Original investigation

Effects of the BDNF Val66Met Polymorphism on Anxiety-Like Behavior Following Nicotine Withdrawal in Mice

Bridgin G. Lee BA¹, Agustin Anastasia PhD², Barbara L. Hempstead MD, PhD², Francis S. Lee MD, PhD³, Julie A. Blendy PhD¹

¹Department of Pharmacology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA;

²Department of Medicine, Weill Cornell Medical College of Cornell University, New York, NY; ³Department of Psychiatry, Weill Cornell Medical College of Cornell University, New York, NY

Corresponding Author: Julie A. Blendy, PhD, Department of Pharmacology, University of Pennsylvania, TRL, 125 South 31st Street, Philadelphia, PA 19104, USA. Telephone: 215-898-0730; Fax: 215-573-2236; E-mail: blendy@mail.med.upenn.edu

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 single nucleotide polymorphism in the translated region of the brain-derived neurotrophic factor (BDNF) gene that substitutes a valine (Val) for a methionine (Met) amino acid (Val66Met) to examine the relationship between the Val66Met single nucleotide polymorphism 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 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.¹ However, despite the numerous health risks less than 10% of quit attempts result in continuous abstinence for 1 year.² Nicotine, the primary addictive component of tobacco, acts on brain nicotinic

acetylcholine receptors to increase neuronal dopamine activity within the mesolimbic reward circuit.³ Altered homeostasis of this brain reward system results in complex behaviors such as dependence, tolerance, sensitization, and craving.⁴ 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,⁵ differentiation,⁶ and survival⁷ of neurons. Genetic modulation of BDNF or its receptor, TrkB, alters the rewarding effect of drugs of abuse such as cocaine and alcohol,^{8–10} linking BDNF to addiction. Smoking also increases plasma BDNF levels in humans^{11,12} and administered nicotine increases BDNF gene expression in critical regions of the mesolimbic reward circuit of rodents.¹³

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.¹⁴ 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.¹⁵ The BDNF Val66Met SNP reduces activity-dependent secretion of BDNF¹⁶ 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.¹⁷ This SNP has been associated with anxiety,¹⁷ hypothalamic-pituitary-adrenal axis reactivity to stress,^{18,19} depression,²⁰ posttraumatic stress disorder,²¹ and substance abuse.²² 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.^{23,24} 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 successful quitting.²⁵ Furthermore, studies utilizing a knock-in mouse containing the BDNF Val66Met variant demonstrate that this SNP can alter affective behavior. Specifically, mice harboring the 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 maze¹⁷ as compared to wildtype control mice harboring a 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.^{26,27} 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.¹⁷ 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 12 hour/12 hour 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 novelty-induced hypophagia (NIH) paradigm where mice were housed in groups of two. Equal numbers of male and female 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) were placed subcutaneously using aseptic surgery techniques. (-)-Nicotine hydrogen tartrate salt (Sigma, St. Louis, MO) was dissolved in sterile 0.85% saline and administered in osmotic minipumps at a dose of 18 mg kg⁻¹ day⁻¹ (dose expressed as the freebase weight) for 2 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: NIH, 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 9:00 AM and 2:00 PM. Mice were acclimated to the behavioral testing facility 1 hour prior to testing for all studies.

Novelty Induced Hypophagia

The NIH test was conducted as previously described^{29–30} 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 1 hour prior to presentation of a palatable food (peanut butter chips; Nestle, Glendale, CA). Training was repeated over 14 days with latency to consume the food measured on days 4–14. Following training, mice were assigned to a 2-week treatment group and implanted with osmotic minipumps (Figure 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.³¹ Mice were placed in a small cage (26×20×14 cm) with 5-cm deep bedding, on top of which 20 equally distributed marbles lay. Following a 1-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 Locomotor Activity

Locomotor activity was measured in a plexiglas arena (43×43 cm²) containing 32 photobeams in a 16×16 formation (MED Associates, St. Albans, Vermont), and was monitored using Photobeam Activity System software. The 6×6 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), and all data are presented as mean ± standard error of mean. Data analysis for

Figure 1. Behavioral effects of the mouse brain-derived neurotrophic factor (BDNF) Val66Met variant in the novelty-induced hypophagia (NIH) test. Mice from the following groups: BDNF^{Val/Val}, saline ($n = 17$), nicotine ($n = 12$), 24-hour withdrawal (WD) ($n = 13$) and BDNF^{Met/Met}; saline ($n = 9$), nicotine ($n = 10$), 24-hour 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 1 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 day 1 to day 14. (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—**** $P < .0001$. Significant compared with BDNF^{Val/Val} chronic nicotine—## $P < .01$. Significant compared with BDNF^{Met/Met} vehicle treated mice—†† $P < .01$.

