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Amygdala-dependent fear conditioning in humans is modulated by the BDNFval66met polymorphism

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Abstract

The Brain derived neurotrophic factor (BDNF) is critically involved in neuroplasticity as well as the acquisition, consolidation, and retention of hippocampal- and amygdala-dependent learning. A common functional A→G single nucleotide polymorphism (*BDNF*val66met) in the pro-domain of the human *BDNF* gene is associated with abnormal intracellular trafficking and reduced activity-dependent BDNF release. We studied the effect of *BDNF*val66met in an aversive differential fear conditioning, and a delayed extinction paradigm in fifty-seven healthy participants. Pictures of male faces were used as stimuli and fear learning was quantified by fear potentiated startle (FPS) and skin conductance responses (SCR).

Aware *BDNF* met-carriers show a behavioral deficit in amygdala-dependent fear conditioning as indicated by an absence of FPS responses in the last acquisition block. This deficit was maintained in the first block of extinction. No genotype differences were found in conditioned SCR discrimination. These data provide evidence for the involvement of BDNF signaling in human amygdala-dependent learning. We suggest that the *BDNF* met-allele may have a protective effect

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for the development of affective pathologies that is mediated via reduced synaptic plasticity induced by negative experience.

Keywords

Brain-derived neurotrophic factor; synaptic plasticity; learning; anxiety; fear-potentiated startle

Learning is a major adaptive function that has assisted organisms in tailoring their behavior to environmental contingencies for hundreds of millions of years. This behavioral adaptation reflects evolutionary preserved mechanisms of synaptic plasticity involving cellular/molecular changes in the central nervous system. Pavlovian fear conditioning is a basic learning paradigm for studying how organisms make use of signaling relationships between environmental events where some stimuli (conditioned stimuli, CSs) serve as informative warnings that a potentially damaging stimulus (the unconditioned stimulus, US) is imminent, thus allowing early recruitment of defense responses (conditioned responses, CRs). This emotional memory formation requires synaptic plasticity via activity dependent changes in synaptic strength.

The brain-derived neurotrophic factor (BDNF) is the most abundant neurotrophin in the mammalian central nervous system (CNS), and it is a major player in mediating synaptic plasticity (e.g. Bramham and Messaoudi, 2005). The involvement of BDNF in hippocampus-dependent cognitive learning and memory has been known from studies in both rodents (for a review see Tyler et al., 2002) and humans (Egan et al., 2003; Hariri et al., 2003). More recently, a similar role of BDNF for Pavlovian fear conditioning, which depends on the amygdala, has been shown (Rattiner et al., 2004a, 2005; Ou and Gean, 2006) as well as an association between a functional polymorphism in the *BDNF* gene and human fear generalization (Hajcak et al., 2009). Using pharmacological and genetic approaches in rats, it was demonstrated that the binding of mature BDNF to the TrkB receptor within the basolateral amygdala was necessary for acquisition and consolidation of conditioned fear (Rattiner et al., 2004b; Rattiner et al., 2005) as well as for the consolidation of extinguished fear (Chhatwal et al., 2006). The conditioning measure in the animal studies were fear potentiated startle (FPS) responses which reflect direct and indirect effects of the amygdala on the brain-stem startle circuitry (Davis and Whalen, 2001).

As the human *BDNF* gene harbors a common single nucleotide polymorphism (SNP) that has been shown to affect activity-dependent secretion of BDNF and thereby synaptic plasticity, this genetic variation is an excellent candidate for an attempt to bridge the gap between animal and human research. The SNP (*BDNF*val66met) in the prodomain of the human *BDNF* gene converts the aminoacid valine (val) to methionine (met) at codon 66 (*BDNF*val66met). The met-allele leads to a selective impairment in intracellular BDNF trafficking and regulated secretion in neuronal cells without compensatory increase in constitutive secretion (Egan et al., 2003; Chen et al., 2004) and occurs in 20-30% of the Caucasians (Shimizu et al., 2004).

