Behavioral/Systems/Cognitive

**Variant BDNF Val66Met polymorphism affects extinction of conditioned aversive memory**

**Abbreviated title:** BDNF Val66Met and extinction of aversive memory

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Abstract

Brain derived neurotrophic factor (BDNF) plays important roles in activity dependent plasticity processes such as long-term potentiation, learning, and memory. The recently reported human BDNF Val66Met (BDNF_{Met}) polymorphism has been shown to lead to altered hippocampal volume and impaired hippocampal-dependent memory and is associated with a variety of neuropsychiatric disorders. There are few studies, however, that investigate the effect of the BDNF_{Met} polymorphism on hippocampal-independent memory processes. A conditioned taste aversion (CTA) task was employed for studying the mechanisms of long-term, hippocampal-independent, nondeclarative memory in the mammalian brain. Using the CTA paradigm, we found a novel impairment in extinction learning, but not acquisition or retention, of aversive memories resulting from the variant BDNF_{Met}. BDNF_{Met} mice were slower to extinguish an aversive CTA memory compared to wild-type counterparts. Moreover, the BDNF_{Met} was associated with smaller volume and decreased neuronal dendritic complexity in the ventromedial prefrontal cortex (vmPFC), which plays a significant role in extinction of CTA. Finally, this delay in extinction learning could be rescued pharmacologically with a cognitive enhancer, D-cycloserine (DCS). To our knowledge, this is the first evidence that the BDNF_{Met} polymorphism contributes to abnormalities in memory extinction. This abnormality in extinction learning may be explained by alterations in neuronal morphology, as well as decreased neural activity in the vmPFC. Importantly, DCS was effective in rescuing this delay in extinction, suggesting that when coupled with behavior therapy, DCS may be an effective treatment option for anxiety disorders in humans with this genetic variant BDNF.
Brain derived neurotrophic factor (BDNF), a molecule known to regulate neuronal survival and differentiation, plays an important role in activity dependent plasticity processes such as long-term potentiation (LTP), learning, and memory (Huang and Reichardt, 2001; Chao, 2003). Genetic, as well as pharmacologic, inhibition of BDNF signaling leads to a reduction in LTP, resulting in impairments on several different memory tests such as Morris water maze, passive avoidance, and fear conditioning (Linnarsson et al., 1997; Mizuno et al., 2000; Liu et al., 2004; Heldt et al., 2007). Recently, a common single nucleotide polymorphism (SNP) in the BDNF gene, resulting in a valine to methionine substitution at position 66 in the prodomain (Val66Met), was identified and was shown to influence human hippocampal volume and hippocampal-dependent memory (Egan et al., 2003; Hariri et al., 2003; Bueller et al., 2006). This BDNF SNP (BDNF_{Met}) exists only in humans and has been associated with altered susceptibility to a variety of neuropsychiatric disorders including anxiety and depression (Momose et al., 2002; Sklar et al., 2002; Ventriglia et al., 2002; Sen et al., 2003). Understanding the memory phenotypes associated with BDNF_{Met} along with their detailed molecular, cellular, and anatomical bases will prove useful for prevention and treatment of neuropsychiatric disorders. Given the wide distribution of BDNF in the CNS, it is likely that other brain regions in addition to the hippocampus might also be affected by this BDNF_{Met} polymorphism. Indeed, studies have shown decreased gray matter volume in the dorsolateral prefrontal cortex, an area associated with planning and higher order cognitive functioning, as well as decreased volume in temporal and occipital lobar regions in carriers of the Met allele (Pezawas et al., 2004; Ho et al., 2006). While studies have begun to investigate the effects of BDNF_{Met}
polymorphism on hippocampal-dependent memory processes, few have looked at the effects on memory systems that are largely hippocampal-independent.

Recently, a variant $\text{BDNF}_{\text{Met}}$ knock-in mouse, which reproduces the phenotypic hallmarks of humans with this BDNF SNP, was generated (Chen et al., 2006). In this current study, we utilized the $\text{BDNF}_{\text{Met}}$ knock-in mice to investigate the effect of the $\text{BDNF}_{\text{Met}}$ polymorphism on hippocampal-independent memory processes. A conditioned taste aversion (CTA) task, which is thoroughly independent of hippocampal and striatal activity, was employed (Cui et al., 2005). Anatomical and pharmacological data highlight the involvement of several brain structures (e.g. parabrachial nucleus, amygdala, insular cortex), and cellular processes (e.g. expression of immediate early genes, Ras-MAP kinase signaling pathway, protein synthesis) in CTA learning (Welzl et al., 2001). CTA represents a valuable memory paradigm for studying the phenomena and mechanisms of long-term, nondeclarative memory in the mammalian brain (Rosenblum et al., 1997). The task is highly reproducible and the resulting memory is long lasting, permitting analysis of multiple memory phases including acquisition, retention and extinction. Here, the effect of the $\text{BDNF}_{\text{Met}}$ SNP on CTA memory processes, its potential mechanism, and pharmacological intervention are investigated.