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 (Figure 1B). For the NIH test, a repeated measures two-way analysis of variance (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 (Figure 1D). For the marble-burying experiment and open field test a two-way ANOVA with genotype and treatment as the two factors was used (Figures 3 and 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-hour withdrawal to saline-treated mice in both genotypes. ANOVA was used to analyze genotype and treatment differences in proBDNF and the BDNF prodomain (Figure 4). The threshold for statistical significance was set as $P < .05$, and Fishers least significant difference test was used for all post-hoc analysis of multiple comparisons when an interaction was detected.

Western Blot

Protein analysis was performed as previously described.^{32,33} Hippocampi from BDNF^{Val/Val} and BDNF^{Met/Met} mice treated with either saline, chronic nicotine (18 mg kg⁻¹ day⁻¹) or undergoing 24-hour withdrawal was homogenized and lysed in 1% triton X-100 (Sigma), 1% nonidet P-40 (Roche, Indianapolis, IN) in Tris-buffered saline pH 7.4 with protease inhibitor cocktail (Sigma). Protein

concentration was quantified by Bradford (Bio-Rad, Hercules, CA). 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 phosphate buffered saline pH 7.4 as described previously.^{32,34} Membranes were blocked with 5% bovine serum albumin, then incubated with the prodomain antibody (mAb287, 1:2000, 12–16 hours at 4°C, GeneCopoeia, Rockville, MD),³⁵ BDNF antibody (Santa Cruz Biotechnology), and actin antibody (Sigma). Membranes were then incubated with anti-mouse or anti-rabbit horseradish peroxidase secondary antibody for 1 hour at 20°C–25°C. Visualization and quantification of the protein was performed using enhanced chemiluminescence (Amersham-GE, Pittsburgh, PA) and ImageJ (1.45, NIH, Bethesda, MD). proBDNF and BDNF prodomain densities were normalized to actin for each sample.

Results

NIH: Anxiolytic Effect of Nicotine and Anxiogenic Effect of Withdrawal are Blunted in BDNF^{Met/Met} Mice

To examine the effect of the BDNF Val66Met polymorphism on anxiety-like behavior we used the NIH test. In the NIH test, reduction in feeding in response to a novel environment is a well-established measure for anxiety-related behaviors.³⁶ 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 ($P < .0001$; Figure 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 (Figure 1C). Latencies to consume increased on novel day as indicated by a main effect of day ($F_{(1,64)} = 109.6$, $P < .0001$; Figure 1D). Two-way ANOVA also identified a main effect of treatment by genotype ($F_{(5,64)} = 3.528$, $P = .007$) and an interaction of day by treatment ($F_{(5,64)} = 3.976$, $P = .0033$). 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 ($P < .01$). 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 ($P < .0001$). 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 ($P < .0001$). 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 ($P < .01$). 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.

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 (Figure 2). In the BDNF Val66Met mice, there was a significant genotype $\times$ treatment interaction on the number of marbles buried ($F_{(1,46)} = 4.164$, $P = .047$). 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 ($P = .046$). 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 ($P = .815$). 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 ($P = .033$).

