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Effects of the BDNF Val66Met polymorphism on anxiety-like behavior following nicotine withdrawal in mice

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Abstract

INTRODUCTION: Nicotine withdrawal is characterized by both affective and cognitive symptoms. Identifying genetic polymorphisms that could affect the symptoms associated with nicotine withdrawal are important in predicting withdrawal sensitivity and identifying personalized cessation therapies. In the current study we used a mouse model of a non-synonymous SNP in the translated region of the BDNF gene that substitutes a valine (Val) for a methionine (Met) amino acid (Val66Met) to examine the relationship between the Val66Met SNP and nicotine dependence.

METHODS: This study measured proBDNF and the BDNF prodomain levels following nicotine and nicotine withdrawal and examined a mouse model of a common polymorphism in this protein ($BDNF^{Met/Met}$) in three behavioral paradigms: novelty-induced hypophagia, marble burying, and the open-field test.

RESULTS: Using the BDNF knock-in mouse containing the BDNF Val66Met polymorphism we found (1) blunted anxiety-like behavior in $BDNF^{Met/Met}$ mice following withdrawal in three behavioral paradigms: novelty-induced hypophagia, marble burying, and the open-field test (2) the anxiolytic effects of chronic nicotine are absent in $BDNF^{Met/Met}$ mice and (3) an increase in BDNF prodomain in $BDNF^{Met/Met}$ mice following nicotine withdrawal.

CONCLUSIONS: Our study is the first to examine the effect of the BDNF Val66Met polymorphism on the affective symptoms of withdrawal from nicotine in mice. In these

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mice, a single-nucleotide polymorphism in the translated region of the BDNF gene can result in a blunted withdrawal, as measured by decreased anxiety-like behavior. The significant increase in the BDNF prodomain in $BDNF^{Met/Met}$ mice following nicotine cessation suggests a possible role of this ligand in the circuitry remodeling after withdrawal.

INTRODUCTION

Smoking is the leading cause of preventable death in developed countries (Adhikari, Kahende, & Malarcher, 2008). However, despite the numerous health risks less than 10% of quit attempts result in continuous abstinence for one year (Trosclair, Caraballo, Malarcher, & Husten, 2002). Nicotine, the primary addictive component of tobacco, acts on brain nicotinic acetylcholine receptors (nAChRs) to increase neuronal dopamine activity within the mesolimbic reward circuit (Wu, 2010). Altered homeostasis of this brain reward system results in complex behaviors such as dependence, tolerance, sensitization, and craving (De Biasi & Dani, 2011). These behaviors all contribute to the symptoms of withdrawal, which include irritability, depressed mood, and anxiety.

One key molecule acting in the reward system is brain-derived neurotrophic factor (BDNF), a secreted factor of the neurotrophin family necessary for the growth (Segal, Pomeroy, & Stiles, 1995), differentiation (Leschik et al., 2013) and survival (Hofer & Barde, 1988) of neurons. Genetic modulation of BDNF or its receptor, TrkB, alters the rewarding effect of drugs of abuse such as cocaine and alcohol (Hall, Drgonova, Goeb, & Uhl, 2003; Hensler, Ladenheim, & Lyons, 2003; Unterwald et al., 2013), linking BDNF to addiction. Smoking also increases plasma BDNF levels in

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humans (Bhang, Choi, & Ahn, 2010; T.-S. Kim, Kim, Lee, & Kim, 2007) and administered nicotine increases BDNF gene expression in critical regions of the mesolimbic reward circuit of rodents (Kivinummi, Kaste, Rantamäki, Castrén, & Ahtee, 2011).

Recently, the relationship between smoking and a non-synonymous single nucleotide polymorphism (SNP) in the translated region of the BDNF gene was investigated. This SNP substitutes a valine (Val) for a methionine (Met) amino acid (Val66Met) and is fairly common with 37% of individuals of European ancestry carrying the Met allele (1000 Genomes Project Consortium et al., 2012). The SNP occurs within the prodomain of the BDNF gene, a region where the chaperone protein sortilin is known to bind and mediate intracellular trafficking in the secretory pathway (Chen et al., 2005). The BDNF Val66Met SNP reduces activity-dependent secretion of BDNF (Egan, Kojima, Callicott, & Goldberg, 2003) without affecting constitutive secretion or mature levels of the peptide, and leads to alterations in the dendritic complexity of hippocampal neurons and total hippocampal volume (Chen et al., 2006). This SNP has been associated with anxiety (Chen et al., 2006), HPA axis reactivity to stress (Alexander et al., 2010; Colzato, Van der Does, Kouwenhoven, Elzinga, & Hommel, 2011), depression (Tsai, Hong, & Liou, 2010), PTSD (Zhang et al., 2014) and substance abuse (Gratacòs et al., 2007). The importance of the Val66Met SNP and nicotine dependence remains unclear as studies report conflicting results on the association between the SNP and smoking behavior (Lang et al., 2007; Montag, Basten, Stelzel, Fiebach, & Reuter, 2008). However, a recent study looking at the association between the BDNF Val66Met SNP and smoking cessation found that the SNP is associated with an increased percent of

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successful quitting (Breetvelt et al., 2012). Furthermore, studies utilizing a knock-in mouse containing the BDNF Val66Met variant demonstrate that this SNP can alter affective behavior. Specifically, mice harboring the methionine (Met) amino acid substitution on both chromosomes ($BDNF^{Met/Met}$) show increased baseline anxiety-like behavior as measured in the open field and elevated plus maze (Chen et al., 2006) as compared to wildtype control mice harboring a valine (Val) amino acid substitution on both chromosomes ($BDNF^{Val/Val}$). A more thorough investigation into the role of the Val66Met SNP on behavioral affect following smoking cessation is needed, as symptoms including depressed mood and anxiety are contributing factors for relapse (Hughes, 1992; 2007). The current study utilized a knock-in mouse containing the BDNF Val66Met variant to examine the role of this SNP in mediating the negative behavioral affect following withdrawal from nicotine.

