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Female Carriers of the Met Allele of the BDNF Val66Met Polymorphism Develop Weaker Fear Memories in a Fear-Potentiated Startle Paradigm

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Abstract

The val66met polymorphism of the *bdnf* gene, which is associated with compromised brain-derived neurotrophic factor (BDNF) signaling, impaired synaptic plasticity, and impaired learning, may increase one's susceptibility to stress- and anxiety-related disorders. Indeed, previous work has reported greater anxiety-related behaviors and impairments of fear conditioning and extinction in individuals who carry the met allele that results from this polymorphism. Nevertheless, findings in this area of research have been equivocal. Thus, we examined the influence of the val66met polymorphism on fear conditioning, extinction, and extinction memory testing. One hundred and twenty healthy participants completed differential fear conditioning in a fear-potentiated startle paradigm, followed by extinction and extinction memory testing 24 and 48 hr later, respectively. Participants were genotyped for the val66met polymorphism and divided into met allele carriers and non-carriers. Results revealed that, although both met-carriers and non-carriers developed conditioned fear, met-carriers exhibited significantly weaker fear acquisition than non-carriers. This difference persisted throughout extinction and extinction memory testing and, during these last two days of testing, was primarily evident in females. These results are consistent with previous work demonstrating that this polymorphism is associated with impaired amygdala-dependent fear learning and extend such findings by demonstrating that females may be more sensitive to such effects.

Keywords

Fear Conditioning, Extinction, Polymorphism, BDNF, Val66Met, Startle

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that is important for neuron development and synaptic plasticity [1]. BDNF acts on pre- and post-synaptic tropomyosin receptor kinase B (TrkB) receptors to facilitate neurotransmission and has been implicated in the induction of long-term potentiation (LTP) [2] [3]. Extensive work has shown that BDNF plays a role in learning and memory, and it is unsurprisingly concentrated in brain areas such as the hippocampus, amygdala, and prefrontal cortex (PFC). Multiple studies have reported increased expression of BDNF or BDNF mRNA as a result of hippocampus-, amygdala-, and PFC-dependent learning or that disruption of BDNF-related signaling impairs such processes [4] [5] [6] [7].

Aberrations of synaptic plasticity are thought to be involved in the etiology of clinical depression and anxiety, and many psychological disorders involve impairments of cognition. Consistent with BDNF's role in synaptic plasticity and cognition, these psychological disorders have been associated with reduced levels of BDNF and irregular expression of TrkB receptors [8] [9] [10] [11] [12]. In fact, many effective psychotropic medications increase BDNF levels, suggesting that such a mechanism might account for some of their therapeutic efficacy [13] [14] [15] [16]. Given the apparent involvement of BDNF in psychological disorders, developing a better understanding of its role in cognitive processes would be of great value.

One extensively studied genetic variant of the *bdnf* gene has been linked to cognitive deficits and susceptibility for multiple psychological disorders [17] [18]. The val66met polymorphism (rs6265) is a common single nucleotide polymorphism in the prodomain of the *bdnf* gene that converts the amino acid valine (val) to methionine (met) at codon 66 [19]. The polymorphism occurs in approximately 20% - 30% of Caucasians [20] and has been associated with reduced BDNF release, compromised intracellular BDNF trafficking, and impaired synaptic plasticity [21] [22] [23] [24]. Research has shown that people or rodents carrying the met allele exhibit impairments in learning and memory, smaller hippocampal volumes, abnormal hippocampus cytoarchitecture, and reduced hippocampal activity during learning tasks [22] [25]-[30].

Of particular relevance for psychological disorders is the finding that rodents carrying the met allele exhibit heightened anxiety-like behavior [30]. Although similar work examining anxiety levels in humans has been inconsistent [31] [32], research has demonstrated that people carrying the met allele exhibit an attentional bias for and greater amygdala responses to emotional stimuli [33] [34] [35] [36] [37]. Many studies have also reported altered fear conditioning processes in met allele carriers. One relatively consistent finding in both rodents and humans is that carriers of the met allele exhibit impaired fear extinction [38] [39] [40] [41], an effect that has been associated with reduced activity in the ventromedial PFC and hippocampus and increased activity in the amygdala [23] [38] [40]. Such findings are particularly relevant for understanding susceptibility

to clinical anxiety, especially post-traumatic stress disorder (PTSD), as an impaired ability to extinguish fear could result in a stronger, more durable traumatic memory. One study revealed that PTSD patients carrying the met allele displayed less fear extinction in a laboratory-based paradigm than PTSD patients who were homozygous for the val allele, and the amount of fear extinction in met allele carriers was inversely correlated with the severity of their PTSD symptoms [42]. An impaired ability to extinguish fear could also adversely impact therapeutic approaches to clinical anxiety that are based on the principles of fear extinction, such as exposure therapy. Indeed, two studies have shown that, in samples of patients with obsessive-compulsive disorder (OCD) and PTSD, patients carrying the met allele exhibited poorer responses to exposure-based therapy than patients homozygous for the val allele [43] [44].

Additional research has suggested that the val66met polymorphism may influence the acquisition of fear. However, the findings from these studies have been mixed. Some studies, in both humans and rodents, have reported that carriers of the met allele exhibit impaired contextual [30] [45] [46], but intact cue [30] [38] [46] [47], fear learning. Because of the neurobiological dissociation between these two processes, such findings have suggested that the val66met polymorphism adversely influences hippocampus-dependent (*i.e.*, context) fear learning, while sparing amygdala-dependent (*i.e.*, cue) fear learning. Nonetheless, other studies have reported impaired cue-dependent fear learning and greater cue fear generalization in met allele carriers [19] [45] [48]. Impaired fear learning in met-carriers could predispose them to clinical anxiety by hindering their ability to discriminate between signals of danger and safety or overgeneralizing their learned fear to non-threatening contexts or cues. Because of the inconsistent findings and methodological approaches in this area of research, we examined the influence of the val66met polymorphism on fear acquisition, fear extinction, and the retention of fear extinction using a fear-potentiated startle paradigm. We hypothesized that met allele carriers would exhibit impaired fear learning and/or fear extinction.

2. Materials and Methods

2.1. Participants

One hundred and twenty healthy undergraduate students (57 males, 63 naturally cycling females; age: M = 19.43, SD = 1.85; predominantly Caucasian) from Ohio Northern University volunteered to participate in the experiment. Individuals were excluded from participating if they met any of the following conditions: history of syncope or vasovagal response to stress; history of any heart condition or cardiovascular issues (e.g., high blood pressure); history of severe head injury; current treatment with psychotropic medications, narcotics, beta-blockers, steroids, or any other medication that was deemed to significantly affect central nervous or endocrine system function; mental or substance use disorder; regular tobacco use; regular use of recreational drugs; regular night-

shift work; auditory disorder; hearing impairment. All experimental procedures were approved by the Institutional Review Board at Ohio Northern University, carried out in accordance with the Declaration of Helsinki, and undertaken with the understanding and written consent of each participant. Participants were awarded class credit and $20 cash upon completion of the study.

