

## Effect of 7,8-Dihydroxyflavone, a Small-Molecule TrkB Agonist, on Emotional Learning

Raul Andero, Ph.D.

Scott A. Heldt, Ph.D.

Keqiang Ye, Ph.D.

Xia Liu, B.S.

Antonio Armario, Ph.D.

Kerry J. Ressler, M.D., Ph.D.

**Objective:** Despite increasing awareness of the many important roles played by brain-derived neurotrophic factor (BDNF) activation of TrkB, a fuller understanding of this system and the use of potential TrkB-acting therapeutic agents has been limited by the lack of any identified small-molecule TrkB agonists that fully mimic the actions of BDNF at brain TrkB receptors *in vivo*. However, 7,8-dihydroxyflavone (7,8-DHF) has recently been identified as a specific TrkB agonist that crosses the blood-brain barrier after oral or intraperitoneal administration. The authors combined pharmacological, biochemical, and behavioral approaches in a preclinical study examining the role of 7,8-DHF in modulating emotional memory in mice.

**Method:** The authors first examined the ability of systemic 7,8-DHF to activate TrkB receptors in the amygdala. They then examined the effects of systemic

7,8-DHF on acquisition and extinction of conditioned fear, using specific and well-characterized BDNF-dependent learning paradigms in several models using naive mice and mice with prior traumatic stress exposure.

**Results:** Amygdala TrkB receptors, which have previously been shown to be required for emotional learning, were activated by systemic 7,8-DHF (at 5 mg/kg *i.p.*). 7,8-DHF enhanced both the acquisition of fear and its extinction. It also appeared to rescue an extinction deficit in mice with a history of immobilization stress.

**Conclusions:** These data suggest that 7,8-DHF may be an excellent agent for use in understanding the effects of TrkB activation in learning and memory paradigms and may be attractive for use in reversing learning and extinction deficits associated with psychopathology.

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**B**rain-derived neurotrophic factor (BDNF) is widely distributed throughout the adult brain. BDNF binds to the nonselective p75 receptor, common to a variety of neurotrophins, as well as to its specific high-affinity TrkB receptor. There is evidence that BDNF plays a critical role in long-term potentiation (1) and is a molecular mediator of learning and memory. Intrahippocampal infusion of BDNF *in vivo* and BDNF infusion in hippocampal slices induce long-lasting enhancement of synaptic plasticity (2–4). In addition, BDNF has been shown to be involved in declarative and spatial memory processes (5, 6). For these reasons, BDNF is an attractive candidate for modulating learning and memory processes.

Deprivation of endogenous BDNF causes impairment of spatial learning and memory in rats (7), and forebrain-restricted deletion of BDNF severely impairs spatial learning (8). Similarly, BDNF and TrkB are both known to play an important role in fear memory acquisition and extinction (9, 10). We previously demonstrated that disruption of TrkB activation using lentiviral expression of a dominant-negative form of TrkB into the basolateral amygdala blocked the acquisition of fear (11) and the consolidation

of extinction (12), which suggests that BDNF-dependent activation of TrkB in the amygdala regulates the learning of fear and extinction memories.

Recent studies indicate that BDNF polymorphisms may be implicated in emotionality and anxiety disorders (13, 14). In addition to the role of the BDNF/TrkB system in learning, memory, and synaptic plasticity, alterations within the system have been implicated in affective disorders (15). Despite this increasing awareness of the many important roles played by BDNF activation of TrkB, a fuller understanding of this system and the use of potential TrkB-acting therapeutic agents has been limited by the lack of any identified small-molecule TrkB agonists that fully mimic the actions of BDNF at brain TrkB receptors *in vivo*.

Jang *et al.* (16) recently screened a chemical library for compounds that activate TrkB *in vitro* and discovered that a series of flavone derivatives, most potently 7,8-dihydroxyflavone (7,8-DHF), significantly activated TrkB. They found that 7,8-DHF binds *in vitro* with high affinity to the TrkB receptor and provokes its dimerization and autophosphorylation, leading to downstream signaling

cascade activation. Systemic administration of 7,8-DHF protects neurons in wild-type but not TrkB-deficient or mutated (F616A) mice from apoptosis, substantially activates TrkB in the brain 2 hours after injection, inhibits kainic acid-induced neuronal cell death, decreases infarct volumes in stroke in a TrkB-dependent manner, is neuroprotective in an animal model of Parkinson's disease (16), and rescues a BDNF deficit in a BDNF cortex-specific knockout murine model (17). To more fully examine the effects of this newly identified TrkB agonist, we examined the effects of 7,8-DHF on the acquisition and extinction of cue-dependent conditioned fear among the most simple yet robust rodent models of emotional learning and memory.

## Method

### Animals

All experiments were performed on adult (2–4 months old) wild-type strain C57BL/6J male mice from Jackson Laboratory (Bar Harbor, Me.). All procedures were approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with National Institutes of Health guidelines. Separate cohorts of animals were used in each experiment.

### Drugs

We administered 7,8-DHF (obtained both from coauthor K.Y. and from Tokyo Chemical Industry, catalog no. D1916) intraperitoneally at a dose of 5 mg/kg in a vehicle of 17% dimethylsulfoxide in phosphate-buffered saline; the same vehicle was also used in control groups. In those experiments in which 7,8-DHF was given, mice received a single dose 1 hour before the appropriate behavioral procedure.

### Immunoblotting, Immunohistochemistry, and Autoradiography

For Western blots, mice were injected with 7,8-DHF at 5 mg/kg i.p.; they were sacrificed 1 hour or 2 hours later, and amygdala tissue was collected bilaterally. Immunoblot analysis was performed with anti-TrkB Y706 (phosphorylated TrkB; Santa Cruz Biotechnology, Santa Cruz, Calif.; 1:200 in 5% bovine serum albumin/phosphate-buffered saline), and anti-TrkB (Cell Signaling Technology, Danvers, Mass.; 1:500 in 5% bovine serum albumin/phosphate-buffered saline), anti-*p*-mitogen-activated protein kinase (MAPK; Cell Signaling Technology; 1:500 in 5% milk/phosphate-buffered saline), and anti-MAPK (Cell Signaling Technology; 1:5,000 in 5% milk/phosphate-buffered saline). For immunohistochemistry, fixed rat brain sections from prior studies (11) were incubated and blocked in phosphate-buffered saline, goat serum, and Triton X-100 and incubated with TrkB rabbit polyclonal antibody (1:500, SC-12, Santa Cruz Biotechnology) and an anti-rabbit biotinylated secondary antibody (1:500) for 2 hours. Avidin-biotin complexes were amplified using a standard Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.). For regional localization of 7,8-DHF binding to TrkB, 7,8-DHF binding was performed as previously described (18).

### Cue Fear Conditioning and Extinction

Fear conditioning was conducted in nonrestrictive acrylic cylinders (SR-LAB startle response system, San Diego Instruments) located in a ventilated, sound-attenuated chamber. The footshock unconditioned stimulus was delivered through a stainless steel grid floor. Shock reactivity was defined as the peak activ-

ity (measured with a piezoelectric accelerometer) that occurred during the 200 msec after the onset of the unconditioned stimulus. The tone-conditioned stimulus was generated by a Tektronix function generator audio oscillator and delivered through a high-frequency speaker (9, 19).

Note that fear conditioning includes a longer intertrial interval (5 minutes) between each of five conditioned stimuli to maximize fear learning, as compared to a greater number of stimuli (ranging from 15 to 30) for fear expression and extinction protocols, which use shorter intertrial intervals of 30–90 seconds. On each of 2 days prior to training, mice were given a 10-minute startle chamber exposure session to habituate them to handling and context.

During cued fear conditioning, mice received five trials of a conditioned stimulus tone (30 seconds, 12 kHz, 70 dB) coterminating with an unconditioned stimulus footshock (500 msec, 0.5 mA in experiments 1 and 2; 500 ms, 1 mA in experiments 3 and 4) with an intertrial interval of 5 minutes. The expression of fear (in a different context from training) was assessed 24 hours after fear conditioning and consisted of 15 conditioned stimulus tone trials of 30 seconds each, with a 1.5-minute intertrial interval. For extinction testing in experiments 2 and 4, mice were given 30 conditioned stimulus tone trials with a 30-second intertrial interval. Fifteen conditioned stimuli were presented in experiment 3 in the extinction session. Stimulus presentation and data acquisition were controlled and digitized by, and stored in, an interfacing desktop computer using SR-LAB and analyzed with the FreezeView software program (Coulbourn Instruments, Whitehall, Pa.).

### Immobilization Stress

Immobilization procedures were conducted in a room separate from fear training and testing apparatus. Each animal was immobilized by gently restraining its four limbs in a prone position to metal arms attached to a wooden board for 2 hours. All animals of the same cage received the same treatment—either immobilization or handling. After treatment, animals were returned to their home cage and remained undisturbed until fear training.

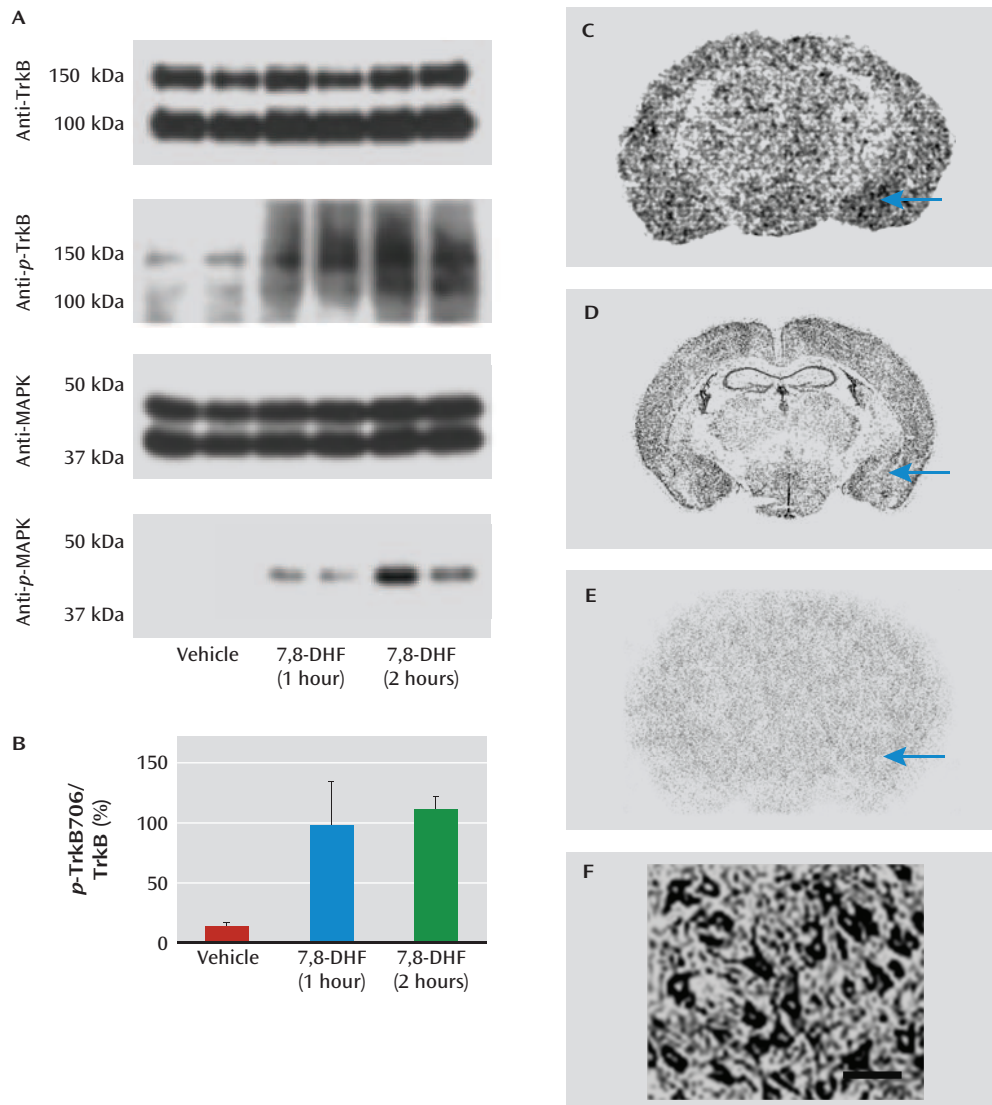
More methodological details are provided in the data supplement that accompanies the online edition of this article.

## Results

### Activation Effect of Systemic 7,8-DHF on TrkB Receptors in the Amygdala

We first confirmed that 7,8-DHF activated amygdala *p*-TrkB in vivo. Mice were given vehicle or 7,8-DHF systemically (5 mg/kg i.p.) either 1 or 2 hours prior to sacrifice. Immunoblots were then performed in duplicate on vehicle-treated and 7,8-DHF-treated amygdala tissue (Figure 1A,B). We found that the total level of TrkB protein did not change, but systemic 7,8-DHF led to robust increases in *p*-TrkB (Y706). To further demonstrate activation of the TrkB signaling pathway, we found that *p*-MAPK was activated within the amygdala 1 hour and 2 hours after administration of 7,8-DHF, again with no change in total MAPK.

We next confirmed the autoradiography receptor binding pattern of <sup>3</sup>H-7,8-DHF ( $K_d=320$  nM [16]) as shown in Figure 1C. The binding pattern resembles the known mRNA expression pattern of the TrkB receptor (Figure 1D), and this binding was prevented with excess cold 7,8-DHF

FIGURE 1. Activation of *p*-TrkB and MAPK in Mouse Amygdala by Systemic 7,8-Dihydroxyflavone (7,8-DHF)<sup>a</sup>

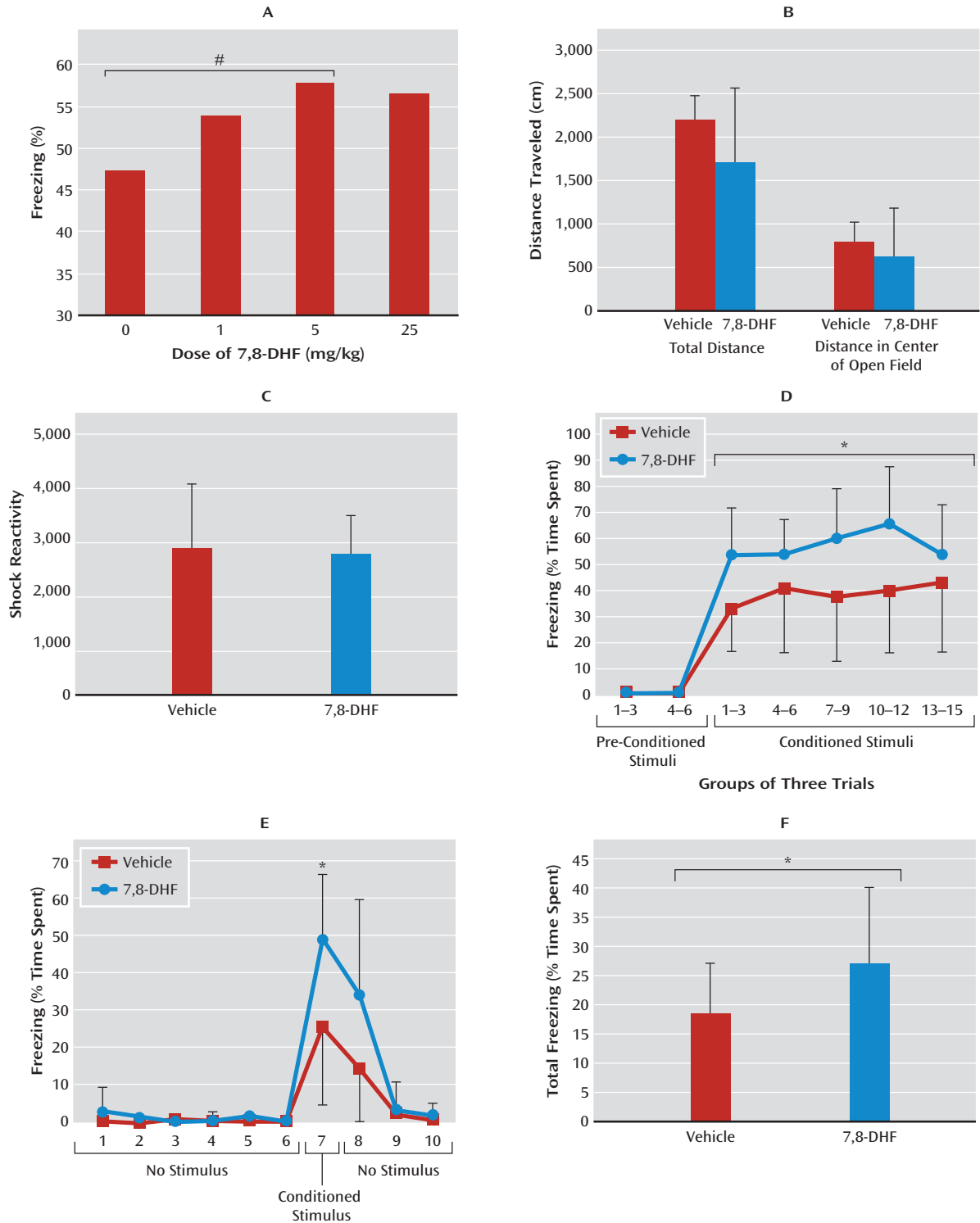
<sup>a</sup> MAPK=mitogen-activated protein kinase. Panel A shows immunoblots of mouse amygdala punches examining total TrkB protein (top), activated *p*-TrkB (second row), total MAPK (third row), and activated *p*-MAPK (bottom row). Each condition is represented in duplicate, with amygdala punches from mice injected intraperitoneally with vehicle (control) or 7,8-DHF (5 mg/kg) 1 hour or 2 hours prior to sacrifice. Full-length TrkB is detected at both ~95 kDa (nonglycosylated) and ~140–145 kDa (glycosylated) forms. Although total levels of TrkB and MAPK do not change, systemic 7,8-DHF led to robust activation/phosphorylation of amygdala TrkB (*p*-TrkB Y706) and MAPK (*p*-MAPK). Panel B shows the quantification (mean values with standard deviations) of the *p*-TrkB706:TrkB ratio represented in the top two immunoblots of panel A, demonstrating increased *p*-TrkB706 with 7,8-DHF treatment. In panel C, receptor autoradiography demonstrates that <sup>3</sup>H-7,8-DHF binds to brain regions known to express TrkB protein, including the amygdala (arrow). Panel D shows in situ hybridization of TrkB mRNA expression from the Allen Brain Atlas ([www.allenbrainatlas.com](http://www.allenbrainatlas.com)). In panel E, <sup>3</sup>H-7,8-DHF shows no significant binding when tissue is pretreated with excess cold 7,8-DHF, suggesting relative specificity. Panel F shows dense expression of TrkB protein on pyramidal neurons in basolateral rat amygdala identified with immunocytochemistry. Scale bar represents ~50 μm.

(Figure 1E). These data are consistent with previous data indicating that systemic 7,8-DHF activates *p*-TrkB at endogenous TrkB receptors in the mouse brain (16). Additionally, pyramidal neurons in the rodent amygdala appear to express high levels of TrkB receptor, as determined by immunohistochemistry (Figure 1F). Together, these data confirm that amygdala TrkB receptors are prevalent, bind to 7,8-DHF in vivo, and are activated by systemic administration of 7,8-DHF.

### Effect of Systemic 7,8-DHF on Learning of Conditioned Fear

Based on previous in vitro and in vivo studies (16, 17), we anticipated that 5 mg/kg of 7,8-DHF would be the optimal dose for mouse learning experiments. However, we performed a dose-response study to directly compare the effects of 0, 1, 5, and 25 mg/kg i.p. of 7,8-DHF during the averaged first three fear-conditioning stimuli in mice (Figure 2A). We found an overall significant drug effect with

FIGURE 2. Effects of 7,8-Dihydroxyflavone (7,8-DHF) on Fear Conditioning in Wild-Type Mice<sup>a</sup>



<sup>a</sup> Panel A shows results of a dose-response study performed with 7,8-DHF (N=10 in each dose group). The graph shows the average proportion of animals freezing during the first three conditioned stimulus trials (<sup>#</sup>p<0.05 relative to vehicle [control]). Panel B shows the mean total distance and the distance traveled in the center of the open field for 10 minutes with 7,8-DHF or vehicle injected 1 hour before testing; there were no differences between groups. Panel C shows the mean shock reactivity in the startle apparatus during the cue-dependent fear conditioning; there were no differences between groups. Panel D shows the mean percentage of time spent freezing, in three conditioned stimulus trials per group (across the entire 15 conditioned stimuli), in testing for cue-dependent fear memory 24 hours after fear conditioning. Mice that had received 7,8-DHF prior to fear conditioning showed increased freezing. Panel E shows the mean percentage of time spent freezing within-session for each test trial for the first 10 trials of session. Within-session fear differences were particularly pronounced in the first conditioned stimulus trial of the testing session. Panel F shows that the mean total time spent freezing during testing (conditioned stimulus and no-stimulus intertrial periods) was significantly greater in the group of mice that had received 7,8-DHF 1 hour prior to fear conditioning. Error bars in panels B–F indicate standard deviations. In panels D–F, \*p<0.05 between 7,8-DHF and vehicle groups.

analysis of variance (ANOVA) (linear trend,  $F=2.7$ ,  $df=1$ , 39, one-tailed  $p \leq 0.05$ ). Post hoc analyses revealed that mice treated with 5 mg/kg were significantly more fearful than those that received only vehicle (one-tailed  $t$  test,  $p \leq 0.05$ ). Thus, we used 5 mg/kg for the remaining studies.

We initially examined the behavioral effects of 7,8-DHF in an open-field maze after 7,8-DHF injection (5 mg/kg i.p., 1 hour prior to test). We found no differences between 7,8-DHF and vehicle on total distance traveled as a measure of baseline locomotion, nor did we find any differences in time traveled in center or surround (Figure 2B; less time spent in the center indicates anxiety-like behavior). These data suggest that acute dosing of systemic 7,8-DHF does not have any significant effects on locomotion or innate anxiety-like behavior.

We next performed Pavlovian fear conditioning (see behavioral experiment 1 in the online data supplement), pairing auditory cues to footshocks to examine whether this TrkB agonist would directly enhance fear learning. There were no differences in footshock reactivity in vehicle and drug groups when 7,8-DHF was administered 1 hour prior to training (Figure 2C), suggesting that 7,8-DHF does not affect pain sensitivity or the unconditioned stimulus representation. Twenty-four hours after fear conditioning, we examined the expression of conditioned fear in the absence of drug. When we examined freezing only on conditioned stimulus trials (grouping trials in bins of three; Figure 2D), we found overall fear increases in the 7,8-DHF group ( $F=5.1$ ,  $df=1$ , 41,  $p \leq 0.05$ ). This effect was particularly pronounced in the very first conditioned stimulus trial of the session (Figure 2E; repeated-measures ANOVA, group effect,  $F=4.8$ ,  $df=1$ , 41,  $p < 0.05$ ; group-by-drug effect,  $F=3.2$ ,  $df=9$ , 41,  $p < 0.01$ ). Examination of freezing during the first conditioned stimulus trial (trial 7) revealed differences between the vehicle and 7,8-DHF groups ( $p < 0.05$ ). We also found that the overall fear (freezing during both conditioned stimulus and intertrial no-stimulus periods) was significantly increased in the animals that had been treated with 7,8-DHF (Figure 2F;  $F=4.3$ ,  $df=1$ , 41,  $p \leq 0.05$ ). These results suggest that 7,8-DHF enhances new fear learning, a BDNF-dependent learning process, with no effect on overall activity, locomotion, anxiety-like behavior, or pain sensitivity.

#### ***Effect of 7,8-DHF on Extinction of Fear, a BDNF-Dependent Process***

Extinction of fear is the gradual reduction of conditioned fear behavior to a conditioned stimulus when it is repeatedly presented in the absence of the unconditioned stimulus. Extinction has previously been shown to be BDNF dependent and to require new learning processes. We examined whether extinction in previously conditioned mice would also be enhanced with 7,8-DHF. Mice were first fear-conditioned and matched for similar levels of freezing behavior to the conditioned stimulus tone (see experiment 2 and Figure S1B in the online data supplement)

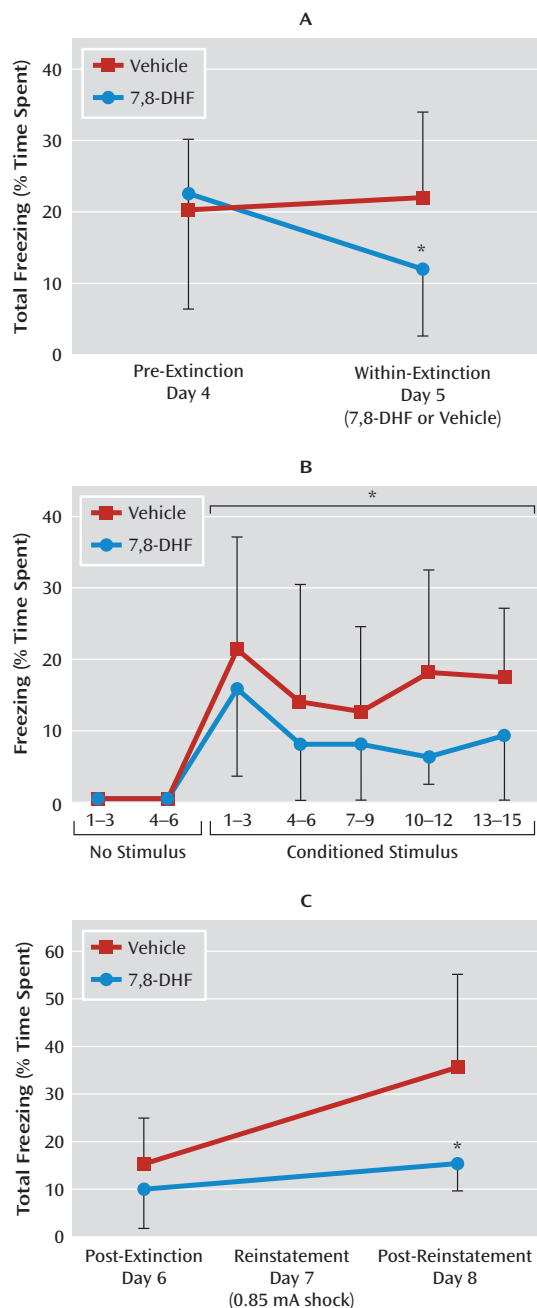
when tested 24 hours later (pre-extinction). On the next day, groups were given either vehicle or 7,8-DHF (5 mg/kg i.p.) 1 hour prior to a suboptimal (15-trial) extinction training, which normally would not support robust extinction of fear. Compared to vehicle, 7,8-DHF supported extinction, as shown by a repeated-measures ANOVA comparing pre-extinction and within-extinction freezing ( $F=4.4$ ,  $df=1$ , 24,  $p < 0.05$ ) (Figure 3A). Least-significant-difference post hoc analysis showed that the 7,8-DHF group froze less in the within-extinction session than did the vehicle group ( $p < 0.05$ ). More detailed analysis of the within-extinction session can be seen in Figure 3B, revealing a decreased level of conditioned freezing in drug-treated mice ( $F=5.2$ ,  $df=1$ , 24,  $p < 0.05$ ). An additional post-extinction session performed 24 hours later led to a floor effect, with no subsequent freezing differences between groups (day 6, post-extinction session) (Figure 3C).