MATERIALS AND METHODS

Animals

BDNF Val66Met mice were generated using a knock-in allele with a point mutation (G to A at position 196) in the coding region of the mouse BDNF gene as described previously (Chen et al., 2006). These mice were bred at the University of Pennsylvania for use in these experiments. Mice (23-38 g; 2-4 months old) had *ad libitum* access to food and water, and were maintained on a 12hr/12hr light/dark cycle (lights on at 07:00 hours). All experimental procedures were conducted during the light cycle at the 24-hour nicotine withdrawal time point. Animals were housed in groups (3-5) except for the NIH paradigm where mice were housed in groups of two. Equal numbers of male and female

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mice were used for all studies. All experimental procedures were approved by the University of Pennsylvania Animal Care and Use Committee and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Surgical Procedures and Drugs

Mice were anesthetized with an isoflurane/oxygen vapor mixture (1-3%) and placed in a stereotaxic frame. Osmotic minipumps (model 2002; Alzet, Cupertino, CA, USA) were placed subcutaneously using aseptic surgery techniques. (-)-Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO, USA) was dissolved in sterile 0.85% saline and administered in osmotic minipumps at a dose of 18mg/kg/day (dose expressed as the freebase weight) for two weeks. Following chronic nicotine or saline treatment minipumps were surgically removed from mice receiving nicotine to initiate withdrawal as well as from ½ of the saline treated mice to control for the effects of surgery.

Behavioral Experiments

For behavioral testing, independent cohorts were used to evaluate anxiety-like behavior in each of the three tests: novelty-induced hypophagia, marble burying, and open field. All behavioral testing occurred at the 24-hour withdrawal time point (Figure 1A) and was conducted between the hours of 9:00AM and 2:00PM. Mice were acclimated to the behavioral testing facility one-hour prior to testing for all studies.

Novelty Induced Hypophagia

The novelty-induced hypophagia (NIH) test was conducted as previously described

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(Turner et al., 2010; Turner, Castellano, & Blendy, 2010; Turner et al., 2013; Yohn, Turner, & Blendy, 2014) with mice housed in groups of two. Mice were placed in a testing room with dividers separating their home cage, and allowed to acclimate for one hour prior to presentation of a palatable food (peanut butter chips; Nestle, Glendale, CA, USA). Training was repeated over 14 days with latency to consume the food measured on days 4-14. Following training, mice were assigned to a two-week treatment group and implanted with osmotic minipumps (Fig. 1A). Following treatment, latency to consume food chips over 15 minutes was measured in the home cage as a baseline, and then measured again 24-hours later in a novel environment. The novel environment consisted of a standard cage with no bedding placed within a white box with bright light (~2150 Lux) and a novel smell (1:10 pine sol).

Marble Burying

The marble burying task was conducted as previously described (Turner, Castellano, & Blendy, 2011). Mice were placed in a small cage (26 x 20 x 14cm) with 5-cm deep bedding, on top of which 20 equally distributed marbles lay. Following a one-hour acclimation to the testing room mice were placed into the cage with the marbles and left undisturbed for 15 minutes. After 15 minutes an investigator blind to the treatment and genotype group removed the mouse and counted the number of marbles that were buried in bedding (3/4 or more coverage).

Open Field and Locomoter Activity

Locomoter activity was measured in a plexiglas arena (43 x 43cm^2) containing 32

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photobeams in a 16x16 formation (MED Associates, St. Albans, Vermont, USA), and was monitored using Photobeam Activity System software (PAS). The 6x6 square region in the middle of the box was defined as the central region. Three parameters were measured in 5-minute increments over 30 minutes: total distance travelled, entries into center, and time spent in central region.

Statistical Analysis

Statistical analyses were carried out using the GraphPad Prism 5.0 software package (GraphPad Software,San Diego, CA, USA), and all data are presented as mean ± standard error of mean (SEM). Data analysis for training during the NIH was performed using the Log-rank test (Mantel-Cox) to identify significant genotype differences in the time to first food consumption in the NIH training paradigm (Fig. 1B). For the novelty-induced hypophagia test a repeated measures two-way ANOVA was used to determine significant differences with genotype and treatment as between subject factors and time (Home day, Novel day) representing the within, repeated-measures factor (Fig. 1D). For the marble-burying experiment and open field test a two-way ANOVA with genotype and treatment as the two factors was used (Fig. 3,4). In the open field a cross-over interaction was detected, but as our predicted hypothesis was that withdrawal would be anxiogenic in $BDNF^{Val/Val}$ mice a one-tailed *t*-test was used to compare 24-hr WD to saline-treated mice in both genotypes. Analysis of variance (ANOVA) was used to analyze genotype and treatment differences in proBDNF and the BDNF prodomain (Fig. 4). The threshold for statistical significance was set as $p<0.05$, and Fishers LSD test was used for all post-hoc analysis of multiple comparisons when an interaction was detected.