Genetic association studies optimally study simple behavioral paradigms with a well-defined underlying neural circuitry eliciting robust behavioral responses that are easy to measure and quantify. Fear conditioning is a prototype of such a model as the underlying neural network is well delineated and FPS is used in animal (whole body startle) and human studies (startle blink) to quantify fear learning which facilitates the translation of the findings. In humans, FPS can be measured by the potentiation of the startle reflex to short auditory probes presented during the CS as compared to the interval between CSs (Hamm et al., 1993). The basis for this measurement is that the startle blink response triggered by a sudden burst of noise (startle probe) is larger when the individual is in an aversive or fearful state. The startle blink reflex is particularly appropriate for the study of amygdala-dependent learning as the startle reaction

reflects the influence of direct and indirect connections from the amygdala to the startle-reflex pathway in the brainstem (Davis and Whalen, 2001). Animal studies have identified the basolateral amygdala as the primary site of BDNF action during fear conditioning (Rattiner et al., 2004b) and thus FPS can be expected to optimally index BDNF dependent processes related to amygdala-dependent fear conditioning. The (baso-)lateral amygdala receives input from cortical and subcortical sensory sites and houses the molecular machinery for forming associations between the CS and the US (LeDoux, 2000; Fanselow and Poulos, 2005). As a result of this associative process, the CS alone may activate the central nucleus of the amygdala. The central nucleus then recruits pathways to the brainstem, striatum, diencephalon and the midbrain (Davis and Whalen, 2001), which for example promotes the potentiation of the startle response.

The purpose of the present study was to examine whether a functional genetic variation in the pro-domain of the human gene coding for the BDNF (BDNFval66met) affects human amygdala-dependent fear learning and extinction by using fear potentiated startle and skin conductance measurements in an aversive differential conditioning and a 24h-delayed extinction paradigm.

Method

Participants

Sixty-three German university students were recruited by advertisements. They participated in two days of testing, donated 20ml of blood for DNA extraction and genotyping and filled out informed-consent forms (approved by ethics committees at the Karolinska Institute and the University of Greifswald). This is the same sample as reported in a previous publication (Lonsdorf et al., 2009). The experiment and data scoring were performed blind to genotype. Six participants were excluded from all data analysis due to technical problems, leaving 57 participants for analysis. Further nine participants were excluded from major analyses because they were classified as unaware of the CS-US contingencies (see also Procedure and Results for more details). The final sample for the major analyses consisted of 48 participants (25 male).

Genotyping

DNA extraction from whole blood was performed using standard methods (Lindblom and Holmlund, 1988). Genotyping for BDNFval66met was performed using the Taqman allelic discrimination method (5' nuclease assay, Livak, 1999). PCR reactions, with a total volume of 5 μ l, were performed in 384-well plates containing 20ng dried-down genomic DNA using the Applied Biosystems (ABI, Foster City, CA) standard protocol and the ABI rs6265 genotyping assay (ABI, Foster City, CA). End-point fluorescence was measured and genotype calling was carried out by the allelic discrimination analysis module an ABI HT7900 (ABI, Foster City, CA) which resulted in clear identification of the three genotypes for the BDNFval66met polymorphism. All genotypes were determined in duplicates.

Stimulus Material

Four different color pictures from the Karolinska Directed Emotional Faces (Lundqvist, Flykt & Öhman, 1998) depicting two different male faces (both with an angry or a neutral expression) were selected to serve as CSs. Each participant saw both male faces and valence of facial expression (angry vs. neutral) was kept constant within participants. A white fixation-cross on black background was presented during the intertrial interval (ITI, 10-18 s). The pictures (visible size: 126 cm \times 93 cm) were projected for six seconds onto a screen approximately 2 m in front of the participant using a projector (Sanyo PLC-XU86) located in an adjacent room. Participants were randomly assigned to one of eight stimulus sequences. The US was a 500-Hz monopolar DC-pulse electric stimulation applied above the right ankle in a 10 ms train of

1-ms single pulses. It was generated by a commercial stimulator (Grass Instruments S48K, West Warwick, RI), isolated (SIU5), and transmitted via a constant-current unit (CCU1) to a bipolar electrode (F-E10S2). Startle probes were 50-ms bursts of 95-dB[A] white noise (rise time < 1 ms) presented binaurally over Sony (MDRCD 170) headphones.