MATERIALS AND METHODS

Animals

The Institutional Animal Care and Use Committee approved all animal procedures. In order to reduce experimental variability, age-matched littermate pairs resulting from heterozygous crossings were used for all experiments. Adult wild-type and littermate
BDNF\textsuperscript{Met/Met} male mice (2-3 months old) derived from heterozygous BDNF\textsuperscript{+/Met} x BDNF\textsuperscript{+/Met} crossed parents were used for all experiments. Adult BDNF\textsuperscript{+/−} male mice (2-3 months old) were generated as previously described (Liebl et al., 1997). All behavioral measurements were performed by raters blind to genotype. All animals were kept on a 12:12 light-dark cycle at 22°C with food and water available ad libitum unless noted otherwise. All experimental manipulations were performed during the light-on phase of the cycle in accordance with institutional guidelines.

**Unconditioned Taste Preferences**

The ability of mice to discriminate between simple tastes was tested using two-bottle, two-day intake tests. Mice were housed in cages in which they had ad libitum access to food, but restricted (from 09:00 to 9:30 h) access to water presented in two bottles. Mice were given one empty tube and one tube containing water. Every 24 h, the tubes were switched in order to train the mice to drink from either tube position. The body weights of the mice before and after the water restriction were measured. There was no significant effect of the water restriction on mice body weight. After one week, mice were given two tubes: one tube containing one of four taste solutions (0.5% sodium saccharin, 75 mM NaCl, 0.02% quinine sulfate, or 0.01 M HCl), and a second tube containing only water. Intake from each tube was recorded on the first day and again on the second day when tube position was switched. All mice were tested with all four taste solutions (n = 9-10 for each taste solution).
Conditioned taste aversion (CTA) test

Mice from each genotype were randomly assigned to receive either an unconditioned (saline injection after sodium saccharin presentation, n=8 for each genotype) or a conditioned (0.15M LiCl injection after sodium saccharin presentation, n=10 for each genotype) treatment. Each day for one week prior experimental onset, mice were transferred to individual cages in which they had ad libitum access to food, but restricted (from 09:00 to 9:30 h) access to water presented in two bottles. Water intake during the 30-min drinking interval was recorded separately for each mouse. At the end of a 7-day adaptation phase, mice reliably consumed more than 1ml of water during the 30-min interval.

On day of conditioning, mice were allowed to drink only 0.5% sodium saccharin (0.5% w/v) (Sigma Chemical Co.) provided in two bottles during the 30-min interval. To induce malaise, each mouse in the conditioned group was given an intraperitoneal injection of LiCl (0.15 M, 2% of body weight, Sigma-Aldrich Chemical Co.) 40 min after removal of the bottle containing sodium saccharin. After LiCl injection, the animals were visibly unwell and ceased all activity for the following several hours. Mice in the unconditioned control group were injected with corresponding amounts of saline.

On test day, two bottles were inserted into each cage simultaneously, one containing water and the other containing sodium saccharin. Placement of saccharin bottles with reference to the water bottles was counterbalanced. Liquid consumption was determined by weighing both bottles before and after drinking. An aversion index (AI) for the sodium saccharin was calculated as follows: AI (%)=[water intake (g)]/[sodium saccharin intake (g)+water intake (g)]×100. On days 1, 3, 7 and 30 post-training, the two bottle choice test
was administered to determine the degree of CTA acquisition and retention (n=10 for each genotype per each retention test). To determine the degree of CTA extinction, the two bottle choice test was repeated for 15 consecutive days post-conditioning. At the beginning of 16th day, all mice were given water only for 9 days. On the 25th day, the two-bottle selective test was repeated in order to evaluate spontaneous recovery of the extinguished CTA memory.

**Pharmacologic Treatment**

D-cycloserine (Sigma-Aldrich, TaiWan, PRC) was freshly dissolved in saline (0.9% wt/vol) and injected intraperitoneally at a dose of 15.0 mg/kg. Control animals were injected intraperitoneally with corresponding amounts of saline. The drug dose was chosen on the basis of the results of our preliminary tests and other behavioral studies implicating DCS’s involvement in extinction facilitation after a single administration (Ledgerwood et al., 2005).