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Western Blot

Protein analysis was performed as previously described (Anastasia et al., 2013; Portugal, Wilkinson, Turner, Blendy, & Gould, 2012). Hippocampi from $BDNF^{Val/Val}$ and $BDNF^{Met/Met}$ mice treated with either saline, chronic nicotine (18mg/kg/day) or undergoing 24-hour withdrawal was homogenized and lysed in 1% triton X-100 (Sigma, St Louis, MO, USA), 1% nonidet P-40 (Roche, Indianapolis, IN, USA) in Tris-buffered saline pH7.4 with protease inhibitor cocktail (Sigma). Protein concentration was quantified by Bradford (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were run on SDS-polyacrylamide gels (SDS-PAGE). After transference to PVDF membranes, proteins were fixed to the membrane with 2.5% glutaraldehyde in PBS pH 7.4 as described previously (Anastasia et al., 2013; Dieni et al., 2012). Membranes were blocked with 5% bovine serum albumin (BSA), then incubated with the prodomain antibody (mAb287,1:2000, 12-16h at 4°C, GeneCopoeia, Rockville, MD, USA; (Yang et al., 2009)), BDNF antibody (Santa Cruz Biotechnology), and actin antibody (Sigma) . Membranes were then incubated with anti-mouse or anti-rabbit horseradish peroxidase (HRP) secondary antibody for 1-hour at 20-25°C. Visualization and quantification of the protein was performed using enhanced chemiluminescence (Amersham-GE, Pittsburg, PA, USA) and ImageJ (1.45, NIH, Bethesda,MD, USA). Pro-BDNF, and BDNF prodomain densities were normalized to actin for each sample.

RESULTS

Novelty-induced hypophagia: Anxiolytic effect of nicotine and anxiogenic effect of withdrawal are blunted in $BDNF^{Met/Met}$ mice.

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To examine the effect of the BDNF Val66Met polymorphism on anxiety-like behavior we used the novelty-induced hypophagia (NIH) test. In the NIH test, reduction in feeding in response to a novel environment is a well-established measure for anxiety-related behaviors (Dulawa and Hen, 2005). When training mice to eat a palatable food we found that the $BDNF^{Met/Met}$ mice took a significantly longer time to first approach and eat the food when compared with the $BDNF^{Val/Val}$ mice (log-rank test ($P<0.0001$; Fig. 1B). The $BDNF^{Val/Val}$ mice all ate the food within 3 days, while the $BDNF^{Met/Met}$ mice did not all eat until day 9 of training. However, total food intake of PB chips was the same across genotypes and treatment groups (Fig. 1C). Latencies to consume increased on novel day as indicated by a main effect of day ($F_{(1,64)}=109.6$, $p<0.0001$; Fig. 1D). Two-way ANOVA also identified a main effect of treatment by genotype ($F_{(5,64)} = 3.528$, $p=0.007$) and an interaction of day by treatment ($F_{(5,64)}=3.976$, $p=0.0033$). Fisher's LSD post-hoc identified a significant decreased latency to consume in $BDNF^{Val/Val}$ mice treated with nicotine as compared to saline treated mice ($P<0.01$). In contrast, following 24-hour withdrawal from nicotine, $BDNF^{Val/Val}$ mice displayed a significant increase in latency to consume when compared to chronically treated nicotine mice ($P<0.0001$). The anxiogenic effect of withdrawal seen in $BDNF^{Val/Val}$ mice was not seen in $BDNF^{Met/Met}$ mice. As shown in Figure 1D, there was a significant decrease in latency to consume in the $BDNF^{Met/Met}$ mice undergoing 24-hour withdrawal as compared to $BDNF^{Val/Val}$ mice undergoing withdrawal ($P<0.0001$). $BDNF^{Met/Met}$ mice also showed a significant decrease in latency to consume during 24-hour withdrawal when compared to $BDNF^{Met/Met}$ saline-treated mice ($P<0.01$). In addition, the anxiolytic effect of chronic nicotine treatment observed in the $BDNF^{Val/Val}$ mice was not significant in the $BDNF^{Met/Met}$ mice.

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Marble Burying: BDNF$^{Met/Met}$ mice exhibit heightened anxiety-like behavior, which is attenuated following 24-hour withdrawal from nicotine

We used the marble burying test to further investigate the effect of the BDNF Val66Met polymorphism on anxiety-like behavior following 24-hour withdrawal (Fig. 2). In the BDNF Val66Met mice, there was a significant genotype x treatment interaction on the number of marbles buried ($F_{(1,46)}$=4.164, p=0.047). Further post-hoc analysis for multiple comparisons revealed that BDNF$^{Met/Met}$ saline-treated mice exhibited greater baseline anxiety-like behavior as measured by an increased number of marbles buried compared to BDNF$^{Val/Val}$ saline-treated mice (P=0.046). Following 24-hour withdrawal there were no differences in the number of marbles buried between the BDNF$^{Met/Met}$ and the BDNF$^{Val/Val}$ mice (p=0.815). However, there was a significant decrease in the number of marbles buried in the BDNF$^{Met/Met}$ mice following 24-hour withdrawal as compared to BDNF$^{Met/Met}$ saline-treated mice (P=0.033).