Physiological recordings

Startle responses were measured by recording electromyographic (EMG) activity of the left orbicularis oculi muscle using miniature Ag/AgCl surface electrodes. The raw EMG signal was amplified, filtered through a 30-Hz high-pass (Coulbourn S75-01) and a 400-Hz low-pass filter (Kemo KEM-VBF8-03; Beckenham, Kent, United Kingdom), rectified, and integrated with a time constant of 10 ms. Skin conductance was recorded using Hellinge Ag/AgCl standard electrodes placed adjacently on the hypothenar eminence of the right hand (see Weike et al., 2007 for more detailed description of the recording procedure).

Procedure

On both days, participants were instructed to attend to the pictures but no information about the CS-US contingencies was provided. Before the presentation of the pictures, four acoustic startle probes were presented for habituation on both days. During the experimental sessions, startle probes were presented 4 or 5 s after picture onset for two thirds of the CS presentations and during one third of the inter-trial intervals (ITI).

Day 1 (Fear acquisition)—The experimental procedure was divided into 5 min familiarization with the lab situation followed by habituation (presentation of six different facial stimuli, four of which startled), individual adjustment of the US intensity (“highly annoying but not painful”), and conditioning. During *conditioning*, participants viewed a mixed series of nine presentations (6 s) of two CS pictures each. One of the pictures (CS+) co-terminated with the US (100% reinforcement), whereas the other picture (CS-) was never coupled with the US. The conditioning phase ended with a standardized post-experimental awareness interview (cf. Bechara et al., 1995), to assess CS-US contingency awareness.

Day 2 (Extinction, approx. 24h later)—During extinction, the CS+ and CS- were presented 18 times each without administration of any further USs. After completing the experiment participants were debriefed and paid 15 Euro.

Data reduction and response definition

The magnitude of the startle eyeblink (in microvolts) was measured from onset to peak, as described previously in detail (Weike et al., 2007). Blink magnitudes were normalized using z-standardization and converted to T scores to ensure that all participants contributed equally to the group means. The T-score calculation, $50+(z \times 10)$, results in a distribution with an overall mean of 50 and a standard deviation of 10 for each participant. SCR magnitude (in microSiemens) was scored as the first response occurring 0.9 to 4.0 s after picture onset. Logarithms were computed for all values to normalize the distribution (Venables and Christie, 1980) and these log values were range-corrected (individual score/individual maximum response) to account for interindividual response level differences (Lykken and Venables, 1971). Startle and SCR measurements showing recording artifacts or excessive baseline activity were discarded.

Data analysis

The acquisition session was split into an early and late block and the extinction session was split into four blocks. Each block consisted of the mean of 3 (startle) or 4 (SCR) consecutive reactions within a stimulus category. Repeated Measures Analyses of Variance with stimulus

(CS+, CS-, ITI for startle; CS+, CS- for SCR) and trialblock (acquisition=2 blocks, extinction=4 block) as the repeated factors and *BDNF*val66met genotype (val/val vs. met-carrier) as the between-subject variable were used to analyze startle responses and SCRs. A significance level $<.05$ was considered significant and Greenhouse-Geisser adjustments of degrees of freedom were used when appropriate. We report η^2 as the estimate of effect size. Hardy Weinberg Equilibrium was calculated via Exact Test using a website (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>).

Results

Genotype frequencies

In total we observed 43 individuals homozygous for the *BDNF* val/val genotype and 14 carriers of the *BDNF* met-allele (N=3 met/met). Genotype frequencies did not differ significantly from Hardy-Weinberg Equilibrium, $p(\text{Exact Test})=0.10$.

