Variant Brain-Derived Neurotrophic Factor (Valine66Methionine) Polymorphism Contributes to Developmental and Estrous Stage-Specific Expression of Anxiety-Like Behavior in Female Mice

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Background: Most anxiety and depressive disorders are twice as common in women compared with men, and the sex difference in prevalence typically emerges during adolescence. Hormonal changes across the menstrual cycle and during the postpartum and perimenopausal periods are associated with increased risk for anxiety and depression symptoms. In humans and animals, reduced brain-derived neurotrophic factor (*BDNF*) has been associated with increased expression of affective pathology. Recently, a single nucleotide polymorphism (SNP) in the *BDNF* gene (*BDNF* Valine66Methionine [Val66Met]), which reduces *BDNF* bioavailability, has been identified in humans and associated with a variety of neuropsychiatric disorders. Although *BDNF* expression can be directly influenced by estrogen and progesterone, the potential impact of the *BDNF* Val66Met SNP on sensitivity to reproductive hormone changes remains an open question.

Methods: As a predictive model, we used female mice in which the human SNP (*BDNF* Val66Met) was inserted into the mouse *BDNF* gene. Using standard behavioral paradigms, we tested the impact of this SNP on age and estrous-cycle-specific expression of anxiety-like behaviors.

Results: Mice homozygous for the *BDNF* Val66Met SNP begin to exhibit increased anxiety-like behaviors over prepubertal and early adult development, show significant fluctuations in anxiety-like behaviors over the estrous cycle, and, as adults, differ from wild-type mice by showing significant fluctuations in anxiety-like behaviors over the estrous cycle—specifically, more anxiety-like behaviors during the estrus phase.

Conclusions: These findings have implications regarding the potential role of this SNP in contributing to developmental and reproductive hormone-dependent changes in affective disorders in humans.

Key Words: Anxiety, *BDNF*, behavior, development, estrous, female, Val66Met

The development of anxiety and depressive disorders peaks during adolescence and early adulthood, with females being at significantly greater risk than males (1–4). Specifically, females show a significant increase in the expression of affective disorders that often coincides with the onset of ovarian cycling (4). Periods of reproductive hormone flux, including the menstrual cycle, the postpartum period and perimenopause are associated with exacerbations in mood and anxiety symptoms (5–12). Significant questions remain regarding the mechanisms through which changes in estrogen and progesterone could affect mood and anxiety and the reasons why only a subset of women demonstrate particularly robust changes in emotional state in response to reproductive events (9). Twin studies and family studies suggest that genetic factors may contribute to susceptibility to reproductive-related affective illness (13–16).

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Brain-derived neurotrophic factor (*BDNF*) has been implicated in the development as well as treatment of affective disorders in both humans and animal models (17). In rodent and humans, decreased *BDNF* expression is associated with the development of affective pathology (18–21). In the rodent brain and human serum, the expression of *BDNF* is augmented by nearly every antidepressant regimen tested to date (reviewed in Duman and Monteggia [17]). In animal models, the direct administration of *BDNF* into the brain has antidepressant effects (22,23). Furthermore, the increase in *BDNF* expression and signaling in response to antidepressants appears to be a key step in decreasing the expression of anxiety and depressive-like behaviors on established marker tasks (24,25). Interestingly, the expression or release of *BDNF* is significantly increased in response to estrogen (26–28) with progesterone serving to counteract the effects of estrogen on *BDNF* expression (29). The modulation of *BDNF* by estrogen and progesterone has been proposed to contribute to alterations in cognitive and emotional functioning across the estrous cycle and during the transitional period of perimenopause (16,30,31).

