Supporting Online Material for

Genetic Variant BDNF (Val66Met) Polymorphism Alters Anxiety-Related Behavior

Zhe-Yu Chen,* Deqiang Jing, Kevin G. Bath, Alessandro Ieraci, Tanvir Khan, Chia-Jen Siao, Daniel G. Herrera, Miklos Toth, Chingwen Yang, Bruce S. McEwen, Barbara L. Hempstead, Francis S. Lee*

*To whom correspondence should be addressed. E-mail: zheyuchen@sdu.edu.cn (Z.-Y.C.) or fslee@med.cornell.edu (F.S.L.)

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Supporting Online Material

Materials and Methods

Generation of $BDNF_{Met}$ mutant mice. The replacement targeting vector consisted of a 7.8kb EcoRV-XbaI 129SV mouse genomic fragment containing the single-exon BDNF-coding sequence. In an ApaI-ApaI fragment containing the BDNF prodomain, the Val66Met mutation was introduced and put back into the targeting vector. A carboxyl terminal His tag was added by a PCR mutagenesis strategy to monitor protein expression. The loxP-Neo cassette was introduced into a EagI site as a positive selectable marker. A pGK-thymidine kinase cassette was used as a negative selectable marker. The targeting vector was comprised of a 1.5kb short arm, a 4.8kb long arm, a 1.5kb targeted sequence carrying the Val66Met mutation, and the Neo cassette flanked by two loxP sites. Linearalized targeting vectors were electroporated into 129 mouse strain embryonic stem (ES) cells. DNA derived by G418/FIAU-resistant ES clones were screened using a diagnostic BglII + BamHI restriction enzyme digestion using the 5' probes external to the targeting vector sequence indicated in Fig. 1A. Recombinant clones containing the predicted 7.4kb rearranged band were obtained at a frequency of 1 in 40. Four positive ES clones were injected into C57BL/6 blastocysts, which were then introduced into pseudopregnant females. Chimeric animals were mated with C57BL/6 to produce heterozygous animals, and these mice were subsequently crossed with mice expressing Cre recombinase in germ cells to excise the neo cassette. All analyses were performed on mice with a 129J and C57BL/6 hybrid genetic background. All procedures relating to animal care and treatment conformed to institutional and NIH guidelines.

Genotyping. All animal procedures were conducted in accord with U.S. Public Health Service guidelines and with the approval of the Weill Medical College Institutional Animal Care and Use Committee. To eliminate the PGK-neomycin selectable marker, $BDNF_{Met}$ mice were mated to the EIIa-Cre transgenic strain, which expresses Cre recombinase at the one-cell zygote stage of embryonic development in all tissues of the developing animal, including the germ cells that transmit the genetic alteration to progeny *(S1)*. Analyses were performed on mice backcrossed to C57BL/6 for 6-7 generations.

Neuronal cell cultures. Dissociated primary cultures of hippocampal and hippocampo-cortical neurons from embryonic day 18 (E18) rats were prepared from timed-pregnant Sprague Dawley rats, as described previously *(S2)*. Fetuses were removed under sterile conditions and kept in PBS on ice for microscopic dissection of the hippocampi. The meninges were removed and the tissue was placed in Neurobasal media. The tissue was briefly minced with fine forceps and then triturated with a fire-polished Pasteur pipette. Cells were counted and plated on culture wells coated with 0.01 mg/ml poly-D-lysine overnight in a Neurobasal medium containing B27 supplement and L-glutamine (0.5 mM). Experiments were conducted 3-4 d after plating.

Enzyme-linked immunoadsorbant assay (ELISA). For endogenous BDNF secretion studies, hippocampal-cortical neurons ($1x10^7$) were seeded to 10 cm dishes. On DIV 4, cells were washed three time with Krebs-Ringer-Henseleit (KRH) buffer with the following composition: 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 25 mM HEPES, 1.2 mM $MgSO_4$, 5.6 mM glucose, 1 mM sodium ascorbate, and 1.2 mM KH_2PO_4, adjusted to pH7.4 with NaOH. The conditioned media were collected after a 12 hour incubation at 37°C and used as a measure of constitutive secretion. To determine regulated secretion, cells were washed 3 times with KRH buffer, followed by a 10 min incubation at 37°C in stimulated media (KRH buffer with an increased KCl concentration (56 mM) and decreased NaCl concentration (75 mM). All conditioned media were collected and concentrated by Amicon Ultra-15 Centrifugal filter devices. Secretion results from the constitutive secretion condition was

normalized to 10 minutes. The BDNF protein concentrations in the respective media samples were determined using the BDNF Emax immunoassay system with recombinant mature BDNF as a standard. This ELISA is based on an antibody to the carboxy terminal region of BDNF, can also recognize proBDNF (data not shown). Standards and samples were performed in duplicates, and each group contains 6 independent samples. For BDNF protein measurements in brain lysates, tissue was dissected and weighed and protein was extracted and quantitated following the manufacturer's protocol. Standards and samples were performed in duplicates, and each group contains 3 independent samples.