Contingency Awareness

Whereas only four of 43 participants with the val/val genotype (9.3%) failed to report the CS-US contingency after acquisition, five out of 14 met-carriers (35.7%) failed to do so, resulting in a significant association between *BDNF*val66met genotype and awareness of the CS-US contingency, Fisher's Exact Test, $p=0.032$. Moreover, a significant interaction between the participants' contingency awareness and conditioning performance was observed for both conditioning measures, i.e., the fear potentiated startle, $F(2,106)=5.14$, $p=0.008$, $\eta^2=0.09$, and the skin conductance responses, $F(1,53)=5.00$, $p=0.03$, $\eta^2=0.09$ (see supplementary figure 1). Importantly, this interaction was further qualified by the *BDNF*val66met genotype for the fear potentiated startle, $F(2,106)=4.49$, $p=0.015$, $\eta^2=0.08$, but not for SCR conditioning. Because of this strong effect of awareness on both startle and SCR in our dataset (see supplementary figure 1) and because the unaware group (N=9) is too small to allow for reliable testing of *BDNF*val66met genotype associations, which was the major aim of this study, we limited our major analyses to participants that were aware of the contingencies.

The final sample of 48 participants consisted of 9 carriers of the *BDNF*66met allele (2 met/met) and 39 homozygous for the *BDNF*66val allele. Carriers of one or two *BDNF*66met alleles were combined as a *BDNF* met-carrier group due to the low frequency of participants homozygous for this allele and according to the literature (e.g. Hariri et al., 2003; Hajcak et al., 2009).

Fear potentiated startle

In aware subjects, repeated measures ANOVAs with stimulus (CS+, CS-, ITI) and trial block (conditioning = 2, extinction = 4) as repeated factors revealed robust startle potentiation as indicated by a significant main effect of stimulus during conditioning, $F(2,92)=5.60$, $p=0.005$, $\eta^2=0.11$, and extinction, $F(2,92)=3.49$, $p=0.038$, $\eta^2=0.07$. The significant main effect of stimulus during conditioning was due to significant CS+ potentiation (CS+ vs. ITI), $p=0.003$, as well as CS discrimination (CS+ vs. CS-), $p=0.034$. During extinction the significant main effect for stimulus was due to significant CS discrimination only, $F(1,46)=8.74$, $p=0.005$, $\eta^2=0.16$.

This robust main effect for stimulus was further qualified by a significant trial block x stimulus x *BDNF*val66met genotype interaction during conditioning, $F(2,92)=4.73$, $p=0.017$, $\eta^2=0.09$, and extinction, $F(6,276)=3.22$, $p=0.013$, $\eta^2=0.07$ (see Figure 1 and 2).

During acquisition, the trial block x stimulus x *BDNF*val66met genotype interaction reflected more pronounced CS+ potentiation, $F(1,46)=4.40$, $p=0.042$, $\eta^2=0.09$, and CS discrimination,

$F(1,46)=6.98$, $p=0.011$, $\eta^2=0.13$, in the val/val group as compared to met-carriers in the second trial block. This was confirmed by a statistically significant stimulus x *BDNF*val66met genotype interaction only during the last block of acquisition, $F(2,92)=6.20$, $p=0.004$, $\eta^2=0.12$. During extinction, the trial block x stimulus x *BDNF*val66met genotype interaction reflected significantly larger CS+ potentiation in the val/val group as compared to the met-carriers, $F(1,46)=5.25$, $p=0.027$, $\eta^2=0.10$, only during the first trial block. Follow-up analyses showed a statistically significant stimulus x *BDNF*val66met genotype interaction in the first block of extinction, $F(2,92)=3.50$, $p=0.04$, $\eta^2=0.07$ (see Figure 1B).

Within group analyses for the last conditioning block for the *BDNF* genotype groups showed a significant main effect for stimulus in the *BDNF* val/val group, $F(2,76)=20.04$, $p<0.000$, $\eta^2=0.35$, indicating robust CS+ potentiation, $F(1,38)=24.82$, $p<0.000$, $\eta^2=0.40$, and robust CS discrimination, $F(1,38)=34.26$, $p<0.000$, $\eta^2=0.47$. In the *BDNF* met-carrier group no main effect for stimulus was found, $F(2,16)<1$ (see Figure 1A).