Recently, a common single nucleotide polymorphism (SNP) in the human *BDNF* gene was identified (*BDNF* Valine66Methionine) (32). This SNP leads to a change from a Valine (Val) to a Methionine (Met) at position 66 within the prodomain of *BDNF* (thus *BDNF* Val66Met). Approximately 30% of the Caucasian population carry the Met allele, with approximately 4% being homozygous (33). The Met allele leads to a reduction in the activity dependent release of *BDNF* (32,34,35) and has been associated with subtle changes in memory function (36) as well as with a variety of neuropsychiatric disorders. Human association studies have begun to investigate the

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potential role of this SNP in the development of affective disorders with somewhat mixed results (17,37,38). To reduce the potential variability related to environmental factors and other mood-related genetic variations in humans, we have developed an animal model of the *BDNF* Val66Met polymorphism by inserting the analogous SNP into an inbred strain of mice. Mice homozygous for the Met allele recapitulate the hallmark effects that have been reported in human Met allele carriers (35) and serves as a translational tool to assess the potential effects of this SNP on cognitive and emotional functioning (39).

Here, we used this animal model to investigate whether the previously demonstrated association of the Met allele insertion with anxiety-like behaviors is evident in female mice and whether the expression of these behaviors varies across development or the estrous cycle. We find here that the Met allele is associated with increased expression of anxiety- and depressive-like behaviors in female adults; that anxiety-like behaviors increase over the course of early development in Met allele carriers but not wild-type mice, and the anxiogenic effects of the Met allele are most apparent during the stage of the estrous cycle in which estrogen has just rapidly fallen. These findings have potential relevance for understanding genetic factors that may contribute to the increased expression of affective pathology in women during times of hormonal cycling (puberty and premenopausal years) and during reproductive events associated with declines in reproductive hormones (postpartum and perimenopause).

Methods and Materials

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committees of Weill Cornell Medical College and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All female mice aged 3 to 22 weeks were housed in standard shoebox caging on a 12-hour light–dark cycle (lights on 7 AM) with food and water available ad libitum. *BDNF* Val66Met mice were generated by 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 in Chen *et al.* (36). This mutation changes the valine at position 66 to a methionine (e.g., Val66Met). Mice were generated from heterozygous Val/Met mothers, were weaned and sex segregated at postnatal Day 21, and in nearly all cases Val/Val, Val/Met, and Met/Met females were mixed housed in a single cage. This allowed testing of Val/Val and Met/Met mice during the same testing session. The mice were crossed onto a C57BL/6J background (13th generation backcross at the time of these experiments). All mice were bred at Weill Cornell Medical College, underwent tail-tipping for collection of DNA at the time of weaning (postnatal Day 20), were then sex segregated and were genotyped using previously described methods (35).

Estrous Cycling

Adult (9- to 30-week old) animals in which cycle status was assessed, received a single vaginal swabbing following the completion of behavioral testing. Swabs were taken within 10 min of the end of testing and occurred between the hours of 9:30 AM and 12:30 PM. Vaginal cytology was observed under a microscope after staining with the Hema 3 Stain Set from Fisher Scientific (Pittsburgh, Pennsylvania). Cycle stage was determined as proestrus, estrus, metestrus, or diestrus according to previously published criteria (40,41). Any animals in which cycle status could not be definitively identified were excluded from the experiment (5 mice).

Behavioral Testing

Elevated Plus Maze. The elevated plus-maze was constructed of white Lexan, raised 70 cm above the floor, and consisted of two opposite enclosed arms with 14-cm-high opaque walls and two opposite open arms of the same size (30 cm × 5 cm). The maze was set up under an infrared sensitive digital camera connected to a video recorder and computer under the control of Ethovision software (Noldus, Leesburg, Virginia). A single testing session lasted 10 min and was carried out under low light (~4 Lux). To begin a trial, the test animal was placed in the center of the plus-maze facing an open arm, and their behavior was recorded for 10 min. The maze was cleaned with a 70% ethanol solution and dried after each trial to eliminate possible odor cues left by previous subjects. The time spent in both the open and enclosed arms was recorded and analyzed using Ethovision software. Measures of anxiety-like behavior were assessed by calculating the relative amount of time spent in the open arms relative to the enclosed arms. Mice were tested only once on the elevated plus maze task.