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 (Figure 3, A–D). A two-way ANOVA indicated a treatment by genotype interaction for time in center ($F_{(1,27)} = 4.445$, $P = .04$; Figure 3A) and entries into center ($F_{(1,27)} = 4.680$, $P = .03$; Figure 3B) but no main effect of genotype or treatment. During 24-hour withdrawal BDNF^{Val/Val} mice exhibited decreased time spent in the center ($t_{16} = 1.910$, $P < .05$; Figure 3A) while BDNF^{Met/Met} did not show this decrease. To further analyze the genotype $\times$ treatment effect on novelty induced locomotion in the open field the first 5 minutes of the session were analyzed and revealed a genotype by treatment interaction ($F_{(1,27)} = 4.950$,

Figure 2. Effect of chronic nicotine, 24-hour withdrawal and the brain-derived neurotrophic factor (BDNF) Val66Met genotype on behavior in the marble burying task. Data represents the mean number of marbles buried over 15 minutes $\pm$ SEM from mice in the following groups: BDNF^{Val/Val}; saline ($n = 13$), 24-hour withdrawal (WD) ($n = 12$) and BDNF^{Met/Met}; saline ($n = 11$), 24-hour 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, * $P < .05$.

$P = .03$; Figure 3C) with BDNF^{Met/Met} mice showing increased time spent in the center for the first 5 minutes following 24-hour withdrawal ($t_{10} = 1.960$, $P < .05$; Figure 3C), with no difference evident in BDNF^{Val/Val} mice. No effect of genotype or treatment was observed on total locomotive activity (Figure 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 (one-way ANOVA $P = .025$; Figure 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 (one-way ANOVA: $P < .0002$; Figure 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 nicotine. Nicotine withdrawal induces an increased anxiety state in both human^{37–41} and animal models.^{42–46} 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

Figure 3. Open field behavior in the brain-derived neurotrophic factor (BDNF) Val66Met variant during baseline (saline-treated) and 24-hour withdrawal. Open field parameters were measured over 30 minutes with data presented $\pm$ SEM in the following groups: BDNF^{Val/Val}; saline ($n = 9$), 24-hour withdrawal (WD) ($n = 10$) and BDNF^{Met/Met}; saline ($n = 6$), 24-hour WD ($n = 7$). (A) BDNF^{Val/Val} mice undergoing 24-hour 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 locomotor activity measured over a 30-minute time frame are seen between genotypes and treatment, $*P < .05$.

Figure 4. Western blot analysis of proBDNF and brain-derived neurotrophic factor (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, 2 weeks of chronic nicotine ($18\text{mg kg}^{-1}\text{ day}^{-1}$) 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 $\pm$ SEM of proBDNF (A) and BDNF prodomain (B) densitometry data normalized to actin; $n = 4$ per group, $*P < .05$.

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.⁴⁷ Withdrawal symptoms such as anxiety are not only related to the success of quitting but are also related to the time to relapse.⁴⁸ 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.^{49–53} Hippocampal volume can also be correlated with anxiety levels in patients with anxiety disorders,⁵⁴ and is associated with successful quit attempts.⁵⁵ Of interest, irregular activation in the hippocampus has been associated with affect in smokers during smoking cue presentation.⁵⁶ Furthermore, the BDNF Val66Met polymorphism is associated with altered hippocampal function and activity.^{16,57} 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.^{28–30} 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.^{28–31,58}

The increased time observed in the BDNF^{Met/Met} mice to initially approach and eat the palatable food during training for the NIH test (Figure 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.⁵⁹ 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.

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 (Figure 1). This effect was further reflected in the marble-burying and open-field tests (Figures 2 and 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 (Figure 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.¹⁷ 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 (Figure 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 (Figure 1D). Drugs other than 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.¹⁷ The behavioral responses in the NIH test are not due to altered spontaneous locomotion or food consumption on novel day (Figure 1C; Figure 3D).

No changes in proBDNF were found across treatments, with overall lower levels of proBDNF displayed in the hippocampus of BDNF^{Met/Met} mice (Figure 4A). These results duplicate a previous finding that the Val66Met SNP reduces activity dependent secretion of proBDNF.⁶⁰ ProBDNF is critically involved and abundant in early postnatal development.³⁵ However, one of the cleaved products of proBDNF, the BDNF prodomain, is present and abundant during adulthood.³² A recent study demonstrated that the BDNF prodomain is not only abundant in the hippocampus but also secreted in an activity dependent manner.³² 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.^{32,61} Of interest, we saw an increase in the BDNF prodomain during 24-hour withdrawal in the hippocampus of BDNF^{Met/Met} mice (Figure 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 studies have shown the effect of the BDNF Val66Met variant on anxiety-related behaviors, its effect on smoking initiation and dependence has elicited mixed clinical results.^{23,24} 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.²⁵ 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.