Behavioral overview. In order to reduce experimental variability, age-matched littermate pairs resulting from heterozygous crossings were used for all experiments. Only male mice were used for all experiments. Mice were derived in a hybrid SV129/Bl6 background and subjected to at least six backcrosses onto C57BL/6J prior to behavioral characterization. All behavioral measurements were performed by raters blind to genotype. All animals care was in accordance with institutional guidelines.

Elevated Plus-Maze. The elevated plus-maze was constructed of black Plexiglas, and raised 70 cm above the floor, and consisted of two opposite enclosed arms with 14 cm high opaque walls and two opposite open arms of the same size (30 cm x 5 cm). The elevated plus-maze was set up under an infrared sensitive digital camera connected to a video recorder. A single testing session lasting 10 minutes was carried out in a dark room. To begin a trial, the test animal was placed in the center of the plus-maze facing an open arm, and their behavior was recorded for 10 min. The maze was cleaned with a 50% ethanol solution and dried after each trial to eliminate possible odor cues left by previous subjects. The number of entries into both the open and enclosed arms was recorded (an entry is when the animal puts all four paws into one arm); the time spent in those two areas; and the frequency of center crosses was also recorded. Anxiety levels were measured by the relative amount of exploration devoted to the open arms relative to that to the enclosed arms. This was quantified by two indices: (i) percentage of time spent in the open arms and (ii) percentage of entries into in the open arms.

Open-Field Test. The open field apparatus consisted of a (40 cm x 40 cm x 49 cm) clear Plexiglas arena with a white floor marked with time-tape dividing it into twelve equal quadrants. The arena was set up in a dim room under a digital camera, connected to a video recorder and a computer under the control of the EthoVision tracking system. A single mouse was placed into the center of open-field arena and their behavior was recorded over a 20-min session. Anxiety level were measured by the relative amount of exploration devoted to the center quadrants relative to those located adjacent to the walls of the arena. This was quantified by two indices: (i) percentage time spent in the center quadrants and (ii) percentage of entries into in the center quadrants. An entry into a given quadrant was only registered if all four paws were placed inside the quadrant.

Novelty Induced Hypophagia Test. Prior to testing, mice were housed 2 per cage and received 3 consecutive days of training (Day1-3) in a darkroom. Training consisted of presenting the mice with a standard dual bearing sipper tube (5 oz. bottle) inserted between the wire bars of the cage roof and containing diluted (1:3; milk:water) sweetened condensed milk. On day 4 mice underwent home cage testing. For testing, one mouse was removed from their home cage and placed into a holding cage containing shavings taken from their home cage. While the cage top was removed, the sipper bottle containing sweetened milk was positioned in the wire mesh cage top and then placed back onto the home cage initiating the beginning of the trial. The latency to drink, amount of time spent drinking, and number of drinking sessions was recorded over a 10 minute period. All home cage testing occurred in a dark room, and was scored with the aid of an infrared camera. Following completion of the 10-minute trial, the second animal was rotated into the home cage and tested in the same

manner. On day 5, novel cage testing was conducted by placing a single mouse into a clean cage of the same dimensions as their home cage, but with no shavings and under bright light conditions. Mice were again be presented with a sipper tube containing diluted sweetened milk and the latency to drink, duration of drinking, and number of times drinking was recorded. Anxiety was measured by the latency to drink in the novel cage. No differences in latency to drink, time spent drinking, or number of drinking sessions, were found between the first and second mouse of a pair tested on this task.

Fear conditioning. The conditioning apparatus consisted of a mouse shock-chamber set up in a sound attenuated box and scented with peppermint odor (0.1% peppermint). On day 1, the conditioning day, following a 2.5 min acclimation period to the conditioning chamber, mice received three conditioning trials consisting of a 30 second presentation of a (5 kHz, 70-dB) tone (CS) that coterminated with a 0.7-mA foot shock (US) delivered through the grid floor during the last 1.0 sec of the tone. Each conditioning trial was separated by a 40 second inter-trial interval. Following conditioning, mice were returned to their home cages. Mice were videotaped during CS presentations for subsequent quantification of behavior. Time spent "freezing" prior to and during the presentation of the tone CS was measured during the CS presentation as well as during a 30-sec baseline period prior to the first tone trial. This latter measure served as an assay for both unconditioned effects on general activity levels. Following tone testing, mice were returned to their home cages. Memory for the context and the tone were evaluated on day 2 and 3 respectively (~24 and 48 hr following conditioning). For the context test, mice were placed in the conditioning chamber and allowed to explore for 1 min, after which freezing to the context was assessed for the remaining 4.5 min. For the tone test, mice were placed in a novel chamber (circular in shape, with red walls, and scented with Lemon odor), allowed to acclimate to the chamber for 2.5 min, and then presented with 3 tones (30 sec, 5 kHz, 70 dB, ITI = 40 sec). Freezing was evaluated during the 2.5 min acclimation period, during each presentation of the tone CS, and during the 40-sec inter-trial interval. Following memory tests, animals were returned to their home cage and colony.