Open Field. The open-field apparatus consists of a (40 cm × 40 cm × 49 cm) white Lexan arena with a white floor. With the aid of tracking software (Noldus EthoVision XT), the arena was digitally divided into 12 equal quadrants. The arena was set up in a dim room (~4 Lux) under a digital camera and connected to a video recorder and a computer under the control of the EthoVision software (Noldus). A single mouse was placed into the center of the arena, and its behavior was recorded over a 10-min session. Measures of anxiety-like behavior included the relative amount of time spent exploring the center quadrants relative to those located adjacent to the walls of the arena. Mice were tested only once on the open field task.

Forced Swim. The forced swim apparatus consisted of a 2-L beaker filled with 1500 mL of room-temperature water. Beakers were set up in isolation cubicles and positioned in front of a digital camera connected to a computer running Noldus Ethovision tracking software. Mice were placed in the water, and their behavior was recorded for 5 min. Using the mobility measurement function on EthoVision software, we calculated immobility of the mice on this task. Measures of depressive-like behavior included the total amount of time spent immobile during the 5-min test.

Statistics. To assess the effect of genotype on the expression of anxiety-like and depressive-like behavior in female mice independent of estrous status, a simple Student's *t* test was used. To assess the correlation between age and levels of anxiety-like behavior, we used the Pearson correlation test. For comparisons of the potential contribution of estrous status to the differential expression of anxiety-like behavior in *BDNF* Val66Met mice, we used analysis of variance (ANOVA) to assess within genotype effects and Tukey's least significant difference (LSD) for post hoc comparisons, controlling for multiple tests. For comparisons of genotype by estrous stage, due to power limitations comparison's of anxiety-like behavior between genotype were carried out using independent planned *t* tests for each stage of the estrous cycle, with Bonferroni corrections for multiple tests, and an adjust alpha of $<.01$. Consistent with standard statistical practices, any data point that was greater than 2 SD from the group mean was eliminated (one instance). For all statistics alpha was set at $<.05$. SPSS software (IBM, Armonk, New York) was used for all analyses.

Results

Female *BDNF* Val66Met Display Increased Anxiety-Like and Depressive-Like Behavior

To investigate the potential contribution of the *BDNF* Val66Met SNP to the expression of anxiety- and depressive-like behavior in

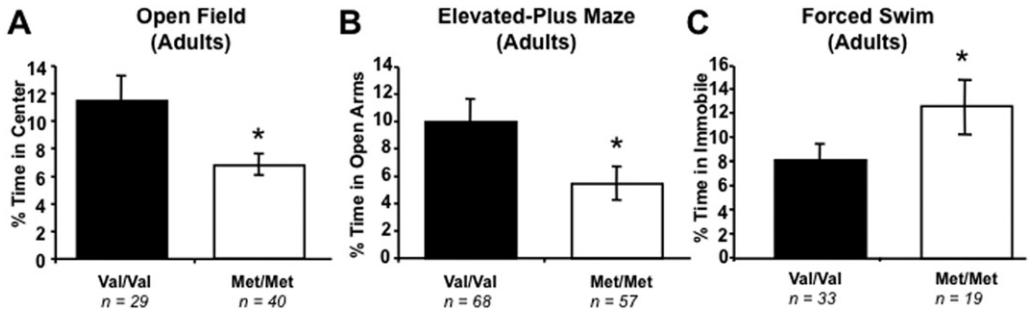

Figure 1. Histograms depicting measures of anxiety-like or depressive-like behavior in adult (> 6 weeks of age) wild-type (Val/Val) and *BDNF* Val66Met homozygous (Met/Met) female mice. **(A)** Met/Met mice ($n = 40$) spent a significantly lower percentage of their time in the center of the open field relative to Val/Val ($n = 29$) controls (Student's *t* test; $p < .005$). **(B)** Met/Met female mice ($n = 57$) also spent a significantly lower percentage of their time in the open arms of the elevated plus maze relative to Val/Val ($n = 68$) controls (Student's *t* test; $p < .005$). **(C)** Met/Met female mice ($n = 19$) spent a significantly greater percentage of their time immobile in the forced swim task relative to Val/Val ($n = 33$) control (Student's *t* test; $p < .04$). Error bars represent standard error of the mean. *$p < .05$. *BDNF*, brain-derived neurotrophic factor; Met, Methionine; Val, Valine.