2.2. Experimental Procedures

2.2.1. Differential Fear Conditioning Paradigm

Paradigm summary. The differential fear conditioning paradigm used in the present study followed that which has been studied extensively in previous work (e.g., [49]-[56]), but with a modified timeline. Specifically, unlike previous work with this paradigm, each fear-potentiated startle session was separated by a period of 24 hr. The paradigm included fear-potentiated startle as the primary dependent measure and consisted of three phases: Day 1—fear acquisition, Day 2—fear extinction, and Day 3—extinction memory testing.

Stimuli. The startle probe was a 40-ms, 108-dB burst of broadband noise with near instantaneous rise time, delivered binaurally through headphones. The conditioned stimuli (CSs), which were two geometric shapes, were presented on a white background via a computer monitor (via SuperLab software; Cedrus Corporation, San Pedro, CA) that was situated in front of participants. The CS+ was a blue square (9.5 × 9.5 cm), and the CS− was a purple triangle (11 × 9.5 cm). The unconditioned stimulus (US) was a 250-ms, 140-p.s.i. airblast directed at the larynx. This US has been used in several previous studies and consistently produces robust fear-potentiated startle (e.g., [49]-[56]). On CS+ trials, the startle probe was presented 6 s after CS onset, followed 500 ms later by the US; the CS+ terminated 500 ms following US onset. On CS− trials, the startle probe was presented 6 s after CS onset, without any US presentation; the CS− terminated 250 ms following the startle probe. On noise alone (NA) trials, the startle probe was presented alone as participants stared at a white background on the computer monitor; NA trials were the length of the startle probe (*i.e.*, 40-ms).

Startle response measurement. The eyeblink component of the acoustic startle response was measured by electromyographic (EMG) recordings of the right orbicularis oculi muscle. Ag/AgCl electrodes (5-mm) filled with electrolyte gel were positioned 1 cm below the pupil of the right eye, 1 cm below the lateral canthus, and behind the right ear over the mastoid (ground). Impedance levels were less than 6 kΩ for each participant. Startle response data were acquired using the Acqknowledge data acquisition and analysis software (Biopac Systems, Inc., Aero Camino, CA) and the EMG module of the Biopac MP150 system (Biopac Systems, Inc., Aero Camino, CA). The EMG signal was sampled at a frequency of 1 kHz.

US expectancy measurement. A three-button response keypad (SuperLab software, Cedrus Corporation, San Pedro, CA) was used during each fear-potentiated startle session to collect trial by trial ratings of US expectancies. During each CS presentation, participants pressed one of three buttons: an "AIR" key

when they expected the CS to be followed by an airblast, a "NO AIR" key when they did not expect the CS to be followed by an airblast, and a "?" key when they were uncertain of what to expect. For the purposes of data analysis, participant responses of "AIR" were scored as +1, responses of "?" were scored as 0, and responses of "NO AIR" were scored as −1 [49]-[56].

Days 1 - 3 (*fear acquisition*, *extinction*, *extinction memory*). Fear acquisition training began with three NA trials, followed by a habituation segment that consisted of four CS+, CS−, and NA trials. Importantly, no CS during the habituation segment was reinforced with an airblast US. An ensuing conditioning phase consisted of three blocks with four trials of each type (CS+, CS−, NA) for a total of 12 trials per block and 36 total trials. During the conditioning phase, the CS+ was always followed by the airblast US, resulting in a 100% reinforcement rate.

On Days 2 and 3 of the experiment, participants underwent fear extinction and extinction memory testing, respectively. Each of these phases began with three NA trials. Then, participants were exposed to four blocks with four trials of each type (CS+, CS−, NA) for a total of 12 trials per block and 48 total trials. None of the CS presentations during these phases were reinforced with an airblast US.

A fixed trial order was used for all participants, with the only restriction being that there were 4 trials of each trial type (*i.e.*, CS+, CS−, NA) presented during each block of 12 trials. The initial preparation of the fixed trial order involved randomizing the order of trial type within each block. The intertrial intervals were randomized to be between 9 and 22 s in duration. **Figure 1** depicts the timeline, stimuli, and trial block composition that made up each experimental session.

Startle data preprocessing. Acqknowledge data acquisition files were imported into the MindWare EMG analysis program (MindWare Technologies, Ltd., Gahanna, OH), which was used to filter and rectify the EMG signals that occurred between 20 - 200 ms following presentation of each startle probe. The EMG signal was amplified by a gain of 2000 and filtered with low- and high-frequency cutoffs at 28 and 500 Hz, respectively. A 60-Hz notch filter was also applied. The resulting data were then exported for analysis. The peak EMG signal 20 - 200 ms after presentation of the startle probe was used as a measure of the acoustic startle response. EMG responses were excluded from data analysis only if instrument or human error occurred and the signal was not acquired.

2.2.2. Genotyping

On Day 3, following assessment of extinction retention, a saliva sample was collected from participants via the OGR-500 Oragene (DNA Genotek, Inc.; Ottawa, ON, Canada). The sample was stored at room temperature, until shipped to DNA Genotek, Inc. for genotyping of the val66met polymorphism (rs6265) of the *BDNF* gene. Genotyping was performed by single tube Taqman® chemistry. The Taqman® assay is an allele discrimination assay using PCR amplification and a pair of fluorescent dye detectors that target the polymorphism. One fluorescent