Funding

This work was supported by T32 GM-008076 (BGL), National Cancer Institute (P50-CA143187) (JAB), NINDS (NS052819) (FSL), and NIH-NINDS R01NS030687 (BLH).

Declaration of Interests

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

Acknowledgments

We thank Ted Huang for technical assistance.

References

- Adhikari B, Kahende J, Malarcher A. Centers for Disease Control and Prevention. Smoking-Attributable Death, Years of Potential Life lost, Productivity loss – United States, 2000–2004. *Morbidity Mortality Weekly Report*. 2008;56(45):1226–1228.
- Trosclair A, Caraballo R, Malarcher A, Husten C. Centers for Disease Control and Prevention. Cigarette smoking among adults—United States, 2011. *Morbidity Mortality Weekly Report*. 2012;61(44):889–894.
- Wu J. Double target concept for smoking cessation. *Acta Pharmacol Sin*. 2010;31(9):1015–1018. doi:10.1038/aps.2010.137.
- De Biasi M, Dani JA. Reward, addiction, withdrawal to nicotine. *Annu Rev Neurosci*. 2011;34:105–130. doi:10.1146/annurev-neuro-061010-113734.
- Segal RA, Pomeroy SL, Stiles CD. Axonal growth and fasciculation linked to differential expression of BDNF and NT3 receptors in developing cerebellar granule cells. *J Neurosci*. 1995;15(7, pt 1):4970–4981.
- Leschik J, Eckenstaler R, Nieweg K, et al. Embryonic stem cells stably expressing BDNF-GFP exhibit a BDNF-release-dependent enhancement of neuronal differentiation. *J Cell Sci*. 2013;126(1, pt 21):5062–5073. doi:10.1242/jcs.135384.
- Hofer MM, Barde YA. Brain-derived neurotrophic factor prevents neuronal death in vivo. *Nature*. 1988;331(6153):261–262. doi:10.1038/331261a0.
- Hall FS, Drgonova J, Goeb M, Uhl GR. Reduced behavioral effects of cocaine in heterozygous brain-derived neurotrophic factor (BDNF) knockout mice. *Neuropsychopharmacology*. 2003;28(8):1485–1490. doi:10.1038/sj.npp.1300192.
- Hensler JG, Ladenheim EE, Lyons WE. Ethanol consumption and serotonin-1A (5-HT1A) receptor function in heterozygous BDNF (+/–) mice. *J Neurochem*. 2003;85(5):1139–1147.
- Unterwald EM, Page ME, Brown TB, et al. Behavioral and transcriptome alterations in male and female mice with postnatal deletion of TrkB in dorsal striatal medium spiny neurons. *Mol Neurodegener*. 2013;8(1):47. doi:10.1186/1750-1326-8-47.
- Bhang S-Y, Choi S-W, Ahn J-H. Changes in plasma brain-derived neurotrophic factor levels in smokers after smoking cessation. *Neurosci Lett*. 2010;468(1):7–11. doi:10.1016/j.neulet.2009.10.046.
- Kim T-S, Kim D-J, Lee H, Kim Y-K. Increased plasma brain-derived neurotrophic factor levels in chronic smokers following unaided smoking cessation. *Neurosci Lett*. 2007;423(1):53–57. doi:10.1016/j.neulet.2007.05.064.
- Kivimäki T, Kaste K, Rantamäki T, Castrén E, Ahtee L. Alterations in BDNF and phospho-CREB levels following chronic oral nicotine treatment and its withdrawal in dopaminergic brain areas of mice. *Neurosci Lett*. 2011;491(2):108–112. doi:10.1016/j.neulet.2011.01.015.
- 1000 Genomes Project Consortium; Abecasis GR, Auton A, Brooks LD, et al. An integrated map of genetic variation from 1,092 human genomes. *Nature*. 2012;491(7422):56–65. doi:10.1038/nature11632.
- Chen ZY, Ieraci A, Teng H, et al. Sortilin controls intracellular sorting of brain-derived neurotrophic factor to the regulated secretory pathway. *J Neurosci*. 2005;25(26):6156–6166. doi:10.1523/JNEUROSCI.1017-05.2005.
- Egan M, Kojima M, Callicott J, Goldberg T. ScienceDirect—cell: the BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function. *Cell*. 2003;112(2):257–269.
- Chen Z-Y, Jing D, Bath KG, et al. Genetic variant BDNF (Val66Met) polymorphism alters anxiety-related behavior. *Science*. 2006;314(5796):140–143. doi:10.1126/science.1129663.