We then examined reinstatement in previously treated 7,8-DHF and vehicle extinction groups by administering a footshock without the conditioned cue prior to cue fear retesting (Figure 3C; repeated-measures ANOVA, drug effect,  $F=6.0$ ,  $df=1$ , 24,  $p < 0.05$ ). Least-significant-difference post hoc analysis showed that the vehicle group had spent more time freezing than the 7,8-DHF group in the post-reinstatement test on day 8 ( $p < 0.05$ ). Thus, mice that had received 7,8-DHF 1 hour before the within-extinction session (day 5) showed less conditioned freezing following reinstatement than the group that had received vehicle only. Note that no differences were observed in locomotor activity prior to and between tone exposure in this context. Together these data suggest that 7,8-DHF may enhance the extinction of conditioned fear and subsequently reduce the level of post-reinstatement fear following a footshock stressor.

#### ***Effect of 7,8-DHF on Fear Extinction in a Mouse Stress Model With Diminished Extinction***

Fear-related psychopathology, such as posttraumatic stress disorder (PTSD), may be due in part to deficits in extinction of fear. We sought to examine whether 7,8-DHF might enhance extinction in a stress model in which a prior traumatic event produced a deficit in fear extinction. We used a mouse version of immobilization stress in which naive mice are restrained to immobilization boards for 2 hours. In rats this procedure has been shown to be quite stressful, detectable in the hypothalamic-pituitary-adrenal (HPA) axis and behaviorally for several weeks following a single session (20, 21). In the present study, naive mice with no pharmacological manipulation were fear-conditioned as described above, with tone-shock pairings 6 days after the immobilization procedure (see experiment 3 and Figure S1C in the online data supplement). We found that in mice, as in rats, immobilization stress leads to a rapid but transient increase in plasma corticosterone levels (Figure 4A;  $t=13.06$ ,  $df=30$ ,  $p < 0.001$ ), demonstrating the robust HPA-axis effects of this one-time stressor.

**FIGURE 3. Effect of 7,8-Dihydroxyflavone (7,8-DHF) on Fear Extinction in Wild-Type Mice<sup>a</sup>**



<sup>a</sup> Panel A shows the mean percentage of total time spent freezing during the testing. Pre-extinction was performed 24 hours after cue-dependent fear conditioning, and within-extinction (in the presence of drug) was performed 24 hours after that. Mice that received 7,8-DHF showed enhanced extinction of fear relative to those that received vehicle. Panel B shows the mean percentage of time spent freezing in response to conditioned stimulus presentation during the within-extinction session, represented in blocks of three trials. Mice that received 7,8-DHF 1 hour beforehand had similar initial freezing but showed rapid extinction of fear compared to those that received vehicle. In panel C, after all animals had been extinguished to a minimal level of fear (post-extinction test in the absence of drug, 24 hours after within-session), they were then subject to reinstatement (24 hours after post-extinction). Total freezing in post-extinction and post-reinstatement was compared between animals that had received 7,8-DHF or vehicle 1 hour before the extinction training. Error bars indicate standard deviations. \* $p < 0.05$  between the 7,8-DHF and vehicle groups.

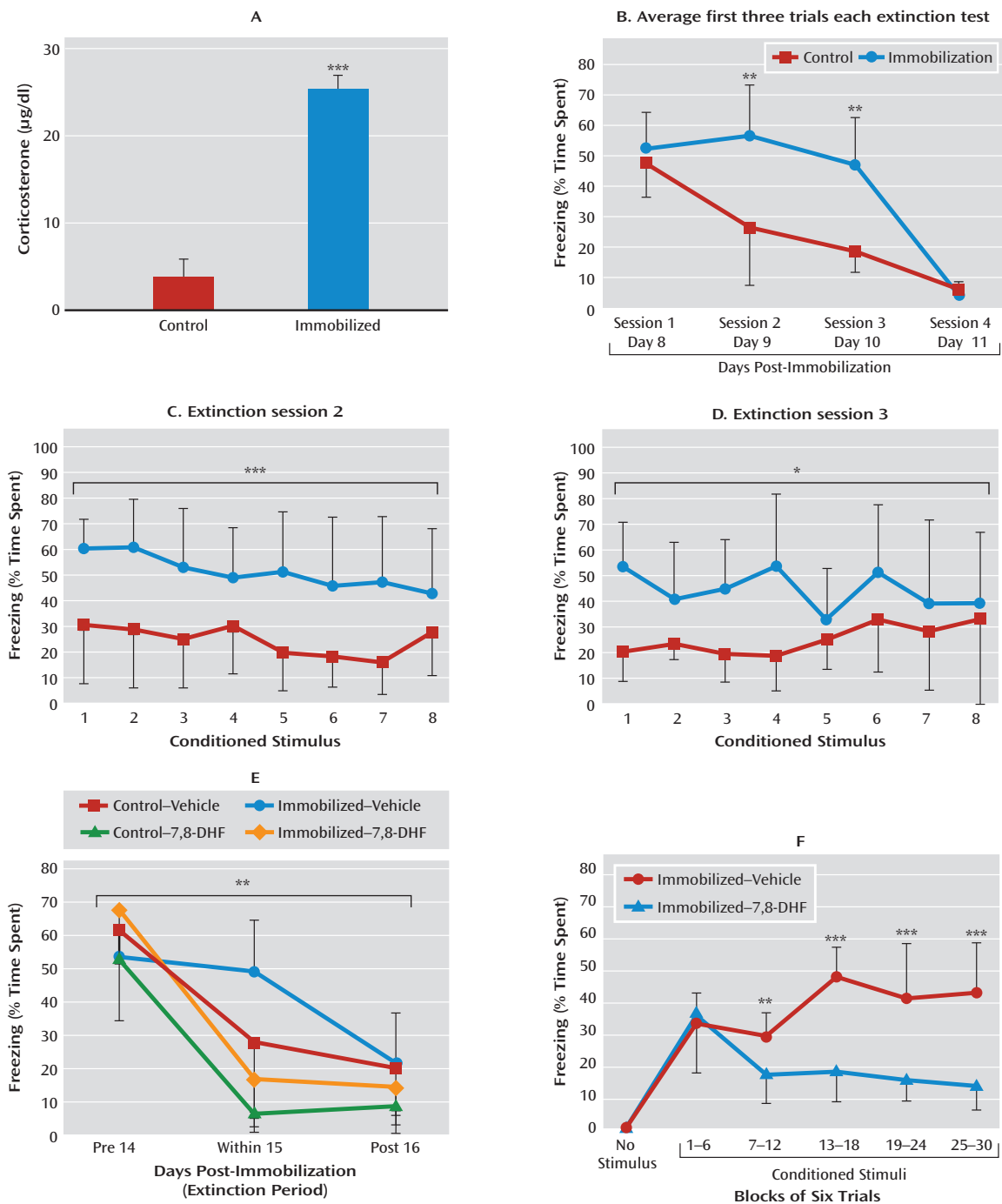
Mice that have previously undergone immobilization have a significant deficit in fear extinction that is apparent when analyzing the first three trials of each session across the four extinction sessions (Figure 4B; repeated-measures ANOVA, session-by-group effect,  $F=59$ ,  $df=3, 42$ ,  $p < 0.01$ ; least-significant-difference post hoc analyses of sessions 2 and 3 revealed greater levels of freezing in the immobilization group than the control group,  $p < 0.01$ ). After examination of freezing sessions 2 and 3 with repeated-measures ANOVA, we found that the immobilization group showed more freezing than the control group in session 2 ( $F=616.52$ ,  $df=1, 14$ ,  $p < 0.001$ ) (Figure 4C) and session 3 ( $F=383.84$ ,  $df=1, 14$ ,  $p < 0.05$ ) (Figure 4D), but with no differences between groups in session 4.

We next examined whether 7,8-DHF would enhance extinction of conditioned fear in mice with a prior history of immobilization. We used a longer undisturbed period (12 days) after stress than in the previous experiment (6 days) to evaluate the longevity of the stress-dependent delayed extinction effect (see experiment 4 and Figure S1D in the online data supplement). Fifteen days after the immobilization session, mice were given vehicle or 7,8-DHF (5 mg/kg i.p.) 1 hour before their first period of extinction training. There were no differences among groups in non-cue-related locomotor activity, shock reactivity during training, or conditioned freezing prior to extinction training. However, when we examined previously immobilized animals with and without 7,8-DHF treatment, we found that extinction was significantly enhanced with 7,8-DHF (Figure 4E; session-by-drug interaction,  $F=17.6$ ,  $df=2, 39$ ,  $p < 0.001$ ). When we included the two control groups (same fear and extinction protocols, but no prior immobilization), we still found an overall between-session extinction effect (Figure 4E; session-by-group interaction,  $F=3.95$ ,  $df=6, 84$ ,  $p < 0.01$ ), with significant post hoc differences between immobilization with and without 7,8-DHF ( $p < 0.001$ ), control with and without 7,8-DHF ( $p < 0.05$ ), and immobilization and control groups ( $p < 0.01$ ). These data suggest that 7,8-DHF is effective both in naive animals and in those with prior traumatic stress. We then analyzed freezing in the within-extinction session specifically in immobilization-stressed animals (Figure 4F). We observed a drug effect, suggesting that the immobilization-vehicle group presented more freezing (or a deficit in extinction) than the immobilization-7,8-DHF group ( $F=21.6$ ,  $df=1, 13$ ,  $p < 0.001$ ; block-by-group interaction,  $F=9.7$ ,  $df=5, 80$ ,  $p < 0.001$ ).

Together with the other experiments, these data suggest that 7,8-DHF enhances extinction of fear in naive animals as well as in animals with extinction deficits due to prior traumatic exposure.

## Discussion

We have demonstrated that 7,8-DHF, a systemic TrkB agonist, enhances emotional learning. We have also

FIGURE 4. Effect of 7,8-Dihydroxyflavone (7,8-DHF) on Extinction in a Traumatic Stress Model<sup>a</sup>

<sup>a</sup> Mice underwent 2-hour immobilization 6 days before the first session of fear conditioning, followed by repeated sessions of extinction. Panel A shows that shortly after immobilization, there is a robust activation of the hypothalamic-pituitary-adrenal axis, as demonstrated by a greater mean acute level of plasma corticosterone relative to control mice. In panel B, the immobilized group showed delayed extinction in analyses of conditioned freezing in extinction sessions 2 and 3 (panels C and D), whereas both groups had equivalent low levels of freezing by session 4, suggesting a delay in extinction following immobilization stress. (In panels A–D, \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  between control and immobilization groups.) In panel E, animals also showed impaired extinction when fear-conditioned 12 days after immobilization stress. Because of the delayed nature of extinction in immobilization-treated animals, we examined the last session to compare differential freezing during pre-extinction, within-session extinction, and post-extinction sessions across groups (with and without immobilization, and with and without 7,8-DHF). Mice that were given 7,8-DHF prior to extinction showed a significant drug-by-session effect of enhancement of extinction across sessions (\*\* $p < 0.01$  session-by-group interaction between pre-extinction, within-extinction, and post-reinstatement). Panel F shows that 7,8-DHF was associated with decreased freezing in the within-session extinction trials in animals with a prior history of immobilization. Freezing (average of six trials within each bin) is shown during within-extinction session, 1 hour after administration of 7,8-DHF or vehicle (\*\*\* $p < 0.001$ , \*\* $p < 0.01$  for the immobilization-vehicle compared with immobilization-7,8-DHF groups). Error bars indicate standard deviations.

built on prior *in vitro* data demonstrating that 7,8-DHF is a novel specific agonist at the TrkB receptor and that it crosses the blood-brain barrier to activate TrkB receptors in the brain, even in the absence of endogenous BDNF (16, 17). Our data show that 7,8-DHF enhances several well-defined emotional learning and memory paradigms that have previously been shown to be BDNF dependent.

We first demonstrated that a single systemic dose of 7,8-DHF enhances the learning of cue-dependent fear conditioning in wild-type animals. This potentiated the fear response to the cue, suggesting that 7,8-DHF is likely targeting TrkB receptors in the brain that are normally critical for fear learning.

Extinction, or the specific new learning of fear inhibition, has also been shown to be BDNF dependent. We showed that 7,8-DHF could enhance extinction in wild-type mice. Clinical fear-related disorders have been shown to be related to a deficit in extinction, which may be related to the decreased BDNF found in a number of models of chronic stress. In our final experiment, we demonstrated that 7,8-DHF “rescued” a deficit in extinction of conditioned fear found in animals with a prior history of a single traumatic stress exposure.

Patients with PTSD and other anxiety disorders are thought to have deficits in extinction of aversive memories (22, 23). Similarly, rodents with anxiety-like behavior or trauma exposure demonstrate a deficit in extinction of conditioned fear (24–26). Notably, the finding that a single stressor is sufficient to impair later extinction is still quite novel and may have only one precedent in the literature (27). This disruption of extinction is thought to be due to both abnormalities in HPA-axis function and possible alterations in BDNF signaling. In mice, 2-hour immobilization led to a decrease in hippocampus BDNF protein levels at 5 and 10 hours after stress, but levels had returned to control levels when evaluated at 24 hours (28). In rats, a single 2-hour period of stress immobilization has been found to be more stressful than high-intensity footshocks (20). Similar stress has been shown to decrease BDNF mRNA in the hippocampus (29), and immobilization has been shown to decrease BDNF mRNA levels in the amygdala and cortex (30). In both cases, no difference was found in the TrkB receptor levels in any area. Together, these data suggest that even individual episodes of significant traumatic stress can alter BDNF function, which may affect later extinction processing.

Our data suggest that 7,8-DHF can fully “rescue” the deficit in extinction produced by prior immobilization stress. In fact, we found that 7,8-DHF exerts similar effects in both control and immobilization-stressed groups (Figure 4E). This finding suggests that the TrkB receptor pathways may be intact after immobilization stress. Thus, the extinction deficit observed in previously immobilization-stressed mice may be due to defective release of BDNF or to a defect in factors other than the BDNF-TrkB pathway, but which can be rescued with additional activation of TrkB.

We found no effects on baseline behaviors or pain sensitivity following acute 7,8-DHF. These data suggest that 7,8-DHF did not have indirect effects that could alter the experience of fear training. Notably we found augmented learning for both fear and extinction, further supporting the role of the TrkB agonist in potentiating learning, and it was not due to enhancing or reducing sensory/motor function in a certain direction. Also, all of the learning paradigms performed here followed a single acute administration of systemic 7,8-DHF, which suggests that the emotional learning and memory enhancements were a function of direct and rapid activation of brain TrkB, as previously shown *in vitro* and *in vivo* (16) and demonstrated for the first time here with autoradiography. Our data do not yet indicate where 7,8-DHF is acting in our fear acquisition and extinction studies. However, this study’s immunoblots, autoradiography studies, and prior data from our group all suggest that TrkB in the amygdala, and possibly in the hippocampus and prefrontal cortex, could be involved in the systemic 7,8-DHF effects. Therefore, 7,8-DHF is likely augmenting emotional learning and plasticity, in the presence of endogenous BDNF, for both fear learning and extinction.

Overall, our findings suggest that this TrkB agonist may be an excellent research tool for understanding the effects of TrkB activation in a variety of learning and memory paradigms. Notably, the BDNF val/met polymorphism has been associated with increased anxiety-like behavior in humans (31, 32) and mice (33), as well as with altered extinction in both species (34). Recently this mouse model was shown to have an extinction deficit that was reversed with D-cycloserine (35), an NMDA [*N*-methyl-D-aspartic acid]-dependent cognitive enhancer (22). It will be fascinating to examine whether 7,8-DHF or similar TrkB-specific small-molecule agonists are able to reverse these effects in animal models and humans with the BDNF val/met polymorphism. Recent data also suggest that BDNF infusion into infralimbic cortex enhances extinction of fear (36), and there is a burgeoning literature suggesting that decreased BDNF signaling may be a critical component in the pathophysiology of mood disorders (15). Future research on the examination of systemically available BDNF agonists may lead to important, clinically relevant findings for the treatment of depression and anxiety disorders. Moreover, 7,8-DHF may be an attractive agent for improving extinction and other emotional learning deficits associated with psychopathology in humans.

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Md. Address correspondence and reprint requests to Dr. Ressler, Department of Psychiatry and Behavioral Sciences, Emory University, 954 Gatewood Dr., Atlanta, GA 30329; kressle@emory.edu (e-mail).

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# Association of *FKBP5* Polymorphisms and Childhood Abuse With Risk of Posttraumatic Stress Disorder Symptoms in Adults

Elisabeth B. Binder, MD, PhD

Rebekah G. Bradley, PhD

Wei Liu, PhD

Michael P. Epstein, PhD

Todd C. Deveau, BS

Kristina B. Mercer, MPH

Yilang Tang, MD, PhD

Charles F. Gillespie, MD, PhD

Christine M. Heim, PhD

Charles B. Nemeroff, MD, PhD

Ann C. Schwartz, MD

Joseph F. Cubells, MD, PhD

Kerry J. Ressler, MD, PhD

**P**OSTTRAUMATIC STRESS DISORDER (PTSD) is a debilitating stress-related psychiatric disorder, with prevalence rates of at least 7% to 8% in the US population, and with much higher rates among combat veterans and those living in high-violence areas.<sup>1-3</sup> Initially viewed as a potentially normative response to traumatic exposure,<sup>4</sup> it became clear that not everyone experiencing trauma develops PTSD. Thus, a central question in research on PTSD is why some individuals are more likely than others to develop the disorder in the face of similar levels of trauma exposure.<sup>5-8</sup> Although PTSD is the single disorder within the *Diagnostic and Statistical Manual of Mental Disorders* (Fourth Edition) (*DSM-IV*)<sup>9</sup> that requires a specific environmental insult within its diagnostic criteria, it is becoming increasingly clear that there are critical roles

**Context** In addition to trauma exposure, other factors contribute to risk for development of posttraumatic stress disorder (PTSD) in adulthood. Both genetic and environmental factors are contributory, with child abuse providing significant risk liability.

**Objective** To increase understanding of genetic and environmental risk factors as well as their interaction in the development of PTSD by gene × environment interactions of child abuse, level of non-child abuse trauma exposure, and genetic polymorphisms at the stress-related gene *FKBP5*.

**Design, Setting, and Participants** A cross-sectional study examining genetic and psychological risk factors in 900 nonpsychiatric clinic patients (762 included for all genotype studies) with significant levels of childhood abuse as well as non-child abuse trauma using a verbally presented survey combined with single-nucleotide polymorphism (SNP) genotyping. Participants were primarily urban, low-income, black (>95%) men and women seeking care in the general medical care and obstetrics-gynecology clinics of an urban public hospital in Atlanta, Georgia, between 2005 and 2007.

**Main Outcome Measures** Severity of adult PTSD symptomatology, measured with the modified PTSD Symptom Scale, non-child abuse (primarily adult) trauma exposure and child abuse measured using the traumatic events inventory and 8 SNPs spanning the *FKBP5* locus.

**Results** Level of child abuse and non-child abuse trauma each separately predicted level of adult PTSD symptomatology (mean [SD], PTSD Symptom Scale for no child abuse, 8.03 [10.48] vs ≥2 types of abuse, 20.93 [14.32]; and for no non-child abuse trauma, 3.58 [6.27] vs ≥4 types, 16.74 [12.90];  $P < .001$ ). Although *FKBP5* SNPs did not directly predict PTSD symptom outcome or interact with level of non-child abuse trauma to predict PTSD symptom severity, 4 SNPs in the *FKBP5* locus significantly interacted (rs9296158, rs3800373, rs1360780, and rs9470080; minimum  $P = .0004$ ) with the severity of child abuse to predict level of adult PTSD symptoms after correcting for multiple testing. This gene × environment interaction remained significant when controlling for depression severity scores, age, sex, levels of non-child abuse trauma exposure, and genetic ancestry. This genetic interaction was also paralleled by *FKBP5* genotype-dependent and PTSD-dependent effects on glucocorticoid receptor sensitivity, measured by the dexamethasone suppression test.

**Conclusions** Four SNPs of the *FKBP5* gene interacted with severity of child abuse as a predictor of adult PTSD symptoms. There were no main effects of the SNPs on PTSD symptoms and no significant genetic interactions with level of non-child abuse trauma as predictor of adult PTSD symptoms, suggesting a potential gene-childhood environment interaction for adult PTSD.

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for predisposing genetic and environmental influences in differentially mediating psychological risk to the traumatized individual.<sup>10-13</sup>

**Author Affiliations** are listed at the end of this article.  
**Corresponding Author:** Kerry J. Ressler, MD, PhD, Howard Hughes Medical Institute and Department of Psychiatry and Behavioral Sciences, Yerkes National Primate Research Center, Emory University, 954 Gatewood Dr, Atlanta, GA 30329 (kressle@emory.edu).

Child abuse occurs at disturbingly high rates and is a major public health problem.<sup>14</sup> Despite the resilience of many abused children, child abuse significantly increases risk for impaired physical and psychological health and decreases adaptive functioning in adulthood.<sup>15-17</sup> A matter of central importance to public health is the identification of factors related to risk and resilience in the wake of child abuse. Of particular relevance to this study is the well-established relationship of child abuse with adult PTSD.<sup>18-22</sup> A number of factors may account for this relationship. PTSD in adults may represent a prolonged symptomatic reaction to traumatic child abuse.

The experience of child abuse is associated with an increased number of traumatic experiences across the lifespan. Child abuse may also increase vulnerability for the later development of PTSD by altering psychological (eg, attachment) and biological (eg, hypothalamic-pituitary-adrenal [HPA] axis function) developmental processes, including interaction with genetic factors. Although other non-child abuse types of traumatic experiences in childhood<sup>23</sup> (eg, a house fire or being in a vehicle crash) might be expected to negatively affect development, the most robust research to date points to child abuse and related family/interpersonal stressful life events in predicting a wide range of later psychological and physical health problems.

The reasons for this are not fully understood but some possible explanations are (1) compared with other types of traumatic events, child abuse is more likely to occur in the family context<sup>24</sup>; (2) any 1 type of child abuse is associated with an increased likelihood of exposure to other types of abuse and to increased levels of family-related stressful events/parental dysfunction (eg, parental substance abuse)<sup>25</sup>; and (3) compared with some other types of trauma exposure, child abuse may be more likely a repeated experience rather than a single event (eg, multiple incidents of sexual abuse by the same perpetrator over a number of years).<sup>25</sup> Our study focuses on the interplay between child

abuse and polymorphisms in the *FKBP5* gene, which is involved in the glucocorticoid-mediated stress response, in the prediction of adult PTSD.

The literature to date on the genetics of PTSD has recently been reviewed,<sup>12,13</sup> with the resulting suggestion that gene  $\times$  environment studies are needed to focus more on distinct endophenotypes and influences from environmental factors. A number of studies suggest that genetic factors contribute to the development of PTSD, with heritability estimates ranging from 30% to 40%.<sup>11,26-29</sup> Candidate gene studies, however, have been inconclusive so far, usually limited by extremely low power to detect any but the strongest possible genetic effects (current published studies include sample sizes of  $<100$ ).

A recent review<sup>12</sup> covering candidate genes in the serotonin, dopamine, glucocorticoid receptor (GR),  $\gamma$ -aminobutyric acid, apolipoprotein E, brain-derived neurotrophic factor, and the neuropeptide Y system finds that (1) the support for a relationship between the serotonin transporter gene and PTSD exists only in research on the interaction of this system with stressful life events in predicting depressive symptoms; (2) results on the dopamine system are inconsistent; (3) there is a lack of evidence for relationships with brain-derived neurotrophic factor, neuropeptide Y, or GR polymorphisms and PTSD with the exception of a finding between GR genotype and basal cortisol levels in a subgroup of patients with PTSD<sup>30</sup>; and finally, (4) limited evidence for the  $\gamma$ -aminobutyric acid system and the apolipoprotein E system.

One of the first studies finding an interaction between a genetic polymorphism and child abuse in predicting psychopathology was the study by Caspi et al,<sup>31</sup> which found that maltreated children with a monoamine oxidase A (MAOA) genotype conferring low levels of MAOA expression were more likely to develop conduct disorder and antisocial-personality disorder and to commit violent crimes as adults compared with those children with the high-

activity MAOA genotype. A recent study<sup>32</sup> has replicated this result, and a second study<sup>33</sup> replicated the effect in white participants but not in other participants (blacks, Hispanics, American Indians, Pacific Islanders, and others) in the sample. The largest group of genotype  $\times$  environment studies has examined the interaction between variation at the *5HTTLPR* (a complex-repeat polymorphism in the 5' upstream region of *SLC6A4*, which encodes the serotonin transporter the *5HTTLPR*) and stressful life events, including child abuse, in predicting depression.<sup>34-40</sup> Very recently, Kilpatrick et al<sup>41</sup> reported an interaction of this polymorphism with severity of trauma and level of social support with the development of PTSD following hurricane exposure as outcome, supporting the relevance of gene  $\times$  environment interactions for this disease.

From a developmental perspective, HPA axis genes are strong candidates with respect to altering susceptibility to PTSD. Exposure to trauma and stress increase HPA axis activity, and PTSD has been associated with long-lasting alterations in HPA axis reactivity<sup>42,43</sup> and specifically higher GR sensitivity.<sup>44,45</sup> Polymorphisms in genes regulating GR activity may impact the acute effects of trauma on the HPA axis and thereby possibly impact long-term HPA axis regulation affecting the development of PTSD. A number of studies suggest that child abuse and neglect affect HPA axis functioning (reviewed by Watts-English et al<sup>46</sup>). Several studies suggest that the depression-related HPA axis hyperactivity may be related to early life stress. For example, plasma corticotropin and cortisol, as well as cerebrospinal fluid corticotropin-releasing hormone (CRH) concentrations, correlate with perceived early life stress more than with current depression.<sup>47,48</sup> Preclinical studies indicate that the persistent hyperactivity of the HPA axis associated with early life stress is mediated by a hyperactive CRH receptor 1 (CRHR1) system, with chronic overactivity of CRHR1 in limbic brain regions.<sup>49,50</sup>

FKBP5 is a co-chaperone of hsp90. It directly interacts with hsp90, which binds to the GR. FKBP5 also has been shown to regulate GR sensitivity. FKBP5 is part of the mature GR heterocomplex.<sup>51</sup> On hormone binding, FKBP5 is replaced by FKBP4, which then recruits dynein into the complex, allowing translocation into the nucleus where the complex regulates expression of glucocorticoid-responsive genes by functioning as a transcription factor.<sup>52</sup> FKBP5 expression is induced by glucocorticoids as part of an intracellular ultra-short negative feedback loop for GR activity.<sup>53</sup>

Overexpression of human FKBP5 *in vitro* reduces hormone binding affinity<sup>54</sup> and nuclear translocation of GR.<sup>55</sup> Naturally occurring overexpression of FKBP5 causes GR resistance in New World monkeys,<sup>54,56</sup> which is accompanied by increased plasma cortisol levels. Furthermore, potentially functional single-nucleotide polymorphisms (SNPs), or SNPs in very strong linkage disequilibrium with a functional variant, appear to alter FKBP5 function. The rare homozygous genotypes of FKBP5 SNPs (rs4713916, rs1360780, and rs3800373) were associated with higher FKBP5 expression in human blood monocytes as well as with a stronger induction of FKBP5 messenger RNA (mRNA) by cortisol. This was accompanied by less corticotropin release measured in patients who were depressed with the combined dexamethasone-CRH test.<sup>57</sup>

The same alleles of rs3800373 and rs1360780 were associated with increased peritraumatic dissociation in children after medical trauma.<sup>58</sup> Higher levels of peritraumatic dissociation have been shown to be predictors of PTSD in adults.<sup>10</sup> In addition, the extent of up-regulation of FKBP5 mRNA in peripheral blood mononuclear cells only hours after a trauma has been shown to correlate with the development of PTSD at 4 months.<sup>59</sup> These data suggest that FKBP5 could be an important candidate gene in trauma-related HPA axis disturbances. We therefore hypothesized that the

putative functional SNPs in FKBP5 moderate the development of PTSD. Because early trauma, PTSD, and FKBP5 SNPs have all been shown to influence GR resistance, we also hypothesize that variants in this gene may alter the impact of early trauma or PTSD on GR sensitivity and address this by investigating the dexamethasone suppression test (DST) in a subsample of individuals.<sup>44,57,60</sup>

Our study addresses the role of polymorphisms in FKBP5 in predicting PTSD, as well as the PTSD symptom-associated changes in GR sensitivity, in a highly traumatized, inner city sample. Specifically, we address whether these polymorphisms interact with increasing levels of child abuse and increasing levels of non-child abuse trauma exposure to predict PTSD symptomatology during adulthood.