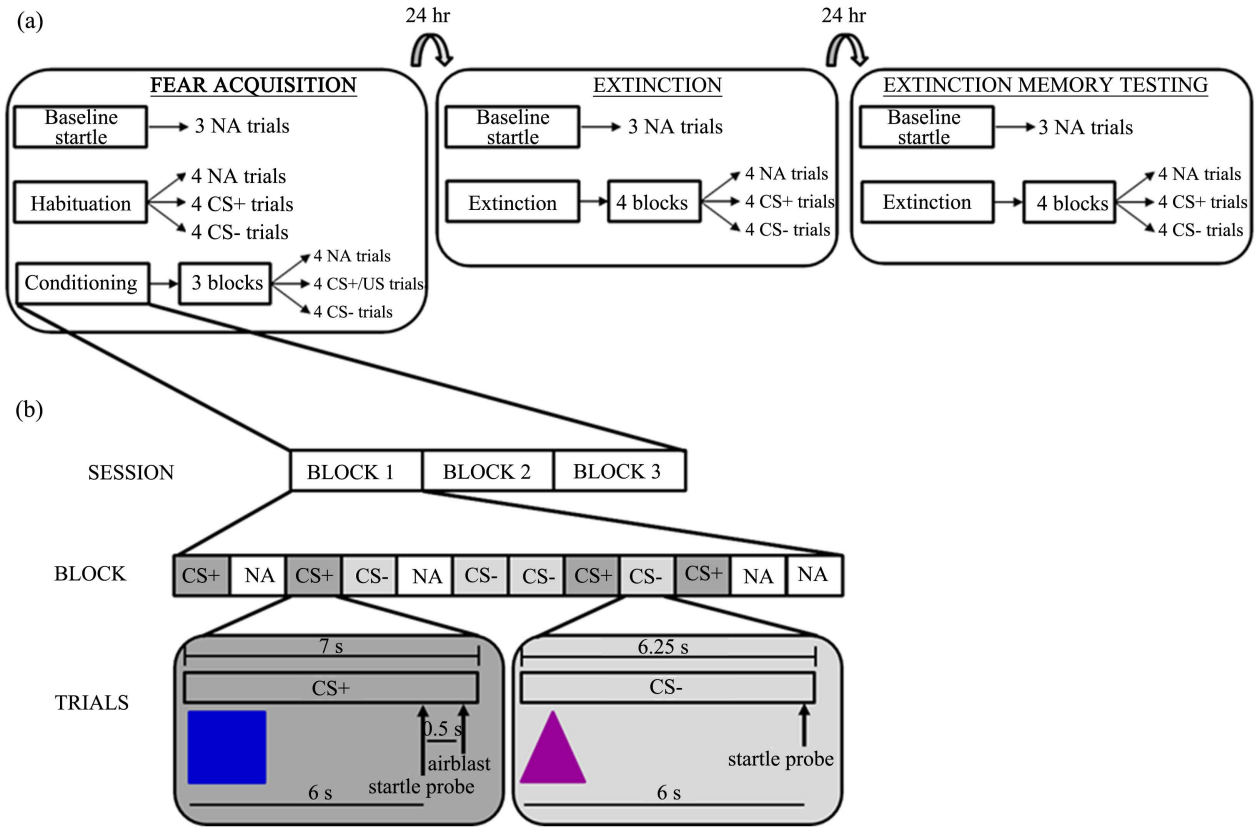

Figure 1. The top part of the figure provides a schematic illustration of the fear-potentiated startle paradigm (a). Fear acquisition, fear extinction, and extinction retention sessions were separated by 24 hr, and each began with 3 exposures to the startle probe alone [noise alone (NA) trials]. Fear acquisition included a habituation phase (during which no stimulus was followed by the aversive US) and a conditioning phase (3 blocks of trials during which the CS+ was always reinforced by the aversive US airblast). Fear extinction and extinction retention sessions consisted of 4 blocks of NA, CS+, and CS− trials, during which no stimulus was followed by the aversive US. The lower part of the figure provides a representative breakout diagram of the conditioned stimuli [reinforced conditioned stimulus (CS+) and nonreinforced conditioned stimulus (CS−)] trial types during the conditioning phase of fear acquisition (b). Within each block, participants were exposed to 12 trials of varying trial types. The timelines for CS+ and CS− stimulus exposure, relative to the startle probe and US, are depicted under these trial types.

dye is attached to the detector that is a perfect match to the first allele (e.g., valine) and a different fluorescent dye is attached to the detector that is a perfect match to the second allele (e.g., methionine). During PCR, the polymerase will release the fluorescent probe into the solution where it is detected using end-point analysis in a Life Technologies, Inc. (Foster City, CA) 7900HT Real-Time instrument. Primes and probes were obtained through Life Technologies design and manufacturing. Life Technologies Taqman Genotyper v1.0.1 software—Taqman® single tube assay was used for analysis. The call rate for the polymorphism was 98.6%.

2.3. Statistical Analyses

Based on previous work, participants were divided into met allele carriers [(met/met (N = 3; 3 males, 0 females), met/val (N = 32; 14 males, 18 females)] and non-carriers [(val/val (N = 85; 40 males, 45 females)] for the purpose of data

analysis. Similar to previous work employing the fear-potentiated startle paradigm (e.g., [50] [51] [52] [54] [57]), we quantified fear-potentiated startle by computing a difference score for the EMG recordings [(startle magnitude to the CS+ or CS− in each block) − (startle magnitude to the NA trials in each block)]. The use of raw difference scores allows one to calculate fear-potentiated startle relative to each participant's baseline startle response (*i.e.*, NA trials) and is supported by work evidencing their superiority to standardized difference scores and percent change scores [58]. Because of the variable nature of the startle response, difference scores were calculated for each trial type within each block (*i.e.*, average of 4 trials of each trial type) in order to obtain a more accurate representation of fear-potentiated startle within each block [59] [60] [61].

Separate mixed-model ANOVAs were used to analyze baseline startle responses (*i.e.*, responses to the first 3 NA trials), fear-potentiated startle, and US expectancies on Days 1 - 3, with genotype and sex serving as the between-subjects factors and, for the analyses of fear-potentiated startle and US expectancies, stimulus (CS+, CS−) and trial block (4 levels for each phase) serving as the within-subjects factors. Late acquisition was defined as block 4 of acquisition on Day 1, when discrimination learning was at maximum, and late extinction was defined as block 4 of extinction on Day 2 and provided a measure of extinction success. The first blocks of extinction on Day 2 and extinction memory testing on Day 3 were considered measures of fear memory and extinction memory, respectively. Initial analyses of startle responses during extinction and extinction memory testing were followed up by additional analyses in which participants' startle responses during late acquisition were included as a covariate to control for group differences in fear learning. To conclude the analyses of fear-potentiated startle and US expectancies, we employed mixed-model ANOVAs to analyze each set of data across all three days of testing. Alpha was set at 0.05 for all analyses, and Bonferroni-corrected post hoc tests were employed when the omnibus F indicated the presence of a significant effect. If the assumption of sphericity was violated, Greenhouse-Geisser corrections were employed, with reduced degrees of freedom reported in the analyses.

3. Results

3.1. Genotype Characteristics

Chi-square goodness-of-fit analyses revealed that there was no significant deviation from the Hardy-Weinberg equilibrium for the *BDNF* genotype ($\chi^2(1, N = 120) = 3.26$, $p = 0.99$). This suggests that the genotype distribution in our sample did not significantly deviate from the expected genotype distribution in the population.

3.2. Fear-Potentiated Startle

3.2.1. Day 1—Fear Acquisition

During acquisition, female non-carriers exhibited significantly greater baseline startle responses to the first 3 NA trials than all other groups (effect of sex: $F(1,$

114) = 4.23, $p < 0.05$, $\eta_p^2 = 0.04$; Genotype × Sex interaction: $F(1, 114) = 4.17$, $p < 0.05$, $\eta_p^2 = 0.04$; Figure 2(a), Figure 2(b)). No other main effects or interactions were significant (all $F < 2.06$, all $p > 0.15$).