Open field: 24-hour withdrawal in BDNF$^{Met/Met}$ mice differentially alters anxiety-like behavior when compared to BDNF$^{Val/Val}$ mice undergoing 24-hour withdrawal

The open field test was used to further evaluate spontaneous locomotor activity and anxiety-like behavior in BDNF$^{Val/Val}$ and BDNF$^{Met/Met}$ mice following 24-hour withdrawal (Fig. 3A-D). A two-way ANOVA indicated a treatment by genotype interaction for time in center ($F_{(1,27)}$ = 4.445, p=0.04; Fig. 3A) and entries into center ($F_{(1,27)}$ = 4.680, p=0.03; Fig. 3B) but no main effect of genotype or treatment. During 24-Hr withdrawal BDNF$^{Val/Val}$ mice exhibited decreased time spent in the center (t_{16}=1.910, p<0.05; Fig 3A)

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while BDNF$^{Met/Met}$ did not show this decrease. To further analyze the genotype x treatment effect on novelty induced locomotion in the open field the first five minutes of the session were analyzed and revealed a genotype by treatment interaction ($F_{(1,27)}$ = 4.950, p=0.03; Fig. 3C) with BDNF$^{Met/Met}$ mice showing increased time spent in the center for the first 5 minutes following 24-hour withdrawal (t_{10}=1.960, p<0.05; Fig 3C), with no difference evident in BDNF$^{Val/Val}$ mice. No effect of genotype or treatment was observed on total locomotive activity (Fig. 3D).

BDNF prodomain is upregulated following 24-hour withdrawal in the hippocampus of BDNF$^{Met/Met}$ mice, while proBDNF remains unchanged across genotypes and treatments.

We used western blot analysis to determine if proBDNF (~32kDa) or BDNF prodomain (~15.5kDa) levels were dysregulated during chronic nicotine treatment and 24-hour withdrawal. As shown in Figure 4, proBDNF levels are significantly lower in hippocampi from BDNF$^{Met/Met}$ compared to BDNF$^{Val/Val}$ mice regardless of treatment (1-way ANOVA *p*=0.025; Fig.4A). The BDNF prodomain was also decreased in BDNF$^{Met/Met}$ mice in the saline and nicotine-treated groups. However, in BDNF$^{Met/Met}$ mice, BDNF prodomain levels were significantly elevated at the 24-hour withdrawal time point (1-way ANOVA: p<0.0002; Fig.4B). No increase in BDNF prodomain levels was observed in BDNF$^{Val/Val}$ mice.

DISCUSSION

In this study we utilized a BDNF Val66Met mouse model to examine the effects of this polymorphism on mediating anxiety-like behavior following withdrawal from

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nicotine. Nicotine withdrawal induces an increased anxiety state in both human (Dani & Harris, 2005; Hogle, Kaye, & Curtin, 2010; Picciotto, Brunzell, & Caldarone, 2002; Piper, Cook, Schlam, Jorenby, & Baker, 2011; Pomerleau et al., 2005) and animal models (Irvine, Cheeta, & File, 2001; Jackson, Martin, Changeux, & Damaj, 2008; Jackson, McIntosh, Brunzell, Sanjakdar, & Damaj, 2009; Jonkman, Henry, Semenova, & Markou, 2005; Stoker, Semenova, & Markou, 2008). By utilizing three behavioral paradigms known to assess anxiety-like behavior, we found that $BDNF^{Met/Met}$ mice have reduced anxiety-like behavior following nicotine withdrawal. This is the first finding to our knowledge that uses this mouse model of a human genetic variant to examine nicotine withdrawal phenotypes and suggests that this model could be used to identify molecular markers for withdrawal sensitivity in humans.

In humans, the negative affect experienced during withdrawal is directly associated with early smoking relapse with relapsers exhibiting a greater decrease in positive affect as compared to successful quitters (Al'absi, Hatsukami, Davis, & Wittmers, 2004). Withdrawal symptoms such as anxiety are not only related to the success of quitting but are also related to the time to relapse (al'Absi, Hatsukami, & Davis, 2005). For this reason, it is important to examine anxiety-like behavior at this early withdrawal timepoint in mice.

Studies in rodents have shown that lesions to the hippocampus impact anxiety-like behaviors, including hyponeophagia (Bannerman et al., 2003; 2004; Kalisch et al., 2006; McHugh, Deacon, Rawlins, & Bannerman, 2004; McHugh, Fillenz, Lowry, Rawlins, & Bannerman, 2011). Hippocampal volume can also be correlated with anxiety levels in patients with anxiety disorders (Bremner et al., 1995), and is associated with

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successful quit attempts (Froeliger et al., 2010). Of interest, irregular activation in the hippocampus has been associated with affect in smokers during smoking cue presentation (McClernon, Kozink, & Rose, 2008). Furthermore, the BDNF Val66Met polymorphism is associated with altered hippocampal function and activity (Egan et al., 2003; Hariri et al., 2003). Thus, alterations of BDNF in the hippocampus together with the known behavioral phenotypes observed in the $BDNF^{Met/Met}$ mice suggested that this anxiety-behavior might be dysregulated following nicotine withdrawal.

The NIH test is sensitive to both the effects of chronic nicotine and withdrawal from nicotine in mice (Turner et al., 2010; 2013; Yohn et al., 2014). In particular, studies have shown an anxiolytic effect of chronic nicotine as measured by a decreased latency to consume a palatable food in a novel environment when compared to saline-treated mice. In contrast, an anxiogenic effect of withdrawal at 24-hours is observed as an increased latency to consume a palatable food in a novel environment when compared to nicotine-treated mice (Hussmann et al., 2014; Turner et al., 2010; 2011; 2013; Yohn et al., 2014).