Locomotor Activity. A single mouse was placed in an open field environment equipped with photocells to detect the presence and location of the animal. Mice were allowed to freely explore the novel environment for a total of two hours. Through an automated system, the number of beam breaks, as well as distance traveled, for ten-minute blocks during this two-hour period was collected. Number of beam breaks was compared across genotypes for each ten-minute block to assess differences in locomotor activity.

Resident-Intruder Aggression Assay. For isolation-induced aggression, male mice were singly housed for ten days. Aggressive behaviors in 8 week old mice were monitored during 5 minute exposures to WT C57BL/6 male intruder mice that had been group-housed (five per cage) and matched with resident mice for approximate age and body weight. Five test sessions were conducted (one trial per day). The latency to first biting attack and the total number of biting attacks were recorded from videotapes of each test session.

Fluoxetine administration. Fluoxetine was dissolved in tap water and delivered ad libitum in the drinking water (tap) in glass bottles. Fluoxetine mixtures were changed every 48 hours to insure delivery of fresh drug. A dose of 18 mg/kg per day fluoxetine were given, which corresponded to 160 mg/L and reported to produce therapeutic serum levels of fluoxetine (*S3*). Administration occurred over a period of 21 days. Afterwards, mice continued to receive fluoxetine during the behavioral testing.

Perfusion & preparation of sections for volume measurement. Postnatal day 60 (P60) male mice were anesthetized with pentobarbital, transcardially perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were extracted and postfixed for 1h in paraformadehyde. Using a freezing

microtome, 40µm sections were cut and every third section was immediately mounted on Superfrost Plus glass slides. Sections were Nissl stained, dehydrated through graded alcohol, and coverslipped with Krystalon.

Hippocampal volume estimation. Using Steroinvestigator software the entire volume of the hippocampus was measured at 4X objective magnification. The external capsule, alveus of hippocampus, and white matter were used as boundary landmarks. All sections throughout each hippocampus were traced and reconstructed. The Cavalieri estimator function was used to calculate the volume of each hippocampus. Following total hippocampal measurements, the cellular layer of each subregion of the hippocampus (DG, CA1, CA2/3) was traced separately and analyzed in the same manner.

Rapid Golgi impregnation. Golgi impregnation of all brains was conducted using FD Rapid GolgiStain Kit. Golgi-Cox (G-C) solution (mixture of A and B solutions from kit) was mixed a minimum of 12 hours prior to use, and stored in a dark place at room temperature. Care was taken during all steps to insure that solutions did not come in contact with metal surfaces. Following extraction from the skull, brains were immersed in G-C solution in a glass bottle for 14 days at room temperature in a dark place (the G-C mixture was changed after the initial 12 hours of impregnation). Following 14-days of incubation in G-C, brains were transferred to solution C (10mL/brain), and incubated for a minimum of 3 days at 4C, again with the solutions having been changed after the initial 12 hours. Brains were then embedded in a 3% agarose solution, blocked, and cut at room temperature on a vibratome (150µm sections). Serial sections were immediately mounted onto 0.3% gelatin coated slides. Once on the slides, prior to complete drying of tissue, sections were brushed with solution C, and allowed to air dry for 48 hours. Slides were then immersed in ddh20 3 times for 5 minutes with gentle shaking, transferred into a solution of D & E (Golgi kit) (25ml D, 25ml E and 150mL dH20) for 5-10 minutes at 4C, and again rinsed 3x5 min in ddh20. Slides were then dehydrated through graded ethanols, cleared with Histoclear (3x5 min), and coverslipped with DPX mounting medium.

Golgi tracing: DG neuron inclusion criteria and analyses. Slides containing the Golgi impregnated brain sections were coded prior to quantitative analysis; the code was not broken until the analysis was complete. Hippocampal DG neurons were examined in the dorsal hippocampus. To be selected for analysis of dendritic arborization, Golgi-impregnated DG granule cells needed to satisfy the following criteria: *(i)* isolated cell body with a clear relationship of the primary dendrite to the soma *(ii)* presence of untruncated dendrites *(iii)* consistent and dark impregnation along the extent of all of the dendrites; *(iv*) relative isolation from neighboring impregnated cells that could interfere with analysis. For each brain, 50 neurons from the hippocampal DG subregion were selected. Cells were traced under 40X magnification using Neurolucida software. The morphological traits of cells (Sholl analysis and Fractal dimension analysis) were analyzed using Neuroexplorer, and data were processed and statistical analyses were done using Prism 4.0.

Statistics. When two means were compared, statistical significance of their difference was calculated using paired or nonpaired Student's *t* test. In multiple comparisons, data were analyzed by one way ANOVA with a Bonferroni post hoc test to determine statistical significance between genotypes.

Reference and Notes

S1. M. Lakso *et al.*, *Proc. Natl. Acad. Sci. U S A* **93**, 5860 (1996).
S2. Z. Y. Chen *et al.*, *J. Neurosci.* **25**, 6156 (2005).
S3. S. C. Dulawa, K. A. Holick, B. Gundersen, R. Hen, *Neuropsychopharmacology* **29**, 1321 (2004).