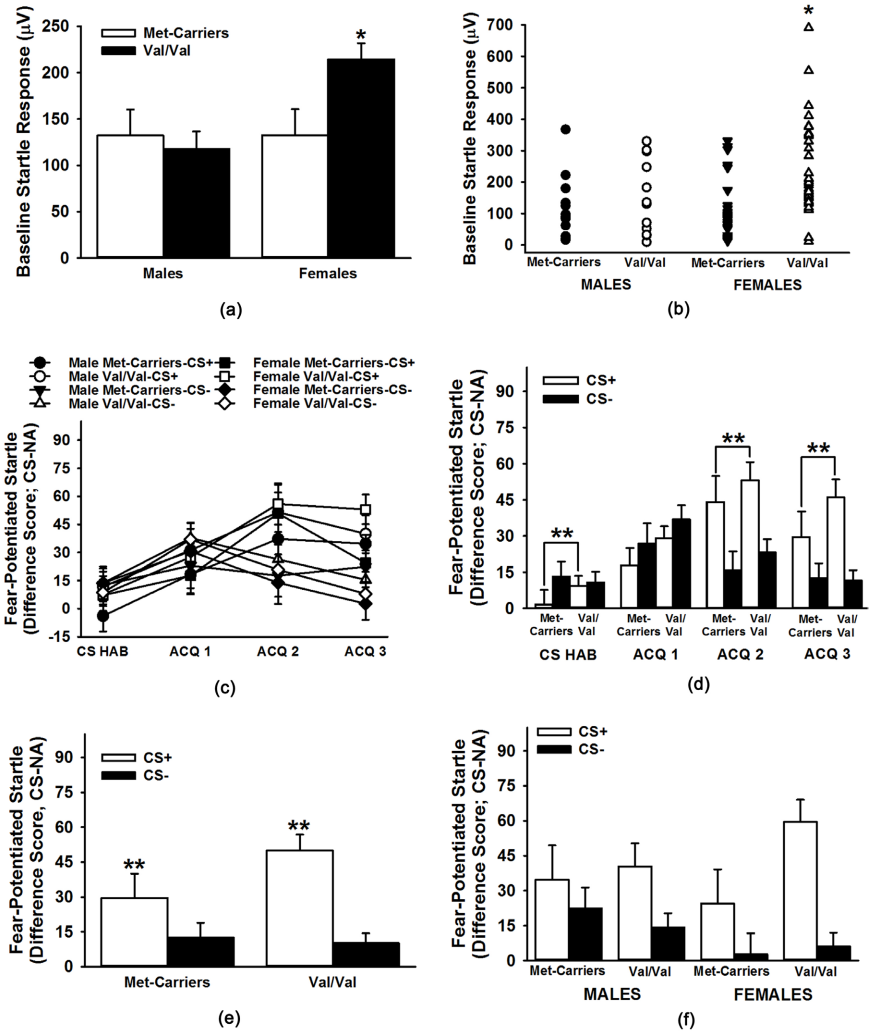

Figure 2. Female non-carriers (Val/Val) exhibited significantly greater startle responses to the startle probe alone (NA = noise alone) than all other groups during fear acquisition (a). The scatterplot for this effect is shown in inset (b) and did not reveal heterogeneity of variance. Inset (c) depicts fear-potentiated startle responses during acquisition on Day 1 for the most complex, Genotype × Sex × Stimulus × Trial Block interaction. Participants exhibited significantly greater fear-potentiated startle responses to the CS− than to the CS+ during the CS habituation phase (CS HAB). During the final two reinforced blocks of acquisition, participants demonstrated significantly greater fear-potentiated startle responses to the CS+ than to the CS−, indicating successful acquisition of differential fear conditioning ((c), (d)). Both met-carriers and non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−, but non-carriers displayed significantly greater CS discrimination than met-carriers (e). Although no significant sex-dependent effects were observed, female non-carriers appeared to exhibit greater CS discrimination than the other groups during the last block of acquisition (f). Data are presented as means ± SEM. *$p < 0.05$ relative to all other groups; **$p < 0.05$ relative to CS−.

Figure 2(c) displays the fear-potentiated startle responses for all trial blocks during acquisition based on sex, genotype, and stimulus. During the nonreinforced habituation segment of acquisition, participants exhibited significantly greater fear-potentiated startle responses to the CS− than to the CS+. Fear-potentiated startle responses to the CS+ and CS− did not differ during the first block of reinforced acquisition trials (ACQ 1). However, in the final two blocks of reinforced acquisition trials (ACQ 2 and 3), participants exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−, demonstrating successful acquisition of differential fear conditioning (effect of stimulus: $F(1, 93) = 21.01$, $p < 0.001$, $\eta_p^2 = 0.18$; effect of trial block: $F(2.72, 279) = 8.46$, $p < 0.001$, $\eta_p^2 = 0.08$; Stimulus × Trial Block interaction: $F(2.41, 279) = 19.97$, $p < 0.001$, $\eta_p^2 = 0.18$; Figure 2(c), Figure 2(d)). No other main effects or interactions were significant (all $F < 2.99$, all $p > 0.08$).

The analysis of late acquisition revealed that, by the end of training, both met-carriers and non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−. However, non-carriers exhibited significantly greater discrimination between the CS+ and CS− than did met-carriers (effect of stimulus: $F(1, 101) = 30.54$, $p < 0.001$, $\eta_p^2 = 0.23$; Genotype × Stimulus interaction: $F(1, 101) = 4.89$, $p < 0.05$, $\eta_p^2 = 0.05$; Figure 2(e)). This effect appeared to be largely driven by female non-carriers, despite no significant Sex × Genotype × Stimulus interaction (Figure 2(f)). No other main effects or interactions were significant (all $F < 3.15$, all $p > 0.07$).

3.2.2. Day 2—Fear Extinction

Baseline startle responses to the first three NA trials on Day 2 were significantly greater in females ($M = 177.78$ μV; $SEM = 16.16$) than in males ($M = 105.92$ μV; $SEM = 16.73$), $F(1, 115) = 9.55$, $p < 0.01$, $\eta_p^2 = 0.08$. No other main effects or interactions were significant (all $F < 2.02$, all $p > 0.15$).

Figure 3(a) displays the fear-potentiated startle responses for all trial blocks during extinction based on sex, genotype, and stimulus. The analysis of the first block of extinction trials on Day 2, which provides an assessment of long-term fear retention, revealed that non-carriers exhibited significantly greater fear-potentiated startle responses to both CSs than did met-carriers (effect of genotype: $F(1, 115) = 8.76$, $p < 0.01$, $\eta_p^2 = 0.07$). This effect was largely driven by significantly greater fear-potentiated startle responses to both CSs in female non-carriers, relative to all other groups (Genotype × Sex interaction: $F(1, 115) = 4.04$, $p < 0.05$, $\eta_p^2 = 0.03$; Figure 3(b)). There was a statistical trend for the Genotype × Stimulus interaction, $F(1, 115) = 3.42$, $p = 0.067$, $\eta_p^2 = 0.03$, suggesting that non-carriers, but not met-carriers, demonstrated greater fear-potentiated startle responses to the CS+ than to the CS− (Figure 3(c)). No other main effects or interactions were significant (all $F < 2.71$, all $p > 0.10$).