## METHODS

### Sample, Recruitment, and Procedure

Data were collected to investigate the roles of genetic and environmental factors in predicting the development of PTSD in a population of urban, low-income, predominantly black men and women. To determine the race/ethnicity composition of the sample as part of the screening procedures, we asked participants to self-identify their race/ethnicity. Their response was coded into 5 common categories (black, white, Hispanic or Latino, Asian, mixed) or other (participants checked "other" when they thought their race/ethnicity was not included in the other 5 categories) (TABLE 1). The mean (SD) age in the sample was 40.8 (13.8) years, ranging from 18 to 81 years.

**Table 1.** Sample Demographics

Demographics	No. (%) of Participants
Sex (n = 900)	
Male	384 (42.7)
Female	516 (57.3)
Self-identified race/ethnicity (n = 898)	
Black	855 (95.2)
White	20 (2.2)
Hispanic or Latino	5 (0.6)
Asian	1 (0.1)
Mixed	8 (0.9)
Other <sup>a</sup>	9 (1.0)
Education (n = 897)	
<12th grade	245 (27.3)
High school graduate	324 (36.1)
Graduate equivalency diploma	52 (5.8)
Some college/technical school	176 (19.6)
College/technical school graduate	85 (9.5)
Graduate school	15 (1.7)
Employment status (n = 898)	
Currently unemployed	606 (67.5)
Currently employed	292 (32.5)
Disability status (n = 896)	
Not currently receiving disability	692 (77.2)
Currently receiving disability	204 (22.8)
Household monthly income, US \$ (n = 884)	
0-249	278 (31.4)
250-499	75 (8.5)
500-999	238 (26.9)
1000-1999	205 (23.2)
≥2000	88 (10.0)

<sup>a</sup>Because race/ethnicity was self-identified, participants checked "other" when they thought their race/ethnicity was not included in the other 5 categories (eg, American Indian).

**Table 2.** Percentage of Sample Reporting Exposure to Non-Child Abuse Traumatic Experiences Assessed by Traumatic Events Inventory<sup>a</sup>

Trauma Type Experienced	No. (%) of Participants		
	Total Sample (N = 900)	Male (n = 384)	Female (n = 516)
Natural disaster	170 (19.4)	101 (26.9)	69 (13.7)
Serious accident or injury	351 (42.9)	183 (53.0)	168 (35.4)
Sudden life-threatening illness	226 (25.9)	113 (30.1)	113 (22.7)
Military combat	28 (3.2)	26 (7.0)	2 (0.4)
Attacked with knife, gun, or other weapon by someone other than spouse, romantic partner, or boyfriend/girlfriend	324 (37.2)	204 (54.5)	120 (24.2)
Attacked without a weapon by someone other than spouse, romantic partner, or boyfriend/girlfriend	261 (30.5)	142 (38.5)	119 (24.4)
Witness of murder of friend or family member	79 (9.2)	43 (11.6)	36 (7.3)
Forced sexual contact $\geq 14$ y of age	132 (15.5)	18 (5.1)	114 (23.9)
Any significant trauma	746 (84.9)	337 (89.6)	409 (81.3)

<sup>a</sup>Because some participants declined to answer some questions, the total number in each trauma-type category may vary slightly. A subset of the trauma types queried within the Traumatic Events Inventory are listed.

Screen interviews were completed with 900 participants approached while in the waiting rooms of primary care or obstetrical-gynecological clinics of Grady Memorial Hospital in Atlanta, Georgia, between 2005 and 2007. Approximately 58% of those approached to participate in the study agreed to do so. Participants completed a battery of self-report measures that took 45 to 75 minutes to complete (dependent in large part on the extent of the participant's trauma history and symptoms). All measures were obtained by verbal interview. Each person was paid US \$15.00 for participation in this phase of the study. Each participant also provided a saliva sample for DNA extraction (participants were paid the same amount for completion of screening data even if they declined to provide a DNA sample). Written and verbal informed consent was obtained for all participants, and all procedures in this study were approved by the institutional review boards of Emory University School of Medicine and Grady Memorial Hospital, Atlanta, Georgia.

### Main Outcome Measures

**Modified PTSD Symptom Scale.** The modified PTSD Symptom Scale (PSS) is a psychometrically valid 17-item self-report scale assessing PTSD symptomatology<sup>61-65</sup> over the prior 2 weeks. Con-

sistent with prior literature, we summed the PSS frequency items (0 indicates not at all to 3 indicates  $\geq 5$  times a week) to obtain a continuous measure of PTSD symptom severity ranging from 0 to 51. For this sample, the PSS frequency items had standardized  $\alpha = .90$  (mean [SD], 13.81 [11.96]). No clearly established PSS cutoff score for PTSD diagnosis has been established; however, *DSM-IV* criteria for PTSD can be applied to PSS frequency items to create a proxy variable for PTSD diagnostic status.

**Clinician Administered PTSD Scale.** The Clinician Administered PTSD Scale (CAPS) was also administered to a subset of 240 participants within 2 to 6 weeks after completing the screening assessment. We found a significant difference ( $F_{1,239} = 56.55, P < .001$ ) between average PSS score (mean [SD], 18.20 [12.82]) for participants positive for current PTSD based on the CAPS (applying *DSM-IV* decision rules with a symptom considered as present with a CAPS frequency score of  $\geq 1$  and intensity score of  $\geq 2$ ) compared with those participants not meeting current CAPS PTSD criteria (mean [SD], 7.51 [9.23]). In addition, 70% of those participants identified as PTSD positive by using the PSS-based proxy variable were also positive for current PTSD at the time of CAPS administration (the criterion A traumatic experiences used for CAPS diagnosis was

not necessarily the same one used for obtaining PSS data). Study participants were asked to respond to the PSS items based on the trauma exposure (inclusive of child physical and sexual abuse and other life trauma exposure) that they believed had impacted them the most. Because we recorded data on the type of trauma identified for PSS score but not the age at which it occurred and because many study participants reported several incidents of the same type of trauma across the lifespan, we were unable to determine if the PSS data was based on a traumatic event occurring in childhood vs adulthood.

**Beck Depression Inventory.** The 21-item Beck Depression Inventory (BDI)<sup>66</sup> is a psychometrically validated, commonly used measure of depressive symptoms.<sup>67</sup> In our sample, the BDI had a standardized  $\alpha$  coefficient of .99 and a mean (SD) of 14.4 (13.2).

**Traumatic Events Inventory.** The traumatic events inventory (TEI)<sup>64,65</sup> assesses lifetime history of trauma exposure and is our primary measure of both child abuse and non-child abuse trauma. The TEI assesses past experience and frequency of 13 separate types of traumatic events as well as feelings of terror, horror, and helplessness with such events.

For the measure of child abuse, 2 of the TEI questions assessed physical abuse and sexual abuse occurring before age 14 years. Based on these questions, 17.6% of the sample reported a history of childhood physical abuse and 18.8% reported a history of childhood sexual abuse. With these data, we created a 3-level categorical variable reflecting number of types of child abuse: no child abuse (70.5% of sample), 1 type of either physical or sexual abuse (22.7%), or 2 types of both physical and sexual abuse (6.8%).

For the measure of non-child abuse trauma, the remaining TEI questions addressed other types of trauma exposure (TABLE 2). To summarize level of exposure to trauma other than child abuse, we summed total number of different types of non-child abuse trauma exposure reported by each participant. The mean number of types of non-child

abuse trauma reported in our sample was 2.46 types (SD, 1.92). Using the same data, we created a 4-level categorical variable reflecting number of types of non-child abuse trauma experienced reported with 19.9% of participants in the no non-child abuse trauma exposure category, 22.3% of participants reporting 1 type, 31.7% of participants reporting 2 to 3 types, and 26.7% of participants reporting 4 or more types of non-child abuse trauma. The total number or types of trauma exposure variable was chosen because in our prior research<sup>64,65</sup> and in other research on impact of trauma exposure<sup>68</sup> it relates in a predictable and consistent manner with a number of measures of adaptive functioning and trauma-related functioning.

Although the non-child abuse variable assesses traumatic experience other than child abuse across the lifespan, data from our sample suggests that these non-child abuse traumatic experiences primarily occur in adulthood. Specifically, we have data from a subset of study participants ( $n=322$ ) asking them the earliest age at which all of the traumatic events assessed by the TEI occurred. Averaged across all of the non-child abuse trauma categories, the mean (SD) for youngest age of trauma exposure in the sample was 25.1 (10.6) years and the median youngest of trauma exposure was 23 years.

**Childhood Trauma Questionnaire.** The Childhood Trauma Questionnaire (CTQ)<sup>69</sup> is a 28-item psychometrically validated, self-report inventory assessing self-reported level of child abuse and neglect.<sup>70</sup> We used the total score from this scale in our secondary analyses as a separate continuous measure of child abuse exposure.

**Dexamethasone Suppression Test.** A total of 80 participants were characterized using a low-dose DST.<sup>60</sup> Blood samples in the fasting state were obtained for baseline serum cortisol concentration and were drawn between 8 AM and 9 AM on day 1. Participants then received 0.5-mg dexamethasone by mouth at 11 PM before the second day blood draw, and blood samples for serum cortisol concentration was collected again in the fasting state between 8 AM and 9

AM for the day 2 collection. Using the cortisol concentration values from day 1 and day 2, the percentage suppression was calculated by  $100 \times [(cortisol\ day\ 1 - cortisol\ day\ 2) / cortisol\ day\ 1]$ . Serum cortisol concentration was measured by using commercially available radioimmunoassay kits (Diagnostic Systems Laboratories, Webster, Texas) from replicate samples and with interassay quality control measures. Significance of the interaction effect of FKBP5 SNPs and PTSD on percentage suppression was determined by using permutation-based methods with age and sex as covariates (10 000 permutations) to avoid inflated  $P$  values due to outlier or small cell sizes. In addition, we used repeated measures analysis of variance to examine the interaction between probable PTSD diagnosis and FKBP5 genotypes on the change in serum cortisol suppression from day 1 to day 2. For cortisol analyses, a probable PTSD diagnosis was determined from the DSM-IV criteria applied to the modified PSS interview, as described previously.<sup>64,65</sup>

The individuals with available DST present only a subset of the whole sample. However, these 80 individuals are not different compared with the rest of the sample in PSS total score for samples with and without DST (mean [SD], 11.30 [11.5] vs 10.15 [11.4];  $P=.40$ ), BDI total score (13.55 [12.1] vs 14.57 [12.1],  $P=.45$ ), race (93.7% vs 96.0% black,  $P=.92$ ), degree of childhood trauma (28.8% and 10.0% in the 1 and  $\geq 2$  types of severe child abuse vs 23.7% and 6.7%, respectively;  $P=.28$ ), and also non-child abuse trauma and sex. We also found no differences in the monthly household income and relationship status. However, the endocrinologically characterized individuals are older (mean age [SD], 43.3 [11.1] vs 39.3 [11.1];  $P=.01$ ). Due to the high comparability of all other variables and because all our analyses are corrected for age and sex, this subsample is representative of the sample as a whole.

### Genetic Data

**DNA Extraction.** DNA was extracted from saliva collected into Scope mouthwash ( $n=46$ ) or into Oragene saliva kits

(DNAGenotek, Ottawa, Ontario, Canada) by using the Qiagen M48 automated extraction system. DNA was available for 762 individuals. One hundred thirty-eight individuals with no genotype information consisted of 11 in which DNA was not collected, 88 in which we attempted to collect DNA using Oragene-spit samples but DNA extraction failed completely in 2 separate extraction trials (these failures were either due to noncompliance of the study participants or failure to correctly break the seal that releases the stabilizing solution in the Oragene saliva kits), and 39 for which DNA had been collected but not extracted at the time of analysis. When comparing the 762 individuals with genotypes to those 138 participants without, we found no significant difference in age, sex, self-described race, income, employment status, relationship status, child abuse, and non-child abuse trauma exposure as well as PTSD and depression severity.

**SNP Genotyping.** Eight SNPs within FKBP5 were selected from dbSNP (<http://www.ncbi.nlm.nih.gov/projects/SNP/>) to include the 3 potentially functional SNPs (rs4713916, rs1360780, and rs3800373)<sup>57</sup>; the other 5 SNPs were selected to span 120 kb covering the remainder of the FKBP5 locus (NM\_004117). We also genotyped 3 SNPs within the CRHR1 gene (rs110402, rs242924, and rs7209436), which have been recently reported to show strong interaction with childhood abuse on adult depression symptoms.<sup>71</sup> All SNPs were genotyped using a TaqMan allelic discrimination assay<sup>72</sup> on an ABI 7900HT instrument (Applied Biosystems, Foster City, California), using predesigned and validated TaqMan assay reagent kits containing 1 pair of polymerase chain reaction primers and 1 pair of fluorescently labeled probes (Applied Biosystems). Polymerase chain reactions were performed in 5- $\mu$ L reaction volumes in a 384-well plate and contained 5 ng of DNA. The standard protocol that was provided with the kit was followed. Thermal cycler conditions were 95°C for 10 minutes, 40 cycles of 92°C for

**Table 3.** List of Tested *FKBP5* SNPs, Their Positions on Human Chromosome 6 According to University of California Santa Cruz Genome Browser Version hg17, Hardy-Weinberg Equilibrium Test *P* Value, Minor Allele Frequency, and Call Rate

dbSNP ID	Position	Hardy-Weinberg Equilibrium Test <i>P</i> Value	Minor Allele Frequency	Call Rate, %
rs3800373	35650460	.45	0.45	95.0
rs992105	35663160	.94	0.18	97.1
rs9296158	35675060	.60	0.49	95.3
rs737054	35683460	>.99	0.07	97.3
rs1360780	35715550	.51	0.42	97.5
rs1334894	35723110	>.99	0.02	96.3
rs9470080	35754410	.34	0.47	93.3
rs4713916	35777960	.53	0.11	94.5

Abbreviation: SNPs, single-nucleotide polymorphisms.

15 seconds, and 60°C for 1 minute. The SDS version 2.2 software (Applied Biosystems) was used for allelic discrimination. For quality control, 9% of the samples were genotyped as duplicates across and within a 384-well plate. Only 0.03% of discordances were recorded and excluded from the analyses. Call rates for SNPs ranged from 93% to 97%, which included some samples that failed all assays and for which DNA quality was inferior. We used Haploview<sup>73</sup> to determine the linkage-disequilibrium structure of the SNPs within the *FKBP5* gene and to test for Hardy-Weinberg Equilibrium. The SNP identifications, their location, the Hardy-Weinberg Equilibrium test *P* values, minor allele frequencies, and call rate percentages are shown in TABLE 3.

**Ancestry Informative Markers.** Population stratification is a potential confounder in association studies of complex traits. If such allele-frequency heterogeneity within a population is coupled to similar population heterogeneity in the outcome of interest, naive tests of interaction could yield spurious results if the resulting confounding is neglected in analysis. Because 95.2% of our sample participants were self-identified as being of black race (which is known to contain varying degrees of genetic admixture of sub-Saharan African, European, and Native American ancestry), we took steps to ensure our results were robust to potential confounding from population stratification by genotyping 134 ancestry informative markers<sup>74,75</sup> in a

subset of 280 individuals from our sample using single-base primer extension and a Beckman SNPstream instrument (Beckman-Coulter, Fullerton, California). Genotyping of ancestry informative markers and data analysis for estimates of ancestry were performed by DNA Print Genomics, Sarasota, Florida (<http://www.dnapi.com>). Maximum likelihood estimates of individual biogeographical ancestry admixture were determined by using methods described previously<sup>76</sup> and by using a 4-continental population model. The choice of a 4-continental population model was based on previously published hypothesis-free cluster analyses of worldwide populations.<sup>77,78</sup> This model lent itself to use the terms European (genetic ancestry shared among Europeans, Middle Eastern, and to a lesser extent South Asians), sub-Saharan African, East Asian, and Indigenous American (genetic ancestry shared among American Indians, Latin and South American Indians, and certain Central Asian populations). The names of these parental populations were chosen to describe extant elements of genetic structure and are arbitrary in that they are reflective of modern population distributions—not necessarily the distributions of the original parental populations 15 000 to 50 000 years ago. Individuals with more than 35 failed ancestry informative marker genotypes were excluded from the analysis (7%), because their individual biogeographical ancestry admixture could not be estimated with sufficient certainty.

## Statistical Analyses

**Primary, Descriptive, and Secondary Analyses.** Our primary analyses were the main *FKBP5* SNP effects on PTSD symptom severity and their interaction effects with child abuse and non-child abuse trauma (690 participants for interaction with non-child abuse trauma and 678 participants for interaction with child abuse). Secondary analyses included BDI scores as outcome (number same as for PSS outcome), *FKBP5* SNP genotype dependent correlation of child abuse severity and PTSD symptom severity (*n* = 728), and effects on the DST (*n* = 80), as well as analysis using *CRHR1* genotypes (number same as for *FKBP5* SNP interactions). Descriptive analyses were run on a sample of 900 individuals with valid PTSD measures.

**Regression Analyses.** We used a variation of linear regression to examine the effects of trauma exposure, *FKBP5* genotypes, and their potential interaction on PSS scores. For *FKBP5* genotype, we modeled a participant's genotype at each SNP under an additive model that is equivalent to coding of the number of copies of a reference allele that the participant possesses. For trauma exposure, we included both child abuse (the 3-level child abuse TEI score) and non-child abuse trauma exposure (using the 4-level non-child abuse trauma TEI score) as predictors. We chose to separate these 2 types of trauma exposure based on the above described research, suggesting that exposure to child abuse increases risk for development of PTSD both as a response to the child abuse itself and in response to other non-child abuse stressors. Because child abuse is related to increased likelihood of subsequent exposure to additional traumatic stressors, we wanted to control for the possibility that effects associated with our child abuse variables were not artifacts of increased overall trauma exposure alone. Within the regression model, we further modeled the potential confounding effects of age, sex, level of depressive symptoms (BDI total score), total number of experienced non-child abuse trauma types, and genetic ancestry (by incorporating the ancestry estimates based on analysis of the ancestry informative markers). Due to missing phenotype and genotype information on some

participants, a maximum number of 676 individuals and a minimum of 633 individuals were informative in the regression models for the interaction effects. The individuals in the group with 676 and 633 that actually entered the analyses were not different in age, sex, self-described race, income, employment status, relationship status, child abuse trauma exposure, as well as PTSD and depression severity from the remainder of the total 900 individuals. They did, however, have higher child abuse rates. In the individuals entering the analyses, the no abuse group size ranged from 67.5% to 68.6% (and were not different) vs 77.1% to 79.0% in the excluded individuals ( $P = .01$  to  $P = .14$ ).

**Permutation Analyses.** As the non-normality of PSS and possible sparse-cell counts in the *FKBP5*-trauma interaction strata could each invalidate the asymptotic  $P$  values produced by regression models, we established the significance of main environment, genotype, or genotype-trauma interaction effects using permutation procedures that randomly assigned the sample PSS scores to participants (sampled without replacement), while holding each participant's genotype and environmental variables fixed.<sup>79,80</sup> For each analysis, we based inference on 10 000 to 100 000 permutations. We conducted these analyses by using appropriate components of SAS version 9.1 (SAS

Institute, Cary, North Carolina). Due to smaller cell sizes, we also used these permutation-based analyses to establish the main genotype or genotype and PTSD interaction effects on the DST.

**Correlation Analyses.** For descriptive and quantitative examination of genotype  $\times$  continuous environment (CTQ total score) interaction of PTSD symptom severity, we used partial correlation controlling for age and sex, correlating the semiquantitative child abuse measures of the CTQ total score with the current continuous PSS score in the whole sample as well as the sample stratified by *FKBP5* SNP genotype. The significance of the interaction of *FKBP5* SNP genotype and CTQ total scores on PSS was established using permutation-based methods, and the post hoc genotype-dependent differences of the correlation coefficients were established by converting the correlation coefficients of each genotype group to Fisher  $Z$  scores by  $Z = \ln[(r+1)/(r-1)]/2$ . We then estimated the standard error of difference between the 2 correlations by  $SE = \{[1/(n_1-3)] + [1/(n_2-3)]\}^{1/2}$  and divided the difference between the 2  $Z$  scores by the standard error. If the  $Z$  value for the difference was 1.96 or higher, the difference in the correlation was significant at  $P = .05$  level. If the difference was 2.58 or higher, the difference in the correlation was significant at  $P = .01$  level.<sup>81</sup>

**Correction for Multiple Testing.** To ensure an overall significance level of  $P \leq .05$  for the primary analyses (main *FKBP5* SNP effects on PTSD symptoms, interaction of *FKBP5* SNPs with child abuse or non-child abuse trauma exposure on adult PTSD symptoms), we corrected for the examination of 24 tests (8 tests of the main effects of the *FKBP5* SNPs, 8 tests of interaction between each SNP and non-child abuse trauma exposure, and 8 tests of interaction between each SNP and categorical measure of child abuse). We used the conservative Bonferroni method for such a multiple-testing correction, which yielded a significance threshold of  $\alpha = .002$ . In an exploratory approach, we also genotyped 3 CRHR1 SNPs based on previous results from our group in examining depression.<sup>71</sup> When we corrected for this additional set of 9 tests (3 CRHR1 SNPs, main effect and interaction of SNP  $\times$  child abuse or non-child abuse trauma exposure), the  $\alpha$  level after correction was  $.05/33 = .0015$ .

## RESULTS

### Descriptive Analyses

**Non-Child Abuse Trauma Exposure and PTSD Symptoms.** To analyze the differential roles of gene  $\times$  environment interaction with PTSD in adult participants, we first had to demonstrate that within our participant population, the level of total non-child abuse

**Table 4.** Non-Child Abuse Trauma Exposure and PTSD Symptoms

PTSD Symptom Scale	Level of Non-Child Abuse Trauma <sup>a</sup>			
	None (n = 159)	1 Type (n = 183)	2-3 Types (n = 265)	$\geq 4$ Types (n = 215)
Mean (SD) [95% CI]	3.58 (6.27) [2.60-4.56] <sup>b,c</sup>	7.30 (10.04) [5.83-8.76] <sup>c,d</sup>	11.57 (11.66) [10.16-12.98] <sup>c</sup>	16.74 (12.90) [15.00-18.47] <sup>d</sup>

Abbreviations: CI, confidence interval; PTSD, posttraumatic stress disorder.

<sup>a</sup>No. of types of non-child abuse (primarily adult) trauma experienced.

<sup>b</sup> $P < .005$  difference in PTSD Symptom Scale from 1 type of trauma.

<sup>c</sup> $P < .001$  difference in PTSD Symptom Scale from other groups.

<sup>d</sup> $P < .005$  difference in PTSD Symptom Scale from no trauma.

**Table 5.** Child Abuse Trauma Exposure and PTSD Symptoms

PTSD Symptom Scale	Level of Child Abuse Trauma <sup>a</sup>		
	None (n = 566)	1 Type (n = 189)	2 Types (n = 54)
Mean (SD) [95% CI]	8.03 (10.48) [7.17-8.90] <sup>b</sup>	14.65 (11.92) [12.94-16.36] <sup>c</sup>	20.93 (14.32) [17.02-24.84] <sup>d</sup>

Abbreviations: CI, confidence interval; PTSD, posttraumatic stress disorder.

<sup>a</sup>No. of types of child abuse trauma experienced.

<sup>b</sup> $P < .001$  difference in PTSD Symptom Scale from other groups.

<sup>c</sup> $P < .001$  for both differences in PTSD Symptom Scale from no abuse and 2 types.

<sup>d</sup> $P < .001$  for both differences in PTSD Symptom Scale from no abuse and 1 type.

**Table 6.** Interaction of Child Abuse Trauma and Non-Child Abuse Trauma<sup>a</sup>

Non-Child Abuse Trauma	PTSD Symptom Scale, Mean (SEM) <sup>b</sup>		
	No Child Abuse	1 Type of Child Abuse	2 Types of Child Abuse
None	3.18 (0.50)	5.24 (1.73)	11.00 (5.57)
1 Type	5.80 (0.78)	12.32 (1.98)	14.44 (2.21)
2-3 Types	9.90 (0.81)	15.78 (1.42)	18.00 (4.49)
≥4 Types	13.77 (1.15)	17.70 (1.45)	23.97 (2.49)

Abbreviation: PTSD, posttraumatic stress disorder.

<sup>a</sup>Note that significant effects are observed for both non-child abuse trauma ( $F_{3,806}=16.2$ ,  $P<.001$ ) and child abuse trauma ( $F_{2,806}=14.4$ ,  $P<.001$ ).

<sup>b</sup>The PTSD Symptom Scale ranges from 0 to 51.

traumatic exposure contributes significantly to current PTSD symptoms. To perform these analyses, we first used a general linear model (controlling for age and sex), using the PSS total score as the dependent variable and the TEI categorical variable representing number of types of non-child abuse traumatic experiences as the independent variable. We found a significant sex effect ( $F_{1,818}=29.5$ ,  $P<.001$ ) and a very robust non-child abuse trauma exposure effect ( $F_{3,818}=61.9$ ,  $P<.001$ ) (TABLE 4). In the zero types of non-child abuse trauma group, the mean (SD) PSS score was 3.58 (6.27) and in the 4 or more types of trauma group, we found continuous increases by more than 5-fold to 16.74 (12.90).

**Child Abuse Trauma Exposure and PTSD Symptoms.** We next examined whether child abuse exposure also predicted the level of current PTSD symptomatology (TABLE 5). We performed the same analyses as above with the categorical child abuse variable (none, 1 type [physical or sexual], and 2 types [physical and sexual]). Similar to the effect of non-child abuse trauma, we also found a significant effect in the presence of child abuse ( $F_{2,806}=50.9$ ,  $P<.001$ ). The experience of child abuse increased the mean (SD) PSS scores from 8.03 (10.48) in the no child abuse group to 14.65 (11.92) in the 1 type and 20.93 (14.32) in the 2 types of child abuse group.

**Non-Child Abuse Trauma Exposure, Child Abuse, and PTSD Symptoms.** Using a general linear model that included both non-child abuse trauma and child abuse trauma (TABLE 6) and their interaction term as

predictors of adult PTSD symptoms, we observed significant main effects of the 2 terms (non-child abuse trauma:  $F_{3,806}=16.2$ ,  $P<.001$ ; child abuse trauma:  $F_{2,806}=14.4$ ,  $P<.001$ ) and sex ( $F_{1,806}=20.2$ ,  $P<.001$ ) as described above, but no significant interaction ( $F_{6,806}=0.51$ ,  $P=.79$ ) between the 2 main predictors. The presence of 1 type of child abuse increases the PSS score at each non-child abuse trauma level by 4.58 points on average (range, 2.03-6.51) and the presence of 2 types of child abuse increases the PSS score at each non-child abuse trauma level by 9.04 points on average (range, 7.79-10.20), suggesting that exposure to child abuse increases risk for higher levels of PTSD symptoms in response to non-child abuse trauma exposure.

### Primary Genetic Analyses

**Main Effect of FKBP5 Polymorphisms on PTSD Symptoms.** Based on previous data supporting a clear role of FKBP5 in modulating the glucocorticoid response to stress, as well as evidence supporting association of FKBP5 variants with risks for and rate of recovery from affective disorders,<sup>52</sup> we hypothesized that genetic variation at FKBP5 may influence liability to PTSD. We examined 8 SNPs spanning 120 kb of the FKBP5 locus (Table 3) but found no significant main effect of FKBP5 genotypes on PSS total score (FIGURE 1).