Neither a main effect for *BDNF*val66met genotype on either day $F(1,46)<1$, nor a significant main effect of *BDNF*val66met genotype on mean ITI raw-score magnitudes (in microvolts) was found, even though met-carriers showed higher magnitudes on a descriptive level during acquisition and extinction. Entering valence of the faces presented as a factor in the analyses had no effect on the results.

We have recently reported an association of the 5-HTTLPR and the COMTval158met polymorphisms on FPS during fear acquisition and extinction respectively on the same group of subjects (Lonsdorf et al., 2009). Additional analyses (see supplementary material) provide support that the present results are not due to stratification of the *BDNF*val66met genotype groups by these other polymorphisms.

Skin conductance responses

In aware participants, robust conditioned SCR discrimination was demonstrated by a significant main effect for stimulus, indicating differential conditioning, during both conditioning, $F(1,46)=10.57$, $p=0.02$, $\eta^2=0.19$, and extinction $F(1,45)=13.31$, $p=0.01$, $\eta^2=0.23$. This effect was however not further qualified by the *BDNF*val66met genotype nor was any main effect for *BDNF* genotype found, $F(1,46)<1$ (see Figure 1C and D). Furthermore *BDNF* genotype did not differ in their unconditioned SCR to the US, $F(1,46)<1$.

Discussion

Our data are consistent with a role of BDNF signaling in the acquisition and retention of conditioned fear in humans. Carriers of the *BDNF*66 met-allele were behaviorally impaired in amygdala-dependent learning as measured by FPS in a classical differential fear conditioning paradigm and showed no retention of fear approximately 24 hours later. During fear conditioning we show during late acquisition, when learning-dependent changes typically are observed, deficient CS+ Potentiation and Discrimination of FPS in aware carriers of the *BDNF* met-allele, but also more frequent failures to cognitively learn and report the US-CS contingencies when considering the whole sample. Furthermore, during early extinction, which most likely reflects retention of acquisition, *BDNF* met-carriers show attenuated CS+ and CS – Potentiation as compared to val/val individuals.

In contrast to FPS reactions, we did not find an effect of the *BDNF*val66met polymorphism on SCRs. While FPS has been suggested to reflect an affective level of learning (Öhman and Mineka, 2001; Hamm and Weike, 2005), SCRs predominantly reflect cognitive contingency awareness and general arousal (Lovibond and Shanks, 2002). In line with this proposition,

SCR can be dissociated from amygdala activation during human fear conditioning (Tabbert et al., 2006).

These results replicate and extend a recent report of impaired FPS in carriers of the *BDNF*66met allele during a generalization fear conditioning paradigm in which participants were explicitly instructed about the CS-US contingency (Hajcak et al., 2009). Even though the paradigm chosen by Hajcak et al. differs in several respects from the paradigm chosen by us, we report similar findings: an attenuated potentiation of the FPS (specifically to the CS+) but also attenuated CS discrimination in the *BDNF* met-carrier group.

In contrast to the generalization conditioning task used by Hajcak's group, our task represents a fear conditioning paradigm much more similar to the paradigms used in animal studies. Keeping as close as possible to the behavioral methods used in animal studies is critical for translational research opting to generalize conclusions about physiological mechanisms from animals to humans. However, most animal studies demonstrating a critical involvement of BDNF signaling for successful fear conditioning use methods that are not available for human studies due to ethical constraints. Thus, when studying the specific biological underpinnings of fear learning and extinction we are dependent on the study of genetic polymorphisms, pharmacological challenge tests and molecular imaging methods.