The analysis of the entire extinction session on Day 2 (*i.e.*, all four blocks of trials) revealed that non-carriers displayed significantly greater fear-potentiated startle responses to both CSs than did met-carriers, particularly early in

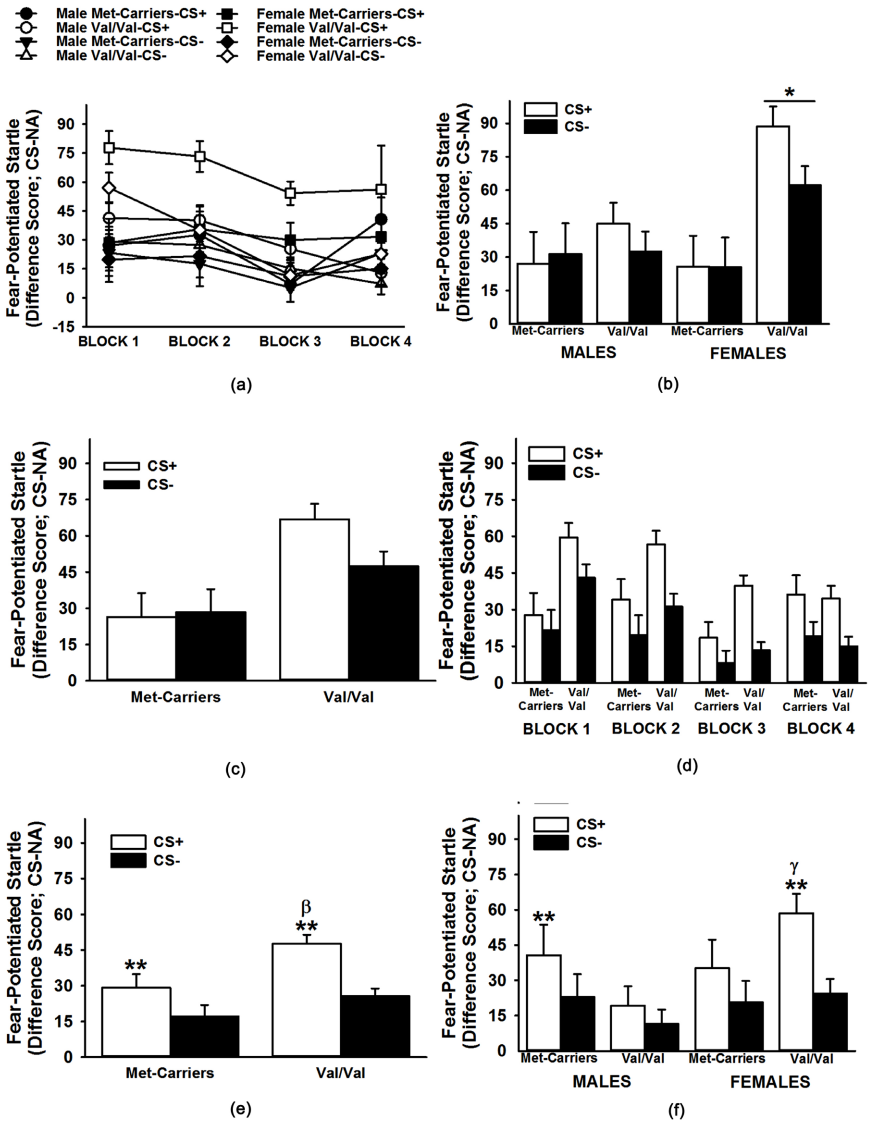

Figure 3. Inset (a) depicts fear-potentiated startle responses during fear extinction on Day 2 for the most complex, Genotype × Sex × Stimulus × Trial Block interaction. During the first block of trials, female non-carriers (Val/Val) exhibited significantly greater fear-potentiated startle responses to both CSs, relative to all other groups (b). Also during the first block of trials, a statistical trend suggested that non-carriers, but not met-carriers, displayed greater fear-potentiated startle responses to the CS+ than to the CS− (c). Across the entire fear extinction session (*i.e.*, all four blocks of trials), participants exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−, and non-carriers displayed significantly greater fear-potentiated startle responses to both CSs than did met-carriers ((c), (d)). Also across the entire fear extinction session, both met-carriers and non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−, but non-carriers displayed significantly greater fear-potentiated startle responses to the CS+ than did met-carriers (e). During the last block of fear extinction, male met-carriers and female non-carriers both exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−; such differences were not observed in male non-carriers and female met-carriers (f). Data are presented as means ± SEM. *$p < 0.05$ relative to all other groups, **$p < 0.05$ relative to CS−; $\beta = p < 0.05$ relative to met-carriers; $\gamma = p < 0.05$ relative to female met-carriers.

extinction training (effect of genotype: $F(1, 105) = 5.31$, $p < 0.05$, $\eta_p^2 = 0.05$; effect of trial block: $F(2.65, 315) = 7.13$, $p < 0.001$, $\eta_p^2 = 0.06$; Genotype × Trial Block interaction: $F(2.65, 315) = 3.89$, $p < 0.05$, $\eta_p^2 = 0.04$; **Figure 3(a)** and **Figure 3(d)**). Both met-carriers and non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−. However, non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than did met-carriers (effect of stimulus: $F(1, 105) = 49.16$, $p < 0.001$, $\eta_p^2 = 0.32$; Genotype × Stimulus interaction: $F(1, 105) = 4.22$, $p < 0.05$, $\eta_p^2 = 0.04$; **Figure 3(e)**). These effects were influenced by sex. Although both males and females exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−, females demonstrated significantly greater fear-potentiated startle responses to the CS+ than did males (effect of sex: $F(1, 105) = 4.78$, $p < 0.05$, $\eta_p^2 = 0.04$; Sex × Stimulus interaction: $F(1, 105) = 8.62$, $p < 0.01$, $\eta_p^2 = 0.08$). Moreover, there was a statistical trend for the Genotype × Sex × Stimulus interaction, $F(1, 105) = 3.70$, $p = 0.057$, $\eta_p^2 = 0.03$, suggesting that the Sex × Stimulus interaction described above was selective to non-carriers. In other words, female non-carriers exhibited greater fear-potentiated startle responses to the CS+ than did female met-carriers; such a difference was not observed in males. No other main effects or interactions were significant (all $F < 3.36$, all $p > 0.07$).

The analysis of the last block of extinction on Day 2, which provides an assessment of extinction success, revealed that participants still exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS− (effect of stimulus: $F(1, 109) = 29.98$, $p < 0.001$, $\eta_p^2 = 0.22$). The Genotype × Sex × Stimulus interaction was significant, $F(1, 109) = 4.76$, $p < 0.05$, $\eta_p^2 = 0.04$. Male met-carriers and female non-carriers both exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−; such differences were not observed in male non-carriers and female met-carriers. Moreover, female non-carriers displayed significantly greater fear-potentiated startle responses to the CS+ than did female met-carriers (**Figure 3(f)**). No other main effects or interactions were significant (all $F < 3.44$, all $p > 0.06$).

Including fear-potentiated startle responses from late acquisition as a covariate did not influence the effects observed for the first block of extinction on Day 2. However, for the analysis of the entire extinction session on Day 2, the effect of genotype ($F(1, 92) = 3.82$, $p = 0.054$, $\eta_p^2 = 0.04$) was reduced to a statistical trend, and the effect of sex and the Genotype × Stimulus interaction were no longer significant. For the analysis of the last block of extinction on Day 2, the Genotype × Sex × Stimulus interaction was reduced to a statistical trend, $F(1, 95) = 2.96$, $p = 0.088$, $\eta_p^2 = 0.03$.