Fig. S1. Striatal volume in BDNF$^{Met/Met}$ mice. Total striatal estimations were obtained from Nissl stained sections of adult (P60) hippocampi from WT (+/+), heterozygous (+/Met), homozygous (Met/Met) and heterozygous BDNF KO (+/-) mice by Cavalieri analyses. All results are presented as a mean ± SEM determined from analysis of 4 mice/genotype.

Fig. S2. Effect of variant BDNF$_{Met}$ on hippocampal neuronal soma morphology. Cell soma area was measured from dentate gyrus neurons from P60 mice (n = 5 mice/genotype, 10 neurons/mouse). All results are presented as a mean ± SEM determined from analysis of 5 mice/genotype and statistics as comparisons to WT controls.

Fig. S3. Effect of variant BDNF$_{Met}$ on hippocampal neuronal dendritic morphology. Fractal dimension analyses of dentate gyrus neurons from P60 mice; n= 5 mice/genotype, 10 neurons/mouse. All results are presented as a mean ± SEM determined from analysis of 5 mice/genotype and statistics as comparisons to WT controls (***p < 0.001).

Fig. S4. **BDNF$^{Met/Met}$ mice display increased intermale aggression**. Aggressive behavior in 8 week old male BDNF$^{Met/Met}$ (Met/Met), BDNF$^{+/-}$ (+/-) mice and their respective WT (+/+) littermates was assessed in a resident-intruder assay. (A) Latency to first biting attack. (B) Number of biting attacks measured during five consecutive trials. All results are presented as a mean ± SEM determined from analysis of 8 mice/genotype and statistics as comparisons to WT controls ((*p < 0.05, **p < 0.01).

Fig. S5. BDNF$^{Met/Met}$ mice develop elevated body weight. Body weight curves for BDNF$^{Met/Met}$ (Met/Met), BDNF$^{+/-}$ (+/-) mice and their respective WT (+/+) littermates indicate that BDNF$^{Met/Met}$ mice have a significantly higher body weight than WT littermates, beginning at 2 months of age. All results are presented as a mean ± SEM determined from analysis of 8 mice/genotype and statistics as comparisons to WT controls (**p < 0.01 for both BDNF$^{Met/Met}$ and BDNF$^{+/-}$ mice).

Fig. S6. Basal locomotor activity in BDNF$^{Met/Met}$ mice. Locomotor activity was measured by placing mice in a chamber equipped with photocells to detect the presence and location of the animal. Mean number of beam breaks over 120 minute period. All results are presented as a mean ± SEM determined from analyses of 8 mice/genotype.

Fig. S7. Locomotor activity in BDNF$^{Met/Met}$ mice in behavioral tests. (A) Locomotor activity in the open field test was measured by total distance traveled in a 10 minute session in the absence and presence of chronic fluoxetine. (B) Locomotor activity in the elevated plus maze was measured by total number of closed arm entries in a 10 minute session. All results are presented as a mean ± SEM determined from analyses of 8 mice/genotype.

Fig. S8. Anxiety-related behavior in BDNF$^{+/Met}$ mice in the open field (A, B) and elevated plus maze (C, D). Percentage of time spent in the center (A) and entries to the center (B) in the open field are shown, as well as percentage time spent in the open arm (C) and percentage of open arm entries (D) in the plus maze. All results are presented as a mean ± SEM determined from analysis of 8 mice/genotype.

Fig. S9. Effect of fluoxetine on BDNF$^{Met/Met}$ mice in the open field test. In the open field test, the number of entries into the center compartment in a 10 minute session was measured in BDNF$^{Met/Met}$ and WT littermate control mice in the absence and presence of chronic fluoxetine (Drug). All results are presented as a mean ± SEM determined from analyses of 8 mice/genotype. (** p < 0.01).

Fig. S1

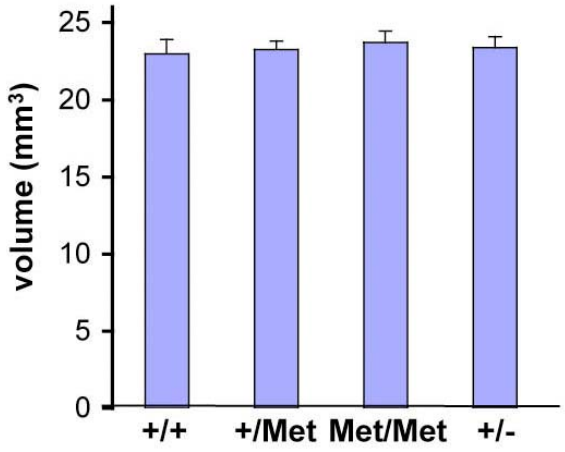
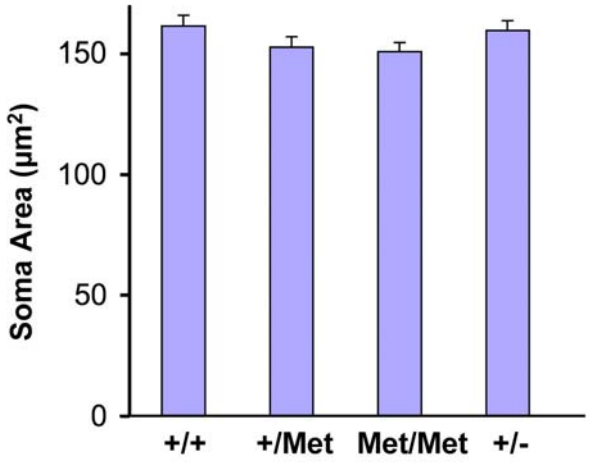