**Tissue collection**

In addition to the mice used for extinction trials, separate cohorts of mice were deeply anesthetized with pentobarbital sodium (40mg/kg, intraperitoneally) 90 minutes post-sodium saccharin (SAC) exposure and perfused transcardially with a solution of 0.9% saline followed by 4% paraformaldehyde in 0.1M phosphate buffer saline (pH 7.4). Brains were dissected out and postfixed in 4% paraformaldehyde for 1 hour. Brains were then placed in 10% sucrose-PBS followed by 20% and 30% sucrose-PBS, each for 24 h at 4°C. Coronal sections were serially cut at 20 µm using a freezing microtome. For stereological
analysis, 40 µm serial coronal sections were cut throughout whole brain using a freezing sliding microtome. One section was taken every third slice and mounted with 0.01% gelatin immediately.

Immunohistochemistry

Every fourth serially obtained section (60 µm interval) was mounted and processed for c-Fos immunohistochemical staining. Sections were incubated for 20 min in 0.3% hydrogen peroxide in absolute methanol to quench endogenous peroxidase, rinsed (3x, PBS), and blocked in 5% normal goat serum in PBS at room temperature for 1 h. Sections were then incubated with polyclonal anti-Fos (1:500; Santa Cruz Biotechnology, Santa Cruz, CA; Catalog No. sc253) at 4°C for 24 h followed by biotinylated goat anti-rabbit antibody (Elite kit, Vector Laboratories) incubation. Bound secondary antibody was then amplified with the Vector Elite ABC kit and visualized by diaminobenzidine reaction.

Microphotographs of c-Fos immunoreactivity were captured by using a Nikon 80i light microscope equipped with a charge-coupled device (CCD) camera interfaced to a personal computer. c-Fos immunoreactive nuclei in vmPFC and hippocampus were counted with the aid of NIS-Elements BR image analysis software and the number of positive nuclei per square millimeter in the vmPFC and hippocampus was calculated for both regions.

Rapid Golgi impregnation

Golgi impregnation of all brains was conducted using FD Rapid GolgiStain Kit from mice that had not undergone any prior behavioral or drug treatments. 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 ensure that solutions did not come in contact with metal surfaces. Brains were immersed in G-C solution for 14 days at room temperature (the G-C mixture was changed after the initial 12 hours of impregnation). Following 14 days of incubation, brains were transferred to solution C (10 ml/brain), and incubated for 3 days at 4°C, 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 (150 µm 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 distilled water 3 times for 5 minutes and then transferred into a solution of D & E (Golgi kit) (25 ml D, 25ml E and 150 ml distilled water) for 5 - 10 minutes at 4°C, and again rinsed 3 x 5 minutes in ddh20. Slides were then dehydrated with ethanol), cleared with Histoclear (3 x 5 min), and coverslipped with DPX mounting medium.

**Golgi tracing**

Slides containing the Golgi impregnated brain sections were coded prior to quantitative analysis; the code was not broken until the analysis was complete. To be selected for analysis of dendritic arborization, Golgi-impregnated vmPFC neurons 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, 25 neurons from the vmPFC subregion were selected. Cells were traced under 40X lens using Neurolucida software. The morphological traits of cells (Fractal dimension analysis) were analyzed using Neuroexplorer, and data were processed and statistical analyses were done using Prism 4.0.

**Volume estimation**

All contouring and measurements were performed on brain sections from mice that had not undergone any prior behavioral or drug treatments by using a Nikon Eclipse 80i microscope attached to an Optronics camera with Microfire (Goleta, CA) software, which was connected to a computer using Stereoinvestigator software (MicroBrightfield, Williston, VT). In order to standardize section positions in different levels, and to facilitate contouring, a reference system - tangential reference point (TRP) system - was designed based on previous methods in rat (Gabbott et al., 2005). Individual sections chosen by systematic random sampling (SRS) were aligned by the tangential reference point (TRP) system and compared to corresponding levels in a mouse brain atlas. The volume of each region was estimated by using the Cavalieri method as previously described (Chen et al., 2006).

**Statistical analyses**

Data were analyzed with Student’s t-test, one-way or two-way repeated-measures ANOVA, followed by Fisher’s LSD post hoc comparisons, where appropriate. The significance level was set to 0.05 for all statistical analyses, and all values in the text and
figures represent means ± S.E.M. Data analyses were performed using SPSS statistical program version 10.0.