The increased time observed in the $BDNF^{Met/Met}$ mice to initially approach and eat the palatable food during training for the NIH test (Fig. 1B) might be indicative of either increased anxiety-like behavior as the food is a novel object introduced into the home cage, or it could be revealing a deficit in training behavior as studies have shown that the $BDNF^{Met/Met}$ mice exhibit impaired spatial and working memory (Wang et al., 2014). However, no significant differences between genotype or between treatment groups by the end of training were observed, suggesting that although acquisition of the task was slow, it did not affect the overall performance of these mice on home or novel day.

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During 24-hour withdrawal, $BDNF^{Met/Met}$ mice showed a decreased latency to consume a palatable food, indicative of a decreased anxiety-like response, when compared to wildtype ($BDNF^{Val/Val}$) mice undergoing 24-hour withdrawal (Fig. 1). This effect was further reflected in the marble-burying and open-field tests (Fig. 2,3). In marble burying there was a significant increase in anxiety-like behavior at baseline in the $BDNF^{Met/Met}$ mice as exhibited by increased number of marbles buried, compared to $BDNF^{Val/Val}$ mice at baseline (Fig. 2). An increase in baseline anxiety-like behavior has previously been reported in $BDNF^{Met/Met}$ mice in elevated plus maze, open field, and NIH tests (Chen et al., 2006). The baseline heightened anxiety phenotype in the $BDNF^{Met/Met}$ mice was lost following 24-hour withdrawal. In the open field test, $BDNF^{Met/Met}$ mice did not show the increased anxiety-like behavior seen in $BDNF^{Val/Val}$ mice following 24-hour withdrawal as exhibited by a decreased time spent in the center of the open field. $BDNF^{Met/Met}$ mice also exhibited increased exploratory behavior during the first 5 minutes of the open field test while undergoing 24-hour withdrawal from nicotine in contrast to the $BDNF^{Val/Val}$ mice undergoing withdrawal (Fig. 3).

Chronic nicotine treatment also had a different effect dependent on the BDNF Val66Met genotype. $BDNF^{Val/Val}$ mice showed the expected decreased latency to consume following chronic nicotine, indicative of decreased anxiety-like behavior. In contrast, while there was a trend, chronic nicotine did not significantly attenuate anxiety-like behavior in the $BDNF^{Met/Met}$ mice as measured by the NIH test (Fig. 1D). Drugs other then nicotine that are known to modulate BDNF, such as fluoxetine, also have dampened sensitivity in $BDNF^{Met/Met}$ mice as measured by the novelty-induced hyperphagia test

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(Chen et al., 2006). The behavioral responses in the NIH test are not due to altered spontaneous locomotion or food consumption on novel day (Fig. 1C;Fig. 3D).

No changes in proBDNF were found across treatments, with overall lower levels of proBDNF displayed in the hippocampus of $BDNF^{Met/Met}$ mice (Fig. 4A). These results duplicate a previous finding that the Val66Met SNP reduces activity dependent secretion of proBDNF (Hashimoto, 2007). ProBDNF is critically involved and abundant in early postnantal development (Yang et al., 2009). However, one of the cleaved products of proBDNF, the BDNF prodomain, is present and abundant during adulthood (Anastasia et al., 2013). A recent study demonstrated that the BDNF prodomain is not only abundant in the hippocampus but also secreted in an activity dependent manner (Anastasia et al., 2013). Of particular interest, the study showed that the Met66 prodomain is biologically active, alters neuronal morphology and induces growth cone retraction. Similar to previous studies we also detected lower levels of BDNF prodomain in the hippocampus of $BDNF^{Met/Met}$ mice compared to $BDNF^{Val/Val}$ mice (Anastasia et al., 2013; Bath et al., 2012). Of interest, we saw an increase in the BDNF prodomain during 24-hour withdrawal in the hippocampus of $BDNF^{Met/Met}$ mice (Fig. 4B). While the functional significance of this upregulation was not studied, it correlates with the blunted anxiety behaviors seen in $BDNF^{Met/Met}$ mice during 24-hour nicotine withdrawal. Thus, it is possible that the Met66 prodomain is a novel ligand that may be required for circuitry remodeling after nicotine withdrawal. Future studies looking at the role of this novel ligand on behavior are of interest.

It is of great clinical significance to understand how the BDNF Val66Met SNP affects the behavior associated with 24-hour nicotine withdrawal. Although multiple

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studies have shown the effect of the BDNF Val66Met variant on anxiety-related behaviors, it's effect on smoking initiation and dependence has elicited mixed clinical results (Lang et al., 2007; Montag et al., 2008). Studies focusing on the effect of the SNP on withdrawal from nicotine are just emerging. A recent study found a significant association of the SNP with smoking cessation where the Met/Met genotype was associated with increased cessation rates as compared to the Met/Val genotype in a large population-based sample (Breetvelt et al., 2012). Given the role of anxiety and negative affect seen in human populations following withdrawal from nicotine it is tempting to hypothesize that the increased cessation rates observed in the Met/Met population could be due to a decreased prevalence of negative affect following withdrawal, as demonstrated in our rodent studies. In conclusion, clinical studies investigating this SNP in nicotine withdrawal, and rodent studies continuing to look at the molecular underpinnings of the behaviors in these mice following withdrawal are necessary to further explain the effect of this SNP on smoking and withdrawal behavior in humans.