volume (mm³)
25
20
15
10
5
0
+/+
+/Met
Met/Met
+/-

Fig. S2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100
50
0
+/+
+/Met
Met/Met
+/-

Fig. S3

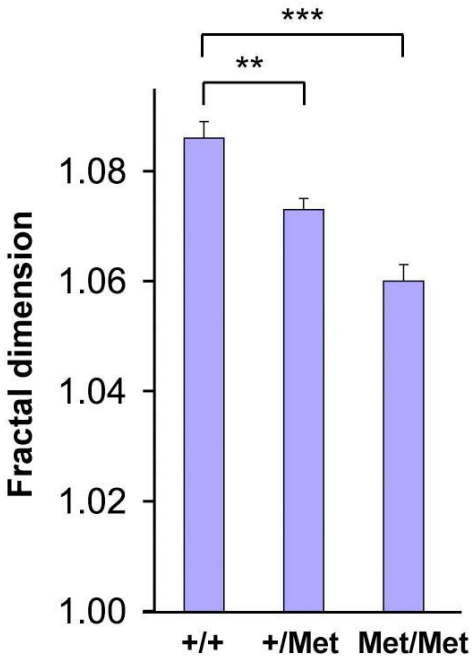


Fig. S4

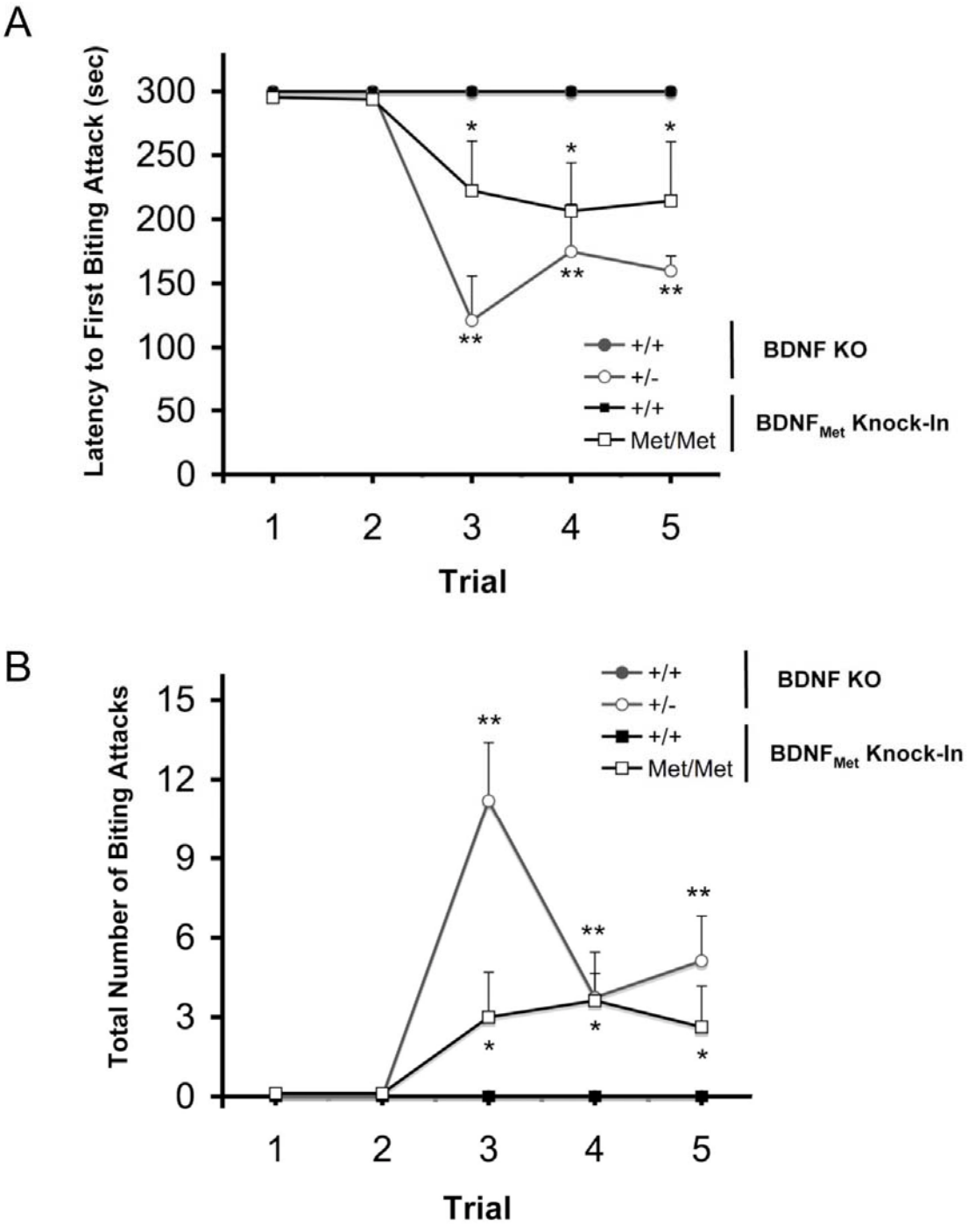

A
Latency to First Biting Attack (sec)
300
250