RESULTS

Unconditioned taste preferences

During the initial two-bottle unconditioned taste preference test, preference of four taste solutions (sweet, salty, bitter, and acidic) over water was measured. BDNF<sup>Met/Met</sup>, BDNF<sup>+/Met</sup>, BDNF<sup>+/−</sup> and wild-type mice showed similar taste preferences, with no effect of genotype on any taste (Figure 1). On the second test day, all genotypes preferred the 0.5% sodium saccharin to water (genotype, F<sub>(3,37)</sub> = 0.35, p = 0.79; Figure 1A), preferred water slightly to 75 mM NaCl (genotype, F<sub>(3,37)</sub> = 0.99, p = 0.43; Figure 1B), and avoided 0.02% quinine sulfate (genotype, F<sub>(3,37)</sub> = 0.39, p = 0.76; Figure 1C) and 0.01 M HCl solutions (genotype, F<sub>(3,37)</sub> = 0.89, p = 0.48; Figure 1D). These results suggested that BDNF<sup>Met/Met</sup>, BDNF<sup>+/Met</sup>, BDNF<sup>+/−</sup>, and wild-type mice did not differ in their level of preference for 0.5% sodium saccharin, 75 mM NaCl, 0.02% quinine sulfate, and 0.01 M HCl solutions. In addition, as homozygous BDNF<sup>−/−</sup> mice have been previously shown to have lingual taste bud deficits (Nosrat et al., 1997), these taste preference results suggest no significant alterations in taste discrimination in the BDNF<sub>Met</sub> and BDNF<sup>+/−</sup> mice compared to wild-type mice in this paradigm.

Acquisition of CTA memory

Mice of all genotypes readily consumed the sodium saccharin solution on the day of conditioning (mean±SEM saccharin solution intake: wild type, 1.53±0.17 ml; BDNF<sup>+/Met</sup>, 1.76±0.23 ml; BDNF<sup>Met/Met</sup>, 2.07±0.46 ml; BDNF<sup>+/−</sup>, 2.10±0.16 ml; genotype, F<sub>(3,71)</sub> = 2.20, p
= 0.15). Across genotypes, mice that received LiCl after sodium saccharin exposure (conditioned) developed similar robust levels of aversion to the sodium saccharin solution, relative to unconditioned controls \((p < 0.01)\) (Figure 2) (LiCl, \(F_{(1,71)} = 262.24, p < 0.01\); genotype, \(F_{(3,71)} = 0.23, p = 0.87\); interaction, \(F_{(3,71)} = 0.81, p = 0.50\). Between subjects comparisons yielded no significant main effect of genotype (genotype, \(F_{(3,39)} = 1.61, p = 0.23\), Figure 2).

**Retention of CTA memory**

To determine degree of retention for the CTA memory in the BDNF<sup>Met</sup> and BDNF haploinsufficient mice, the aversion index was measured during the CTA test by varying the post-LiCl injection time interval at which taste aversion was tested (paired with sodium saccharin solution intake) to 3, 7 or 30 days post-conditioning. All mice exhibited a strong preference for regular water, indicating that mice were capable of retaining the CTA memory for at least one month. Comparison among the groups revealed no significant differences across genotypes (genotype, \(F_{(3,39)} = 0.85, p = 0.48\) for 30-day retention test; Figure 3).

**Extinction of CTA memory**

We then determined whether BDNF<sup>Met</sup> and haploinsufficient BDNF mice could extinguish CTA memories. In striking contrast to the memory acquisition behavior, where BDNF<sup>Met/Met</sup> and BDNF<sup>+/−</sup> mice readily learned to avoid the solution that was associated with the aversive memory, they failed to show a reduction of this aversion from 4<sup>th</sup> day to 10<sup>th</sup> day of the experiment, demonstrating impairments in the extinction of the aversive
memory compared to both conditioned wild type and conditioned BDNF<sup>+/Met</sup> mice (Figure 4A). Post hoc comparisons revealed significant differences between the conditioned BDNF<sup>Met/Met</sup>, BDNF<sup>+/−</sup>, and wild-type mice from day 4 to day 10 (Figure 4). (genotype within conditioned treatment: genotype, F<sub>(3,599)</sub> = 26.89, p < 0.01; day, F<sub>(14,599)</sub> = 71.95, p < 0.01; interaction, F<sub>(42,599)</sub> = 2.62, p < 0.01). No significant differences in the spontaneous recovery of the CTA memory between the genotypes were found (genotype within spontaneous recovery, F<sub>(3,119)</sub> = 1.1, p = 0.35; day, F<sub>(2,119)</sub> = 12.22, p < 0.01; interaction, F<sub>(6,119)</sub> = 0.89, p = 0.58). In the unconditioned mice, there was no effect of genotype on the aversion index (Figure S1), (genotype within unconditioned, F<sub>(3,255)</sub> = 1.83, p = 0.15; day, F<sub>(7,255)</sub> = 2.54, p = 0.16; interaction, F<sub>(21,255)</sub> = 0.49, p = 0.98).