3.2.3. Day 3—Extinction Memory Testing

During extinction memory testing on Day 3, female non-carriers exhibited significantly greater baseline startle responses to the first 3 NA trials than male non-carriers (Genotype × Sex interaction: $F(1, 116) = 4.24$, $p < 0.05$, $\eta_p^2 = 0.04$). No other main effects or interactions were significant (all $F < 3.22$, all $p >$

0.07).

The analysis of the first block of extinction memory testing on Day 3 indicated that participants exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS− (effect of stimulus: $F(1, 110) = 8.40$, $p < 0.01$, $\eta_p^2 = 0.07$). No other main effects or interactions were significant (all $F < 2.35$, all $p > 0.12$).

The analysis of the entire extinction memory testing on Day 3 revealed effects that mirrored those observed during the extinction session on Day 2 (Figure 4(a), Figure 4(b)). In this case, however, only non-carriers continued to exhibit significantly greater fear-potentiated startle responses to the CS+ than to the CS− (effect of stimulus: $F(1, 100) = 9.90$, $p < 0.01$, $\eta_p^2 = 0.09$; Genotype × Stimulus interaction: $F(1, 100) = 15.34$, $p < 0.001$, $\eta_p^2 = 0.13$; Figure 4(c)). Similar to the extinction session on Day 2, non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than did met-carriers. These effects were once again influenced by sex. Female non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than did female met-carriers; such an effect was not observed in males (Genotype × Sex interaction: $F(1, 100) = 4.90$, $p < 0.05$, $\eta_p^2 = 0.05$; Genotype × Sex × Stimulus interaction: $F(1, 100) =$

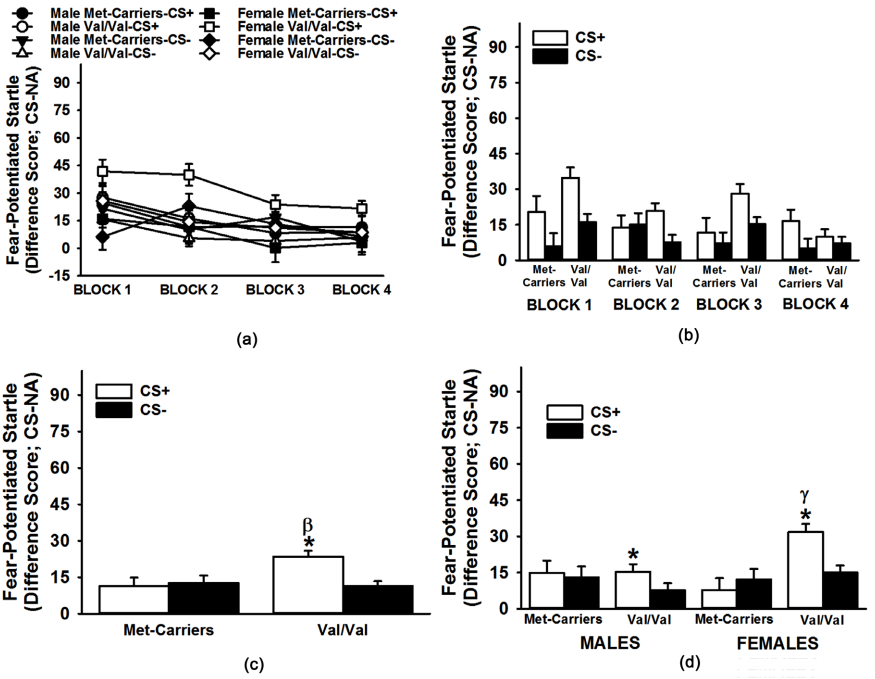

Figure 4. Inset (a) depicts fear-potentiated startle responses during extinction memory testing on Day 3 for the most complex, Genotype × Sex × Stimulus × Trial Block interaction. During extinction memory testing on Day 3, participants exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS− (b), which was selective to non-carriers (c). Only male and female non-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS−, and female non-carriers displayed significantly greater fear-potentiated startle responses to the CS+ than all other groups (d). Data are presented as means ± SEM. *$p < 0.05$ relative to CS−, $\beta = p < 0.05$ relative to met-carriers; $\gamma = p < 0.05$ relative to all other groups.

5.10, $p < 0.05$, $\eta_p^2 = 0.05$; **Figure 4(d)**). Fear-potentiated startle responses to the CS+ and CS− significantly decreased across trial blocks, $F(2.75, 300) = 6.63$, $p < 0.001$, $\eta_p^2 = 0.05$. No other main effects or interactions were significant (all $F < 2.36$, all $p > 0.11$).

The analysis of the last block of extinction memory testing on Day 3 indicated that participants exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS− (effect of stimulus: $F(1, 108) = 5.62$, $p < 0.05$, $\eta_p^2 = 0.05$). No other main effects or interactions were significant (all $F < 2.86$, all $p > 0.09$).

Including fear-potentiated startle responses from late acquisition as a covariate eliminated the significant effects of stimulus that were observed during the first and last blocks of extinction memory testing on Day 3. For the analysis of the entire extinction memory session, the covariate analysis eliminated the significant effect of stimulus and the significant Genotype × Stimulus, Genotype × Sex, and Genotype × Sex × Stimulus interactions. The effect of trial block and the Genotype × Stimulus interaction remained significant.

3.2.4. Effects across Days

The analysis of fear-potentiated startle across all three phases revealed that participants exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS− during acquisition and extinction on Days 1 and 2 than they did during extinction memory testing on Day 3. These differences were particularly evident during the later trial blocks of acquisition on Day 1 and the earlier trial blocks of extinction on Day 2 (effect of day: $F(1.72, 164) = 8.50$, $p < 0.001$, $\eta_p^2 = 0.09$; effect of stimulus: $F(1, 82) = 75.42$, $p < 0.001$, $\eta_p^2 = 0.48$; effect of trial block: $F(3, 246) = 3.61$, $p < 0.05$, $\eta_p^2 = 0.04$; Day × Stimulus interaction: $F(2, 164) = 6.40$, $p < 0.01$, $\eta_p^2 = 0.07$; Day × Trial Block interaction: $F(5.06, 492) = 5.98$, $p < 0.001$, $\eta_p^2 = 0.07$; Stimulus × Trial Block: $F(3, 246) = 6.32$, $p < 0.001$, $\eta_p^2 = 0.07$; Day × Stimulus × Trial Block interaction: $F(4.79, 492) = 10.62$, $p < 0.001$, $\eta_p^2 = 0.12$). Although both met-carriers and non-carriers displayed significantly greater fear-potentiated startle responses to the CS+ than to the CS−, non-carriers exhibited significantly greater CS discrimination across all three phases than met-carriers (effect of genotype: $F(1, 82) = 4.57$, $p < 0.05$, $\eta_p^2 = 0.05$; Genotype × Stimulus interaction: $F(1, 82) = 12.91$, $p < 0.001$, $\eta_p^2 = 0.14$). Although both males and females displayed significantly greater fear-potentiated startle responses to the CS+ than to the CS−, females exhibited significantly greater CS discrimination across all three phases than males (Sex × Stimulus interaction: $F(1, 82) = 8.69$, $p < 0.01$, $\eta_p^2 = 0.10$). This interaction appeared to be influenced by genotype. Although the Genotype × Sex × Stimulus interaction was only a statistical trend, $F(1, 82) = 3.28$, $p = 0.074$, $\eta_p^2 = 0.04$, there was an indication that female non-carriers exhibited greater fear-potentiated startle responses to the CS+ than did female met-carriers. Furthermore, the trend also suggested that male met-carriers were the only participants to not exhibit greater fear-potentiated startle responses to the CS+ than to the CS−

across all three phases. No other main effects or interactions were significant (all $F < 2.52$, all $p > 0.08$).