200
150
100
50
0
1
2
3
4
5
Trial
*
**
+/+
+/-
+/+
Met/Met
BDNF KO
BDNF Met Knock-In
B
Total Number of Biting Attacks
15
12
9
6
3
0
1
2
3
4
5
Trial
**
*
+/+
+/-
+/+
Met/Met
BDNF KO
BDNF Met Knock-In

Fig. S5

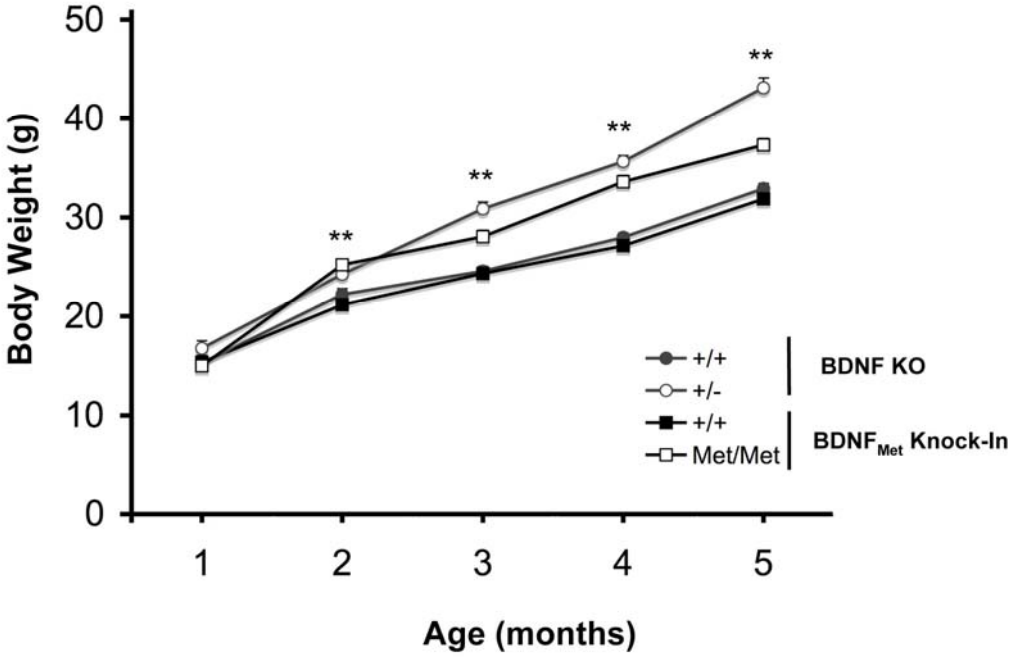
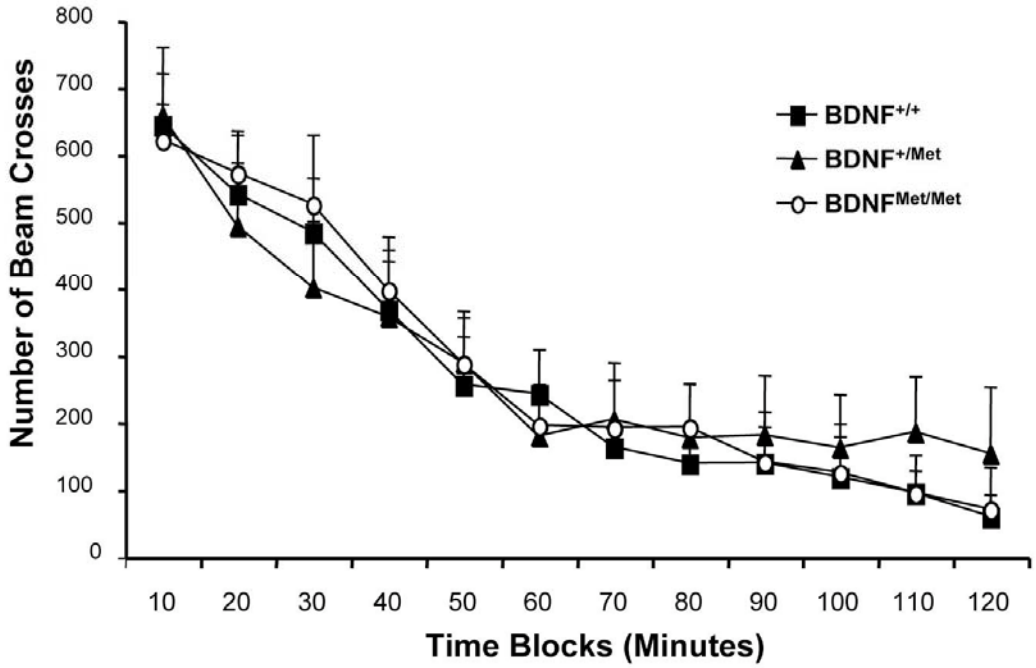