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DECLARATION OF INTERESTS

The authors BGL, AA, BLH, FSL and JAB report no biomedical financial interests or potential conflicts of interest.

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Figure Legends:

Figure 1.

Behavioral effects of the mouse BDNF Val66Met variant in the Novelty-Induced Hypophagia test. Mice from the following groups: $BDNF^{Val/Val}$; saline (n=17), nicotine (n=12), 24-Hr WD (n=13) and $BDNF^{Met/Met}$; saline (n=9), nicotine (n=10), 24-Hr WD (n=9) were run in the NIH test. (A) Experimental paradigm for nicotine delivery and time frame for behavioral testing. Osmotic minipumps were implanted on day one after animals were randomly assigned to a treatment group and counterbalanced for sex and genotype. All behavioral testing occurred on day 14, following 24-hour withdrawal in the withdrawal treatment group. Training for the NIH test training occurred days -14 to day 1. (B) $BDNF^{Met/Met}$ mice take significantly longer to first approach and consume the palatable food during training for the NIH paradigm compared to $BDNF^{Val/Val}$ mice. (C) No difference in food intake is observed on novel day regardless of treatment or genotype. (D) $BDNF^{Val/Val}$ mice chronically treated with nicotine exhibit a reduced latency to feed on novel test day compared with saline controls and 24-hour withdrawal. $BDNF^{Met/Met}$ mice exhibit a reduced latency to feed during 24-hour withdrawal compared with $BDNF^{Val/Val}$ mice undergoing withdrawal and $BDNF^{Met/Met}$ saline-treated mice. Significant compared with $BDNF^{Val/Val}$ 24-hour withdrawal - ****P<0.0001. Significant compared with $BDNF^{Val/Val}$ chronic nicotine - ##P<0.01. Significant compared with $BDNF^{Met/Met}$ vehicle treated mice - ††P<0.01.

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Figure 2. Effect of chronic nicotine, 24-hour withdrawal and the BDNF Val66Met genotype on behavior in the marble burying task. Data represents the mean number of marbles buried over 15 minutes ± SEM from mice in the following groups: $BDNF^{Val/Val}$; saline (n=13), 24-Hr WD (n=12) and $BDNF^{Met/Met}$; saline (n=11), 24-Hr WD (n=14). $BDNF^{Met/Met}$ mice bury more marbles at baseline compared with $BDNF^{Val/Val}$ saline-treated mice. In $BDNF^{Met/Met}$ mice 24-hour withdrawal from nicotine reduces the amount of marbles buried compared to saline-treated $BDNF^{Met/Met}$ mice, *P<0.05.

Figure 3. Open field behavior in the BDNF Val66Met variant during baseline (saline-treated) and 24-hour withdrawal. Open field parameters were measured over thirty minutes with data presented ± SEM in the following groups: $BDNF^{Val/Val}$; saline (n=9), 24-Hr WD (n=10) and $BDNF^{Met/Met}$; saline (n=6), 24-Hr WD (n=7). (A) $BDNF^{Val/Val}$ mice undergoing 24-Hr WD spend less time in the center compared to $BDNF^{Val/Val}$ saline-treated mice. (B) Number of entries into the center is unchanged by genotype and treatment alone but displays a genotype by treatment interaction. (C) $BDNF^{Met/Met}$ mice spend more time in the center for the first 5-minute time block compared to saline-treated $BDNF^{Met/Met}$ mice, while no difference in treatment on $BDNF^{Val/Val}$ mice is observed. (D) No differences in total locomoter activity measured over a 30-minute time frame are seen between genotypes and treatment, *P<0.05.

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Figure 4.

Western blot analysis of ProBDNF and BDNF Prodomain in the hippocampus. (A) Detection of proBDNF (~32kDa) expression in the hippocampus is reduced in $BDNF^{Met/Met}$ mice compared to $BDNF^{Val/Val}$ mice. Treatment with saline, two weeks of chronic nicotine (18mg/kg/day) or 24-hour withdrawal does not alter proBDNF levels. (B) BDNF prodomain (~15.5kDa) expression is decreased in hippocampal lysates of saline-treated and chronic nicotine-treated $BDNF^{Met/Met}$ mice compared to $BDNF^{Val/Val}$ mice. Following 24-hour withdrawal BDNF prodomain levels in $BDNF^{Met/Met}$ mice are increased. Representative blots are shown below each panel, bars represent mean ± SEM of proBDNF (A) and BDNF prodomain (B) densitometry data normalized to actin; n=4 per group, *P<0.05.

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Figure 1.