3.2.5. Influence of Menstrual Cycle on Fear-Potentiated Startle

Because of the sex-dependent effects observed in the overall sample, we performed exploratory analyses to examine whether menstrual cycle activity would influence the effects of the val66met polymorphism on fear-potentiated startle. In order to do so, we divided female participants into follicular [0 - 14 days since last period; $N = 35$ (10 met-carriers, 25 non-carriers)] or luteal [≥15 days since last period; $N = 26$ (8 met-carriers, 18 non-carriers)] phases of the menstrual cycle [62]. In these analyses, menstrual stage had no significant effect on fear-potentiated startle during acquisition, extinction, or extinction memory testing, nor did it significantly interact with genotype to influence the behavioral responses.

3.3. US Expectancies

3.3.1. Day 1—Fear Acquisition

During acquisition on Day 1, US expectancy ratings for the CS+ were significantly greater than US expectancy ratings for the CS− (effect of stimulus: $F(1, 106) = 722.53$, $p < 0.001$, $\eta_p^2 = 0.87$). This difference significantly increased as acquisition progressed (effect of trial block: $F(1.67, 318) = 6.48$, $p < 0.01$, $\eta_p^2 = 0.06$; Stimulus × Trial Block interaction: $F(2.22, 318) = 213.43$, $p < 0.001$, $\eta_p^2 = 0.67$; **Figure 5(a)**). No other main effects or interactions were significant (all $F < 3.41$, all $p > 0.06$).

3.3.2. Day 2—Fear Extinction

During fear extinction on Day 2, participants exhibited significantly greater US expectancy ratings following presentation of the CS+ than following presentation of the CS− (effect of stimulus: $F(1, 107) = 69.10$, $p < 0.001$, $\eta_p^2 = 0.39$). However, US expectancy ratings following presentation of the CS+ significantly decreased during each block (effect of trial block: $F(2.15, 321) = 34.58$, $p < 0.001$, $\eta_p^2 = 0.24$; Stimulus × Trial Block interaction: $F(2.23, 321) = 26.23$, $p < 0.001$, $\eta_p^2 = 0.20$; **Figure 5(b)**). No other main effects or interactions were significant (all $F < 3.47$, all $p > 0.06$).

3.3.3. Day 3—Extinction Memory Testing

During fear extinction memory testing on Day 3, participants exhibited significantly greater US expectancy ratings following presentation of the CS+ than following presentation of the CS−. This effect was evident for all trial blocks. US expectancy ratings following presentation of the CS+ decreased across blocks of trials and were no different from US expectancy ratings following presentation of the CS− by Block 4 (effect of stimulus: $F(1, 106) = 11.56$, $p < 0.001$, $\eta_p^2 = 0.10$; effect of trial block: $F(1.78, 318) = 22.63$, $p < 0.001$, $\eta_p^2 = 0.18$; Stimulus × Trial Block interaction: $F(1.83, 318) = 4.85$, $p < 0.05$, $\eta_p^2 = 0.04$; **Figure 5(c)**). The Genotype × Sex × Stimulus × Trial Block interaction was also significant,

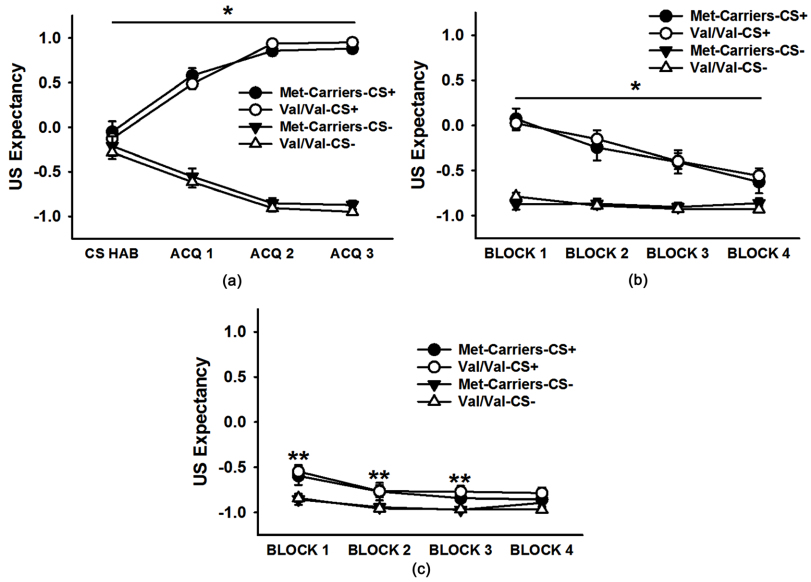

Figure 5. During acquisition (a) and extinction (b), US expectancy ratings for the CS+ were significantly greater than US expectancy ratings for the CS−; these differences significantly increased during acquisition, and significantly decreased during extinction. During Blocks 1, 2, and 3 of extinction memory testing (c), participants exhibited significantly greater US expectancy ratings following presentation of the CS+ than following presentation of the CS−. US expectancy ratings following presentation of the CS+ significantly declined across trial blocks and, by Block 4, were not statistically different from US expectancy ratings following presentation of the CS−. Data are presented as means ± SEM. *$p < 0.001$ main effect of CS+ relative to CS−; **$p < 0.01$ relative to CS−.

$F(1.83, 318) = 5.14$, $p < 0.01$, $\eta_p^2 = 0.05$, indicating that female non-carriers exhibited significantly greater US expectancy ratings following presentation of the CS+ than all other groups, which was particularly evident during the first block of trials. No other main effects or interactions were significant (all $F < 2.39$, all $p > 0.12$).

4. Discussion

The purpose of the present study was to examine the influence of the val66met polymorphism of the *bdnf* gene on fear acquisition, extinction, and extinction memory testing in a differential fear conditioning paradigm. During acquisition, we found that female non-carriers exhibited significantly greater baseline startle responses to the first 3 NA trials than all other groups. By the end of acquisition, both met-carriers and non-carriers demonstrated significantly greater fear-potentiated startle responses to the CS+ than to the CS−; however, met-carriers displayed significantly weaker CS discrimination than non-carriers. This difference between met-carriers and non-carriers persisted throughout extinction and extinction memory testing on Days 2 and 3, and during these days of testing, the difference was primarily evident in females. These findings provide important insight into the ongoing assessment of the val66met involvement in fear learning and, in particular, highlight the possibility of sex as a mediating factor in such

effects.