Body Weight (g)
50
40
30
20
10
0
1
2
3
4
5
Age (months)
**
**
**
**
+/+
+/-
BDNF KO
+/+
Met/Met
BDNFMet Knock-In

Fig. S6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700
600
500
400
300
200
100
0
10
20
30
40
50
60
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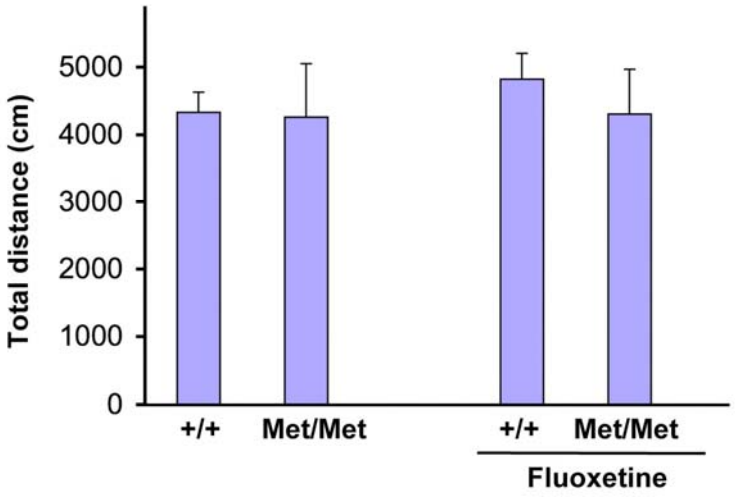
90
100
110
120
Time Blocks (Minutes)
BDNF+/+
BDNF+/Met
BDNFMet/Met

Fig. S7

A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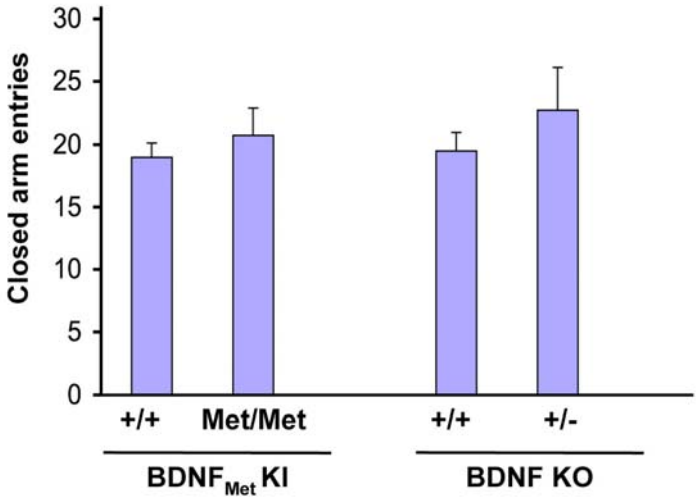
3000
2000
1000
0
+/+
Met/Met
+/+
Met/Met
Fluoxetine

B


Closed arm entries
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25
20
15
10
5
0
+/+
Met/Met
+/+
+/-
BDNF Met KI
BDNF KO