In emotional memory formation, BDNF modulates a neural circuit centered on the amygdala. Elevations of BDNF levels during/after fear conditioning may mediate emotion-induced synaptic plasticity and thereby restructuring of synapses at critical sites of the fear network. BDNF translates environmental stimulation via neuronal activity into structural changes in the brain. However, there may be a dark side of synaptic plasticity as anxiety and affective disorders are known to involve an (over-) activation of the brain's fear circuit and may very well be associated with exaggerated synaptic plasticity in the amygdala (cf. Rattiner et al., 2005). In line with this hypothesis, a recent study (Gatt et al., in press) demonstrated that individuals homozygous for the *BDNF*66val allele that had been exposed to stressful events early in life had increased amygdala and medial prefrontal gray matter volumes and displayed elevated anxiety. Chronic stress enhances dendritic arborisation in the rat amygdala (Vyas et al., 2002), a process clearly reflecting synaptic plasticity and enlarged amygdala volumes have been reported in anxiety disorder patients (e.g. Juranek et al., 2006).

Hippocampal activations appear related to awareness of the CS-US contingency in human fear conditioning (Bechara et al., 1995; Knight et al., 2009), whereas amygdala activation is not (Tabbert et al., 2006; Knight et al., 2009). Therefore, it is interesting to note that our data suggest, that carriers of the *BDNF*66 met-allele also may show impairments in correctly reporting the CS-US contingencies. Thus, our data may provide evidence for a relationship of the *BDNF*val66met genotype with both amygdala (FPS) and hippocampus (contingency awareness) dependent learning in our fear conditioning paradigm. However, even though significantly more *BDNF* met-carriers failed to report the correct CS-US contingency after acquisition, the number of subjects in this comparison was very small and therefore, did not allow for analyses that subdivide this group further by the *BDNF*val66met genotype. Therefore, future studies with larger sample sizes and/or studies experimentally manipulating contingency awareness are warranted to follow up on this interesting suggestive evidence as it may have important implications for individuals placed in situations where it is critical to be able to learn to discriminate between danger and safety (e.g. military personnel or law enforcement officers).

Because our final sample included only two individuals homozygous for the *BDNF* met/met allele we were unable to discriminate between a possible linear effect or a heterosis (Chen et al., 2004) of the *BDNF*val66met genotype on FPS. It is important to note that excluding the two homozygous met-carriers from the analyses yielded the same statistical results. Given the

infrequency of this genotype most human studies to date have merged carriers of one or two met-alleles to one met-carrier group. Despite the fact that in many studies no or very few met/met individuals are observed, authors usually talk about met-carriers when in fact only inferences about heterozygotes can be drawn. It is important for future studies to elucidate if heterozygotes and individuals homozygous for the *BDNF*66met allele in fact share the same phenotype.