- Alexander N, Osinsky R, Schmitz A, Mueller E, Kuepper Y, Hennig J. The BDNF Val66Met polymorphism affects HPA-axis reactivity to acute stress. *Psychoneuroendocrinology*. 2010;35(6):949–953. doi:10.1016/j.psyneuen.2009.12.008.
- Colzato LS, Van der Does AJW, Kouwenhoven C, Elzinga BM, Hommel B. BDNF Val66Met polymorphism is associated with higher anticipatory cortisol stress response, anxiety, and alcohol consumption in healthy adults. *Psychoneuroendocrinology*. 2011;36(10):1562–1569. doi:10.1016/j.psyneuen.2011.04.010.
- Tsai SJ, Hong CJ, Liou YJ. Effects of BDNF polymorphisms on antidepressant action. *Psychiatry Investig*. 2010;7(4):236–242. doi:10.4306/pi.2010.7.4.236.
- Zhang L, Benedek DM, Fullerton CS, et al. PTSD risk is associated with BDNF Val66Met and BDNF overexpression. *Mol Psychiatry*. 2014;19(1):8–10. doi:10.1038/mp.2012.180.
- Gratacòs M, González JR, Mercader JM, de Cid R, Urretavizcaya M, Estivill X. Brain-derived neurotrophic factor Val66Met and psychiatric disorders: meta-analysis of case-control studies confirm association to substance-related disorders, eating disorders, and schizophrenia. *Biol Psychiatry*. 2007;61(7):911–922. doi:10.1016/j.biopsych.2006.08.025.
- Lang UE, Sander T, Lohoff FW, et al. Association of the met66 allele of brain-derived neurotrophic factor (BDNF) with smoking. *Psychopharmacology (Berl)*. 2007;190(4):433–439. doi:10.1007/s00213-006-0647-1.
- Montag C, Basten U, Stelzel C, Fiebach CJ, Reuter M. The BDNF Val66Met polymorphism and smoking. *Neurosci Lett*. 2008;442(1):30–33. doi:10.1016/j.neulet.2008.06.064.
- Breetvelt EJ, Numans ME, Aukes MF, et al. The association of the alpha-5 subunit of the nicotinic acetylcholine receptor gene and the brain-derived neurotrophic factor gene with different aspects of smoking behavior. *Psychiatr Genet*. 2012;22(2):96–98. doi:10.1097/YPG.0b013e32834c0c75.
- Hughes JR. Tobacco withdrawal in self-quitters. *J Consult Clin Psychol*. 1992;60(5):689. doi:10.1037/0022-006X.60.5.689.
- Hughes JR. Effects of abstinence from tobacco: etiology, animal models, epidemiology, and significance: a subjective review. *Nicotine Tob Res*. 2007;9(3):329–339. doi:10.1080/14622200701188919.
- Turner JR, Castellano LM, Blendy JA. Nicotinic partial agonists varenicline and sazetidine-A have differential effects on affective behavior. *J Pharmacol Exp Ther*. 2010;334(2):665–672. doi:10.1124/jpet.110.166280.
- Turner JR, Ray R, Lee B, et al. Evidence from mouse and man for a role of neuregulin 3 in nicotine dependence. *Mol Psychiatry*. 2014;19(7):801–810. doi:10.1038/mp.2013.104.
- Yohn NL, Turner JR, Blendy JA. Activation of $\alpha 4\beta 2^*/\alpha 6\beta 2^*$ nicotinic receptors alleviates anxiety during nicotine withdrawal without upregulating nicotinic receptors. *J Pharmacol Exp Ther*. 2014;349(2):348–354. doi:10.1124/jpet.113.211706.
- Turner JR, Castellano LM, Blendy JA. Parallel anxiolytic-like effects and upregulation of neuronal nicotinic acetylcholine receptors following chronic nicotine and varenicline. *Nicotine Tob Res*. 2011;13(1):41–46. doi:10.1093/ntr/ntq206.
- Anastasia A, Deinhardt K, Chao MV, et al. Val66Met polymorphism of BDNF alters prodomain structure to induce neuronal growth cone retraction. *Nat Commun*. 2013;4:2490. doi:10.1038/ncomms3490.
- Portugal GS, Wilkinson DS, Turner JR, Blendy JA, Gould TJ. Developmental effects of acute, chronic, and withdrawal from chronic nicotine on fear conditioning. *Neurobiol Learn Mem*. 2012;97(4):482–494. doi:10.1016/j.nlm.2012.04.003.
- Dieni S, Matsumoto T, Dekkers M, et al. BDNF and its pro-peptide are stored in presynaptic dense core vesicles in brain neurons. *J Cell Biol*. 2012;196(6):775–788. doi:10.1083/jcb.201201038.
- Yang J, Siao C-J, Nagappan G, et al. Neuronal release of proBDNF. *Nat Neurosci*. 2009;12(2):113–115. doi:10.1038/nn.2244.