**Interaction of FKBP5 Polymorphisms With Non-Child Abuse Trauma to Predict PTSD Symptoms.** We next examined whether FKBP5 polymorphisms interact with increasing levels of non-child abuse (primarily adult

trauma to predict adult PTSD symptoms. We regressed PSS scores on FKBP5 genotype, non-child abuse trauma (using the 4-level variable), and the interaction between genotype and non-child abuse trauma, adjusting for age and sex. We did not find any significant interaction effects using permutation-based methods (Figure 1). These data demonstrate that FKBP5 polymorphisms do not appear to have a simple role in moderating the effects of non-child abuse trauma exposure on PTSD outcomes.

**Interaction of FKBP5 Polymorphisms With Child Abuse to Predict PTSD Symptoms.** We then regressed PSS scores on FKBP5 genotype, child abuse (3-level variable), and the interaction between genotype and child abuse, adjusting for age and sex. In this analysis, we identified 4 SNPs that showed significant interactions with child abuse after correcting for multiple testing (interaction  $P<.002$ ) (Figure 1). All 4 associated SNPs are in fairly high linkage disequilibrium, with pair-wise  $r^2$  values ranging from 0.53 to 0.87 (Figure 1). The most significant SNP, rs9296158, is located in intron 5 with an interaction  $P<.0004$ . Two of the associated SNPs, rs3800373 and rs1360780, have previously been reported to associate with differential glucocorticoid-mediated responses by Binder et al.<sup>57</sup> For all 4 significant SNPs, we observed a similar additive mode of interaction, and there appears to be a gene dose-dependent protection from severe child abuse-associated increases in adult PTSD scores (TABLE 7). In the group of individuals with 2 types of child abuse, individuals homozygous for the protective G allele of rs9296158 had a mean (SEM) PSS score of 13.54 (3.76); in the heterozygous group, the mean PSS score was 21.25 (2.03); and in the group homozygous for the risk allele A, the mean PSS score was 31.11 (5.37). The PSS scores of more than 20 indicate clinical significant PTSD and higher scores indicate more severe PTSD, with a maximum of 51 points that can be reached in that scale.<sup>61-63</sup> Interestingly, the genotypes previously associated with a higher number of previous depressive episodes and faster response to antidepressant treatment<sup>57</sup> were the genotypes with the high-

est level of adult PTSD symptoms in the presence of child abuse.

### Exploratory/Secondary Genetic Analyses

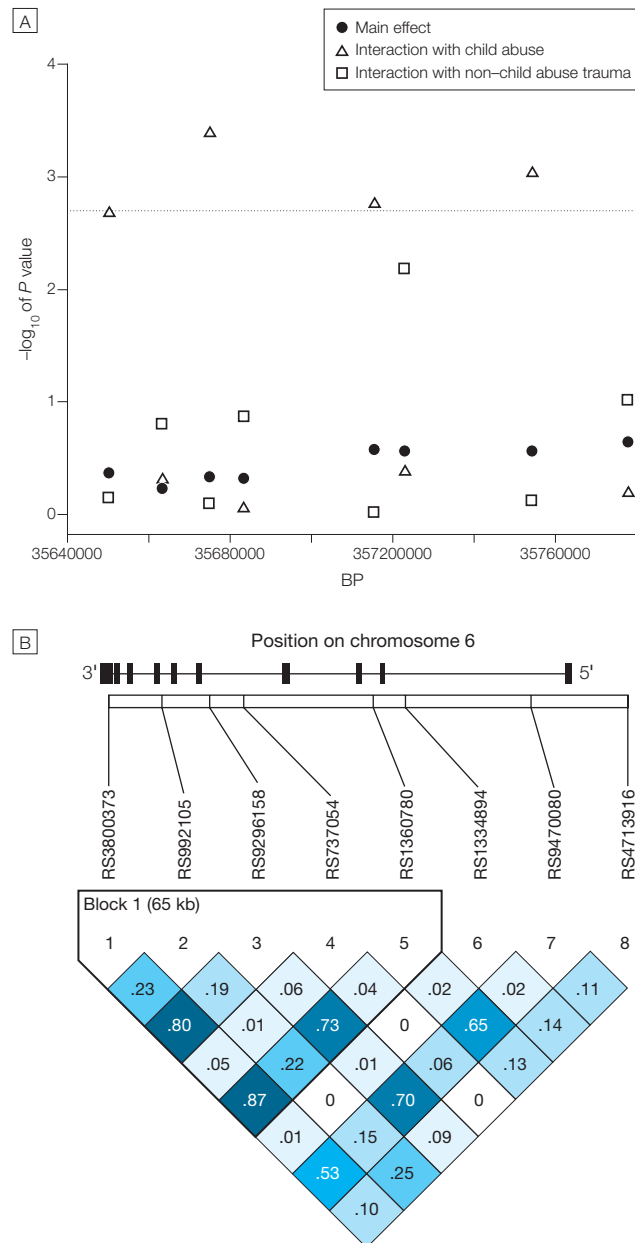
**FKBP5 and Child Abuse Interaction.** Given the prior data on *FKBP5* and major depression, and the observation that in our sample the 3-level child abuse variable is significantly and positively correlated with our 4-level non-child abuse trauma variable ( $n=851$ , Pearson correlation=0.271; controlling for age and sex,  $P<.001$ ), we reexamined the above interaction controlling for BDI score as a continuous measure of depression symptoms or the 4-level non-child abuse trauma variable in addition to age and sex. After adjusting for these variables, the interaction terms of child abuse and *FKBP5* SNPs remained significant for 2 of the 4 SNPs (rs9296158 and rs3800373; interaction  $P=.07$  for rs1360780 and interaction  $P=.06$  for rs9470080). In addition, we also conducted a regression analysis with BDI as the dependent variable. None of the *FKBP5* SNPs showed a significant interaction of genotype and child abuse to predict depression outcome (controlling for age and sex) after controlling for multiple testing. Thus, these data suggest that the *FKBP5* genotype may interact with childhood trauma to predict adult PTSD severity, even when controlling for age, sex, non-child abuse trauma level, and severity of depressive symptoms.

**Genotype-Dependent Correlations of Child Abuse and PTSD Symptom Severity.** We explored the genotype-dependent correlations of child abuse and PTSD symptom severity interaction effect using an ordinal variable of child abuse, which contains 154 points ranging from a CTQ score of 25 to 120. This allowed us to examine correlation analysis without having to group individuals into only 3 child abuse levels, which can lead to small group sizes. There is also importance in validating these effects with a separate, broader, and more continuous measure of child abuse. Thus, we investigated the interaction of the genotypes of the 4-associated

*FKBP5* SNPs with the CTQ total score to predict PTSD symptom severity. Permutation-based analyses showed an in-

teraction effect with rs9296158 genotype and the continuous environment measure with  $P=.01$  and  $P=.03$  for

**Figure 1.** *FKBP5* SNPs and Main Genetic Effect on PTSD Symptoms and Interaction Effects With Non-Child Abuse Trauma Levels and Child Abuse



SNPs indicate single-nucleotide polymorphisms; PTSD, posttraumatic stress disorder. A, The plot shows the negative  $\log_{10}$  of the  $P$  value for the main genetic effect (filled circles), the interaction of *FKBP5* SNP genotypes and non-child abuse trauma level (open squares), and the interaction of *FKBP5* SNP genotypes and child abuse (open triangles) to predict adult PTSD symptoms. The x-axis shows the position of the SNPs on chromosome 6 and the y-axis shows the  $P$  value for the respective effects, plotted as the negative  $\log_{10}$  of the  $P$  value. Dotted line indicates  $P<.002$ . B, The position of the *FKBP5* gene and its exons (filled rectangles) on chromosome 6 as well as a linkage disequilibrium (LD) plot of all tested SNPs using  $r^2$  as the measure of LD is also shown.  $r^2=1$  indicates complete LD and is depicted by the darkest shade of blue.  $r^2<1.0$  are printed in the respective square for compared SNPs, with darker shades of blue representing higher levels of LD.

**Table 7.** Interaction of *FKBP5* Genotype and Level of Child Abuse Predicts PTSD Symptoms in Adults

<i>FKBP5</i> Genotypes	PTSD Symptom Score, Mean (SEM)					
	No. of Participants	No Child Abuse	No. of Participants	1 Type of Child Abuse	No. of Participants	2 Types of Child Abuse
rs9296158						
AA	107	7.28 (0.93)	42	16.92 (1.85)	9	31.11 (5.37) <sup>a</sup>
AG	223	7.89 (0.69)	88	12.89 (1.16)	24	21.25 (2.03)
GG	107	8.80 (1.08)	31	14.33 (2.28)	14	13.54 (3.76)
rs3800373						
CC	89	7.03 (0.98)	35	17.65 (2.04)	8	29.00 (5.60) <sup>b</sup>
AC	210	8.11 (0.74)	77	12.71 (1.24)	23	22.96 (2.35)
AA	137	7.97 (0.89)	49	15.17 (1.75)	16	13.75 (3.31)
rs1360780						
TT	81	7.17 (1.02)	36	16.82 (2.08)	7	31.00 (6.04) <sup>b</sup>
CT	222	7.97 (0.70)	77	13.12 (1.23)	26	21.28 (2.32)
CC	150	8.23 (0.87)	50	14.51 (1.73)	15	14.00 (3.53)
rs9470080						
TT	94	6.76 (0.99)	44	16.40 (1.84)	10	31.20 (4.69) <sup>b</sup>
CT	224	8.25 (0.69)	88	13.23 (1.17)	23	20.05 (2.26)
CC	109	8.42 (1.07)	28	14.90 (2.39)	13	14.38 (3.97)

Abbreviation: PTSD, posttraumatic stress disorder.

<sup>a</sup>Interaction  $P < .001$ .<sup>b</sup>Interaction  $P < .002$ .**Table 8.** *FKBP5* SNP Genotype-Dependent Correlations of CTQ Total Score and PSS Scores

CTQ Correlation With PSS Score Dependent on <i>FKBP5</i> Genotype	Genotype		
	AA	rs9296158 AG	GG
No. of participants	164	360	172
Pearson correlation $R$ value	0.470	0.374	0.169
Z values for difference	AA/AG = 1.17	AG/GG = 2.35 <sup>a</sup>	AA/GG = 3.00 <sup>b</sup>
	rs1360780		
	CC	CT	TT
No. of participants	238	347	125
Pearson correlation $R$ value	0.210	0.390	0.455
Z values for difference	CC/TT = 2.46 <sup>a</sup>	CT/CC = 2.33 <sup>a</sup>	TT/CT = 0.74
	rs3800373		
	AA	AC	CC
No. of participants	171	352	156
Pearson correlation $R$ value	0.248	0.350	0.435
Z values for difference	AA/CC = 2.59 <sup>b</sup>	AC/AA = 2.30 <sup>a</sup>	CC/AC = .83
	rs9470080		
	CC	CT	TT
No. of participants	222	336	135
Pearson correlation $R$ value	0.215	0.392	0.465
Z values for difference	CC/TT = 1.91	CT/CC = 1.20	TT/CT = 1.03

Abbreviations: CTQ, Childhood Trauma Questionnaire; PSS, PTSD Symptom Scale; PTSD, posttraumatic stress disorder; SNP, single-nucleotide polymorphism.

<sup>a</sup> $P < .05$ .<sup>b</sup> $P < .01$ .

rs3800373 and  $P = .07$  for rs1360780. To better describe and quantify these differences, we reinvestigated positive correlations of child abuse severity and PSS and BDI scores stratifying by the genotypes of the 4-associated *FKBP5* SNPs.

Partial correlation (controlling for age and sex) of CTQ total score and PSS scores yielded highly significant correlations of child abuse and PTSD symptom severity in the whole sample ( $r = 0.347$ ,  $P = 3.7 \times 10^{-27}$ ). However,

when stratifying this analysis by the rs9296158 genotypes (Table 7), we observed significantly higher correlation coefficients in the AA vs GG group ( $z$  value for difference of correlation coefficient = 3.00,  $P < .01$ ) and the AG vs GG group ( $z$  value for difference of correlation coefficient = 2.35,  $P < .05$ ) for correlations between CTQ total score and PSS score. Similar effects were observed with 2 other SNPs, rs1360780 and rs3800373 (TABLE 8). Interestingly, although BDI scores and the CTQ total scores also showed highly significant positive correlations ( $R = 0.39$ ,  $P = 6.9 \times 10^{-33}$ ), there were no significant differences of the correlation coefficients among the 3 rs9296158 genotype groups.

**Genetic Admixture and Population Stratification.** Based on statistical analysis of the ancestry-informative marker genotype data from the subsample, we found that sub-Saharan African ancestry estimates for participants ranged between 41% and 100%, with a mean of 83.6%; European ancestry estimates ranged between 0% to 54%, with a mean of 10.9%; Indigenous American ancestry estimates ranged between 0% to 19%, with a mean of 3.3%; and East Asian ancestry estimates ranged from 0% to 33%, with a mean of 2.3%. We found that the degrees of ancestry from

these subpopulations were not significantly correlated with total PSS score, level of non-child abuse trauma exposure or presence of child abuse (all Pearson correlation coefficients between 0.01 and -0.04). In addition, using the percentage ancestry estimates for these 4 populations as covariate in the interaction analysis did not alter the observed SNP  $\times$  environment

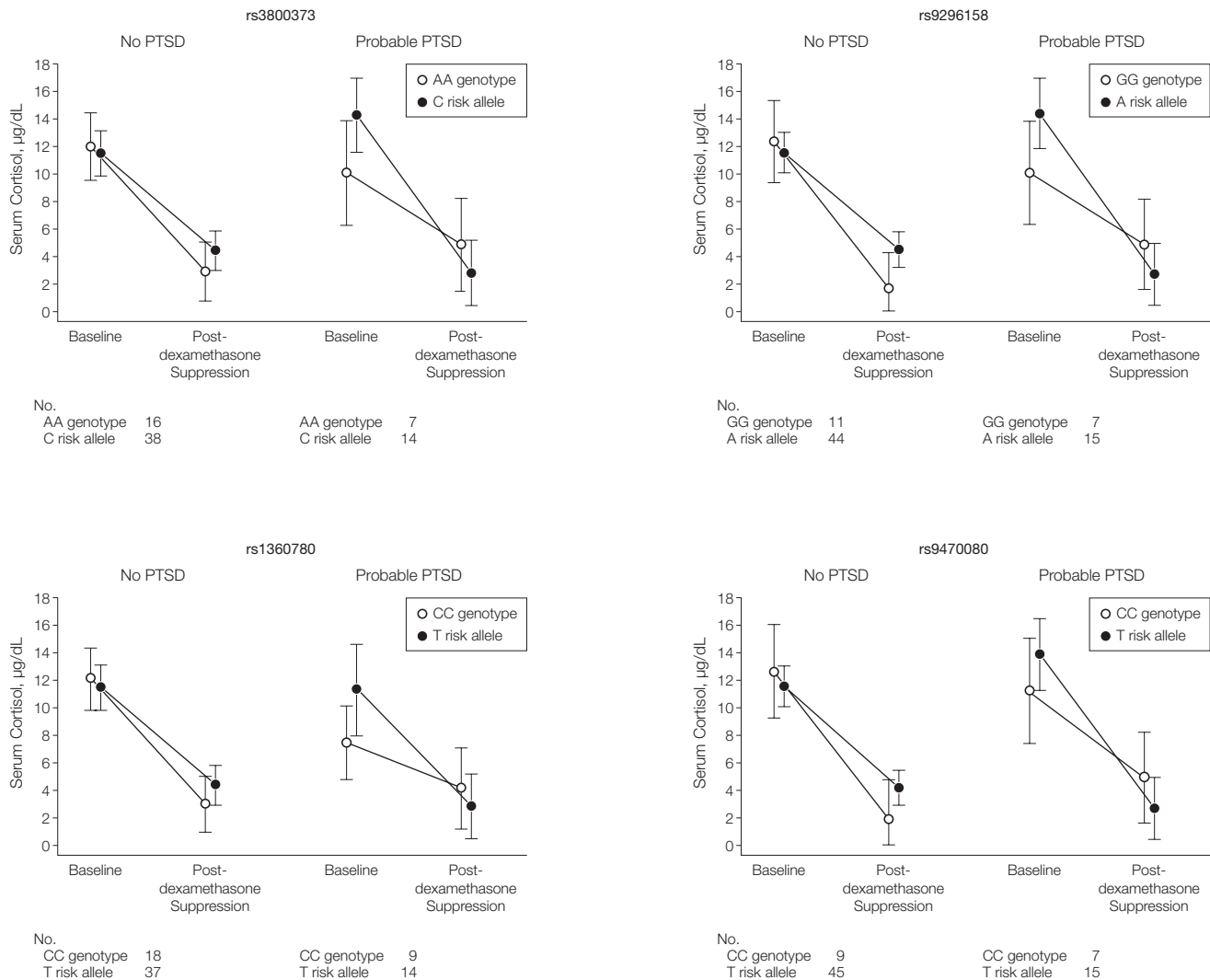
interactions. This evidence suggests that population stratification is not likely confounding in our analyses.

**Effects of CRHR1 SNPs on PTSD Symptom Severity.** Because we have recently reported interactions of CRHR1 SNPs with child abuse on adult depression,<sup>71</sup> we tested the 3 SNPs with the strongest interaction effects (rs110402,

rs242924, and rs7209436) for the effect on PTSD symptom severity. None of the 3 SNPs showed a significant main effect or interaction effect with child abuse or non-child abuse trauma exposure on PTSD symptom severity.

**DST, PTSD, and FKBP5 SNP.** To assess whether FKBP5 SNPs had a functional consequence on GR sensitivity

**Figure 2.** Glucocorticoid Receptor Sensitivity, PTSD, and FKBP5 SNPs



PTSD indicates posttraumatic stress disorder; SNPs, single-nucleotide polymorphisms. Mean serum cortisol concentration with 95% confidence interval (CI) is shown in participants who were tested at baseline and after 0.5 mg of dexamethasone (postdexamethasone suppression). The 4 panels represent the mean cortisol concentrations at baseline and postdexamethasone for individuals without probable PTSD (no PTSD) or with probable PTSD stratified by rs3800373, rs9296158, rs1360780, and rs9470080 genotypes. Individuals were categorized as risk allele carriers when they carried the C, A, T, or T alleles of these SNPs, respectively. Carriers of the AA, GG, CC, or CC homozygote genotypes of rs3800373, rs9296158, rs1360780, and rs9470080, respectively, were labeled as carrying the presumed protective genotypes. We found a significant interaction of genotype carrier- and PTSD-status on cortisol suppression (repeated measures analysis of variance: rs3800373,  $F_{1,76}=6.03$ ,  $P=.02$ ; rs9296158,  $F_{1,78}=8.76$ ,  $P<.004$ ; rs1360780,  $F_{1,79}=3.95$ ,  $P=.05$ ; rs9470080,  $F_{1,77}=5.32$ ,  $P=.02$ ; but also using permutation-based methods on percentage cortisol suppression). Although the risk alleles seem to be associated with less suppression (ie, glucocorticoid receptor resistance) in the no PTSD group, they are associated with greater suppression of cortisol from baseline to postdexamethasone in the group with PTSD. For rs9296158 GG carrier with no PTSD and rs9470080 CC carrier with no PTSD, the lower bound of the 95% CI had a negative value and was truncated at zero.

changes observed in PTSD, we examined the interaction of probable PTSD diagnosis and the 4 *FKBP5* SNPs with significant gene  $\times$  environment interactions on the change in cortisol concentration before and after 0.5-mg dexamethasone. These data were available for 80 individuals and, for any given genotype, we had full genotype, phenotype, and DST data for 75 to 78 individuals. In this sample, the mean (SD) serum concentrations of cortisol on day 1 were 12.08 (5.06)  $\mu\text{g/dL}$  (to convert to nmol/L, multiply by 27.588) and following dexamethasone, mean (SD) concentrations were 3.65 (4.38)  $\mu\text{g/dL}$ . When we examined the change from baseline cortisol to the dexamethasone-suppressed cortisol concentration using repeated measures analysis of variance, we found significant interactions between risk allele carrier status and PTSD categorical diagnosis (probable diagnosis based on PSS inventory), with repeated measure of cortisol (rs9296158:  $F_{1,78}=8.76$ ,  $P<.004$ ; rs3800373:  $F_{1,76}=6.03$ ,  $P=.02$ ; rs1360780:  $F_{1,79}=3.95$ ,  $P=.05$ ; and rs9470080:  $F_{1,77}=5.32$ ,  $P=.02$ ) (FIGURE 2), but no significant main effects. These data suggest that the majority of the patients with the risk alleles with PTSD show enhanced suppression to dexamethasone or enhanced GR sensitivity, which has been reported to be a possible endocrine signature of PTSD. In contrast, those individuals with the putative resilience genotype with PTSD appear to show the opposite effect. These results are supported by permutation-based analyses using percentage suppression as outcome and *FKBP5* risk allele carrier status and probable PTSD as predictors.

## COMMENT

Our results indicate that levels of child abuse and non-child abuse trauma each independently predicted the level of adult PTSD symptomatology. Although polymorphisms in *FKBP5* did not directly predict the level of PTSD symptoms or interact with the level of non-child abuse trauma to predict PTSD symptoms, SNPs within the

*FKBP5* locus robustly interacted with the level of child abuse to predict the level of adult PTSD symptoms.

The most novel and important finding of our study was the interaction between *FKBP5* polymorphisms and child abuse history to predict the levels of adult PTSD symptoms. The polymorphisms seem to belong to the same bin of SNPs, all in high linkage disequilibrium, which is associated with functional differences in *FKBP5* expression and consequent alterations in GR function. Notably, all 4 SNPs showing a significant interaction effect had either been reported to be associated with higher *FKBP5* protein levels or a stronger induction of *FKBP5* mRNA by cortisol in healthy probands (rs1360780 and rs3800373),<sup>57</sup> or were in strong linkage disequilibrium with these SNPs (rs9296158 and rs9470080). The SNP genotypes that were associated with the highest *FKBP5* mRNA induction in peripheral blood mononuclear cells by cortisol<sup>57</sup> were also the ones that were associated with the highest vulnerability to PTSD symptoms following child abuse. Individuals carrying the other allele seemed to be protected from the development of PTSD symptoms in a gene-dose dependent manner. This is in agreement with the finding of Segman et al<sup>59</sup> who showed that trauma-induced increased levels of *FKBP5* mRNA expression in peripheral blood mononuclear cells immediately following medical trauma were predictors of the presence of PTSD at 4 months after the trauma.

*FKBP5* expression is induced by glucocorticoids as part of an intracellular ultrashort negative feedback loop for GR activity,<sup>53</sup> with increased expression of *FKBP5* reducing glucocorticoid binding affinity<sup>54</sup> and nuclear translocation of the GR,<sup>55</sup> resulting in resistance to glucocorticoid activation. Thus, the alleles previously associated with high *FKBP5* protein/mRNA expression<sup>57</sup> should be associated with GR resistance. This is precisely what we observed in individuals without PTSD symptoms. The healthy carriers of these alleles showed less dexamethasone suppression and thus more GR resistance.

This functional association appears to be switched in patients with PTSD symptoms. These same alleles were associated with a higher dexamethasone suppression ratio and thus increased GR sensitivity, which is associated with PTSD,<sup>43,45</sup> while the protective genotype was associated with relative GR resistance in patients with PTSD symptoms. This environment-dependent reversal of the functional association may be similar to the effects previously reported in patients with depression, where less cortisol response in the dexamethasone-CRH test (an indication of increased GR sensitivity) was observed with the same alleles that had been associated with more *FKBP5* protein and thus presumably GR resistance in healthy controls.<sup>57</sup>

Our study is the first to our knowledge to provide evidence directly supporting a developmental or symptom-dependent difference in the functional consequences of these *FKBP5* SNPs on GR sensitivity. We hypothesized that specific *FKBP5* alleles may enhance the effects of acutely released cortisol following child abuse on *FKBP5* mRNA expression and that abnormal *FKBP5* expression leads to maladaptive changes in GR sensitivity. These changes resulted in long-term alterations of HPA axis sensitivity, such as GR hypersensitivity that effect adult response to trauma. Consistent with this notion, alterations of HPA axis responsiveness<sup>43,45</sup> have been previously identified as risk factors for PTSD.

PTSD has been suggested to result in part from initial overconsolidation of traumatic memories<sup>82,83</sup> or, conversely, abnormal extinction of such memories.<sup>8,84</sup> Thus, alterations in *FKBP5* function could conceivably be involved in abnormal GR-mediated signaling in neurons involved with stress response and memory formation. Polymorphisms within the *FKBP5* gene that lead to altered GR responsiveness could promote sensitization of the neural systems involved in stress response and emotional memory processing, thereby placing children who have been abused with specific genetic variants at higher

risk for PTSD or PTSD-spectrum symptoms when exposed to other types of traumas. This hypothesis may be supported by the finding that the alleles of rs3800373 and rs1360780, which are associated with higher risk for PTSD symptoms following severe child abuse in our study, were also associated with higher levels of peritraumatic dissociation in children after medical trauma.<sup>58</sup> Notably, peritraumatic dissociation has previously been identified as another risk factor for the development of PTSD.<sup>10</sup>

It is presently not clear if the associated polymorphisms represent the actual functional variants that lead to the differential FKBP5 expression patterns or are in linkage disequilibrium with a so far untyped, potential functional variant. Denser fine-mapping of this region combined with resequencing and *in vitro* and *in vivo* functional studies may allow definitive identification of the genetic variants mechanistically responsible for the interactions and associations observed herein. The fact that in our study rs4713916, located in a potential regulatory region 5' upstream of *FKBP5*, did not show a significant interaction effect, although in Binder et al<sup>52</sup> it had similar association patterns to rs1360780 and rs3800373, is likely due to the different extent of linkage disequilibrium observed among these SNPs in the present sample of black individuals vs the previous sample of ethnic German individuals (linkage disequilibrium was substantially stronger in the German sample).

Depression and PTSD show a high comorbidity,<sup>85</sup> so that concurrent depressive symptoms could confound our genetic interaction analysis for PTSD symptom outcome. However, controlling for current depressive symptoms, which were measured with the BDI, did not alter the observed interaction effect on PTSD symptoms and there was no gene  $\times$  child abuse effect observed when BDI score was used as the outcome. Although the PSS and BDI scores strongly and significantly correlate in our sample (Pearson correlation=0.68,  $P < .001$ ), our observation of a PTSD symptom-specific effect suggests that

interaction of *FKBP5* genotypes with child abuse history might influence PTSD symptoms not captured by the BDI, and thereby not shared with depression. Additional evidence suggesting separate gene  $\times$  environment interactions for depression and PTSD symptomatology also comes from the lack of an interaction effect between SNPs in the *CRHR1* gene and child abuse on PTSD symptom severity, despite the fact that these SNPs show strong interaction effects on the symptom severity of adult depression.<sup>71</sup>

One major limitation of our study is that we cannot present an independent replication, so that at this level of validation, it can only be considered hypothesis generating. The supporting evidence from functional effects of the polymorphisms in the DST serve to strengthen our finding that awaits independent replication. Another limitation is the lack of ancestry informative marker data on the complete sample. We do, however, feel the existing ancestry results among our sample of 280 participants genotyped for the 134 ancestry informative markers help to alleviate the concern of confounding by population stratification. The ancestry estimates in our subsample genotyped for the ancestry informative markers are not correlated with PSS or BDI scores.<sup>71</sup> Because confounding due to population stratification only occurs when there is both correlation between outcome and ancestry as well as correlation between SNP genotypes and ancestry, our results are not likely susceptible to bias arising from confounding due to population structure.