adult female mice, we used three commonly applied procedures, the open-field task (anxiety), the elevated plus maze (anxiety), and the forced swim task (depression). We wanted to ensure that any observed effects of genotype on basal levels of anxiety- or depressive-like behavior were not due to differential response of the two genotypes to the stress of vaginal swabbing. So, for the initial study, female mice were randomly selected from the colony over a period of several weeks, and vaginal swabbing to assess estrous status was not performed. We anticipate that any effects that we observe would be the composite of female mice from mixed cycle stages. We found that mice homozygous for the *BDNF* Val66Met polymorphism (Met/Met) demonstrated significantly elevated levels of anxiety-like behavior relative to wild-type Val/Val control mice on both anxiety tasks. An anxiety-like phenotype in Met/Met mice was indicated by significantly lower amounts of time spent in the center of the open field (Student's *t* test; $p < .005$; Figure 1A) or in the open arms of the elevated plus maze (Student's *t* test; $p < .005$; Figure 1B) compared with wild-type Val/Val controls. In addition, we found a significant effect of genotype on the expression of depressive-like behavior (Student's *t* test; $p < .05$; Figure 1C) with Met/Met mice showing increased immobility compared with Val/Val controls in the forced swim task.

Anxiety-Like Behaviors in *BDNF* Val66Met Mice Increases During the Transition to Adulthood

In human females, emergence of affective disorders often peaks during or shortly following the transition to sexual maturity (4,42). To assess whether the *BDNF* Val66Met polymorphism might contribute to emergence of affective disorders during this transitional period in mice, we randomly sampled mice across a variety of ages (postnatal day 30 [P30] to postnatal day 153 [P153]) and used the elevated-plus maze to assess levels of anxiety-like behavior. In wild-type (Val/Val) mice, we found no correlation between age and the level of anxiety-like behavior ($R^2 = .02$; Pearson correlation, $p < .294$; Figure 2A). Interestingly, in female mice homozygous for the *BDNF* Val66Met polymorphism (Met/Met), we found a significant correlation between age and the expression of elevated levels of anxiety-like behaviors ($R^2 = .11$; Pearson correlation, $p < .008$; Figure 2B) as indicated by decreased time spent in the open arms of the elevated plus maze.

BDNF Val66Met Effects on Anxiety-Like Behavior Depend on Estrous Cycle Status

To assess the potential contribution of the *BDNF* Val66Met polymorphism to fluctuations in the expression of affective phenotype across the estrous cycle, we again used *BDNF* Val66Met mice. Separate mice underwent testing on the elevated-plus maze or the open-field task and then immediately afterward received a single vaginal swabbing to obtain a cytological smear. Using previously defined criteria (40,41), vaginal smears were analyzed (see Supplement 1, Figure S1, for examples) and behavioral data was binned based on the mouse's stage in the estrous cycle (i.e., proestrus, estrus, metestrus, and diestrus) for each genotype. Based on previous studies, the *BDNF* Val66Met SNP did not affect the length of the estrous cycle or duration in a specific stage of the cycle relative to wild-type mice (30). Furthermore, based on previous assessment of basal and estrus stage specific progestin receptor immunoreactivity in the medial preoptic area of Val/Val

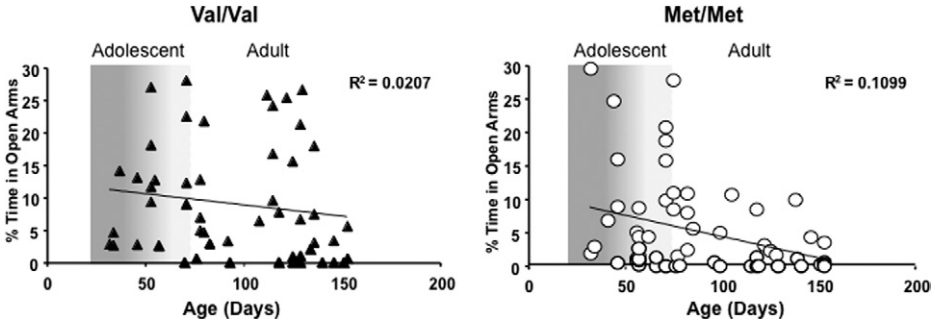

Figure 2. Scatterplots of measures of anxiety-like behavior in the elevated plus maze (y axis) relative to age in postnatal days (x axis) for wild-type (Val/Val) and *BDNF* Val66Met homozygous (Met/Met) mice. Shaded areas indicate best estimates of developmental status based on previous reports (45,46). Trend lines are included along with R^2 values for correlations. *BDNF*, brain-derived neurotrophic factor; Met, Methionine; Val, Valine.