36. Dulawa SC, Hen R. Recent advances in animal models of chronic antidepressant effects: the novelty-induced hypophagia test. *Neurosci Biobehav Rev.* 2005;20(4-5):771-783.
37. Dani JA, Harris RA. Nicotine addiction and comorbidity with alcohol abuse and mental illness. *Nat Neurosci.* 2005;8(11):1465-1470. doi:10.1038/nn1580.
38. Hogle JM, Kaye JT, Curtin JJ. Nicotine withdrawal increases threat-induced anxiety but not fear: neuroadaptation in human addiction. *Biol Psychiatry.* 2010;68(8):719-725. doi:10.1016/j.biopsych.2010.06.003.
39. Picciotto MR, Brunzell DH, Caldarone BJ. Effect of nicotine and nicotinic receptors on anxiety and depression. *Neuroreport.* 2002;13(9):1097-1106.
40. Piper ME, Cook JW, Schlam TR, Jorenby DE, Baker TB. Anxiety diagnoses in smokers seeking cessation treatment: relations with tobacco dependence, withdrawal, outcome and response to treatment. *Addiction.* 2011;106(2):418-427. doi:10.1111/j.1360-0443.2010.03173.x
41. Pomerleau OF, Pomerleau CS, Mehninger AM, Snedecor SM, Ninowski R, Sen A. Nicotine dependence, depression, and gender: characterizing phenotypes based on withdrawal discomfort, response to smoking, and ability to abstain. *Nicotine Tob Res.* 2005;7(1):91-102. doi:10.1080/14622200412331328466.
42. Irvine E, Cheeta S, File S. Tolerance to nicotine's effects in the elevated plus-maze and increased anxiety during withdrawal. *Pharmacol Biochem Behav.* 2001;68(2):319-325.
43. Jackson KJ, Martin BR, Changeux JP, Damaj MI. Differential role of nicotinic acetylcholine receptor subunits in physical and affective nicotine withdrawal signs. *J Pharmacol Exp Ther.* 2008;325(1):302-312. doi:10.1124/jpet.107.132977.
44. Jackson KJ, McIntosh JM, Brunzell DH, Sanjakdar SS, Damaj MI. The role of alpha6-containing nicotinic acetylcholine receptors in nicotine reward and withdrawal. *J Pharmacol Exp Ther.* 2009;331(2):547-554. doi:10.1124/jpet.109.155457.
45. Jonkman S, Henry B, Semenova S, Markou A. Mild anxiogenic effects of nicotine withdrawal in mice. *Eur J Pharmacol.* 2005;516(1):40-45. doi:10.1016/j.ejphar.2005.04.032.
46. Stoker AK, Semenova S, Markou A. Affective and somatic aspects of spontaneous and precipitated nicotine withdrawal in C57BL/6J and BALB/cByJ mice. *Neuropharmacology.* 2008;54(8):1223-1232. doi:10.1016/j.neuropharm.2008.03.013.
47. Al'absi M, Hatsukami D, Davis GL, Wittmers LE. Prospective examination of effects of smoking abstinence on cortisol and withdrawal symptoms as predictors of early smoking relapse. *Drug Alcohol Depend.* 2004;73(3):267-278. doi:10.1016/j.drugalcdep.2003.10.014.
48. al'Absi M, Hatsukami D, Davis G. Attenuated adrenocorticotrophic responses to psychological stress are associated with early smoking relapse. *Psychopharmacology.* 2005.
