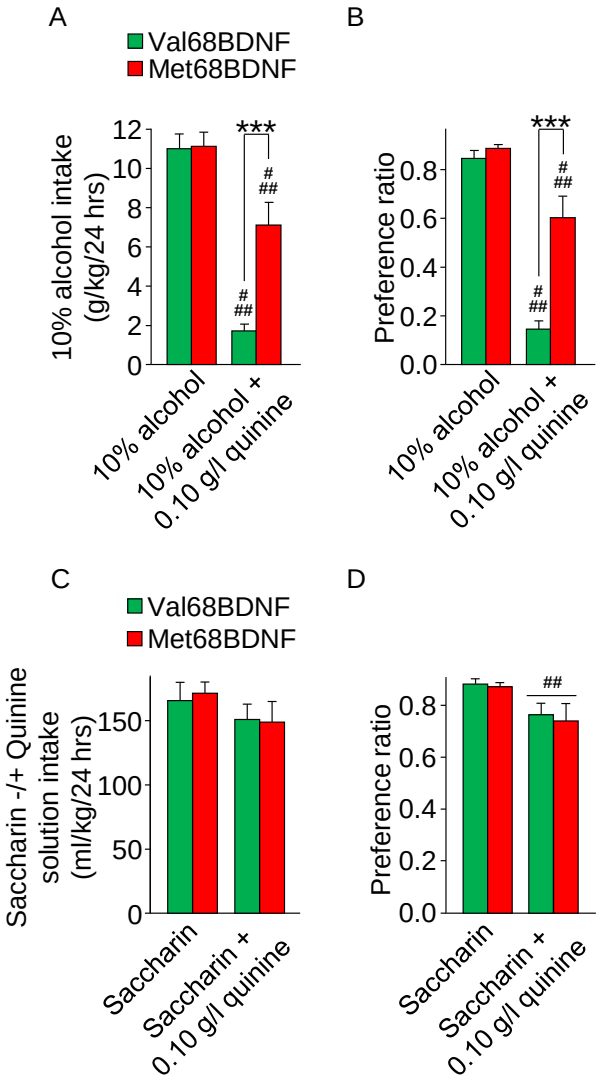

Figure S12. Baseline data of the experiments shown in Figure 7. (**A-B**) Data shown represent the level of alcohol drinking and preference pre- and post-adulteration of the 10% alcohol solution with quinine before testing LM22A-4. (**A**) Two-way RM-ANOVA, significant interaction genotype x quinine-adulteration [$F_{(1,14)} = 20.6$, $P < 0.001$] and preference for alcohol. (**B**) Two-way RM-ANOVA, significant interaction genotype x quinine-adulteration [$F_{(1,14)} = 23.0$, $P < 0.001$]. Results are expressed as mean ± SEM, $^{###}P < 0.001$ compared to 0.0 g/l quinine in the same genotype and $***P < 0.001$, LSD post hoc test; $n = 7$ per genotype. (**C-D**) Data shown represent the level of saccharin solution intake (**C**) and preference (**D**) pre- and post-quinine-adulteration before testing the effect of LM22A-4. (**C**) Two-way RM-ANOVA, no main effect of the genotype [$F_{(1,12)} = 0.02$, $P = 0.89$] and preference for (**D**; two-way RM-ANOVA, no main effect of the genotype [$F_{(1,12)} = 0.17$, $P = 0.68$] and main effect of quinine [$F_{(1,12)} = 9.32$, $P < 0.05$]) the saccharin solution before and after quinine adulteration. Results are expressed as mean ± SEM, $^{#}P < 0.05$ compared to 0.0 g/l quinine, LSD post hoc test; $n = 7$ per genotype.

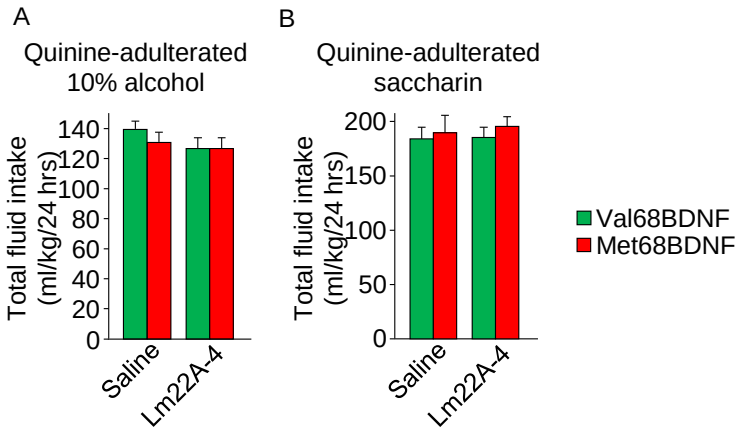

Figure S13. LM22A-4 does not change the level of total volume of fluid consumed in mice drinking alcohol/quinine solution or saccharin/quinine solution. (**A**) Total volume of fluid in LM22A-4-treated mice having an intermittent access to 10 % alcohol/0.10 g/l quinine solution. Two-way RM-ANOVA revealed no main effect of both LM22A-4 treatment ($F_{(1,14)}$ = 2.1, P = 0.17) and genotype ($F_{(1,14)}$ = 0.38, P = 0.55) on total volume of fluid intake. (**B**) Total volume of fluid in LM22A-4-treated mice drinking 0.03% saccharin/0.10 g/l quinine solution. Two-way RM-ANOVA revealed no main effect of both LM22A-4 treatment ($F_{(1,12)}$ = 0.08, P = 0.78) and genotype ($F_{(1,12)}$ = 0.67, P = 0.43) on total volume of fluid intake. Results are expressed as mean ± SEM. (**A**) n = 9 per genotype, (**B**) n = 7 per genotype.

Supplemental References

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