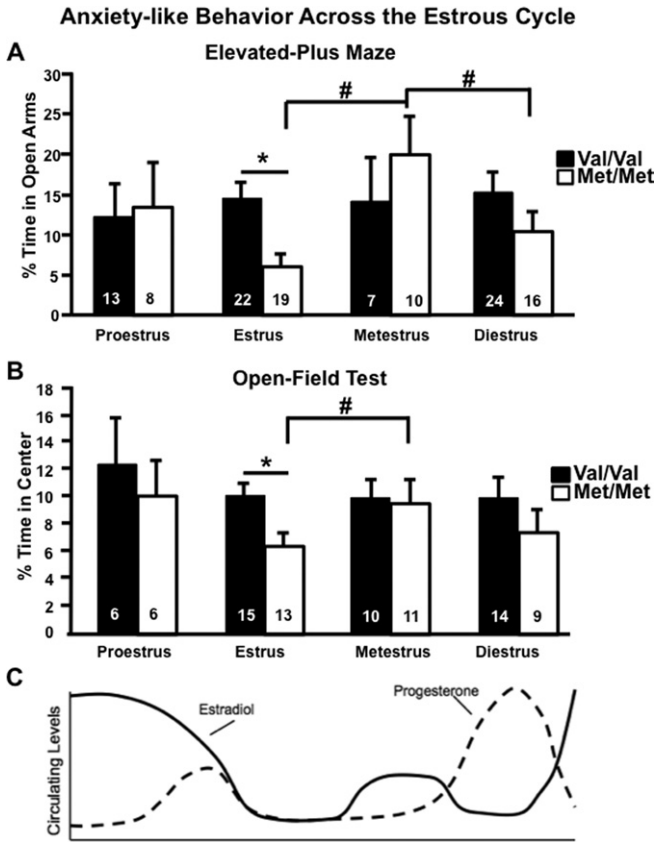

Figure 3. Histograms of behavior on the elevated plus task and open field task of wild-type (Val/Val) and *BDNF* Val66Met homozygous (Met/Met) mice. Numbers inset in the columns indicate the *n* for that group. Using analysis of variance differences in anxiety-like behavior were assessed across the estrous cycle. **(A)** On the elevated plus maze, Met/Met mice showed significantly elevated levels of anxiety during diestrus and estrus relative to metestrus ($^{\#}$significance $p < .05$). Comparisons were also made between Val/Val and Met/Met mice across the various phases of the estrous cycle. *Met/Met mice significantly differed from Val/Val mice primarily during estrus. **(B)** On the open field task, Met/Met mice showed significantly elevated levels of anxiety during estrus relative to metestrus. Met/Met mice also significantly differed from Val/Val mice during the estrus phase of the cycle. **(C)** Line drawings indicating estimates of changes in circulating estrogen and progesterone levels during the different stages of the estrous cycle. Line drawings were based upon previously published reports in CD-1 mice (47). Error bars represent standard error of the mean. *BDNF*, brain-derived neurotrophic factor; Met, Methionine; Val, Valine.

and Met/Met mice, there is no indication that genotype affects basal estradiol levels or estrous-stage specific changes in circulating estradiol levels (30).