Limitations in the phenotype side are that we used retrospective reports of child abuse. It is well-documented that reports of trauma are correlated with PTSD symptoms,<sup>86,87</sup> and we have to acknowledge that the retrospective reports of child abuse and also non-child abuse trauma can therefore be biased in favor of an association between these reports and PTSD symptoms. A similar limitation is the lack of examination of the time since the index trauma. Furthermore, because we

have used PTSD symptom severity and not diagnosis as an outcome measure and our sample has very high levels of multiple trauma exposure, our results may not be comparable with those obtained in studies with PTSD diagnosis as outcome and studies with less variation in trauma type or fewer overall types of trauma exposure. However, several studies have now indicated that multiple trauma exposure across the lifespan is the rule rather than the exception in samples similar to our study<sup>3,88,89</sup> (eg, low income, urban, high percentage of black or Latino participants) and that multiple trauma exposure is related to increased mental and physical health risk.<sup>65,90,91</sup> Understanding risk and resilience in response to multiple trauma exposures is of high public health importance.

Our sample was also derived from primarily impoverished, inner-city outpatient primary care clinics and participants were not presenting for treatment for PTSD so that we cannot directly generalize our findings to a clinical or epidemiological setting. Comparability with other studies and populations may be further limited as methods of assessment and definitions of child abuse vary greatly across studies. Finally, the mechanisms accounting for the relationship of child abuse to increased risk for PTSD in adulthood are not well understood. It may be that child abuse directly impacts psychological and biological developmental processes or that child abuse is associated with other variables (child/parental temperament, broader family environment, attachment) that impact psychological and biological development or an interaction of the 2.

## CONCLUSIONS

Data reported herein suggest that genetic variation in the *FKBP5* gene, which is involved in glucocorticoid signal transduction, may alter sensitization of the stress-response pathway during development, placing those individuals who have had significant child abuse at significant risk for PTSD

in the face of other traumatic experiences. These genotypes potentially serve as predictors of both risk and resilience for adult PTSD among survivors of child physical and sexual abuse.

Our genetic results support the hypothesis that the glucocorticoid response system moderates the effects of early life stress on adult PTSD symptoms and that GR hypersensitivity may be important in the pathophysiology of this disorder. These results suggest the possibility that heritable differences in glucocorticoid-mediated neural functioning exacerbate or dampen the effects of child abuse on the stress hormone system, thus altering HPA axis sensitivity and risk for PTSD in adulthood.

**Author Affiliations:** Departments of Psychiatry and Behavioral Sciences (Drs Binder, Bradley, Liu, Gillespie, Heim, Nemeroff, Schwartz, Cubells, and Ressler, and Mr Deveau) and Human Genetics (Drs Binder, Epstein, Tang, and Cubells, and Ms Mercer), Emory University School of Medicine and Yerkes National Primate Research Center (Dr Ressler), Atlanta, Georgia; Beijing Institute of Microbiology and Epidemiology, Beijing, China (Dr Liu); Atlanta VA Medical Center, Atlanta, Georgia (Dr Bradley); and Howard Hughes Medical Institute, Chevy Chase, Maryland (Dr Ressler).

**Author Contributions:** Dr Ressler had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Drs Binder and Bradley contributed equally to this article.

**Study concept and design:** Binder, Bradley, Epstein, Heim, Schwartz, Cubells, Ressler.

**Acquisition of data:** Binder, Bradley, Deveau, Mercer, Tang, Gillespie, Heim, Cubells, Ressler.

**Analysis and interpretation of data:** Binder, Bradley, Liu, Epstein, Mercer, Heim, Nemeroff, Cubells, Ressler. **Drafting of the manuscript:** Binder, Bradley, Epstein, Tang, Gillespie, Cubells, Ressler.

**Critical revision of the manuscript for important intellectual content:** Liu, Epstein, Deveau, Mercer, Heim, Nemeroff, Schwartz, Cubells, Ressler.

**Statistical analysis:** Binder, Bradley, Liu, Epstein, Tang, Gillespie, Ressler.

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# Influence of Child Abuse on Adult Depression

## Moderation by the Corticotropin-Releasing Hormone Receptor Gene

Rebekah G. Bradley, PhD; Elisabeth B. Binder, MD, PhD; Michael P. Epstein, PhD; Yilang Tang, PhD; Hemu P. Nair, PhD; Wei Liu, PhD; Charles F. Gillespie, MD, PhD; Tiina Berg, PhD; Mark Evces, PhD; D. Jeffrey Newport, MD; Zachary N. Stowe, MD; Christine M. Heim, PhD; Charles B. Nemeroff, MD, PhD; Ann Schwartz, MD; Joseph F. Cubells, MD, PhD; Kerry J. Ressler, MD, PhD

**Context:** Genetic inheritance and developmental life stress both contribute to major depressive disorder in adults. Child abuse and trauma alter the endogenous stress response, principally corticotropin-releasing hormone and its downstream effectors, suggesting that a gene  $\times$  environment interaction at this locus may be important in depression.

**Objective:** To examine whether the effects of child abuse on adult depressive symptoms are moderated by genetic polymorphisms within the corticotropin-releasing hormone type 1 receptor (*CRHR1*) gene.

**Design:** Association study examining gene  $\times$  environment interactions between genetic polymorphisms at the *CRHR1* locus and measures of child abuse on adult depressive symptoms.

**Setting:** General medical clinics of a large, public, urban hospital and Emory University, Atlanta, Georgia.

**Participants:** The primary participant population was 97.4% African American, of low socioeconomic status, and with high rates of lifetime trauma ( $n=422$ ). A supportive independent sample ( $n=199$ ) was distinct both

ethnically (87.7% Caucasian) and socioeconomically (less impoverished).

**Main Outcome Measures:** Beck Depression Inventory scores and history of major depressive disorder by the Structured Clinical Interview for *DSM-IV* Axis I Disorders.

**Results:** Fifteen single-nucleotide polymorphisms spanning 57 kilobases of the *CRHR1* gene were examined. We found significant gene  $\times$  environment interactions with multiple individual single-nucleotide polymorphisms (eg, rs110402,  $P=.008$ ) as well as with a common haplotype spanning intron 1 ( $P<.001$ ). Specific *CRHR1* polymorphisms appeared to moderate the effect of child abuse on the risk for adult depressive symptoms. These protective effects were supported with similar findings in a second independent sample ( $n=199$ ).

**Conclusions:** These data support the corticotropin-releasing hormone hypothesis of depression and suggest that a gene  $\times$  environment interaction is important for the expression of depressive symptoms in adults with *CRHR1* risk or protective alleles who have a history of child abuse.

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**Author Affiliations:** Atlanta VA Medical Center (Dr Bradley), and Departments of Psychiatry and Behavioral Sciences (Drs Bradley, Binder, Nair, Gillespie, Berg, Evces, Newport, Stowe, Heim, Nemeroff, Schwartz, Cubells, and Ressler) and Genetics (Drs Binder, Epstein, Tang, Liu, and Cubells) and Yerkes National Primate Research Center (Dr Ressler), Emory University School of Medicine, Atlanta, Georgia; and Beijing Institute of Microbiology and Epidemiology, Beijing, China (Dr Liu).

**A**LTHOUGH NUMEROUS STUDIES suggest that both genetic and environmental influences contribute substantially to vulnerability to major depressive disorder (MDD), the search for direct genetic influences contributing to the risk for depression has been inconclusive so far. Recent studies<sup>1,2</sup> examining genetic variants predisposing to MDD suggest that they may only do so via interaction with environmental variables. Several reports have now replicated the finding that the short allele of a functional repeat polymorphism located in the promoter region of the serotonin transporter locus *SLC6A4*<sup>3</sup> increases vulnerability to depression only in the pres-

ence of significant adverse life events.<sup>1,4-8</sup> Although the serotonin transporter is an attractive genetic candidate for moderating the adverse effects of life events, epidemiological studies clearly indicate that the vulnerability to depression is influenced by several genes.<sup>9</sup>

Genes regulating the effects of stress via the hypothalamic-pituitary-adrenal (HPA) axis have been implicated in the physiological and pathological regulation of stress reactivity.<sup>10,11</sup> The physiological stress response is primarily mediated by the release of hypothalamic corticotropin-releasing factor, also known as corticotropin-releasing hormone (CRH), from nerve terminals in the median eminence arising in the paraventricular nucleus,

which stimulates adrenocorticotropin release from the anterior pituitary that in turn induces the release of cortisol from the adrenal cortex. Overactivity of the HPA axis is a hallmark of MDD and is at least partly due to a hyperactivity of CRH neurons.<sup>12,13</sup>

In addition to its effects on the HPA axis, CRH activity at the CRH type 1 receptor (CRHR1) in extrahypothalamic regions is also thought to produce symptoms of anxiety and depression. Preclinical animal models of depression<sup>14,15</sup> and clinical studies of patients with depression<sup>16,17</sup> have found increased CRH concentrations in the cerebrospinal fluid as well as altered CRHR1 messenger RNA expression and CRH binding in limbic brain regions (eg, hypothalamus, amygdala, and other areas mediating emotion response), lending support to this theory. Furthermore, a nonblinded study demonstrated clinical efficacy of a CRHR1 antagonist in patients with depression.<sup>18</sup> Finally, single-nucleotide polymorphisms (SNPs) in the *CRHR1* gene (Entrez Gene ID 1394 or RefSeq ID NM\_004382) have been associated with MDD in Han Chinese individuals,<sup>19</sup> and response to antidepressant medications appears to vary with *CRHR1* haplotypes in Mexican American persons.<sup>20</sup>

Studies suggest that HPA axis hyperactivity related to MDD may be a function of early life stress (ELS).<sup>21,22</sup> Given the relationships between ELS, MDD, and the dysregulation of the CRH and CRHR1 system, we hypothesized that genetic polymorphisms altering the function of CRHR1 may moderate the effects of child abuse on adult depression.

## METHODS

### SAMPLING AND PHENOTYPING METHODS

#### Sample and Sample Recruitment

The data from this study were collected as part of a larger study investigating the roles of genetic and environmental factors in predicting response to stressful life events in a predominantly African American urban population of low socioeconomic status. The sample for the replication study is discussed later. Research participants were approached, either while waiting for their medical appointments or while waiting with others who were scheduled for medical appointments, in the waiting rooms of the primary care clinic or obstetrical-gynecological clinic of a large, urban, public hospital. Subjects who indicated willingness to participate provided written informed consent, participated in a verbal interview, and provided a salivary sample for DNA extraction (described later). The data presented in this article are from the first 560 subjects screened (we have incomplete genotype or phenotype data for some subjects, so the total number of subjects in the analysis will be fewer than 560, with exact sample sizes stated in the "Results" section). The final genetic analysis was performed on a subset of 422 of these subjects. All of the procedures in this study were approved by the institutional review boards of Emory University School of Medicine and Grady Memorial Hospital, Atlanta, Georgia. Demographic data on sex, self-identified race/ethnicity, education, employment, disability status, and monthly household income are presented in **Table 1**. The mean (SD) age in the sample was 38.4 (13.3) years (range, 18-81 years).

#### Procedure

Participants verbally completed a battery of self-report measures, including the Beck Depression Inventory (BDI) and the

**Table 1. Primary Sample Demographics**

Characteristic	Participants, No. (%)
Sex	
Male	194 (39.0)
Female	303 (61.0)
Self-identified race/ethnicity	
African American or black	484 (97.4)
Caucasian or white	4 (0.8)
Hispanic or Latino	2 (0.4)
Asian	1 (0.2)
Mixed	5 (1.0)
Other	3 (0.6)
Education	
< 12th Grade	153 (30.8)
High school graduate or GED	217 (43.7)
Some college or technical school	78 (15.7)
Technical school graduate	21 (4.2)
College graduate	21 (4.2)
Some graduate school	9 (1.8)
Employment status	
Currently unemployed	338 (67.6)
Currently employed	162 (32.4)
Disability status	
Not currently receiving disability assistance	394 (79.3)
Currently receiving disability assistance	103 (20.7)
Household monthly income, \$	
0-249	158 (31.8)
250-499	51 (10.3)
500-999	136 (27.4)
1000-1999	106 (21.3)
> 2000	46 (9.3)

Abbreviation: GED, general equivalency diploma.

brief Childhood Trauma Questionnaire (CTQ), which took 45 to 75 minutes to complete (dependent in large part on the extent of the participant's trauma history and symptoms). We read instruments to participants to guard against relatively high rates of impaired literacy. Each person was paid \$15.00 for participation in this phase of the study.

#### Phenotype Measures

**Beck Depression Inventory.** Depressed mood was assessed with the 21-item BDI,<sup>23</sup> a well-validated, commonly used, continuous measure of the level of depressive symptoms. In this sample, the BDI had a standardized  $\alpha$  coefficient of .99 (mean [SD] BDI score, 14.43 [13.11]). In addition, we examined validity of the BDI using data collected in a later phase of the study for which the Structured Clinical Interview for *DSM-IV* Axis I Disorders (SCID-I),<sup>24</sup> a validated interview assessment of *DSM-IV* mood disorders, was administered. In this subsample of participants ( $n=202$ ), 19.3% met the criteria for current MDD and 40.1% met criteria for lifetime MDD. In addition, 1.1% met criteria for current bipolar I disorder and 2.0% met lifetime criteria for bipolar I disorder; 2.2% met criteria for current bipolar II disorder and 3.0% met lifetime criteria for bipolar II disorder. Based on MDD diagnosis with the SCID-I, the BDI total scores for those with current MDD (mean [SD] BDI total score, 28.26 [15.01]) were significantly different from the BDI total scores for those without a current MDD diagnosis (mean [SD] BDI total score, 13.48 [12.73]) ( $F_{1,173}=32.9$ ;  $P<.001$ ). Additionally, because these analyses were performed in subjects identified in a general medical clinic, it is possible that the BDI score is a proxy for medical-related depressive symptoms separate from MDD.

To examine this, we used history of prescribed medications as a proxy variable for the medical illness category. Although we have only limited diagnostic information, we have medical pharmaceutical data for approximately 120 subjects from the full genotype and phenotype data set. Similar to the linear regression analyses with permutations for SNP analyses (described later), we used linear regression with permutation procedures (using 10 000 permutations with BDI as the dependent variable and each of the 13 medical variables as predictors) to establish whether the proxy variables for different types of medical illness predict the BDI score. These permutation procedures randomly assigned the sample BDI scores to subjects (sampled without replacement) while holding each subject's medical prescription variables fixed. We conducted these analyses using appropriate components of SAS version 9.1 statistical software (SAS Institute, Inc, Cary, North Carolina). There were no significant effects found for any of the 13 available classes of medications examined (all  $P > .10$ ). This demonstrates that the moderation effects of child abuse and *CRHR1* genotype were unlikely affecting medical condition as a possible contributor to depressive symptoms.

**Childhood Trauma Questionnaire.** The CTQ<sup>25</sup> is a self-report inventory assessing 3 types of childhood abuse: sexual, physical, and emotional. Studies have established the internal consistency, stability over time, and criterion validity of both the original 70-item CTQ and the current brief version.<sup>26</sup> The CTQ yields a total score and subscale scores for each of the types of child abuse. Our CTQ data demonstrated good internal consistency reliability ( $\alpha = .99$  for physical abuse;  $\alpha = .94$  for sexual abuse;  $\alpha = .93$  for emotional abuse; and  $\alpha = .98$  for the total of these 3 scales). Bernstein and Fink<sup>25</sup> established scores for none, mild, moderate, and severe for each type of abuse. The data from the CTQ were used to classify participants into 2 categories for each type of abuse (physical, sexual, and emotional): (1) those with CTQ scale scores in the none to mild range, and (2) those with CTQ scores in the moderate to severe range. We then created a composite variable across all of the 3 types of abuse. Using this composite, we divided participants into 2 groups with respect to the numbers of types of abuse that fell into the moderate to severe range: (1) those with no type of abuse in the moderate to severe range, and (2) those with at least 1 type of abuse in the moderate to severe range.

## ADDITIONAL SUPPORTIVE SAMPLE

A sample of 204 women recruited at the Women's Mental Health Center at Emory University had been assessed for lifetime psychiatric diagnoses using the SCID-I<sup>24</sup> and for the presence of child abuse using the CTQ. The severity of child abuse was then scored as none to mild vs moderate to severe. In this sample, the mean (SD) age was 31.9 (5.0) years, and 87.7% were Caucasian or white, 7.0% were African American or black, 3.7% were Native American, and 1.6% were Asian. As compared with the previous sample, subjects in this sample were primarily middle-income Americans. The mean (SD) number of years of education in this sample was 16.0 (2.0) years. Among the subjects, 23.6% reported moderate to severe child abuse. Most subjects (96.4%) fulfilled criteria for at least 1 Axis I DSM-IV diagnosis, with 62.6% of the patients presenting with a principal lifetime diagnosis of MDD. The other reported lifetime diagnoses included bipolar I disorder, bipolar II disorder, panic disorder, generalized anxiety disorder, anxiety disorder not otherwise specified, obsessive-compulsive disorder, specific phobias, dysthymia, depressive disorder not otherwise specified, brief psychotic disorder, schizophrenia, mood disorder due to the general medical condition, cannabis abuse, opioid abuse, post-

traumatic stress disorder, adjustment disorder, and binge eating. Women with these latter diagnoses did not fulfill criteria for MDD at any time and represented the group with no MDD.

## GENETIC METHODS

### DNA Extraction

We extracted DNA from saliva collected into Scope mouthwash (Procter and Gamble, Cincinnati, Ohio) ( $n = 46$ ) or into Oragene saliva kits (DNA Genotek Inc, Ottawa, Ontario, Canada) using the Qiagen M48 system (Qiagen, Hilden, Germany). High-quality DNA was available for 502 individuals.

### SNPs and Genotyping

Fifteen SNPs validated in African populations were selected from public databases (dbSNP, <http://www.ncbi.nlm.nih.gov/projects/SNP/>; HapMap, <http://www.hapmap.org>) to span the *CRHR1* gene (NM\_004382) from the promoter region to the 3' end of the gene, with an average intermarker distance of 4.1 kilobases (kb) over a total region of 57 kb. Ten of these SNPs had a minor allele frequency greater than 5.0% and were thus included in the analysis (full SNP information is available from us, and identification numbers of 10 selected SNPs are listed in **Figure 1A**). In our population, we observed moderate linkage disequilibrium (LD) in this gene, especially in the part containing the coding regions with 2 separate blocks of LD (Figure 1A). All of the SNPs were genotyped using a TaqMan allelic discrimination assay<sup>27</sup> developed for use on the 7900HT instrument (Applied Biosystems, Foster City, California) in 502 individuals using predesigned and validated TaqMan assay reagent kits containing 1 pair of polymerase chain reaction primers and 1 pair of fluorescently labeled probes (Applied Biosystems). Assay identification numbers for all of the 15 assays are available on request. Polymerase chain reactions were performed in 5- $\mu$ L reaction volumes in 384-well plates and contained 5 ng of DNA. The standard protocol provided with the kit was followed. Thermal cycler conditions were 95°C for 10 minutes and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The SDS 2.2 software (Applied Biosystems) was used for allelic discrimination. For quality control, 9.0% of the samples were genotyped as duplicates across and within a 384-well plate. Only 2 discordances were recorded with the 6255 unique genotypes (0.03%) and excluded from the analysis. All of the SNP identification numbers, their locations, Hardy-Weinberg equilibrium, and minor allele frequencies are available on request.

### Ancestry-Informative Markers

Genotyping of ancestry-informative markers (AIMs) and data analysis for estimates of ancestry were performed by DNA Print Genomics, Sarasota, Florida (<http://www.dnaprint.com>). One hundred thirty-four AIMs were genotyped in 280 individuals from our sample using single-base primer extension and a Beckman SNPstream instrument (Beckman-Coulter, Fullerton, California). Maximum likelihood estimates of individual biogeographical ancestry admixture were determined using methods described previously by others<sup>28</sup> and 134 AIMs<sup>29-31</sup> chosen from the genome based on their information for a continental, 4-population model. The choice of a 4-population model was based on previously published hypothesis-free cluster analyses of worldwide populations,<sup>31,32</sup> and this model lent itself to use of the terms *European* (genetic ancestry shared among European, Middle Eastern, and, to a lower extent, South Asian populations), *sub-Saharan African*, *East Asian*, and *indigenous American* (genetic ancestry shared among American Indian, Latin and South American Indian, and certain Central Asian popula-

tions). The names of these parental populations were chosen to describe extant elements of genetic structure and are arbitrary in that they are reflective of modern population distributions—not necessarily the distributions of the original parental populations 15 000 to 50 000 years ago. Individuals with more than 35 failed AIM genotypes were excluded from the analysis (7.1%) because their biogeographical ancestry admixture could not be estimated with sufficient certainty.

## STATISTICAL ANALYSIS

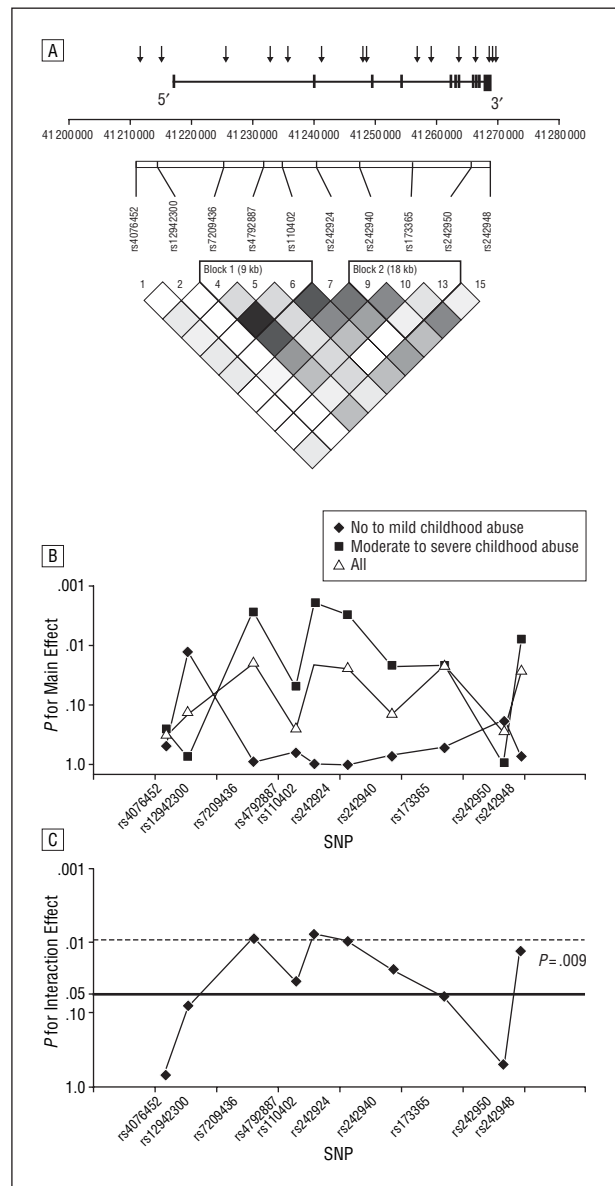
### LD Structure

We used Haploview<sup>33</sup> to determine the LD structure of the SNPs within the *CRHR1* gene and test for Hardy-Weinberg equilibrium (Figure 1A). We also compared the LD structure of a subgroup of *CRHR1* SNPs across our study population and 2 populations genotyped in the HapMap project (Yorubans [a sub-Saharan African population] and Centre d'Étude du Polymorphisme Humain [Caucasian population]). We compared a tag SNP selection for the *CRHR1* locus based on these HapMap populations covering the same area on chromosome 17 using an  $r^2$  cutoff of 0.75 and a minor allele frequency greater than 0.05. We found that across HapMap populations, our SNP sample covers at least 60.0% of the genetic variation described in HapMap. In addition, we used 3 SNPs that have not yet been typed in the HapMap project that cover additional, likely overlapping genetic variation of the gene.

### Regression and Permutation Analyses for Interaction Effects

We used linear regression to assess whether SNPs in the *CRHR1* gene interacted with child abuse to influence BDI scores. For these analyses, a maximum sample size of 422 individuals was informative, but the total sample sizes for the single-SNP analyses vary by a maximum of 5.0% depending on the call rate of each individual SNP. We first considered single-SNP analyses that regressed the BDI outcome (continuous variable) on genotype (coded under an additive model), with child abuse coded into the 2 groups of none to mild and moderate to severe. We further adjusted the regression models for potential confounders including age, sex, and ancestry. We established the significance of genotype-child abuse interaction effects using permutation-based procedures<sup>34,35</sup> that randomly assigned the sample BDI scores to subjects (sampled without replacement) while holding each subject's genotype and environmental variables fixed. This permutation method is robust against nonnormal distribution of the outcome variable as we observed with the BDI scores in this population. For each analysis, the empirical  $P$  value was based on 10 000 permutations. We conducted these analyses using appropriate components of SAS version 9.1 statistical software.

After completing the single-SNP interaction analyses, we next considered whether SNP-based haplotypes interacted with child abuse to influence BDI scores. Using linear regression, we modeled the BDI score as a function of the various haplotypes (coded under an additive model), child abuse, and the haplotype-child abuse interaction effects. We pooled all of the haplotypes with less than 10.0% estimated frequency into a rare category. We further adjusted the regression models for potential confounders, such as age and sex. We assessed the significance of haplotype-environment interaction effects using permutation procedures that randomly shuffled BDI scores within the sample while holding genotype and environmental data for each subject fixed. For each interaction analysis, we based inference on 100 000 permutations.



**Figure 1.** Main and interaction effects of *CRHR1* single-nucleotide polymorphisms (SNPs) with child abuse on adult depression. A, *CRHR1* linkage disequilibrium (LD) map demonstrating the physical location (arrows) and LD pattern of the selected *CRHR1* SNPs. For initial genotyping, we selected 15 SNPs covering the gene from the promoter region to the 3' end of the gene, with an average intermarker distance of 4.1 kilobases (kb) over a total region of 57 kb. The x-axis shows the position on chromosome 17. Ten of these SNPs had a minor allele frequency greater than 5.0% and were thus included in the analysis. The lower part shows an LD plot generated with Haploview using  $r^2$  as the measure of LD, which ranges from 1 (or complete LD, indicated by black squares) to 0 (or absence of LD, indicated by white squares). In our population, we observed moderate LD in this gene, especially in the part containing the coding regions with 2 separate blocks of LD according to the confidence interval method. B, Main genetic effects. The  $P$  values (log scale) for the main genetic effect (for a model containing SNP genotype, Childhood Trauma Questionnaire scores, age, and sex as independent variables and Beck Depression Inventory scores as outcome) for all of the genotyped subjects ( $n=422$ ), subjects with no to mild child abuse ( $n=262$ ), and subjects with moderate to severe child abuse ( $n=160$ ). Concordant with a gene  $\times$  environment interaction effect, the main genetic effect is carried by individuals with moderate to severe child abuse and is stronger than in the whole sample, whereas no SNPs significant in the whole sample remain significant in the group with no to mild child abuse. C, Interaction effects. The y-axis shows the  $P$  value (log scale) for the interaction effect of the 10 SNPs that were present with a minor allele frequency greater than 5.0%. Horizontal lines indicate  $P=.05$  (nominally significant) and  $P=.0094$  (significance level after correction for multiple testing as described in the text).