Though the insular cortex and the amygdala are involved in both acquisition and extinction of CTA (Bahar et al., 2003; Eisenberg et al., 2003; Bermudez-Rattoni, 2004; Belelovsky et al., 2005; Akirav et al., 2006), recent studies reported that the vmPFC plays an important role in CTA extinction. c-Fos protein expression has been shown to be upregulated in the vmPFC 90 min post-SAC exposure during CTA extinction tests, while inhibition of protein synthesis in vmPFC has been shown to result in impaired CTA extinction (Mickley et al., 2005). Here, we show that BDNF<sup>Met/Met</sup> mice demonstrate normal acquisition but delayed extinction of a CTA memory. Thus, to further investigate the potential mechanism of the extinction impairment in BDNF<sup>Met/Met</sup> mice, we used immunohistochemical techniques to measure c-Fos positive cells in the vmPFC during CTA extinction. First, we determined the c-Fos levels after drinking novel SAC in unconditioned wild-type, BDNF<sup>Met/Met</sup>, and BDNF<sup>+/−</sup> mice. Mice of all three genotypes had equivalent density of c-Fos positive cells in the vmPFC and in the hippocampus (ANOVA,
vmPFC: F_{(2,14)} = 0.06, p = 0.95; hippocampus: F_{(2,14)} = 0.40, p = 0.68), Figure S2). c-Fos levels were assessed in all groups on the fourth day of extinction. The density of c-Fos positive cells in the vmPFC but not in the hippocampus increased significantly after extinction (vmPFC, extinction: F_{(1,29)} = 897.87, p <0.01, Figure 4B, 4C). More importantly, wild-type mice showed higher density of c-Fos-positive cells than BDNF^{Met/Met} and heterozygous BDNF knockout mice in the vmPFC during extinction (ANOVA, vmPFC: F_{(2,14)} = 225.62, p<0.01, Figure 4C). These results suggest that increased c-Fos expression in the extinction group was attributable to extinction training per se, while the blunted increase in c-Fos positive cells in BDNF^{Met/Met} and BDNF^{+/−} mice may be an effect of genotype. The c-Fos immunostaining results in the vmPFC are consistent with the behavior results.

**Anatomical basis of CTA extinction deficit**

It was then investigated whether there were anatomical abnormalities in the vmPFC, which has been shown to play a significant role in extinction of both CTA and conditioned fear responses (Morgan et al., 1993; Quirk et al., 2000; Milad and Quirk, 2002; Barrett et al., 2003; Santini et al., 2004; Mickley et al., 2005; Milad et al., 2005; Quirk and Mueller, 2008). BDNF^{Met/Met} and BDNF^{+/−} mice were histologically prepared for stereologic volume estimation of vmPFC from Nissl-stained sections. Using Cavalieri volume estimation, we detected a significant decrease (16.71% for BDNF^{Met/Met} mice; 17.78% for BDNF^{+/−} mice) in ventromedial prefrontal cortical volume in BDNF^{Met/Met} and BDNF^{+/−} mice compared to age-matched, littermate wild-type controls (Figure 5A). We also measured striatal volume, because in human studies this structure has not been reported to be altered by the
BDNF<sub>Met</sub> polymorphism (Pezawas et al., 2004), and found no alteration in mouse striatal volumes across genotype (Figure 5A). Because secreted BDNF regulates neuronal differentiation (Huang and Reichardt, 2001; Chao, 2003), the decreased vmPFC volume may be due to altered neuronal morphology. Golgi staining was used to visualize individual vmPFC neurons in layer 2/3 and to analyze dendritic complexity in BDNF<sup>Met/Met</sup> as well as in BDNF<sup>+/−</sup> mice. Fractal dimension analyses was used to quantify how completely a neuron fills its dendritic field (Caserta et al., 1995), and revealed a significant decrease in dendritic arbor complexity in the vmPFC neurons from BDNF<sup>Met/Met</sup> and BDNF<sup>+/−</sup> as compared to littermate wild-type mice (Figure 5B, 5C). These results suggest that the vmPFC, a brain region that has been shown to play a significant role in mediating extinction behavior, has altered anatomy, as well as altered neuronal morphology in the BDNF<sup>Met/Met</sup> mice.