BDNF wild-type and Val66Met mice had been fully backcrossed onto a C57BL/6 background. Consistent with previous reports for C57BL/6 mice (40), measures of anxiety-like behavior did not change as a function of estrous status for wild-type Val/Val mice in the elevated plus [ANOVA $F(3,61) = .153$, $p < .927$; Figure 3A] or open field task [ANOVA $F(3,41) = .271$, $p < .846$; Figure 3B]. Interestingly, mice homozygous for the *BDNF* Val66Met mutation (Met/Met) showed significant variation in measures of anxiety-like behavior over the estrous cycle on the elevated plus maze [ANOVA $F(3,49) = 3.254$, $p < .029$; Figure 3A]. For the elevated plus maze, anxiety-like behavior of Met/Met mice was significantly elevated during estrus relative to metestrus (Tukey's LSD, $p < .004$; Figure 3A) and less strongly during diestrus relative to metestrus (Tukey's LSD, $p < .04$; Figure 3A). In follow-up testing, using the open field task, we observed a significant group difference in levels of anxiety-like behavior between estrus relative to metestrus (planned Student's *t* test, $p < .05$; Figure 3B), an effect similar to that observed in the elevated plus maze. However, potentially because of the smaller sample sizes, we did not observe an overall main effect of estrous status on anxiety-like behavior for the open field [ANOVA $F(3,36) = 1.014$, $p < .398$].

To assess any potential differences in the expression of anxiety-like behavior of wild-type relative to Met/Met mice over the estrous cycle, we carried out multiple planned *t* tests using Bonferroni correction's for multiple tests. We found that wild-type and Met/Met mice differed in their expression of anxiety-like behavior specifically during the estrus stage of the estrous cycle on the elevated plus [$t(39) = -2.46$, $p < .01$; Figure 3A], and open field [$t(26) = 2.39$, $p < .03$; Figure 3B]. In mice, estrus corresponds to the period following the rapid decline in circulating estrogen levels. To ensure that the effects we observed were not an artifact of genotype specific changes in general activity over the estrous cycle, we also tested for differences in the total distance traveled by these mice during testing on the elevated plus maze. We found no effect of estrous status or genotype on the total distance traveled (Figure S2 in Supplement 1).

Discussion

These experiments represent the first studies to assess the potential impact in females of the *BDNF* Val66Met polymorphism to the expression of depressive- or anxiety-like behavior across early development and over the estrous cycle. On the basis of these data, the *BDNF* Met allele is associated with an increase in anxiety-like behavior in females, which emerges during the period in which female mice are reaching sexual maturity. In addition, expression of anxiety-like behavior is significantly influenced by acute fluctuation in reproductive hormones across the estrus cycle. These results suggest that women carrying the *BDNF* Met allele may be more sensitive to the potential impact of reproductive hormones on anxiety. In addition, in light of the high comorbidity of anxiety, premenstrual and depressive disorders, similar increased prevalence of depressive disorders in females and similar response to antidepressant medications, our findings also suggest that the *BDNF* Met allele may play a role in susceptibility to premenstrual dysphoric disorder and postpartum and perimenopausal depression.

BDNF Val66Met Promotes the Expression of Anxiety- and Depressive-Like Behavior in Adult Female Mice

We found that mice homozygous for the *BDNF* Val66Met polymorphism showed elevated levels of anxiety- and depressive-like behavior as adults in both the elevated plus, open-field, and forced swim tasks. In our initial experiments, we chose not to subject mice to vaginal swabbing as a means to limit their exposure to stress, because stress can significantly affect performance on these tasks. A subset of mice underwent testing on both tasks with an intervening period of at least 1 week. We made the assumption that using a large sample would allow us to randomly select mice from across the entire span of the estrous cycle and assess the mean level of anxiety- or depressive-like behavior. On the basis of our subsequent experiments, in which we did characterize estrous status, we cannot exclude the possibility that there may have been a slight oversampling of the estrus stage of the cycle that may have influenced our results. However, despite this shortcoming, this set of experiments speaks to the contribution of this gene to the overall expression of anxiety-like and depressive-like behavior and the consistency of these effects across behavioral tasks.