**Table 2. Protective Effects of *CRHR1* Alleles and Haplotypes in Second Sample<sup>a</sup>**

<i>CRHR1</i> Alleles and Haplotypes	No MDD (n=74)			Lifetime MDD (n=126)		
	No to Mild Abuse, No. (%) (n=53)	Moderate to Severe Abuse, No. (%) (n=21)	<i>P</i> Value	No to Mild Abuse, No. (%) (n=100)	Moderate to Severe Abuse, No. (%) (n=26)	<i>P</i> Value
rs7209436						
CC	21 (40.4)	5 (23.8)	.04	37 (37.0)	8 (30.8)	.46
CT	22 (42.3)	9 (42.9)		36 (36.0)	13 (50.0)	
TT	9 (17.3)	7 (33.3)		27 (27.0)	5 (19.2)	
rs242940						
AA	18 (34.6)	3 (14.3)	.03	32 (32.0)	9 (36.0)	.20
AG	24 (46.2)	11 (52.4)		35 (35.0)	10 (40.0)	
GG	10 (19.3)	7 (33.3)		33 (33.0)	6 (24.0)	
Protective haplotype block 1, TCA copies, No.						
0	22 (41.5)	5 (25.0)	.04	36 (36.0)	9 (34.6)	.31
1	22 (41.5)	8 (40.0)		36 (36.0)	12 (46.2)	
2	9 (17.0)	7 (35.0)		28 (28.0)	5 (19.2)	
Protective haplotype best SNPs, TAT copies, No. <sup>b</sup>						
0	23 (43.4)	5 (25.0)	.03	36 (36.4)	9 (34.6)	.31
1	21 (39.6)	8 (40.0)		35 (35.3)	12 (46.2)	
2	9 (17.0)	7 (35.0)		28 (28.3)	5 (19.2)	

Abbreviations: MDD, major depressive disorder; NS, nonsignificant; SNPs, single-nucleotide polymorphisms.

<sup>a</sup>Distribution of the estimated copy numbers of the protective alleles (rs7209436 and rs242940) as well as of the protective TCA and TAT haplotypes between women with no to mild child abuse compared with those with moderate to severe child abuse, with or without lifetime diagnosis of MDD as diagnosed with the Structured Clinical Interview for *DSM-IV* Axis I Disorders. The *P* values represent the directional 1-tailed *P* values comparing the respective genotype frequencies between the 2 levels of child abuse for the no MDD and lifetime MDD groups.

<sup>b</sup>The sample size for the TAT haplotype in the lifetime MDD group with no to mild abuse is 99.

## Haplotypes

We estimated the haplotype frequencies for the 3 SNP haplotypes of LD blocks 1 and 2 (Figure 1A) as well as for the 3 SNP haplotypes consisting of the 3 SNPs with the strongest gene × environment interaction effects (rs7209436 and rs110402 located in block 1 and rs242924 between the 2 blocks). The haplotype block structure was determined using the confidence interval method according to Gabriel et al<sup>36</sup> as implemented in the Haploview software. Given that we generated only genotype data, the phase of the underlying haplotypes of a particular subject may be unknown. Therefore, we estimated sample haplotype frequencies using an expectation maximization algorithm<sup>37</sup> and used these frequencies to calculate each subject's posterior haplotype-pair probability given his or her genotype data. This was done using the SNP HAP program (<http://slack.ser.man.ac.uk/progs/snphap.html>). We then applied a conservative haplotype test that used only those subjects whose posterior haplotype-pair probability exceeded 95.0%. However, because the region has a low degree of haplotype complexity (high LD with a few common haplotypes), this excluded only 5.2% of subjects from interaction analysis.

## Correction for Multiple Testing

We applied the program SNPSpD (<http://genepi.qimr.edu.au/general/daledN/SNPSpD/>) to adjust the nominal significance level for the testing of multiple SNP variants within the *CRHR1* gene. The program, based on the work of Nyholt,<sup>38</sup> applies spectral decomposition techniques to determine the effective number of independent SNPs in regions where LD exists. The software then implements a Bonferroni correction that divides the nominal significance level of .05 by this effective number of inde-

pendent SNPs. Using this method, we determined that  $\alpha = .0094$  provides an appropriate threshold to declare significance within the *CRHR1* gene after correcting for multiple testing.

## Statistical Analyses in the Second Supportive Sample

The second supportive sample was clinical in nature, so we tested the protective effects of the protective alleles and haplotypes against development of MDD as opposed to other types of psychiatric disorders in the presence of moderate to severe child abuse (Table 2). Haplotypes with a posterior haplotype probability greater than 95.0% could only be estimated in 199 women for the block 1 haplotype and 198 women for the 3 best SNP haplotypes. Differences in the SNP genotypes of rs7209436, rs4792887, rs110402, rs242940, and rs242924 and in the copy number frequency of the TCA haplotype, formed by rs7209436, rs4792887, and rs110402, respectively, and of the TAT haplotype, formed by rs7209436, rs110402, and rs242924, respectively, between the groups with no to mild vs moderate to severe child abuse were tested separately for patients with a lifetime diagnosis of MDD and patients with no lifetime diagnosis of MDD using contingency tables and an additive genetic model (linear by linear association). Our specific directional hypotheses were that the protective alleles of these SNPs and both the TCA and TAT haplotypes (which were protective in the African American sample) would also be protective, and thus over-represented, in women with moderate to severe child abuse but no lifetime diagnosis of MDD. Thus, we report 1-tailed *P* values for the analyses of these directional hypotheses. The frequencies of the SNPs and for the common haplotypes formed by rs7209436, rs4792887, and rs110402 and by rs7209436, rs110402, and rs242924 in the replication sample are reported in Table 2.

## CHILD ABUSE AND ADULT DEPRESSION

To examine the interaction of the *CRHR1* genotype and ELS, we initially analyzed data from our first sample group of 560 low-income, primarily (97.4%) African American adults living in an urban area. Of the 476 subjects with complete CTQ and BDI phenotypes, we first examined the effects of child abuse on adult depression. As expected based on prior literature, higher CTQ abuse scores predicted higher adult BDI scores ( $F_{3,472}=37$ ;  $P<.001$ ) (**Figure 2A**). Additionally, when child abuse is divided into the none to mild and moderate to severe groups, we see a significant difference in BDI scores (none to mild:  $n=300$ , mean BDI score, 11.58; moderate to severe:  $n=176$ , mean BDI score, 19.74;  $P<.001$ ).

## CRHR1 POLYMORPHISMS AND LD STRUCTURE

The gene encoding *CRHR1* is located on chromosome 17q21.31 and contains 13 exons spanning 51 kb. Of the 502 participants in the study from whom we obtained DNA, 422 were genotyped for 15 SNPs located in a 57-kb region spanning the 5' promoter region to the 3' end of the *CRHR1* gene, with an average intermarker distance of 4.1 kb (Figure 1A). We identified 2 blocks of LD: one within intron 1 spanning at least 8 kb (block 1), and the second from introns 2 through 9 spanning at least 18 kb (block 2). Ten of the SNPs showed a minor allele frequency greater than 5.0% and were thus included in the gene  $\times$  environment regression analysis described later.

## CRHR1 POLYMORPHISMS INTERACT WITH CHILD ABUSE TO PREDICT ADULT DEPRESSION

To test the primary hypothesis that genetic polymorphisms in the *CRHR1* gene moderate the effects of child abuse on adult depression, we used linear regression-based methods. We first performed single-SNP analyses in which we regressed, using permutation analyses, continuous BDI scores on genotype (coded under an additive model), child abuse (none to mild vs moderate to severe), and the interaction between genotype and child abuse, adjusting for potential confounders such as age and sex. Statistical significance of main effects and interactions were evaluated using a permutation-based analysis (see the "Methods" section). Prior to adjusting for multiple comparisons, 7 of the 10 tested SNPs showed a significant interaction (lowest  $P=.008$  for rs110402) with child abuse for the prediction of adult depression (Figure 1B and C).

## INTERACTION OF INDIVIDUAL CRHR1 POLYMORPHISMS, CHILD ABUSE, AND DEPRESSION

To correct for multiple comparisons, we used spectral decomposition techniques to determine the effective number of independent SNPs in regions where LD exists. Using

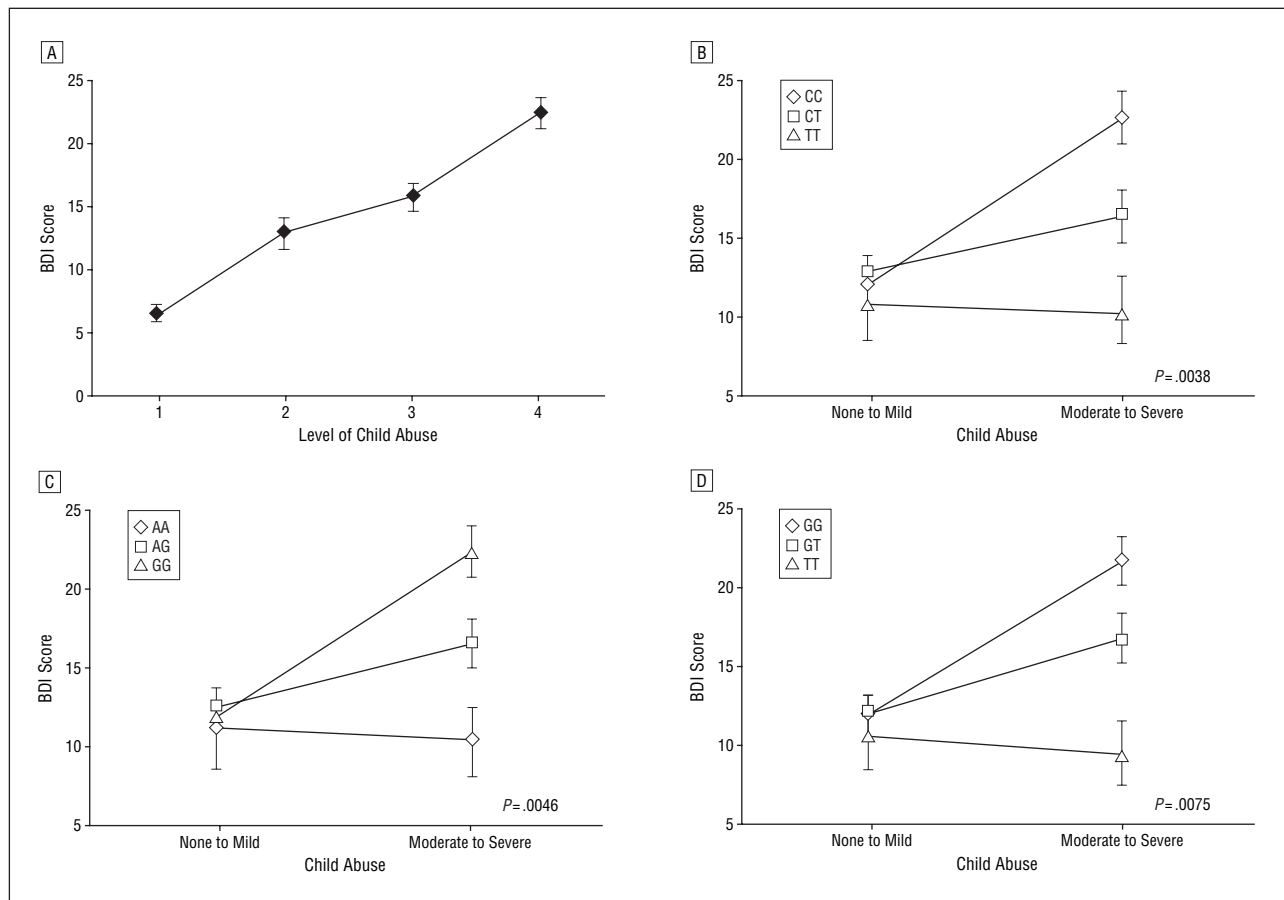
this method, we determined that  $\alpha=.0094$  provides an appropriate threshold to declare significance within the *CRHR1* gene. This  $\alpha$  level is derived from 5.3 independent tests accounting for the moderate extent of LD across the 10 tested SNPs.

The interactions between child abuse and 2 of the *CRHR1* SNPs—rs110402 and rs7209436—remained significant after correction for multiple testing (Figure 2B and C), and 5 others showed significant interaction effects prior to correction (Figure 2D and **Figure 3**). The rs110402 and rs7209436 SNPs both reside in the first LD block of *CRHR1* that spans intron 1 of the gene and are in high LD ( $r^2=0.93$ ). For both SNPs, no significant genotype effect on depression was detected in the no to mild abuse group, with the mean BDI scores from these subjects ranging from 10.91 to 12.67 across genotypes (Figure 2B and C). Among individuals in the moderate to severe abuse groups, the rare allele of both SNPs had a protective effect on the severity of adult depressive symptoms. While individuals homozygous for the common alleles (CC for rs7209436 and GG for rs110402) had average BDI scores of 22.33 to 22.49 (reflective of moderate depressive symptoms), heterozygous individuals had significantly lower BDI scores (mean, 16.31-16.39, reflective of mild depressive symptoms) and individuals homozygous for the rare alleles (TT or AA, respectively) had a mean BDI score of 10.22 (reflective of low to absent levels of depressive symptoms) despite the presence of at least 1 type of moderate to severe child abuse. The data suggest an additive protective genetic effect of the rare allele of these 2 SNPs in those exposed to child abuse. We also note that similar interactive gene dosage patterns were seen across the other SNPs that were significant prior to correction, including rs242924 (Figure 2D), rs4792887, rs242940, rs173365, and rs242948 (Figure 3).

CRHR1 HAPLOTYPES SHOW SIMILAR GENE  $\times$  ENVIRONMENT INTERACTION

A similar permutation-based linear regression approach was applied to investigate gene  $\times$  environment interactions involving common *CRHR1* haplotypes (haplotype frequency  $>10.0\%$ ) in haplotype blocks 1 and 2 (Figure 1A) as well as a haplotype defined by the 3 SNPs carrying the strongest gene  $\times$  environment interactions in the individual SNP analysis. In block 1, we observed 3 common haplotypes of approximately equal frequency (30.4%-34.1%) that summed to account for approximately 98.5% of the observed haplotypes (**Figure 4**). Haplotype interaction analysis showed a significant interaction effect of child abuse and haplotypes within block 1. This association was accounted for by the haplotype formed of alleles T (rs7209436), C (rs4792887), and A (rs110402), which appears to be protective in a dose-dependent manner ( $P<.001$ ) (Figure 4A and C). The other 2 common haplotypes within block 1 and those within block 2 did not show a significant interaction effect.

We then investigated the haplotypes formed by the 3 most significant SNPs in the single-SNP analysis, of which 2 are located in block 1 (rs7209436 and rs110402) and 1 is between blocks 1 and 2 (rs242924). These 3 SNPs form



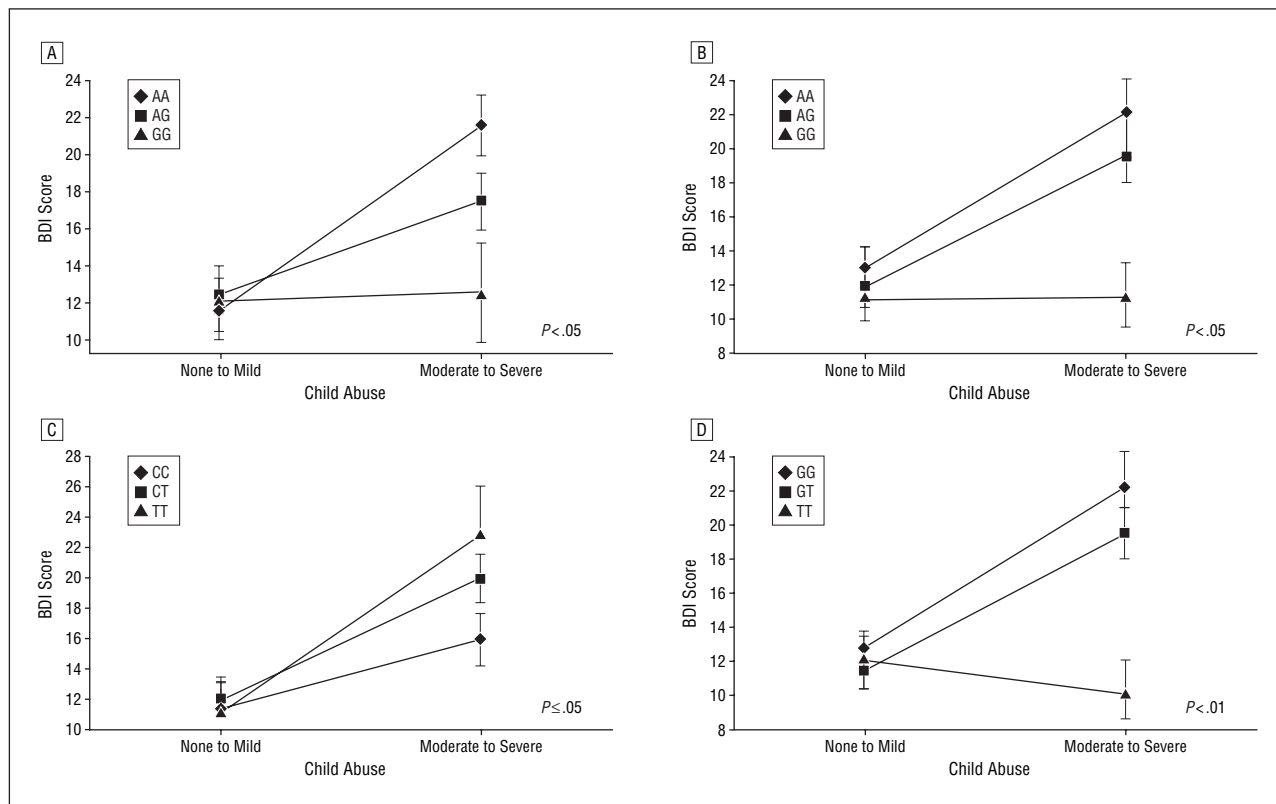
**Figure 2.** Child abuse interacts with the *CRHR1* genotype to enhance risk for depression in adults. A, Mean Beck Depression Inventory (BDI) scores ( $n=476$ ) are predicted by continuous scores on the Childhood Trauma Questionnaire (CTQ) physical, sexual, and emotional abuse scales divided into quartiles (quartile 1: CTQ score=15.0-17.5 [ $n=117$ ]; quartile 2: CTQ score=17.5-22.0 [ $n=112$ ]; quartile 3: CTQ score=22.0-31.0 [ $n=123$ ]; quartile 4: CTQ score > 31 [ $n=124$ ]). The error bars indicate SEM. The overall analysis of variance comparing the 4 groups on the BDI was significant ( $F_{3,472}=37.12$ ;  $P<.001$ ). Post hoc analysis indicated significant differences ( $P<.001$ ) between all pairs of groups except for between groups 2 and 3. Effect of rs7209436 (B), rs110402 (C), and rs242924 (D) genotypes and child abuse on adult depression. Error bars indicate SEM. The sample sizes for no to mild child abuse and moderate to severe child abuse are as follows: rs7209436: CC, 110 and 84, respectively; CT, 115 and 61, respectively; TT, 23 and 9, respectively (B); rs110402: GG, 108 and 84, respectively; AG, 114 and 61, respectively; AA, 23 and 9, respectively (C); and rs242924: GG, 112 and 87, respectively; GT, 111 and 60, respectively; TT, 24 and 10, respectively (D).  $P$  values of significance (based on conservative linear permutation analyses) for the main effect of genotype in the moderate to severe abuse group are shown.

2 common “yin and yang” haplotypes in our sample accounting for more than 95.0% of the observed haplotypes (CGG=66.5% and TAT=28.8%) (Figure 4B and C). The haplotypes formed by these 3 SNPs showed a significant interaction with child abuse on adult BDI scores ( $P=.003$ ). The presence of the rarer haplotype appears to decrease the risk of adult depressive symptoms in an additive manner in those with a history of child abuse.

#### GENETIC ADMIXTURE AND POPULATION STRATIFICATION

Varying degrees of genetic admixture of sub-Saharan African, European, and Native American ancestry have been reported in African American individuals<sup>31,32</sup> and could lead to population stratification and thus spurious association results. To control for potential differences in genetic ancestry, we genotyped 134 AIMs.<sup>31,39</sup> Differences in the extent of admixture in our sample do not account for the observed results because genetic ancestry was correlated with neither the extent of child abuse (Pearson correlations of genetic ancestry with CTQ total abuse

scores were nonsignificant, ranging from  $-0.033$  to  $0.048$  for the 4 different tested populations) nor adult BDI scores (Pearson correlations of genetic ancestry with BDI total scores were nonsignificant, ranging from  $-0.094$  to  $0.099$ ). To confirm that admixture was not an important confounder in our analysis, we reinvestigated the interaction effect of rs110402, the SNP with the lowest  $P$  value, by using linear regression and adding the percentages of sub-Saharan and European ancestry as independent variables. In this model, the interaction effect between the rs110402 genotype and child abuse on BDI scores was less than  $P=.05$  ( $P=.04$  in a smaller sample of 210 individuals). Percentages of sub-Saharan and European ancestry were not significant predictors in this model, with  $P$  values of .38 and .80, respectively. Because the  $\beta$  values for the interaction term were not significantly different with and without ancestry in the model ( $\beta$  [SE], 6.56 [2.70] and 5.59 [2.01], respectively), we also concluded that ancestry is not a confounder in our analysis. Overall, the interaction term remained at  $P\leq .05$  for 5 of the 10 *CRHR1* SNPs when adjusting for admixture.



**Figure 3.** Effect of rs242940 (A), rs173365 (B), rs4792887 (C), and rs242948 (D) genotypes and child abuse on adult depression. Error bars indicate SEM. The sample sizes for no to mild child abuse and moderate to severe child abuse are as follows: rs242940: AA, 98 and 74, respectively; AG, 113 and 69, respectively; GG, 31 and 13 respectively (A); rs173365: GG, 79 and 56, respectively; AG, 114 and 78, respectively; AA, 51 and 21, respectively (B); rs4792887: CC, 107 and 58, respectively; CT, 105 and 70, respectively; TT, 29 and 26, respectively (C); and rs242948: GG, 84 and 57, respectively; GT, 107 and 79, respectively; TT, 64 and 22, respectively (D). *P* values of significance for the main effect of genotype in the moderate to severe abuse group are shown.

### SUPPORTIVE EVIDENCE FROM AN INDEPENDENT SAMPLE

These data suggest that certain alleles of the *CRHR1* gene may provide a protective effect on the risk for depression in adults with a history of child abuse. To confirm this hypothesis, we genotyped rs7209436, rs110402, rs4792887, rs242924, and rs242940 in an independent sample that was ethnically (87.7% Caucasian) and socioeconomically (less impoverished) distinct from the original population described earlier. We analyzed the allele distribution of the previously identified protective SNP alleles and haplotypes TCA (rs7209436, rs110402, and rs4792887) (Figure 4A and C) and TAT (rs7209436, rs110402, and rs242924) (Figure 4B and C) in women with and without a lifetime diagnosis of MDD and with and without child abuse ( $n=199$ ). For haplotype block 1, we found the following frequencies: TCA, 42.1%; CGG, 43.0%; and CTG, 13.1%. For the best SNP haplotype, we found frequencies of 41.6% for TAT and 56.0% for CGG.

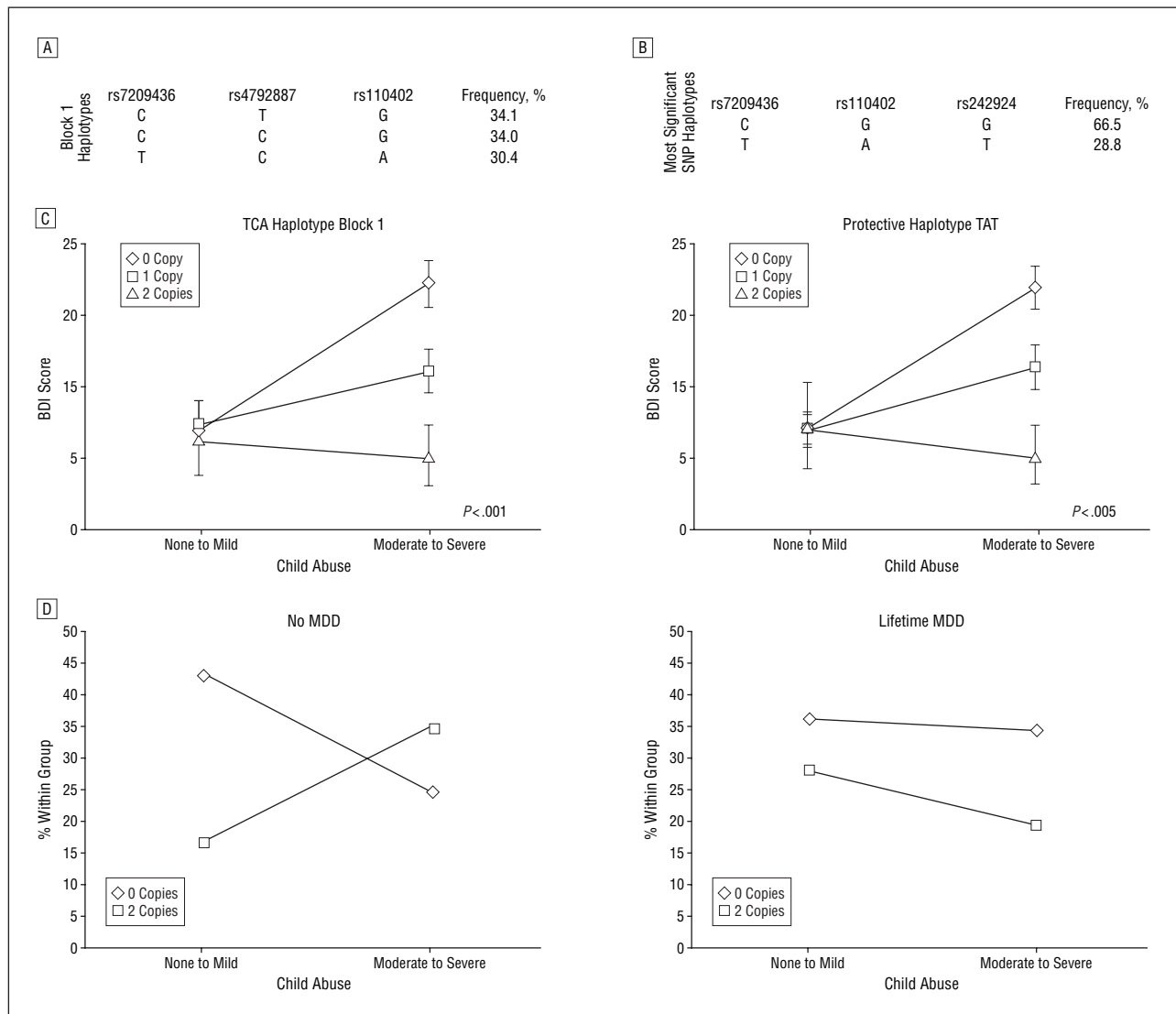
We hypothesized that we would find an overrepresentation of 2 copies of the protective haplotypes in women with a history of moderate to severe child abuse but who had not developed MDD within their lifetimes. Indeed, we found a significant overrepresentation of the protective alleles of the tested SNPs (most significant in replication rs7209436 and rs242940) and of 2 protective copies of both the TCA and TAT haplotypes in this group (Table 2). In the group with child abuse but no

MDD, 35.0% carried 2 copies of these haplotypes; however, in the group with no child abuse and no MDD, only 17.0% had 2 copies (Table 2). In contrast, in the group with a lifetime diagnosis of MDD, there were no significant differences in the distribution of haplotype copy numbers regardless of child abuse history.

The group with a history of child abuse but no MDD appears to be protected from depression despite the increased psychosocial risk. Notably, this group also has a relative increase in the copy numbers of protective *CRHR1* alleles compared with those with a history of lifetime depression (Figure 4D), underscoring the potential protective effects of the identified genotypes in 2 independent and ethnically different samples.