**D-cycloserine rescue of extinction impairment in CTA memory**

In an attempt to rescue the CTA extinction learning delays in BDNF<sup>Met/Met</sup> and BDNF<sup>+/−</sup> mice, mice were injected intraperitoneally with either the NMDA receptor partial agonist, D-cycloserine (DCS) (15 mg/kg i.p.), or vehicle after the third extinction trial. DCS was chosen as acute DCS treatment has been shown to facilitate extinction learning in a variety of paradigms (Davis et al., 2003; Ledgerwood et al., 2005; Davis et al., 2006; Otto et al., 2007), as well as enhance NMDA dependent signaling independent of BDNF. In contrast to our previous CTA extinction findings, which showed genotype effects, no significant genotype effect was observed after DCS administration (Figure 6A). Aversion index scores from extinction day 4 to day 10 showed no significant difference between
genotypes (genotype, $F_{(3,599)} = 1.08, \ p = 0.36$; day, $F_{(14,599)} = 103.13, \ p < 0.01$; interaction $F_{(42,599)} = 0.46, \ p = 0.10$), suggesting rescue by DCS of the delayed extinction learning in BDNF$^{\text{Met/Met}}$ and BDNF$^{+/ -}$ mice. These data revealed that D-cycloserine is able to rescue delayed extinction learning in BDNF$^{\text{Met/Met}}$ and BDNF$^{+/ -}$ mice. In contrast to DCS treatment, saline injections did not enhance CTA extinction in any of the genotypes under study (Figure 6A) and a significant effect of genotype on the aversion index across days 4 to 10 of extinction remained (genotype, $F_{(3,359)} = 317.70, \ p < 0.01$; day, $F_{(14,359)} = 60.33, \ p < 0.01$; interaction, $F_{(42,359)} = 2.38, \ p < 0.01$). Separate cohorts of mice c-Fos positive cells were counted on extinction day 4 after DCS or saline treatment and density of c-Fos positive cells in the saline group was similar to that of the extinction group (Figure 6B, Figure 4B). All genotypes receiving DCS treatment showed equivalent c-Fos levels (vmPFC: $F_{(2,14)} = 2.91, \ p = 0.11$) (Figure 6C). DCS treated BDNF$^{\text{Met/Met}}$ and BDNF$^{+/ -}$ mice showed significantly higher density of c-Fos-labeled cells than saline treated littermates (vmPFC: $F_{(3,19)} = 124.49, \ p < 0.01$).

**DISCUSSION**

The goal of this study was to identify novel behavioral phenotypes associated with the human BDNF SNP (Val66Met) using a mouse model system that contains the variant BDNF$^{\text{Met}}$. Using the CTA paradigm, we provide evidence for a new learning impairment in the BDNF$^{\text{Met}}$ mice: delayed extinction of aversive memories. This learning impairment is specific to the extinction process, as no learning delays were observed in acquisition or retention of the aversive memory. In addition, there are no detectable sensory abnormalities produced by BDNF gene variant that could be responsible for altered CTA
extinction learning. Finally, we demonstrate that the delayed extinction learning can be rescued by employing a cognitive enhancer, DCS, which acts in a BDNF independent manner.