Fig. S8

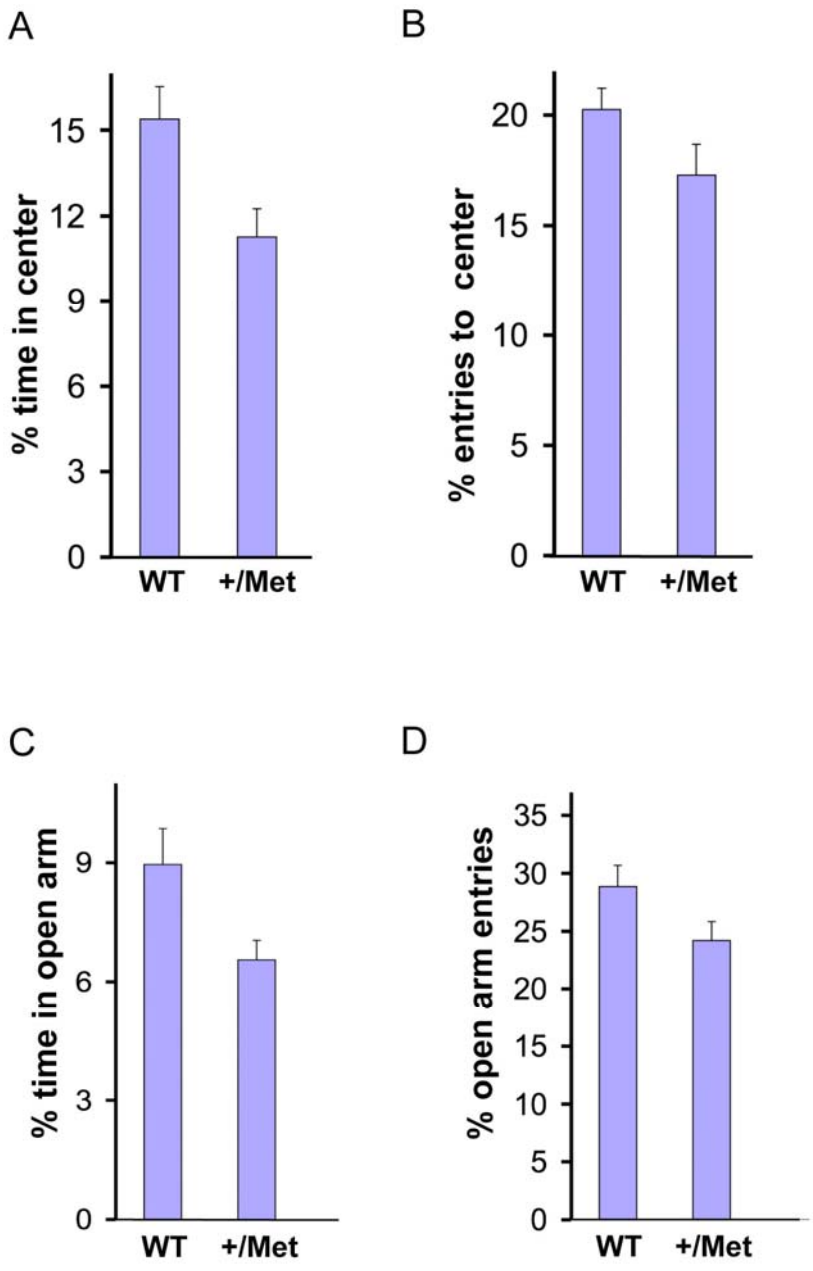
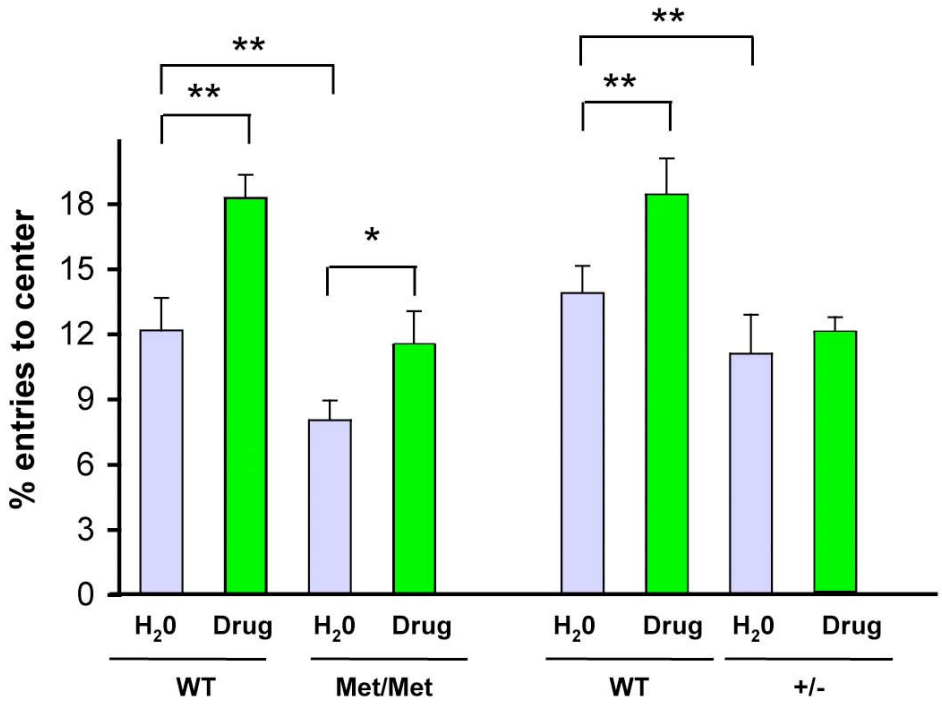

A
% time in center
0
3
6
9
12
15
WT
+/Met
B
% entries to center
0
5
10
15
20
WT
+/Met
C
% time in open arm
0
3
6
9
WT
+/Met
D
% open arm entries
0
5
10
15
20
25
30
35
WT
+/Met

Fig. S9


**
**
**
**
*
% entries to center
0
3
6
9
12
15
18
H20
Drug
H20
Drug
H20
Drug
H20
Drug
WT
Met/Met
WT
+/-