### COMMENT

Our results demonstrate that genetic variants in *CRHR1* are moderators of the effects of child abuse on adult depressive symptoms in 2 independent, ethnically different populations. A haplotype formed by 3 SNPs located in intron 1 of the *CRHR1* gene is most significantly associated with diminished effects of child abuse on adult depressive symptoms. Similar effects were seen on a single-SNP basis and with haplotypes formed by the 3 most significant SNPs. These genotypes and haplotypes potentially serve as predictors of both risk and resilience for adult depression in men and women with a history of child abuse. The pro-



**Figure 4.** Effect of estimated *CRHR1* haplotypes and child abuse on adult depressive symptoms. The frequencies of the estimated individual haplotypes within the first linkage disequilibrium block (A) and of the 3 most significant single-nucleotide polymorphisms (SNPs) (B). C, The effects of the estimated copy numbers of specific haplotypes on adult depression in the presence or absence of moderate to severe child abuse. Error bars indicate SEM. Only haplotypes with an estimation likelihood greater than 95.0% were included in the analysis. The sample sizes for no to mild child abuse and moderate to severe child abuse were as follows: TCA haplotype: 0 copies, 107 and 82, respectively; 1 copy, 109 and 60, respectively; 2 copies, 21 and 9, respectively; TAT haplotype: 0 copies, 118 and 91, respectively; 1 copy, 106 and 54, respectively; 2 copies, 19 and 9, respectively. *P* values of significance for the interaction effect of haplotype in the moderate to severe abuse group are shown. D, A graphical representation of the number of copies of the TAT haplotype in a smaller but ethnically and socioeconomically separate sample. In this separate sample, the best SNP haplotype frequencies were 41.6% for TAT and 56.0% for CGG. The frequencies of TAT haplotypes are shown within child abuse groups for those with no history of major depressive disorder (MDD) and those with a lifetime history of MDD. The protective alleles appear to be present at a higher rate in those with moderate to severe child abuse who have no lifetime MDD compared with those with a lifetime history of MDD.

protective effect was seen in a predominantly African American sample as well as in a sample that was predominantly Caucasian, and the effect was observed with both current depressive symptoms and lifetime diagnosis of MDD.

It is unlikely that any of these SNPs are actually functional variants in the *CRHR1* gene; instead, they are presumably in LD with an as-yet untyped, potentially functional variant. Given the haplotype block structure of the gene, the pattern of association (Figure 1), and the fact that a haplotype in the first LD block showed the most significant interaction effect (Figure 4), it is likely that the causal variant is located in the 5' region of the gene (ranging from the first 2 kb of the promoter to intron 2). Increasing evidence suggests that much of the regula-

tion of gene function is within the noncoding regions.<sup>40,41</sup> Intron 1 of *CRHR1* contains 3 highly conserved regions that may have regulatory functions (according to the UCSC Genome Browser database, <http://genome.ucsc.edu/>). Functional intronic regulatory elements have been reported for several genes<sup>42,43</sup>; therefore, these *CRHR1* intronic regions could affect transcriptional modulation of gene function. Denser fine mapping of this region combined with resequencing followed by in vitro and in vivo studies may allow for the identification of potentially functional variants.

One potential limitation to this study is the use of BDI scores as a continuous variable to measure current depression symptoms, as our genotype effects measure the

interactions with child abuse on adult depression symptoms rather than MDD per se. However, this limitation could also be seen as a strength of the study. As pointed out in the "Methods" section, a significant percentage of our sample received a SCID-I–based DSM diagnosis of MDD, and these data strongly validate our BDI measures of depression. Additionally, current research on the assessment of psychopathology points to a number of advantages of a continuous or dimensional assessment of psychopathology, including depressive symptoms. Other potential limitations are the limited size and different phenotype measure (presence or absence of lifetime MDD) in the second supportive study sample. Despite the limitations of this additional sample, we believe that both having a supporting result within a different socioeconomically based sample and finding the apparent protective effects of the *CRHR1* polymorphisms using the SCID-I diagnoses support the main findings from the larger primary sample. In the future, we would also like to know the differential effects of age, sex, and type of abuse among other factors that may mediate these effects.

Several studies suggest that depression-related HPA axis hyperactivity may be related to ELS, which may form the physiological basis for the finding that abuse during early life substantially elevates adult risk for depression.<sup>21,22</sup> For example, plasma adrenocorticotropin and cerebrospinal fluid CRH concentrations appear to correlate with perceived ELS more strongly than with current depression severity.<sup>44,45</sup> Preclinical studies indicate that the persistent hyperactivity of the HPA axis associated with ELS is mediated by hyperactive CRH neurons, with chronic activation of *CRHR1* in limbic brain regions.<sup>46,47</sup> It is reasonable to hypothesize that alterations in *CRHR1* responsiveness during these early emotional critical periods could alter later risk for HPA axis overactivity and depression.

Our genetic results support the hypothesis that the CRH and *CRHR1* system moderates the effects of ELS on adult psychopathology. Our data also point out that genetic association and linkage studies that fail to take the environment into account may miss important genetic variants involved in the etiology of complex diseases. Given the clinical and preclinical data<sup>12,13,16,21,45,48</sup> that overactivity of CRH-ergic neurotransmission is associated with depressive and anxiety-like symptoms, it is reasonable to expect that the protective polymorphisms identified here associate with either decreased sensitivity of the *CRHR1* or increased negative feedback regulation of its functioning. These results suggest the possibility that heritable differences in CRH-mediated neurotransmission exacerbate or dampen the effects of child abuse on the stress hormone system, potentially modulating HPA axis sensitivity, extrahypothalamic CRH-ergic circuits, and risk for depressive symptoms in adulthood.

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**Correspondence:** Kerry J. Ressler, MD, PhD, Department of Psychiatry and Behavioral Sciences, Yerkes Research Center, Emory University, 954 Gatewood Dr, Atlanta, GA 30329 (kressle@emory.edu).

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# Prelimbic cortical BDNF is required for memory of learned fear but not extinction or innate fear

Dennis C. Choi<sup>a</sup>, Kimberly A. Maguschak<sup>a</sup>, Keqiang Ye<sup>b</sup>, Sung-Wuk Jang<sup>b</sup>, Karyn M. Myers<sup>a</sup>, and Kerry J. Ressler<sup>a,1</sup>

<sup>a</sup>Howard Hughes Medical Institute, Center for Behavioral Neuroscience, Department of Psychiatry and Behavioral Sciences, Yerkes National Primate Research Center, Emory University School of Medicine, Atlanta, GA 30322; and <sup>b</sup>Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322

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**In the medial prefrontal cortex, the prelimbic area is emerging as a major modulator of fear behavior, but the mechanisms remain unclear. Using a selective neocortical knockout mouse, virally mediated prelimbic cortical-specific gene deletion, and pharmacological rescue with a TrkB agonist, we examined the role of a primary candidate mechanism, BDNF, in conditioned fear. We found consistently robust deficits in consolidation of cued fear but no effects on acquisition, expression of unlearned fear, sensorimotor function, and spatial learning. This deficit in learned fear in the BDNF knockout mice was rescued with systemic administration of a TrkB receptor agonist, 7,8-dihydroxyflavone. These data indicate that prelimbic BDNF is critical for consolidation of learned fear memories, but it is not required for innate fear or extinction of fear. Moreover, use of site-specific, inducible BDNF deletions shows a powerful mechanism that may further our understanding of the pathophysiology of fear-related disorders.**

learning | plasticity | prefrontal cortex | Cre/LoxP | inducible knockout

In healthy individuals, the prefrontal cortex and amygdala are critical for processing fearful and other emotional stimuli and for learning to extinguish fears in situations that are no longer threatening (1, 2). In contrast, patients suffering from post-traumatic stress disorder (PTSD) or anxiety disorders describe persistent anxiety-provoking memories that are severely debilitating and cannot be extinguished (3–6). Therefore, the experimental analysis of fear modulation and extinction is critical for an understanding of the neurobiology of fear inhibition. The medial prefrontal cortex (mPFC) is suggested to be an important region for the regulation of fear (7–13). Although it is established that the infralimbic cortex (IL) region of the mPFC is required for fear extinction (9, 11, 14), the role of the prelimbic cortex (PL) in the regulation of fear learning and extinction are yet to be fully understood. Although previous studies have shown that lesions of the PL do not affect acquisition or expression of fear (7, 9, 15), inactivation reduces freezing behavior in previously fear-conditioned rats (16). Additionally, activation of PL neurons are required for the expression of previously learned fears (17, 18), and microstimulation of the PL potentiates expression of conditioned fear (19). Moreover, these neurons have also shown plasticity after fear conditioning (18, 20, 21) and have sustained activity to conditioned tones (22). Overall, these data suggest that the PL is necessary for the expression of previously learned fear, but the mechanisms remain unclear.

One potential candidate may be BDNF and its receptor tyrosine kinase receptor B (TrkB); they are known to regulate neuronal structure and function and are important for synaptic plasticity (23–26). Additionally, *in vivo* studies have shown a role for BDNF in learning and memory, including fear conditioning (27–31). More specifically, we have previously shown that disruption of TrkB activation using lentiviral expression of a dominant-negative form of TrkB (TrkB.T1) into the basolateral amygdala blocked the acquisition of fear (27) and the consolidation of extinction (29), suggesting that BDNF-dependent activation of TrkB within the amygdala regulates the learning of fear and extinction memories.

This role of BDNF has yet to be investigated in the subregions of the mPFC and may play an important role in the plasticity of fear-learning circuitry. Because BDNF is highly expressed in the PL, which projects heavily to basolateral amygdala (32), we hypothesized that prelimbic BDNF-dependent plasticity is necessary for the expression and possibly extinction of learned fear memories. To test our hypothesis, we used different approaches with (i) a neocortical BDNF knockout mouse, (ii) reversal of the BDNF knockout phenotype with a newly identified TrkB receptor agonist, and (iii) viral-mediated BDNF deletions limited to the prelimbic cortex.

## Results

**Neocortical BDNF Knockout Mice Have Impaired Learned Fear.** To examine the role of cortex-specific BDNF in modulating fear, we used a newly developed cortex-specific BDNF knockout mouse model (33). Expression of Cre recombinase in these mice includes the PL and neocortex but spares the more ventral IL, along with all other paleocortical and subcortical regions, with no expression in hippocampus, thalamus, striatum, hypothalamus, or amygdala (Fig. 1*A* and Fig. S1). These Cre-driver mice were crossed with transgenic homozygous BDNF-floxed (fBDNF) mice (34), allowing us to selectively delete BDNF expression in select areas of the neocortex when Cre expression is present (Fig. 1*B* and *C*). In the littermate control mice (Cre<sup>-/-</sup>), BDNF is abundantly expressed in the prefrontal cortex, including PL and IL (Fig. 1*D*), whereas BDNF knockout mice (Cre<sup>+/-</sup>) have loss of BDNF expression in the PL, sparing BDNF expression in the more ventrally located IL (Fig. 1*C*). Cre expression was also confirmed with a LacZ reporter mouse (35) (Fig. 1*A* and *E*). Cortical BDNF deletion in Cre<sup>+/-</sup> overlays directly on the regions expressing LacZ (Fig. 1*C* and *F*). We also generated mutant mice by crossing the Cre<sup>+/-</sup> line with a floxed-stop fluorescent reporter line (36). Membrane-bound eGFP allowed visualization of cellular patterns of the neocortical Cre expression (Fig. 1*G*), confirming neocortical and prelimbic-specific Cre expression.

After confirming the neocortex-specific deletion of BDNF, we examined the effects of this inducible deletion on a number of behavioral tasks. We found that Cre<sup>+/-</sup> BDNF knockout mice were similar to Cre<sup>-/-</sup> littermate controls in locomotor function, baseline startle response, shock reactivity (a control for pain sensitivity), and prepulse inhibition (a control for auditory function and sensorimotor gating) (Fig. 2). These data suggest that neocortical BDNF is not required for these sensory, pain, and startle behaviors and for normal locomotor function. Additionally, there were no differences between Cre<sup>+/-</sup> and Cre<sup>-/-</sup> mice on measures

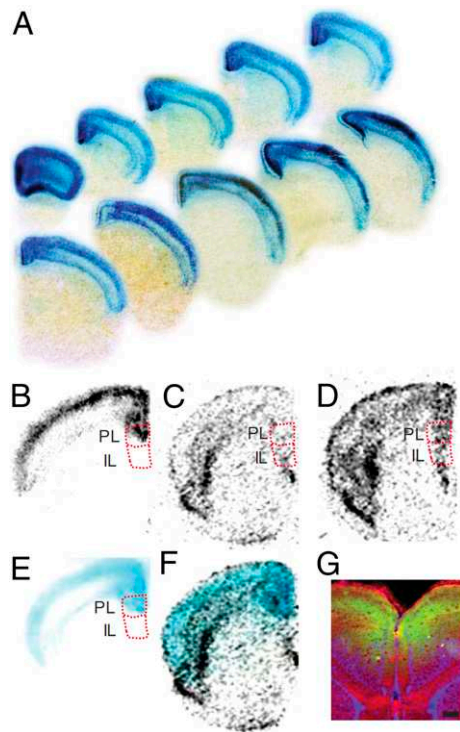
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<sup>1</sup>To whom correspondence should be addressed. E-mail: kressle@emory.edu.

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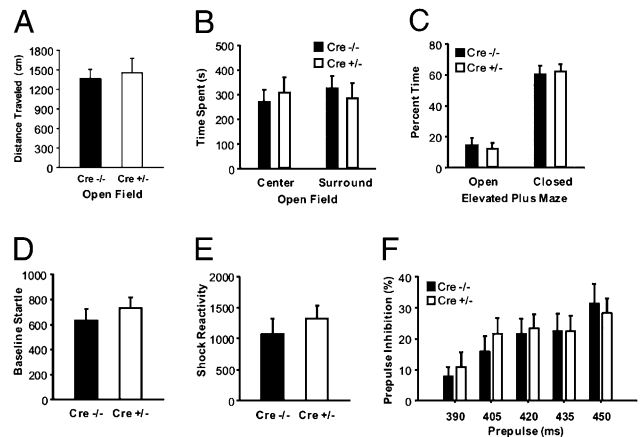
**Fig. 1.** Selective neocortical BDNF knockout mice. (A) Cortex  $Cre^{+/−}$  mice were crossed with the floxed-stop lacZ reporter mouse line. X-gal staining is shown across anterior–posterior coronal slices. (B) In situ hybridization of Cre mRNA in cortex  $Cre^{+/−}$  mice. PL, prelimbic cortex; IL, infralimbic cortex. (C)  $Cre^{+/−}$ , fBDNF neocortex selective knockout mice have loss of cortical BDNF mRNA compared with (D)  $Cre^{+/−}$ , fBDNF littermates with intact BDNF expression. (E) LacZ expression in  $Cre^{+/−}$ , Rosa LacZ mice extends into the PL and (F) overlays with the specific knockout of BDNF in the fBDNF crosses. (G) Cortex  $Cre^{+/−}$  mice were crossed with fluorescent reporter mice (36) with labeled Cre expression (eGFP) in the PL that spares the ventral IL. Red, Td tomato; green, eGFP; blue, DAPI.

of anxiety-like behavior or unlearned/innate fear as measured by the elevated-plus or open-field mazes (Fig. 2B and C).

The neocortical BDNF knockout mice ( $Cre^{+/−}$ ) subsequently underwent cue-dependent fear conditioning, because we hypothesized that BDNF in the PL is required for learning or expression of cued fear. The  $Cre^{+/−}$  had normal acquisition of fear and expression of newly acquired fear memories, which was measured during cue-dependent fear training to five pairings of conditioned stimulus (CS) tones and unconditioned stimulus (US) shocks (Fig. 3A). To test their short-term memory, these mice were tested 1 h after the cessation of fear training; they were placed in a different context and measured for fear behavior (freezing) to the presentation of three CS tones. The  $Cre^{+/−}$  had attenuated fear responses to the tone compared with the  $Cre^{-/-}$  littermate controls ( $t(15) = 2.13$ ;  $*P < 0.05$ ) (Fig. 3B).

To investigate long-term fear memory, another group of neocortical BDNF knockout mice ( $Cre^{+/−}$ ) and controls ( $Cre^{-/-}$ ) were similarly fear-conditioned and tested 24 h later for expression of previously learned fear to the presentation of 15 trials of CS cues in a different context. The  $Cre^{+/−}$  had robustly attenuated freezing to the presentation of the CS-tones test compared with  $Cre^{-/-}$  ( $t(29) = 2.44$ ;  $*P < 0.05$ ) (Fig. 3C) and diminished freezing during the first and third blocks of five CS-tone presentations ( $F_{1,92} = 5.93$ ;  $*P < 0.05$ ) (Fig. 3D).

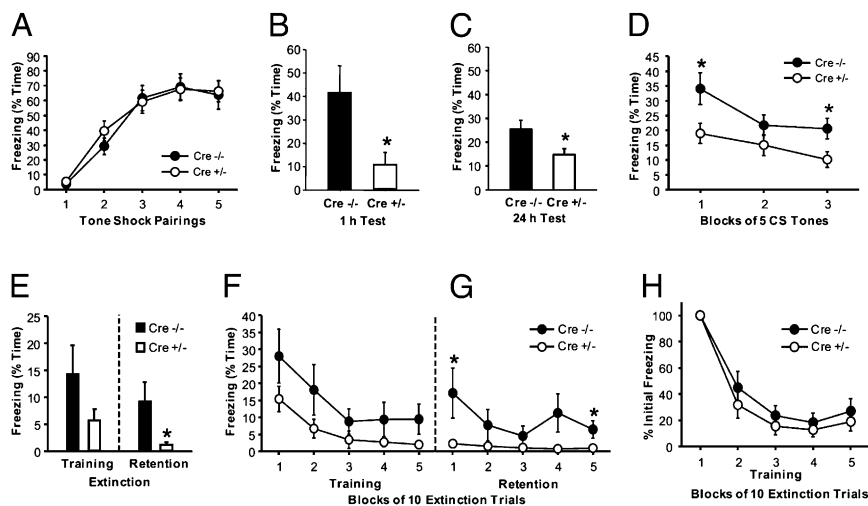
A different set of neocortical BDNF knockout mice were fear-conditioned as above followed by extinction 24 h later with 50 CS tones in the absence of the US. The next day, these mice were



**Fig. 2.** Neocortical BDNF knockout mice have normal locomotor, anxiety-like, and sensory responsiveness. (A) Locomotor activity was assessed in open-field fBDNF,  $Cre^{-/-}$ , and  $Cre^{+/−}$  mice that showed no differences in distance traveled over 10 min. (B) Anxiety-like behavior was also assessed in the open field, where there were no differences between groups in time spent in the field center or surround. There were also no differences in (C) anxiety-like behavior assessed on an elevated-plus maze or in (D) baseline startle, (E) shock reactivity, or (F) prepulse inhibition as measured by startle responses ( $n = 8$  per group).

exposed to another 50 CS tones in the extinction-retention test. All groups significantly extinguished fear during the extinction training period (Fig. 3E and F). Although there were only trends in freezing during the prolonged extinction training period, the  $Cre^{+/−}$  had significantly less average freezing during the retention test ( $t(25) = 2.34$ ;  $*P < 0.05$ ) (Fig. 3E) and less freezing during the first and fifth blocks of 10 tone presentations during the retention test ( $F_{1,134} = 4.22$ ;  $*P < 0.05$ ) (Fig. 3G) compared with animals with normal cortical BDNF expression. Because the  $Cre^{+/−}$  knockout mice consistently showed lower initial levels of fear, the rate of extinction was also examined by percent of initial freezing, which examines relative extinction rates, and there were no differences between groups (Fig. 3H). We also determined that the  $Cre^{+/−}$  still maintained similar levels of freezing to the CS tested at least to 72 h after fear conditioning (Fig. S2), indicating that the low levels of freezing in the  $Cre^{+/−}$  mice after extinction was not caused by decay of their fear learning over the time course of the extinction retention test (48 h postconditioning). Together, these data suggest that neocortical BDNF deletions that involve PL but not IL lead to deficits in rapid short-term and long-term learning and memory to the conditioned cue but do not impair extinction.

**7,8-Dihydroxyflavone Rescues Impairment of Fear Conditioning Seen in Cortical BDNF Knockout Mice.** To investigate BDNF activation of TrkB, use of potential new TrkB-acting therapeutic agents have been limited because of the lack of any identified TrkB agonists that fully mimic the actions of BDNF at brain TrkB receptors in vivo. Most recently, Jang et al. (37) successfully screened a chemical library for compounds that activate TrkB in vitro, revealing a number of flavone derivatives; the most potent of these, 7,8-dihydroxyflavone (7,8-DHF), binds with high affinity to the TrkB receptor and provokes its dimerization and autophosphorylation, leading to downstream-signaling cascade activation (37). Systemic administration of this compound in mice substantially activates TrkB in the brain, inhibits neuronal cell death, decreases infarct volumes in stroke in a TrkB-dependent manner, and is neuroprotective in an animal model of Parkinson's disease. We, therefore, examined whether or not systemic administration of 7,8-DHF would rescue the fear-learning deficit found in these neocortical BDNF knockout mice. Fig. 4A shows that neocortical tissue from these mice expressed significantly less BDNF protein



**Fig. 3.** Neocortical BDNF expression is required for short-term and long-term memory of conditioned fear but not extinction. (A) Neocortical BDNF knockout mice (*Cre<sup>+/-</sup>*) and fBDNF littermate controls (*Cre<sup>-/-</sup>*) acquired fear after cued fear conditioning with five tone-shock pairings. (B) One hour later, *Cre<sup>+/-</sup>* expressed less overall freezing than *Cre<sup>-/-</sup>* during the presentation of a three CS-tone test ( $n = 8$  per group). (C) In separate experiment, *Cre<sup>+/-</sup>* and *Cre<sup>-/-</sup>* were fear trained to a tone cue, and 24 h later, *Cre<sup>+/-</sup>* expressed less overall freezing than *Cre<sup>-/-</sup>* during the 15-tone test ( $n = 15$  *Cre<sup>-/-</sup>*;  $n = 16$  *Cre<sup>+/-</sup>*). (D) *Cre<sup>+/-</sup>* showed less freezing during the first and third blocks of CS tone presentations ( $n = 15$  *Cre<sup>-/-</sup>*;  $n = 16$  *Cre<sup>+/-</sup>*). (E) To investigate extinction learning, additional cortical BDNF knockout mice were fear trained to a tone cue. One day later, they were extinction trained, and 1 day later, they were tested for extinction retention. Average freezing during extinction training and extinction retention tests to 50 CS tones each day was recorded ( $n = 13$  *Cre<sup>-/-</sup>*;  $n = 14$  *Cre<sup>+/-</sup>*). (F) Within-session extinction occurred in both groups, and *Cre<sup>+/-</sup>* had less fear during (G) extinction retention ( $n = 13$  *Cre<sup>-/-</sup>*;  $n = 14$  *Cre<sup>+/-</sup>*). (H) Within-session extinction was normalized to percentage of initial freezing levels for each group with no differences between groups ( $*P < 0.05$  *Cre<sup>+/-</sup>* versus *Cre<sup>-/-</sup>*).

in Western blots and that systemic 7,8-DHF increased the phosphorylation of TrkB in this tissue. These data reveal that despite the lack of cortical BDNF gene expression, endogenous TrkB receptors within the brain are activated with systemic 7,8-DHF.

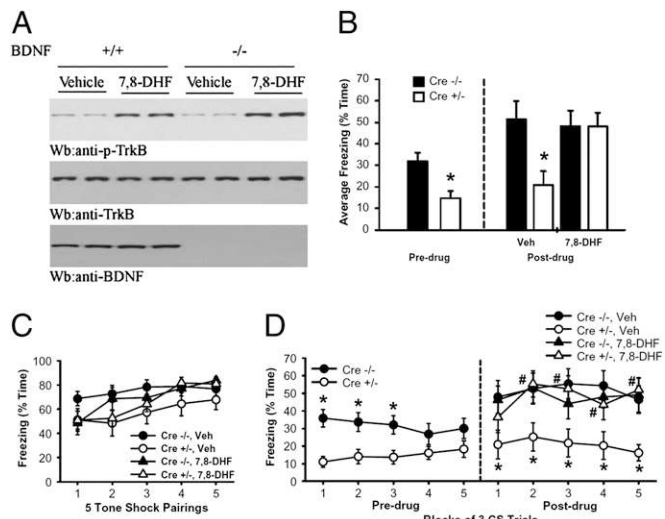
Neocortical BDNF knockout mice (*Cre<sup>+/-</sup>*) and littermate controls (*Cre<sup>-/-</sup>*) were cue fear-conditioned by administering five pairings of tone (6 kHz) coterminating with shock followed 24 h later by fear testing. *Cre<sup>+/-</sup>* showed significantly less total freezing compared with *Cre<sup>-/-</sup>* after the initial predrug fear-conditioning session [ $t(32) = 3.073$ ;  $P < 0.05$ ] (Fig. 4B). This was supported by an overall significant genotype effect ( $F_{1,169} = 9.445$ ;  $P < 0.05$ ) with freezing deficits in the *Cre<sup>+/-</sup>* during predrug testing sessions in the first three blocks of CS trials (Fig. 4B and D, predrug). These mice were then retrained 30 min after the administration of either 7,8-DHF (5 mg/kg, i.p.) or vehicle using a new, different CS tone (12 kHz) and training context. There were no differences in freezing between groups during fear acquisition to the new CS (Fig. 4C). These mice were then tested to the new CS 24 h later in the absence of drug (Fig. 4B and D, postdrug). The *Cre<sup>+/-</sup>* injected with vehicle still had freezing deficits compared with their littermate controls. In contrast, *Cre<sup>+/-</sup>* injected with 7,8-DHF now had equivalently robust fear expression as the control mice (genotype effect  $F_{1,174} = 15.483$ ;  $P < 0.05$ ; drug by genotype interaction  $F_{1,174} = 15.314$ ;  $P < 0.05$ ) (Fig. 4D, postdrug). Notably, the agonist was only present and active during the fear-conditioning period (Fig. 4B and C), but it was no longer present during the fear test. This provides additional evidence that the mPFC BDNF deletion does not affect expression of conditioned fear but is required for learning or consolidating of fear memories. Also of note was that the additional TrkB activation did not increase the fear learning in the *Cre<sup>-/-</sup>* control mice beyond their drug-free level. Together, these data suggest that systemic 7,8-DHF can rescue the learning deficit in mice with a selective neocortical BDNF deletion by activating TrkB receptors in the absence of endogenous neocortical BDNF.

**Targeted Lentivirus-Mediated Inducible BDNF Deletion Within PL.** Based on prior studies suggesting a specific role for the PL in fear

expression (16), we hypothesized that the PL is the likely neocortical subregion requiring BDNF within our transgenic mice. To test this, we used the fBDNF mice in combination with Cre-mediated gene deletion in a localized fashion through bilateral injections of Cre-expressing (LV-Cre) or control eGFP-expressing (LV-GFP) lentivirus vectors (31, 38) targeting the PL. First, we showed that robust Cre expression was observed when this lentivirus was injected into LacZ reporter mice (35) (Fig. 5A and B). Using in situ hybridization for Cre and BDNF mRNA, we then showed that lentivirus-mediated Cre expression (Fig. 5C) overlays with the loss of BDNF expression (Fig. 5D and E) in the PL with only minor dorsal spread in the LV-Cre infected mice. In the LV-GFP control mice, GFP was strongly expressed in the PL (Figs. 5F and G). Nissl stain illustrated minimal cell damage by lentivirus infection into PL in LV-GFP (Fig. 5H) and LV-Cre mice (Fig. 5I).

To examine whether or not specific deletions of prelimbic BDNF would attenuate fear expression, mice were trained with cue-dependent fear conditioning (as described above) 10 days after lentiviral infections. The LV-Cre mice with prelimbic BDNF deletions had no differences in acquisition and expression of newly acquired fear compared with LV-GFP controls (Fig. 6A). As hypothesized, 24 h later, when tested for fear to the cue, the LV-Cre mice had a massive decrease in average freezing compared with the controls [ $t(12) = 3.25$ ;  $*P < 0.05$ ] (Fig. 6B) and throughout the test ( $F_{1,141} = 11.66$ ;  $*P < 0.05$ ) (Fig. 6C and Fig. S3). These findings replicate the deficit of fear observed in the neocortex BDNF knockout mice with an even more dramatic loss of fear expression.