Our data provide several new insights into phenotypes associated with the variant BDNF<sub>Met</sub>. First, the learning impairment in taste aversion is selective only for the extinction process, with no observable impairments in acquisition or retention of CTA in BDNF<sup>Met/Met</sup> and BDNF<sup>+/−</sup> mice. In contrast, previous studies have shown BDNF is essential for memory acquisition and retention in hippocampal-dependent tasks. BDNF<sup>+/−</sup> or hippocampal-specific BDNF deletions block memory acquisition and retention in Morris water maze, radial arm maze, inhibitory avoidance learning and contextual fear test (Linnarsson et al., 1997; Ma et al., 1998; Mizuno et al., 2000; Liu et al., 2004; Heldt et al., 2007). BDNF<sup>Met/Met</sup> mice have also been shown to have a deficit in hippocampal-dependent memory acquisition when tested on a contextual fear paradigm (Chen et al., 2006). Although BDNF has also been shown to be involved in amygdala dependent tasks, such as cued fear conditioning, BDNF does not appear to affect memory acquisition or retention in hippocampal-independent non-declarative CTA memory tests, suggestive of selective roles for BDNF in different learning tasks (Rattiner et al., 2004). Prior to this current study, the BDNF Val66Met polymorphism had not yet been implicated in hippocampal-independent learning tasks. In this context, it is interesting that humans with the BDNF<sub>Met</sub> allele have specific deficits in episodic memory, which is hippocampal-dependent, but no deficits in hippocampal-independent processes such as semantic or working memory (Egan et al., 2003; Hariri et al., 2003).
Second, this is the first study showing an impairment in extinction learning in BDNF\textsuperscript{Met/Met} mice. BDNF has been previously shown to be involved in conditioned fear extinction. Hippocampus-specific deletion of BDNF in adult mice impairs extinction of aversive memories (Heldt et al., 2007), yet an effect of the BDNF\textsubscript{Met} polymorphism on extinction learning has never before been shown. We found that BDNF\textsuperscript{Met/Met} mice and BDNF\textsuperscript{+/-} mice, but not BDNF\textsuperscript{+/-Met} mice, are slower to extinguish a learned aversive response compared to wild-type counterparts. Because BDNF\textsuperscript{Met/Met} and BDNF\textsuperscript{+/-} mice showed similar Aversion Index (AI) scores on day 1 of extinction, it is unlikely that they developed a stronger aversion to LiCl injection. To further confirm that the extinction deficit was not due to the ceiling effects, we altered the LiCl doses and found that higher concentrations could indeed elicit higher AI scores, suggesting that ceiling effects were not reached with the 0.15M dose LiCl (Supplemental Figure 3). Thus, our results further confirm that BDNF is required for the neural plasticity underlying the extinction processes, which is consistent with data obtained from fear conditioning studies (Chhatwal et al., 2006; Bredy et al., 2007; Heldt et al., 2007). In this context, our previous studies have shown that BDNF\textsuperscript{Met/Met} mice displayed increased anxiety-related behaviors in conflict settings (Chen et al., 2006). The inability to properly regulate and inhibit fear responses is a hallmark of most anxiety disorders such as post-traumatic stress disorder (PTSD) (Myers and Davis, 2002; Davis et al., 2006). Our findings suggest that human carriers with BDNF\textsubscript{Met} allele may have a similar impairment in extinguishing aversive or fearful memories after stressful events, and thus may be at increased risk for developing anxiety disorders such as PTSD.
The potential mechanism underlying the observed extinction deficit in BDNF<sub>Met</sub> mice was also investigated in our study. Previous studies have highlighted the importance of the ventromedial prefrontal cortex (vmPFC) in the extinction of conditioned emotional responses (Morgan et al., 1993; Quirk et al., 2000; Milad and Quirk, 2002; Barrett et al., 2003; Santini et al., 2004; Mickley et al., 2005; Milad et al., 2005). Anatomical changes in vmPFC were investigated and revealed that vmPFC volume was significantly decreased in BDNF<sup>Met/Met</sup> mice compared to wild-type mice. This decrease of vmPFC volume is also consistent with the significant decreases in cortical volume in human carriers of the Met allele (Pezawas et al., 2004). Interestingly, thickness of the vmPFC in humans is also correlated with extinction success in fear conditioning studies (Milad et al., 2005). As evidenced with Golgi staining, the neuronal dendritic complexity among BDNF<sup>Met/Met</sup> mice was also significantly decreased compared to wild-type controls (Figure 5). Thus, the neuroanatomical changes in the BDNF<sub>Met</sub> mice may be responsible for the delays in extinction learning. Extinction of previously acquired memories is a new form of learning and a potential target of BDNF-mediated plasticity (Myers and Davis, 2002; Davis et al., 2006). Evidence suggests that the expression of c-Fos (the protein product of the immediate early gene c-fos) is a marker of neural activity (Herrera and Robertson, 1996), which can increase dramatically in brain areas mediating extinction learning when animals have extinguished a CTA response (Herry and Mons, 2004; Mickley et al., 2005). Consistent with these findings, we found extinction induced increases in c-Fos expression in the vmPFC in wild type mice, yet the number of c-Fos positive cells in the vmPFC in BDNF<sup>Met/Met</sup> and BDNF<sup>+/−</sup> mice was less than in wild-type mice after 4 days of extinction. When examining the hippocampus as a control region, the number of c-Fos positive cells
was equivalent for all groups. It is widely accepted that neural activity plays a pivotal role in synaptic plasticity. Therefore, the decreased neural activity in vmPFC is consistent with the abnormal CTA extinction observed in BDNF$^{\text{Met/Met}}$ and BDNF$^{+/\text{-}}$ mice.

Finally, we showed that pharmacological intervention was able to rescue the delay in extinction learning observed in BDNF$^{\text{Met/Met}}$ mice. D-cycloserine, a partial agonist at the glycine recognition site of the glutamatergic NMDA receptor, had been shown to rapidly facilitate extinction learning when administered in single doses prior to or soon after extinction trials in animals (Davis et al., 2003; Ledgerwood et al., 2005; Davis et al., 2006; Otto et al., 2007). As DCS’s onset of action on extinction occurs within 30 minutes (Ledgerwood et al., 2005), it precludes any potential mechanisms involving activity dependent increases in BDNF expression. A single administration of DCS was able to facilitate extinction learning and maintain normalized extinction across subsequent trials as NMDA receptor activation has been implicated in long-term extinction memory (Ledgerwood et al., 2003). In addition, in human studies, DCS has been shown to facilitate the extinction of fear in human phobic patients undergoing behavioral exposure therapy (Ressler et al., 2004). In our study, DCS rescued the CTA extinction deficit in BDNF$^{\text{Met/Met}}$ mice as well as in BDNF$^{+/\text{-}}$ mice (Figure 6). These findings are the first to demonstrate that a pharmacological agent, acting in a BDNF independent manner, can reverse a learning deficit in the BDNF$^{\text{Met}}$ mice.