BDNF Val66Met Contributes to the Developmental Emergence of Anxiety-Like Behavior

We found a significant correlation between age and the expression of anxiety-like behavior in Met/Met but not wild-type mice. The difference in anxiety-like behavior emerged during the transitioning from the adolescent period to young adulthood, when there is an increase in the expression of anxiety-like behaviors. One possible interpretation of these results is that as mice transition to sexual maturity, fluctuations in estrogen and progesterone associated with the onset of estrous cycling begin to unmask the effects of the Val66Met SNP on the expression of anxiety-like behavior. However, because we did not assay changes in circulating gonadal hormones or the onset of stable expression of estrous cycling in these mice, such an interpretation remains speculative. Future work in which gonadal hormone levels in these mice are directly measured or manipulated through surgical or pharmacologic means (such as castration) could advance our understanding of whether and how the presence of this SNP might affect central nervous system response to estrus cycle phase or changing levels of specific gonadal hormones. A second interpretation of these results could be that Met/Met mice are simply displaying elevated levels of anxiety-like behaviors sooner, and, given time, these differences by genotype may diminish. On the basis of our previous work (30) Val/Val and Met/Met display normal adult-like cycling by 80 days of age. We tested mice up to 153 days of age, which would give ample opportunity for both genotypes to express normal cycling and show any developmental effects on anxiety-like behaviors. Testing beyond 153 days of age was not carried out because mice Met/Met mice greater than 6 months of age show highly significant differences in body weight (Met/Met $>$ Val/Val), which may affect locomotor activity and bias measures on standard anxiety tasks.

BDNF Val66Met Leads to Estrous-Cycle-Specific Effects on Anxiety-Like Behavior

We also found that mice homozygous for the *BDNF* Val66Met mutation showed significant variation in the expression of anxiety-like behavior over the estrous cycle, but wild-type mice did not. These data are consistent with a recent report demonstrating that wild-type C57BL/6 mice do not show cycle-dependent variation in anxiety-like behaviors or locomotion (40) and expand on our recent report in which we assessed the impact of this SNP on cognitive functioning in adult females (30). In that report (30), we detected cycle-specific changes in hippocampal memory function, but, in contrast to the study described here, failed to detect a significant effect of genotype on anxiety-like behavior. However, in those prior experiments anxiety-like behavior was assayed following a week of handling and repeated vaginal swabbing, both of which may have reduced our ability to detect reliable differences in anxiety-like behavior across the estrous cycle. Furthermore, anxiety-like behavior was assessed during the habituation phase of the novel object location task, with novel objects present in the arena, which also may have reduced our ability to detect genotypic differences in anxiety behavior. The current study was designed explicitly to assess anxiety-like behavior independent of such variables.

We hypothesize that our current observation of increased anxiety-like behavior of *BDNF* Met/Met mice during estrous may be related to decreased *BDNF* availability conferred by this SNP, affecting *BDNF* signaling in a cycle-dependent fashion. Because estrogen is a positive regulator of *BDNF* expression, the estrus phase would be expected to lead to a reduction in *BDNF* expression. Consistent with such a hypothesis, we have recently also identified a significant reduction in *BDNF* signaling (as assayed by TrkB phosphorylation and downstream markers of activation) in Met/Met mice compared with controls during specific phases of the estrous cycle (43). However, to link alterations in *BDNF* signaling across the cycle to anxiety-like behavior conclusively, further studies in which we restore *BDNF* levels or selectively increase TrkB activation in a cycle-dependent manner are needed. Recent studies using novel TrkB antagonists in male mice have already yielded data that support such a theory (44).

Our current findings suggest that in humans, carriers of the Val66Met SNP may show greater emotional responsivity to onset of puberty and reproductive events associated with estrogen withdrawal, including the postpartum and perimenopausal periods. As estrogen levels fall, the animal data described here suggest that *BDNF* expression will decline. A reduction in *BDNF* expression has been associated with mood disorders in multiple clinical studies (17,31). Because the time course and hormonal pattern of the estrus cycles in mice and the menstrual cycle in humans are so different, it is difficult to comment on possible implications of these data for understanding premenstrual mood changes in women. Clarification of the impact of this genotype on vulnerability to reproductive-associated affective disorders in women may lead to improved strategies for prevention and treatment.

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Supplementary material cited in this article is available online.

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