49. Bannerman DM, Grubb M, Deacon RMJ, Yee BK, Feldon J, Rawlins JNP. Ventral hippocampal lesions affect anxiety but not spatial learning. *Behav Brain Res.* 2003;139(1-2):197-213. doi:10.1016/S0166-4328(02)00268-1.
50. Bannerman DM, Rawlins JN, McHugh SB, et al. Regional dissociations within the hippocampus-memory and anxiety. *Neurosci Biobehav Rev.* 2004;28(3):273-283. doi:10.1016/j.neubiorev.2004.03.004.
51. Kalisch R, Schubert M, Jacob W, et al. Anxiety and hippocampus volume in the rat. *Neuropsychopharmacology.* 2006;31(5):925-932. doi:10.1038/sj.npp.1300910.
52. McHugh SB, Deacon RMJ, Rawlins JNP, Bannerman DM. Amygdala and ventral hippocampus contribute differentially to mechanisms of fear and anxiety. *Behav Neurosci.* 2004;118(1):63-78. doi:10.1037/0735-7044.118.1.63.
53. McHugh SB, Fillenz M, Lowry JP, Rawlins JNP, Bannerman DM. Brain tissue oxygen amperometry in behaving rats demonstrates functional dissociation of dorsal and ventral hippocampus during spatial processing and anxiety. *Eur J Neurosci.* 2011;33(2):322-337. doi:10.1111/j.1460-9568.2010.07497.x.
54. Bremner JD, Randall P, Scott TM, et al. MRI-based measurement of hippocampal volume in patients with combat-related posttraumatic stress disorder. *Am J Psychiatry.* 1995;152(7):973-981.
55. Froeliger B, Kozink RV, Rose JE, Behm FM, Salley AN, McClernon FJ. Hippocampal and striatal gray matter volume are associated with a smoking cessation treatment outcome: results of an exploratory voxel-based morphometric analysis. *Psychopharmacology.* 2010;210(4):577-583. doi:10.1007/s00213-010-1862-3.
56. McClernon FJ, Kozink RV, Rose JE. Individual differences in nicotine dependence, withdrawal symptoms, and sex predict transient fMRI-BOLD responses to smoking cues. *Neuropsychopharmacology.* 2008;33(9):2148-2157. doi:10.1038/sj.npp.1301618.
57. Hariri AR, Goldberg TE, Mattay VS, et al. Brain-derived neurotrophic factor val66met polymorphism affects human memory-related hippocampal activity and predicts memory performance. *J Neurosci.* 2003;23(17):6690-6694.
58. Hussmann GP, DeDominicis KE, Turner JR, et al. Chronic sazetidine-A maintains anxiolytic effects and slower weight gain following chronic nicotine without maintaining increased density of nicotinic receptors in rodent brain. *J Neurochem.* 2014;129(4):721-731. doi:10.1111/jnc.12653.
59. Wang D-D, Tian T, Dong Q, et al. Transcriptome profiling analysis of the mechanisms underlying the BDNF Val66Met polymorphism induced dysfunctions of the central nervous system. *Hippocampus.* 2014;24(1):65-78. doi:10.1002/hipo.22204.
60. Hashimoto K. BDNF variant linked to anxiety-related behaviors - Hashimoto - 2007 - BioEssays - Wiley Online Library. *Bioessays.* 2007; 29(2):116-119.
61. Bath KG, Jing DQ, Dincheva I, et al. BDNF Val66Met impairs fluoxetine-induced enhancement of adult hippocampus plasticity. *Neuropsychopharmacology.* 2012;37(5):1297-1304. doi:10.1038/npp.2011.318.