In conclusion, through the use of a mouse model system, we determined that the variant BDNF$^{\text{Met}}$ allele leads to a specific impairment in extinction of aversive memory. This abnormality in extinction behavior may be explained by abnormalities observed in vmPFC structures and decreased neural activity. To our knowledge, this is the first
evidence that the $\text{BDNF}_{\text{Met}}$ allele contributes to deficits in memory extinction. These findings provide the basis for further studies to determine whether human $\text{BDNF}_{\text{Met}}$ carriers have similar behavioral deficits. In this context, our previous studies have shown that anxiety-related behaviors in $\text{BDNF}_{\text{Met/Met}}$ mice could not be rescued by the common antidepressant fluoxetine (Chen et al., 2006). In this current study of $\text{BDNF}_{\text{Met/Met}}$ mice, we found that DCS was effective in rescuing this learning impairment, suggesting that in humans with this genetic variant $\text{BDNF}$, DCS may be an effective treatment option for anxiety disorders when coupled with behavioral exposure therapy, especially under conditions where serotonin reuptake inhibitor antidepressants may be ineffective. In all, our study demonstrates one strategy for how to use model systems of human SNP’s to identify novel behavioral phenotypes and novel pharmacologic approaches to treat anxiety disorders. Drug discovery strategies based on identifying pharmacological agents that act in a $\text{BDNF}$ independent manner may improve therapeutic responses for humans with this common $\text{BDNF}$ polymorphism.

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There are no conflicts of interests for any of the authors relating to these results.
References


Figure Legend

Figure 1. Unconditioned taste preference. Intakes for four taste solutions (A. saccharin, B. NaCl, C. quinine, D. HCl) were compared against intakes for water (n = 9-10 for each genotype) during two-bottle tests.

Figure 2. Acquisition of CTA. Mice of all genotypes were injected with LiCl (conditioned) or saline (unconditioned) on conditioning day and tests were carried out 24 h later. All results are presented as means ± SEM determined from analysis of 8-10 mice per genotype and statistics are in comparison with the unconditioned same genotype mice (**p<0.01).

Figure 3. Retention of CTA memory. The retention of CTA memory in mice of all genotypes was measured on 3, 7 and 30 days after conditioning. All results were presented as means ± SEM determined from analysis of 10 mice per genotype per test day.

Figure 4. Extinction of CTA memory. A. Twenty-four hours after CTA training, extinction tests were carried out for 15 consecutive days and then spontaneous recovery was evaluated as described in Methods. The aversion indices are presented as means ± SEM from analysis of 10 mice per genotype (**p < 0.01, *p < 0.05, vs. wild-type). B. Representative photomicrographs of extinction induced c-Fos labeling in vmPFC and hippocampus in wild-type, BDNF<sup>Met/Met</sup>, and BDNF<sup>+/−</sup> mice (Scale bar in vmPFC= 100 µm, scale bar in hippocampus= 1000 µm). C. Density of c-Fos positive cells in vmPFC and hippocampus (HIP) of sodium saccharin (SAC) controls and the extinction group are
presented as means ± SEM from five mice per genotype (*p < 0.01, versus wild-type mice).

**Figure 5.** Altered ventromedial prefrontal cortex anatomy in BDNF<sup>Met/Met</sup> mice. **A.** Ventromedial prefrontal cortex (vmPFC) and striatal volume estimations were obtained from Nissl stained sections from P60 wild-type, BDNF<sup>Met/Met</sup> and BDNF<sup>+/−</sup> mice by Cavalieri analyses. All results are presented as means ± SEM determined from analysis of five mice per genotype (*p<0.05, Student’s t test). Representative examples of Golgi-stained vmPFC pyramidal neurons and fractal dimension analyses from P60 **(B)** BDNF<sup>Met/Met</sup> and **(C)** BDNF<sup>+/−</sup> mice. All results are presented as means ± SEM determined from analysis of 5 mice per genotype and statistics are in comparison with wild-type controls (*p < 0.01, Student’s t-test).

**Figure 6.** D-cycloserine rescue of extinction deficit in CTA memory. **A.** Mice of all genotypes were intraperitoneally injected with either saline or D-cycloserine (DCS) after the third extinction trial (arrow denotes time of injection). The aversion indices are presented as means ± SEM from analysis of 6 to 10 mice per genotype. **B.** Representative photomicrographs of extinction induced c-Fos labeling in the vmPFC in wild-type, BDNF<sup>Met/Met</sup>, and BDNF<sup>+/−</sup> mice after saline or DCS treatment (Scale bar = 100 µm). **C.** c-Fos labeled cell density quantitation in the vmPFC after saline or DCS treatment was calculated. The results are presented as means ± SEM from 5 mice per genotype.