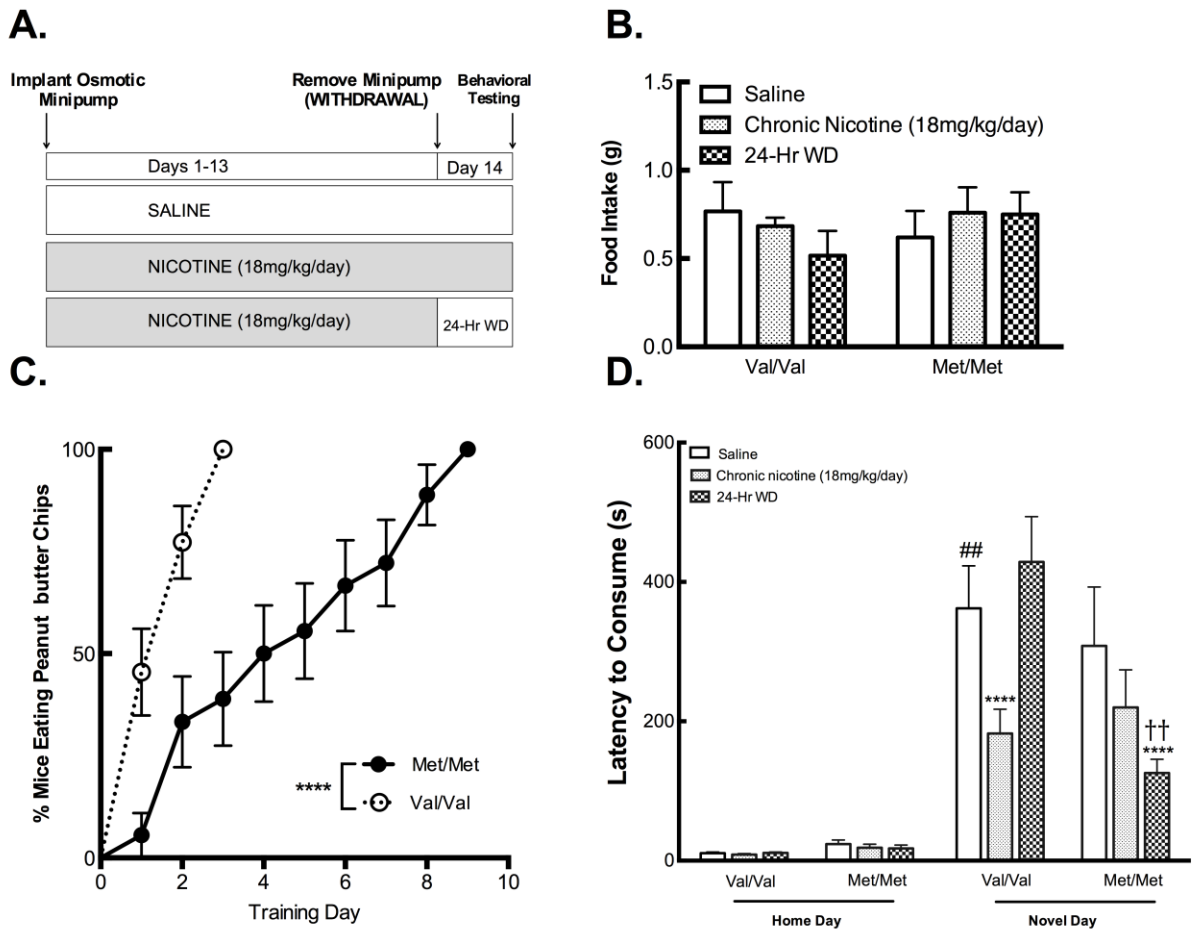

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Figure 2

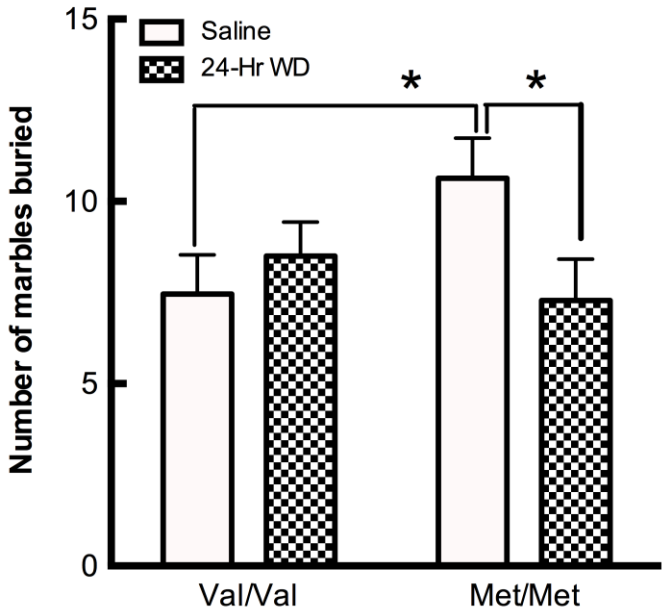

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Figure 3