Prior work examining the influence of the val66met polymorphism on fear conditioning has produced mixed results. Some investigators have reported that carriers of the met allele exhibit impaired fear acquisition [19] [46] [48], while others have observed no significant effect of the polymorphism on fear acquisition [38] [45] [47] [63]. These differences in outcomes could be explained, in part, by the type of fear conditioning employed in such studies. Indeed, some research has demonstrated that met-carriers exhibit impaired contextually-based fear conditioning, which is dependent on the hippocampus, while retaining intact cue-based fear conditioning, which is dependent on the amygdala [30] [45] [46]. The problem with this explanation, however, is that some investigators have observed impaired cue-based fear acquisition and/or fear memory in met-carriers, relative to non-carriers [19] [48], suggesting a more general deficit in fear conditioning processes. Another possibility is that met-carriers do not have impaired fear learning per se, but rather have trouble differentiating threatening stimuli from safe stimuli. Indeed, Soliman and colleagues [38] found that, even though met-carriers exhibited statistically equivalent fear acquisition as non-carriers, met-carriers took significantly longer than non-carriers to recognize that a neutral cue (*i.e.*, CS−) was not associated with the US. Furthermore, other studies (e.g., [45]) have reported greater fear generalization in met-carriers, relative to non-carriers, particularly in novel contexts. In the present study, however, we did not observe any evidence to support these explanations.

A more consistent finding in this area of research is that met-carriers exhibit impaired extinction learning [38] [39] [40] [41]; yet, we did not observe a significant effect of the met allele on the rate of extinction. During extinction training, we observed significantly less fear-potentiated startle responses to the CS+ in met-carriers, relative to non-carriers, but this difference appeared early in extinction training and was likely a result of a weaker fear memory that was consolidated following acquisition. Moreover, met-carriers exhibited significantly greater fear-potentiated startle responses to the CS+ than to the CS− across the entire extinction session, and 24 hr later, this difference was absent, suggesting intact extinction learning and retention in these individuals.

A particularly novel finding from the present study is that the deficit in fear learning observed in met-carriers was, for the most part, selective to females. Although no significant sex-dependent effects were detected for the analyses of acquisition, the deficit in fear learning in female met-carriers appeared to be driven by greater CS discrimination learning in female non-carriers, which was discernable during the last block of acquisition trials (**Figure 2(d)**). It is important to note that female non-carriers also exhibited significantly greater baseline startle responses than all other groups at the beginning of acquisition. Nevertheless, the greater fear-potentiated startle responses observed in female non-carriers cannot be explained by differences in baseline startle, given that fear-potentiated startle was calculated by correcting for each participant's responses to NA trials.

Research examining sex differences in fear conditioning has been inconsistent, especially in humans. Preclinical work has shown that males exhibit greater conditioned fear than females [64] [65] [66]. However, research in humans has revealed no differences [67] [68] or greater fear learning in females [69] or males [70] [71]. To our knowledge, this is the first study to show a sex-dependent effect of the val66met polymorphism on fear conditioning. One other study did report that male non-carriers exhibited greater startle responses, overall, than female non-carriers [47], but that finding lies in contrast to the present observations. The sex differences in fear learning observed here could relate to sex-dependent differences in BDNF levels between met-carriers and non-carriers, as observed in previous work [72] [73]. Research has also shown that BDNF levels in females vary with the menstrual cycle [74], an effect that might relate to an interaction between estrogen and BDNF [75]. However, we did not observe any differences in fear-potentiated startle responses between menstrual stages. The observed sex differences might also relate to sex-dependent differences in brain activity between met-carriers and non-carriers. Wei and colleagues [76] reported that female non-carriers exhibited greater resting cerebral blood flow in the frontal cortex and hippocampus than male non-carriers; the opposite pattern of results was observed in met-carriers (*i.e.*, males > females). Finally, it is possible that female non-carriers respond differently to the stress of fear conditioning, relative to met-carriers. Indeed, previous work has shown that, in females, non-carriers exhibit greater stress-induced cortisol responses than carriers [77] (however, see [78]), which could result in stronger fear learning. These possible explanations for sex-dependent differences in fear learning between met-carriers and non-carriers should be explored in future work.

The alterations of fear conditioning observed in met-carriers may be related to gene-dependent differences in the neural circuitry underlying fear. For instance, Lonsdorf and colleagues [63] observed no significant behavioral differences between met-carriers and non-carriers during fear acquisition; however, they did find that met-carriers exhibited greater amygdala activity than non-carriers, while non-carriers exhibited greater ventromedial PFC (vmPFC) activity than met-carriers. These findings are consistent with additional work showing that, during extinction, met-carriers exhibit greater amygdala activity and reduced vmPFC activity, relative to non-carriers. Several studies have also shown that met-carriers exhibit an attentional bias for and greater amygdala responses to emotional stimuli [33] [34] [35] [36] [37]. Collectively, these findings suggest that met-carriers might retain less PFC-mediated inhibitory control over amygdala-driven fear responses. This would result in impaired extinction processes, and instead of impairing fear learning, per se, it might result in greater fear generalization and more "diffuse" fear [63] [79].

In conclusion, we have shown that the val66met polymorphism is associated with weaker fear memories, especially in females. It is important to note, however, that our sample of participants was obtained from a small, liberal arts university in the Midwest region of the United States. Moreover, most of our par-

ticipants were mostly Caucasian and likely of middle-to-high socioeconomic status. Thus, our results may not be representative of the entire population. Thus, the presented findings should be considered preliminary data that may prompt additional investigations of the relationship between the val66met polymorphism, sex, and fear learning. Although our sample size, particularly for met-carriers, was modest, it is comparable to or larger than most of the sample sizes from previous work in this area [19] [38] [45] [47] [48] [63]. Consistent with other work in this area [63], our findings suggest a relationship between the val66met polymorphism and emotional learning that goes beyond extinction. One might question how weaker fear-potentiated startle responses could serve as a risk factor for stress- or anxiety-related psychological disorders. As suggested by Lonsdorf and colleagues [63], reduced fear-potentiated startle could reflect a deficiency in threat mobilization and result in more diffuse anxiety. Indeed, individuals with anxiety-related disorders that are characterized by long-lasting diffuse anxiety (e.g., generalized anxiety disorder, PTSD) counterintuitively exhibit diminished startle responses to aversive content, relative to individuals with anxiety-related disorders that are characterized by discrete fear (e.g., specific phobias) [79]. Perhaps the met allele results in changes to the neurobiology underlying fear that leads to a reduced ability to differentiate between safe and threatening stimuli, or greater generalization of fear. Future work is warranted to clarify the involvement of the val66met in fear learning, particularly with regard the influence of sex on such effects.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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