Our results may have important implications for the genetic risk of developing affective psychopathologies. Hajcak et al. (2009) argue that carriers of the met-allele may be at risk for affective pathologies. However, following our arguments above, we take a different perspective. We suggest that the *BDNF* met-allele may rather be a protective factor for anxiety disorders (e.g. PTSD) due to the putative impairment of aversive memory acquisition/consolidation via reduced experience-induced synaptic plasticity within the amygdala. Indeed the *BDNF* met-allele has been associated with reduced neuroticism scores (for a meta-analysis see Frustaci et al., 2008) and has been shown to protect for structural abnormalities in the amygdala-anterior cingulate loop which are seen in carriers of the short-allele of the 5-HTTLPR polymorphism, which is a genetic variant associated with negative affect (Pezawas et al., 2008). However, studies on *BDNF* val66met knock-in mice suggest that the met-allele may be associated with anxiety (Chen et al., 2006; Yu et al., 2009). Associative (fear) learning is an adaptive process and impairments certainly threaten behavioral adaptation and ultimately successful living. Thus the role of the *BDNF*val66met polymorphism in clinical anxiety as well as the translation from knock-in mice to human studies needs to be elucidated by future studies using larger sample sizes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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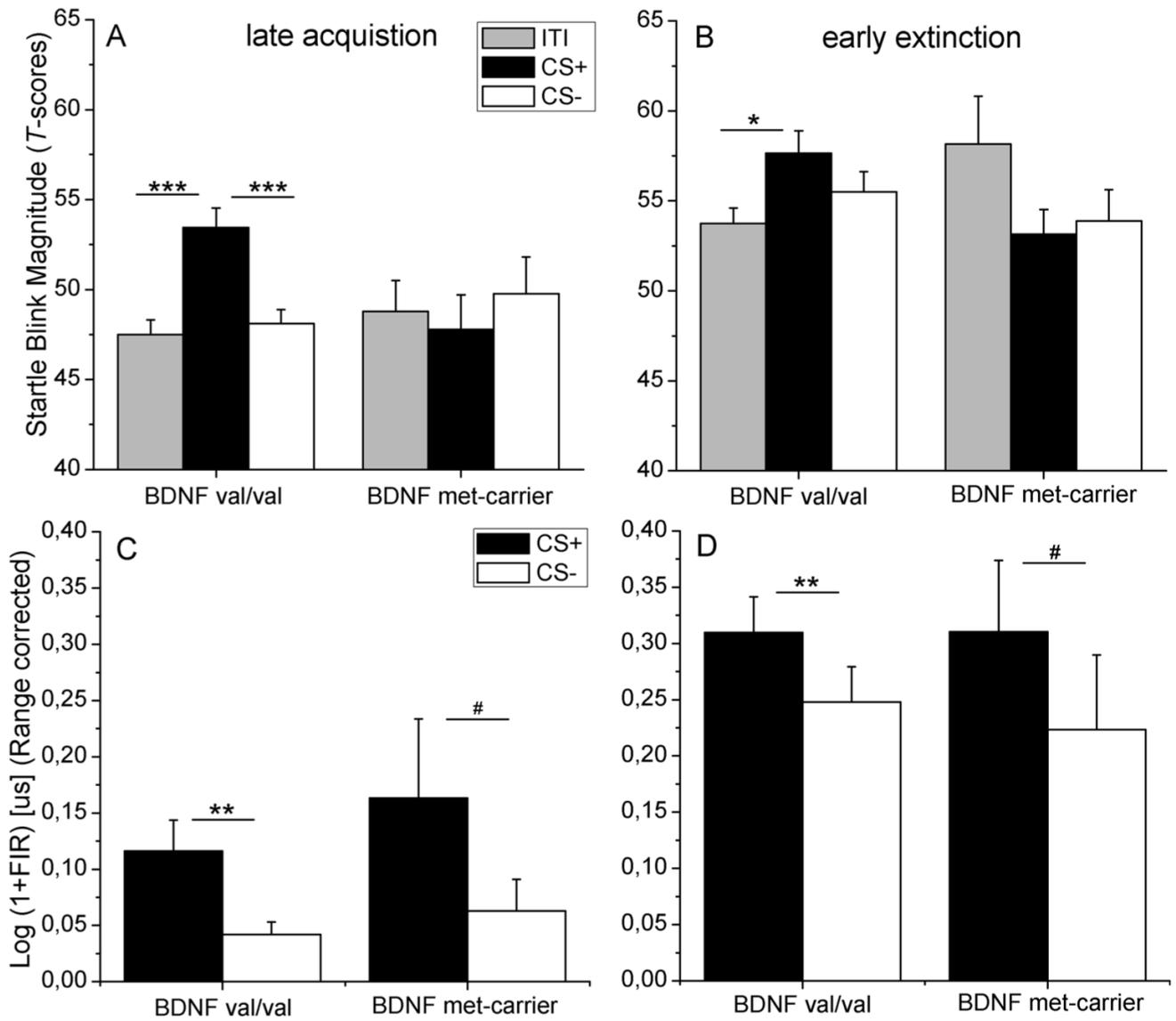
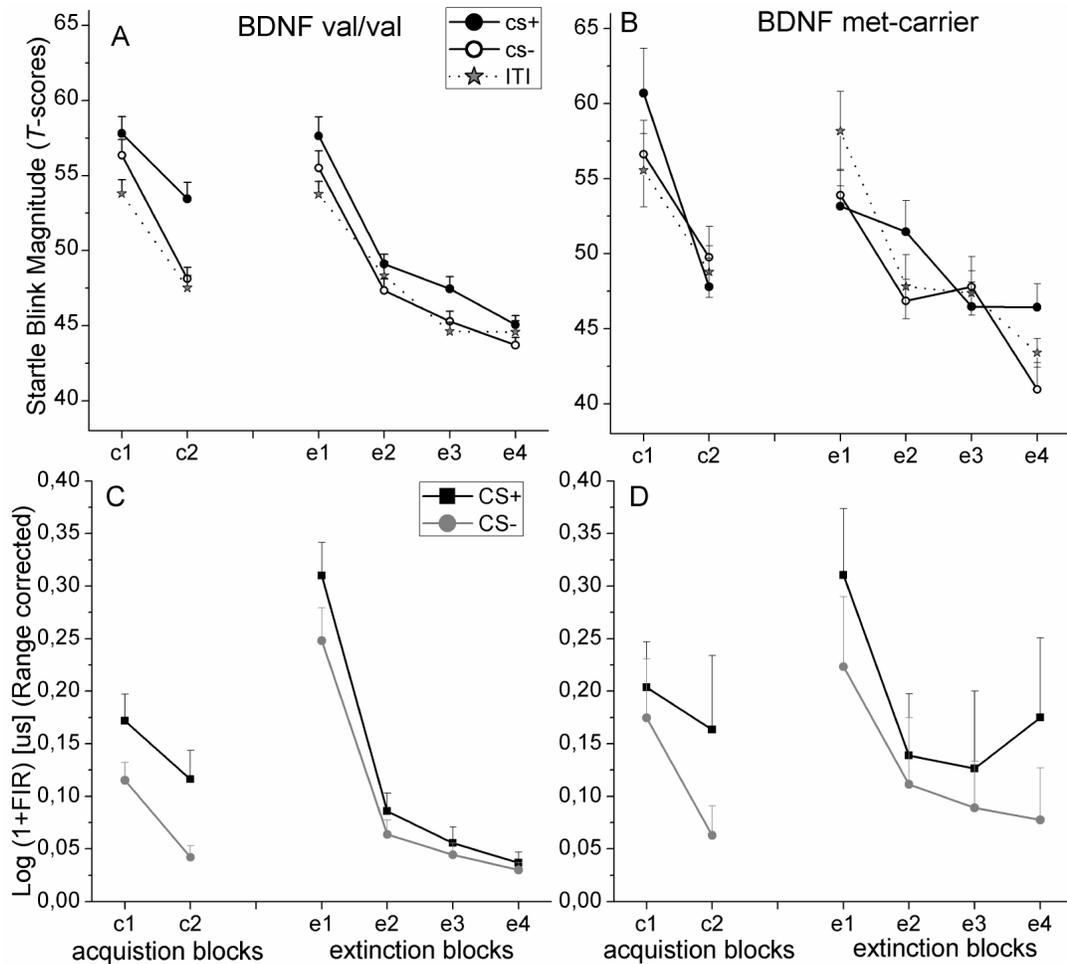


Figure 1.

Fear potentiated startle reactions (T-scores) elicited during the CS+, CS-, and the intertrial interval (ITI) for the *BDNF*val66met genotype groups (val/val vs. met-carrier) during (A) late acquisition and (B) early extinction as well as skin conductance responses to the CS+ and CS- for the *BDNF*val66met genotype groups (val/val vs. met-carrier) during (C) late acquisition and (D) early extinction (# $p < 0.10$, * $p < .05$, ** $p < .005$, *** $p < .001$).

**Figure 2.**

Fear potentiated startle reactions (T-scores) elicited during the CS+, CS-, and the intertrial interval (ITI) for (A) *BDNF* val/val and (B) met-carrier during the two acquisition blocks and the four extinction blocks as well as skin conductance responses to the CS+ and CS- for (C) *BDNF* val/val and (D) met-carrier during the two acquisition blocks and the four extinction blocks.