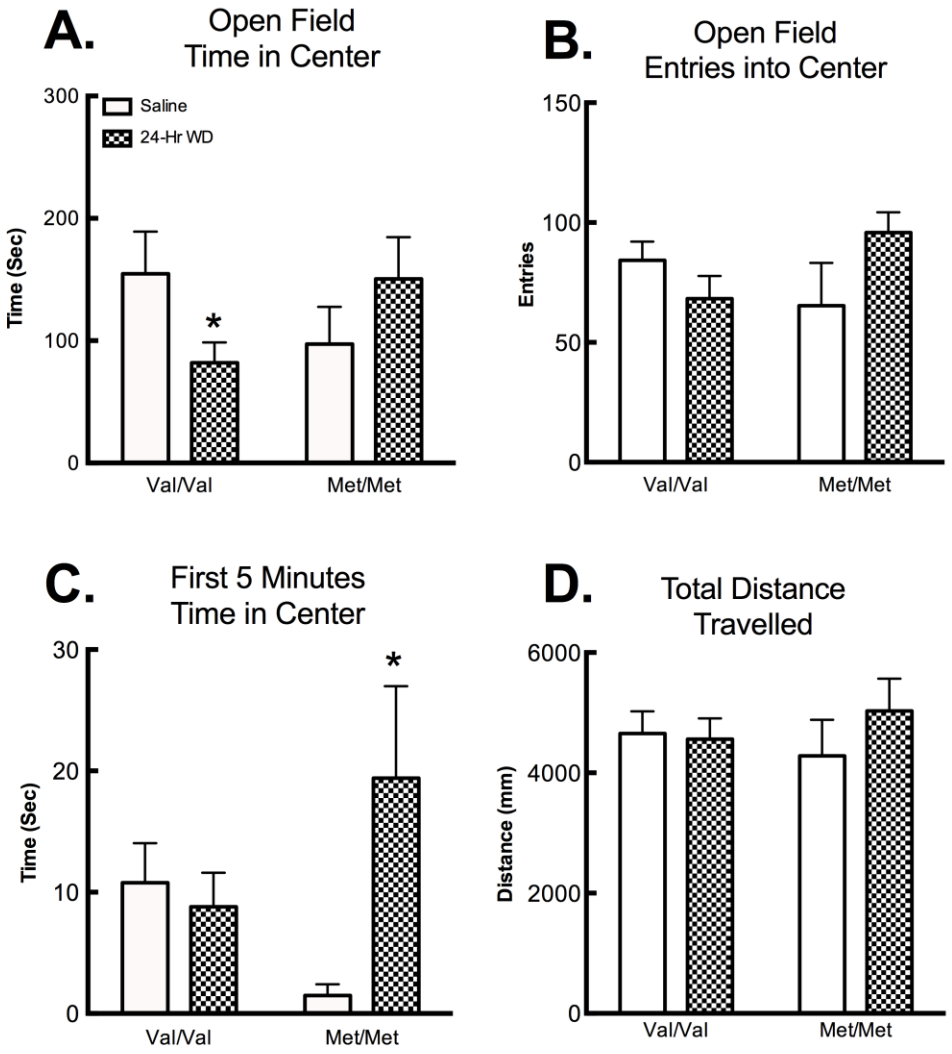

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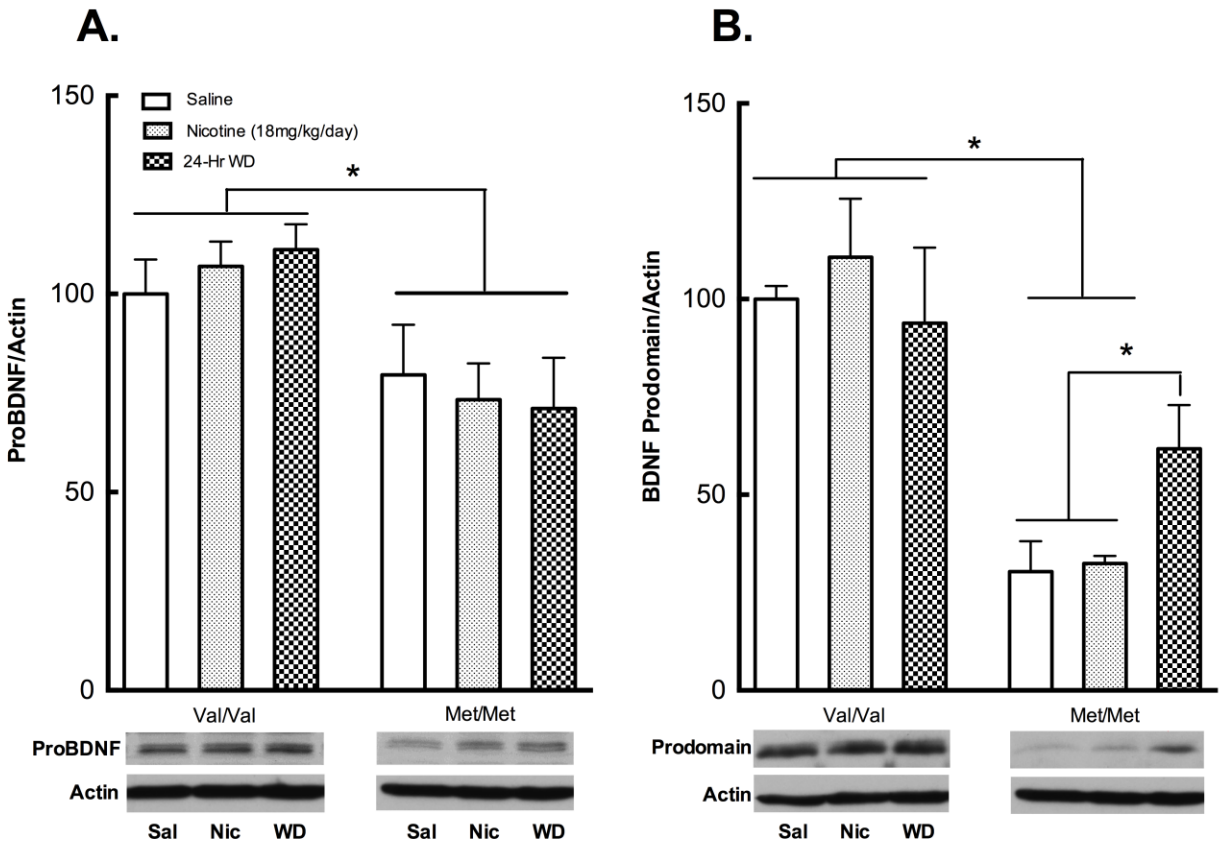
Figure 4.



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