Additionally, the LV-Cre mice showed no differences in locomotor activity in the open-field maze (Fig. 6D). They also showed no difference in baseline anxiety level in terms of time spent in the center of the open-field maze (Fig. 6E), time spent in the open arms of the elevated-plus maze (Fig. 6F), or novelty seeking in the novel-object recognition test, which serves as a measure of both novelty seeking and non-emotional learning (Fig. 6G). These data with virus-mediated, inducible BDNF prelimbic deletions replicate our similar findings in the neocortical BDNF knockout mice, indicating that the deficits in



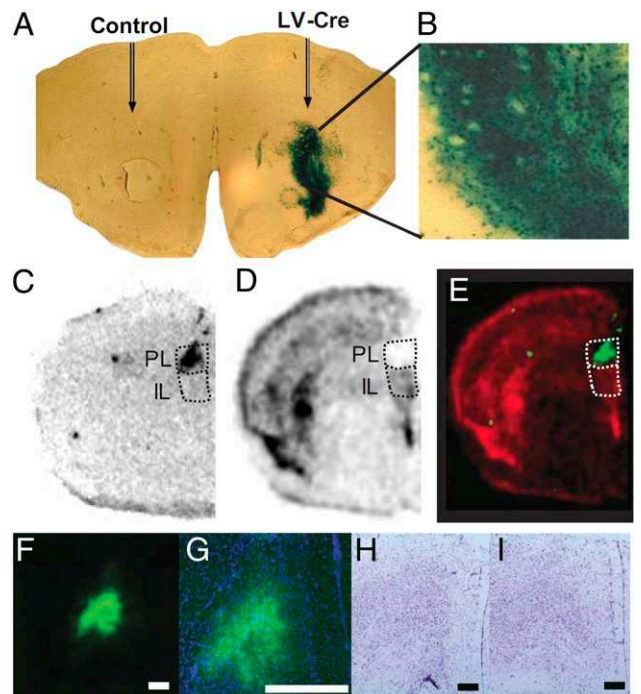
**Fig. 4.** 7,8-DHF rescues a fear-expression deficit in neocortical BDNF knockouts. (A) Immunoblotting revealed that there was TrkB activation in cortical tissue after systemic 7,8-DHF administration but not with vehicle, in cortical BDNF knockout mice, or in controls. Systemic 7,8-DHF lead to phosphorylation of TrkB in cortex independently of the presence of BDNF, which is shown to be minimal in the BDNF knockout mice (anti-BDNF). (B) Average freezing to tone CS 24 h after cued fear conditioning (predrug) and again after reconditioning to a different tone (postdrug) is shown. Cre<sup>+/-</sup> had less freezing than Cre<sup>-/-</sup> (predrug). These mice were then injected (i.p.) with either 7,8-DHF or vehicle followed by reconditioning to a novel tone. Cre<sup>+/-</sup> injected with vehicle had less freezing to the new tone than did Cre<sup>-/-</sup> (\*Student's *t* test; *P* < 0.05). However, Cre<sup>+/-</sup> injected with 7,8-DHF showed equivalent levels of freezing compared with 7,8-DHF-injected controls. (C) Within-training freezing during reconditioning to a new CS tone after injections of 7,8-DHF or vehicle is similar across groups. (D) When analyzing freezing 24 h after conditioning by blocks of three CS tones, Cre<sup>+/-</sup> showed diminished levels compared with Cre<sup>-/-</sup> in the absence of drug. After reconditioning with 7,8-DHF administration, Cre<sup>+/-</sup> had greater freezing compared with vehicle-injected Cre<sup>+/-</sup> but no differences compared with 7,8-DHF or vehicle-injected Cre<sup>-/-</sup> controls (\**P* < 0.05 versus respective Cre<sup>-/-</sup>; #*P* < 0.05 versus respective vehicle; *n* = 12 Cre-vehicle; *n* = 7 Cre<sup>+/-</sup>-vehicle; *n* = 8 Cre<sup>+/-</sup>-DHF; *n* = 7 Cre<sup>-/-</sup>-DHF).

freezing are not caused by altered locomotion or sensory function, and innate fear remained intact.

## Discussion

In summary, the transgenic neocortical BDNF knockout mice had robust deficits in freezing following cued fear conditioning at both 1 h and 24 h after fear conditioning, and extinction learning was moderately enhanced. Notably, the mice acquired fear and expressed immediate fear learning and freezing normally. Additionally, the neocortical BDNF knockout phenotype was rescued with the systemic TrkB agonist, 7,8-DHF, showing that BDNF activation is required for fear learning but not during fear testing for normal expression. Together, these data indicate that the presence of BDNF within PFC is important for learning or consolidation of both short-term and long term-memory. In the absence of BDNF, the knockout mice may potentially have dramatically altered or adapted circuitry, leading to rapid change in the output during the presentation of CS and the deficit observed even at the 1-h short-term test. Therefore, BDNF signaling may be critical for strengthening of neural connections in neocortical regions known to regulate expression of cued fear during learning.

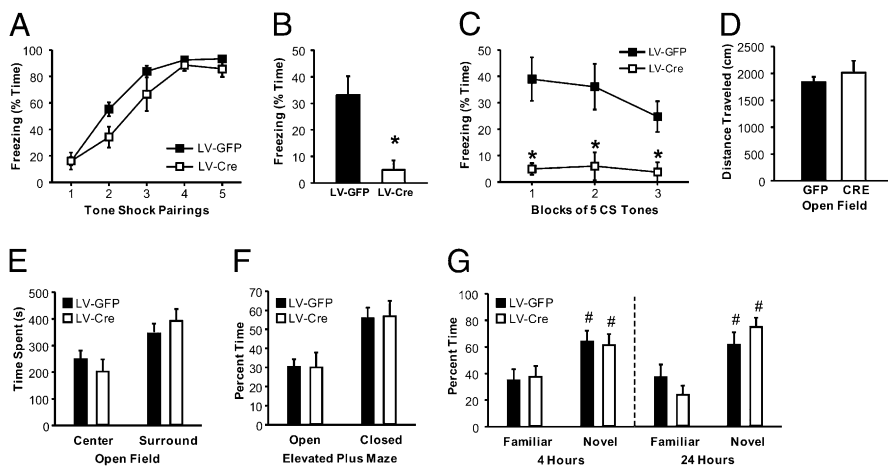
The Western blot analyses of the cortical tissue of the neocortical BDNF knockouts revealed robust loss of BDNF protein in the cortex, whereas cortical TrkB-receptor expression remained unchanged. Moreover, systemic 7,8-DHF administration resulted in increased phosphorylated TrkB in the cortex. This indicates that



**Fig. 5.** Inducible BDNF deletion in PL with Cre lentivirus. (A and B) Robust lentiviral Cre expression was labeled by LacZ when injected into Rosa LacZ reporter mice in striatum. (C) Cre recombinase mRNA expression showed the infection site targeting the PL. (D) BDNF mRNA was specifically deleted in the PL and (E) overlays with the infection of Cre recombinase lentivirus. Red, BDNF mRNA; green, Cre mRNA. LV-GFP control infections in the PL of fBDNF mice at (F) 40 $\times$  magnification and (G) 100 $\times$  magnification. Nissl stain with cresyl violet showed no appreciable cell damage after lentiviral infection of (H) LV-GFP or (I) LV-Cre. (Scale bars: 500  $\mu$ m).

systemic 7,8-DHF does cross the blood–brain barrier and targets TrkB activation, which replicates the initial findings of Jang et al. (37); however, our case was specifically within neocortical tissue of BDNF knockout mice. We then found that 7,8-DHF was able to rescue the fear learning deficit in mice that had a neocortex-specific deletion of BDNF. These behavioral data parallel the protein analyses of 7,8-DHF increasing phosphorylated-TrkB levels in the cortex, both suggesting that cortical TrkB receptor activation is important for fear learning or consolidation. Future studies remain to determine whether or not 7,8-DHF augments fear learning by targeting TrkB receptors in the prefrontal cortex or via downstream projections to TrkB in the basolateral amygdala to enhance plasticity in these mice that lack endogenous prefrontal BDNF expression.

In combination with the neocortical BDNF knockout mouse findings, the prelimbic lentivirus experiments show normal fear expression during the learning/acquisition period of training, but robust deficits in fear after consolidation; this provides further evidence that BDNF expression in the PL is likely necessary for the strengthening of fear-expression circuitry involved during the conditioned learning of fear memories. Notably, the viral-mediated PL BDNF deletions seem to have a more robust deficit in learned fear than the neocortical knockouts, suggesting greater specificity to the role of prelimbic cortical BDNF in regulating fear. Additionally, the neocortical transgenic Cre recombinase is driven by a cholecystokinin (CCK) promoter and likely preferentially targets an interneuron population within the select areas of the neocortex. Thus, the more extensive cellular (excitatory, inhibitory, and glial) BDNF deletion that is obtained with the PL-targeted lentiviral approach using a cytomegalovirus constitutive promoter may lead to more dense behavioral effects because of the enhanced regional



**Fig. 6.** Prelimbic BDNF is critical for consolidation and expression of learned fear. (A) Mice infected with LV-GFP or LV-Cre acquired and expressed fear during cued fear conditioning. (B) One day after fear conditioning, mice were tested with CS tones. LV-Cre mice had attenuated average freezing compared with LV-GFP mice. (C) During fear testing, CS presentations across 15 tones showed less freezing in LV-Cre mice. (D) There were no differences in locomotor activity in the open field. (E) Anxiety-like (innate fear) behavior was assessed in the open field, showing no differences in time spent in the center or surround. (F) Anxiety-like behavior was similar on the elevated-plus maze. (G) All mice learned equally well on an object recognition test where all mice spent significantly more time exploring a novel object during short-term (4 h) and long-term (24 h) testing of memory ( $^{\#}P < 0.05$  versus familiar object). For all figures,  $^*P < 0.05$  versus LV-GFP ( $n = 7$  per group).

specificity combined with a more dense BDNF deletion within this area. Overall, these data are consistent with reports that the IL is the major mPFC site involved with extinction, whereas PL is involved with driving output of learned fear and gating of fear extinction (9, 16, 19, 22). Future studies remain to determine whether or not BDNF is important for the plasticity of PL and IL circuits that may interact together synergistically to modulate expression and extinction of learned fear.

The viral-mediated PL BDNF deletions in adult mice also provide evidence that the deficits in learning or consolidation of fear in the neocortical BDNF knockout mice is unlikely caused by BDNF-induced structural changes in the cortex during development. This is further supported by our studies showing that both the neocortical BDNF knockout mice and the induced PL BDNF deletion mice have generally intact sensory and motor functions; this indicates that the deficits in freezing (fear expression) were not simply caused by alterations in motor or sensory deficits. Future experiments will determine the differential role of PL and IL BDNF in the extinction of fear memories.

These data may seem inconsistent with recent pharmacological inactivation studies indicating that PL is not necessary for long-term fear memory but specifically for output during fear expression (16, 39). One explanation for this difference is that the cortical BDNF knockouts or PL BDNF deletions are permanent, and they may lead to structural changes and adaptations in the existing circuitry between the prefrontal cortex and targets, including the amygdala. However, our data also show that a single systemic dose of the BDNF agonist, 7,8-DHF, rescued this effect, and after this treatment, fear expression is normalized, even in the absence of the drug. Thus, a more parsimonious explanation may be that BDNF, whether acting locally within intracortical circuits or distally (e.g., in PL projections to amygdala), may be required at the time of plasticity to alter the output of cue-specific fear but not for the process of fear expression itself. Note that our data showing normal fear expression to unconditioned shocks during acquisition are consistent with this interpretation. Moreover, future studies remain to determine whether or not prefrontal BDNF may be targeting downstream regions such as the basolateral amygdala, where we have previously shown TrkB in the basolateral amygdala to be critical for fear learning (29).

In addition to animal studies that indicate the importance of mPFC interaction with the amygdala for normal fear learning and extinction, neuroimaging has shown that the human PFC is involved in fear modulation (1, 2). Clinical studies also implicate dysfunction between these two regions in many affective disorders, including anxiety (4), depression (3), and fear-related disorders like PTSD (5, 6). Most relevant to our findings are recent studies indicating that BDNF polymorphisms are correlated with

prefrontal cortex anatomy (40), and they may be implicated in emotionality and anxiety disorders (41, 42). Overall, our studies provide support for BDNF-mediated neuroplasticity as a molecular mechanism underlying prefrontal-specific regulation of fear learning, likely within mPFC-amygdala circuits, which may ultimately lead to improvements in pathophysiology and treatment of uncontrollable fear in patients.

## Methods

**Animals.** Lentivirus experiments used homozygous BDNF-floxed mice (34) (*Bdnf<sup>flm3/ae1</sup>*); Jackson Labs), which possess loxP sites both upstream and downstream of exon 5 of the BDNF gene. The cortex-specific Cre mouse line, previously described as "transgenic line C" (33), was created when the coding sequence for Cre recombinase was placed downstream of a 3-kb CCK promoter. The cortex-specific Cre line was crossed to floxed BDNF mice, floxed-stop [Jackson Labs, *Gt (ROSA)26Sor*] lacZ reporter mice (35), and floxed-stop EGFP-tdTomato mice (36). All experiments were performed on group-housed adult (2–4 months) males. All procedures used were approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with National Institutes of Health guidelines for the care and use of laboratory animals.

**Drugs.** 7,8-DHF (TCI) was dosed systemically (i.p.) at 5 mg/kg in vehicle (17% DMSO in PBS).

**Cre Recombinase Lentivirus Infection.** LV-Cre or a GFP-expressing control vector (LV-GFP), delta8.9, and VSV-g were cotransfected into HEK293T cells to produce replication-incompetent virus, which was concentrated by ultracentrifugation as described previously (27, 29, 31) to  $1 \times 10^9$  infectious particles per milliliter. LV-GFP or LV-Cre virus was bilaterally injected using a Hamilton syringe on a microinjection pump into the PL (Bregma; AP +2.0; ML  $\pm 0.4$ ; DV -1.2) using stereotaxic surgery under ketamine/dormitor anesthesia.

**Anxiety Measures.** The elevated-plus maze is a platform with two walled, closed arms and two nonwalled, open arms connected by an open center. The mice were placed onto the center between the plus maze arms and were recorded exploring the plus maze for 5 min. The open field was an open Plexiglass box. Mice were allowed to explore for 10 min. Activity data were obtained and analyzed using the Activity Software (Med Associates Inc.).

**Novel Object Recognition.** Mice explored an open-field box for 10 min each day for 3 days. On the following day, two identical objects were placed in the open-field box, and the mice explored objects for 5 min. Four hours later, one of the objects was replaced by a novel object, and mice explored the objects for 5 min. Twenty-four hours later, another novel object was introduced along with one familiar object, and mice explored the objects for 5 min. Time spent exploring objects was recorded.

**Cue-Dependent Fear Conditioning.** Mice were preexposed to conditioning chambers (San Diego Instruments) 3 days before training. During cued fear training, mice received five paired CS tones (30 s, 6 or 12 kHz, 90 db) and US shock (500 ms, 1.0 mA) trials with a 5-min intertrial interval (ITI). Startle

response to the shocks and percentage of time spent freezing to the tones was measured by SR-LAB software (San Diego Instruments).

**Cued Fear Expression, Extinction Training, and Retention.** The expression of fear memory was tested 24 h after fear conditioning in a novel context (modular test chambers; Med Associates Inc.). The mice were exposed to 3 (1-h test), 15 (24- and 72-h tests), and 50 (extinction and retention tests) CS tones with a 1.5-min ITI. Freezing during the tone presentations was measured with FreezeView software (Coulbourn Instruments). Extinction retention tests occurred 24 h after extinction training.

**In Situ Hybridization and Histology.** Mice were killed, and brains were collected, flash frozen on dry ice, sectioned on a cryostat (16  $\mu$ m/section), and stored at  $-80^{\circ}\text{C}$ . Cre expression and BDNF deletion were confirmed with in situ hybridization (28). In brief, slides were pretreated and hybridized with  $^{35}\text{S}$ -UTP labeled with antisense riboprobes for BDNF (exon 5) or Cre. After a stringent wash protocol, slides were exposed to Biomax MR film (Eastman Kodak Co.). For LacZ/XGal staining, slide-mounted tissue was fixed in 4% paraformaldehyde and incubated at  $37^{\circ}\text{C}$  overnight in 1 mg/mL X-Gal. For Nissl staining, slide-mounted tissue was counter stained with Cresyl violet.

**Western Immunoblotting.** The BDNF knockout mice were injected with 5 mg/kg 7,8-DHF i.p., and 2 h later, they were killed; cortex tissue was homogenated,

lysed in lysis buffer, and centrifuged, and the supernatant was collected. The normalized proteins were subjected to SDS/PAGE analysis and transferred to a nitrocellulose membrane. Western blotting analysis was performed with anti-TrkB Y816 (gift from Moses Chao, NYU School of Medicine, New York, NY) and TrkB antibodies (Cell Signaling Technology Inc.), both diluted at 1:1,000.

**Statistical Analyses.** Fear acquisition, expression, and extinction data were analyzed by two-way or three-way ANOVA with repeated measures, where appropriate. Other behavioral tests were analyzed by ANOVAs or Student's *t* test, where appropriate. Statistically significant main effects or interactions by ANOVA were followed by post hoc least-squares difference tests for multiple comparisons. Data are presented as mean  $\pm$  SEM; significance was set at  $P < 0.05$ .

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# Physiological markers of anxiety are increased in children of abused mothers

Tanja Jovanovic,<sup>1</sup> Ami Smith,<sup>1</sup> Asante Kamkwala,<sup>1</sup> James Poole,<sup>1</sup>  
Tara Samples,<sup>1,5</sup> Seth D. Norrholm,<sup>1,2</sup> Kerry J. Ressler,<sup>1,3,4</sup> and Bekh Bradley<sup>1,2</sup>

<sup>1</sup>Emory University School of Medicine, Dept of Psychiatry and Behavioral Sciences, Atlanta, GA, USA; <sup>2</sup>Atlanta VA Medical Center, Mental Health Service, Decatur, GA, USA; <sup>3</sup>Howard Hughes Medical Institute, Bethesda, MD, USA; <sup>4</sup>Yerkes National Primate Research Center, Atlanta, GA, USA; <sup>5</sup>Fielding Graduate University, Santa Barbara, CA, USA

**Background:** A growing number of studies indicate that low income, African American men and women living in urban environments are at high risk for trauma exposure, which may have intergenerational effects. The current study employed psychophysiological methods to describe biomarkers of anxiety in children of traumatized mothers. **Methods:** Study participants were recruited from a highly traumatized urban population, comprising mother-child pairs ( $n = 36$ ) that included school-age children. Mothers were assessed for childhood abuse with the Childhood Trauma Questionnaire, as well as symptoms of depression and posttraumatic stress disorder (PTSD). The children were measured for dark-enhanced startle responses and heart-rate variability. **Results:** Dark-enhanced startle was found to be higher in children whose mothers had high levels of childhood physical abuse, as compared to children whose mothers had low levels of physical abuse. During the habituation phase of the startle experiment, children whose mothers had high levels of childhood emotional abuse had higher sympathetic system activation compared to children of mothers with low emotional abuse. These effects remained significant after accounting for maternal symptoms of PTSD and depression, as well as for the child's trauma exposure. **Conclusion:** These results demonstrate that children of mothers who have history of childhood physical and emotional abuse have higher dark-enhanced startle as well as greater sympathetic nervous system activation than children of mothers who do not report a history of childhood physical and emotional abuse, and emphasize the utility of physiological measures as pervasive biomarkers of psychopathology that can easily be measured in children. **Keywords:** Child abuse, maternal trauma, child anxiety, startle response, heart-rate variability.

Childhood maltreatment has pervasive and detrimental neurobiological and psychological consequences; for recent review, see Heim, Shugart, Craighead, and Nemeroff (2010). Studies have suggested that abuse during childhood is associated with higher prevalence of adult mood and anxiety disorders (McCauley et al., 1997). The negative impact of early adverse events on the brain has been established for more than a decade: animal models as well as human clinical studies of early life stress have found long-term neurobiological effects (Carpenter et al., 2009; Heim et al., 2000; Plotsky & Meaney, 1993). Our laboratory has recently found that high levels of child abuse are associated with increased startle reactivity in adulthood (Jovanovic et al., 2009). Furthermore, different types of abuse (i.e., physical, sexual, emotional) appear to have differential effects on neurobiology; emotional and sexual, but not physical, abuse has been found to alter neuroendocrine function (Cicchetti, Rogosch, Gunnar, & Toth, 2010). On the other hand, we found that sexual and physical abuse, but not emotional abuse, were associated with psychophysiological changes (Jovanovic et al., 2009).

There is substantial evidence that maternal trauma exposure and subsequent psychopathology have transgenerational effects and are related to altered biological and psychological outcomes in their children. The effects of maternal trauma may be moderated by negative parenting behavior, parental psychopathology, shared trauma exposure, genetic or epigenetic risk (Grillon et al., 2005; Yehuda, Bell, Bierer, & Schmeidler, 2008). One study found that Holocaust survivors with post-traumatic stress disorder (PTSD) were more likely to engage in emotional abuse/neglect toward their children which, in turn, predicted alterations in their offspring's PTSD symptoms (Yehuda, Halligan, & Bierer, 2001a; Yehuda, Halligan, & Grossman, 2001b). Another study which examined women exposed to September 11 WTC attacks during pregnancy, reported that trauma exposure altered hypothalamic-pituitary-adrenal (HPA) axis function in their infants, suggesting in utero transmission (Yehuda et al., 2005). A recent study examined the effects of maternal child abuse on their infants' cortisol levels and found that children of mothers with a history of abuse had decreased baseline cortisol levels (Brand et al., 2010). Maternal behavior towards her child, such as increased impulsiveness, may mediate the relationship between the mother's history of

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abuse and negative child outcomes (Möhler et al., 2009).

There is a small body of literature on physiological outcomes in children for whom maternal mental illness and/or trauma exposure place them at increased risk for psychopathology. One of the only longitudinal studies of such vulnerability traits in offspring of probands with anxiety disorders found that high-risk offspring had greater autonomic reactivity and increased startle responses compared to low-risk offspring (Merikangas, Avenevoli, Dierker, & Grillon, 1999; Monk et al., 2001). Grillon and colleagues (Grillon, Dierker, & Merikangas, 1997) found that greater startle magnitude delineated children of parents with anxiety disorders from children of parents with alcohol abuse or dependence. A recent multi-generational study of patients with major depression found that heightened startle magnitude was evident in the 3rd generation of the probands, showing a pervasive transgenerational effect (Grillon et al., 2005). Similarly, maternal psychopathology appears to increase sympathetic nervous system activity in their offspring (Dierckx et al., 2009).

Several peripheral psychophysiological measures have been used as indices of hyper-arousal and anxiety, including the acoustic startle response (ASR) and heart-rate variability (HRV). These measures offer non-invasive methods for assessing neurobiological activity, such as neural activity within the central limbic system, and the autonomic nervous system. The acoustic startle response is characterized by an integrative, reflex contraction of the skeletal musculature in response to a sudden intense stimulus (Landis & Hunt, 1939). It is mediated by a simple subcortical three-neuron circuit (Davis, 1992), but is modulated by limbic brain structures such as the amygdala in fearful or anxiogenic situations (Davis, 1992). For example, delivering startle probes in the dark reliably increases startle responses in humans (Grillon, Pellowski, Merikangas, & Davis, 1997). This effect of darkness is an analogue of light-enhanced startle in rodents, which is dependent on the limbic structure of the bed nucleus of the stria terminalis (Walker & Davis, 1996). The availability of such animal models and ability to capitalize on the understanding of underlying neurocircuitry is a great advantage of the startle measure relative to electrodermal measures such as skin conductance. Furthermore, dark-enhanced startle requires no training or delivery of aversive stimuli, and is thus more tolerable for pediatric populations. HRV measures autonomic nervous system control of cardiac activity and can be assayed across different frequency bands using power spectral analysis; the high-frequency band (HF-HRV) is a measure of parasympathetic, or vagal inputs to the heart. The ratio of the low-frequency to the high-frequency band (LF/HF HRV ratio) is a measure of sympathovagal balance. These measures provide more detailed insight into cardiovascular

stress reactivity compared to heart-rate; they have frequently been measured in children and adolescents and have been associated with both acute anxiety (Arai et al., 2009; Hannesdóttir, Doxie, Bell, Ollendick, & Wolfe, 2010; Monk et al., 2001) as well as trait anxiety (Monk et al., 2001) and hyper-arousal (Greaves-Lord et al., 2007).

Based on the above-mentioned transgenerational neurobiological changes associated with maternal trauma, and our previous studies of altered startle reactivity in adults with a history of child maltreatment, we hypothesized that maternal child abuse history would be related to altered startle and autonomic nervous system responses in their children. This is the first study to examine these physiological markers in children of abused mothers; it is the first step to developing laboratory paradigms that measure phenotypes of risk in a highly traumatized population. This step is of great importance in assessing high-risk children for precursors of psychopathology that may lead to early interventions.

## Method

### *Study sample and psychological assessment*

Thirty-six children and their mothers participated in the study. The participants were recruited from the waiting rooms of the Primary Care, Obstetrics Gynecology or Child and Adolescent Psychiatry Outpatient Clinic (CAPOC) at the Grady Health System in Atlanta, GA. Inclusion criteria for the mothers were: 18–65 years of age, primary caretaker of a 6- to 13-year-old child, willing and able to sign informed consent; exclusion criteria were active psychosis, bipolar disorder, suicide ideation, and significant medical illness. Eligible child participants were between 6 and 13 years of age and willing to participate; exclusion criteria were autism spectrum disorders, bipolar or psychotic disorders, or cognitive disability. Prior to their participation, all mothers signed informed consent as well as parental permission for their children, and the children provided study assent approved by the Emory University Institutional Review Board and the Grady Research Oversight Committee.

The Structured Clinical Interview for DSM-IV (First, Spitzer, Williams, & Gibbon, 1995) was administered to all mothers. In addition to the diagnostic interview, all participants completed the Childhood Trauma Questionnaire (CTQ), the PTSD Symptom Scale (PSS), the Beck Depression Inventory (BDI), and the Traumatic Events Screening Inventory Parent Report (TESI). The CTQ is a self-report inventory assessing perceived childhood physical, sexual, and emotional abuse. Bernstein and Fink (1998) established scores for none, mild, moderate, and severe for each type of abuse. The data from the CTQ were used to classify participants into two categories for each type of abuse (physical, sexual, and emotional): (1) low abuse included those with CTQ scale scores in the 'none to mild' range, and (2) high abuse included those with CTQ scores in the 'moderate to severe' range. The PSS is a psychometrically valid 17-item self-report scale assessing PTSD

symptomatology over the two weeks prior to rating (Falsetti, Resnick, Resick, & Kilpatrick, 1993). The PSS provides a continuous measure of PTSD symptom severity and has been validated with the Clinician Administered PTSD Scale (CAPS; (Foa, Riggs, Dancu, & Rothbaum, 1993; Foa & Tolin, 2000). The BDI consists of a 21-item questionnaire (Beck, Ward, Mendelsohn, Mock, & Erbaugh, 1961). This instrument provides a well-validated, commonly used, continuous score of depressive symptoms. The TESI is a 24-item parent-report version of a structured clinical interview that inquires about the child's lifetime experience trauma exposure (Ribbe, 1996).

### *Data acquisition and experimental design*

The physiological data were acquired using Biopac MP150 for Windows (Biopac Systems, Inc., Aero Camino, CA). The acquired data were filtered, rectified, and smoothed using MindWare software (MindWare Technologies, Ltd., Gahanna, OH) and exported for statistical analyses. Startle data were collected by recording the eyeblink muscle contraction using the electromyography (EMG) module of the Biopac system. The startle response was recorded with two Ag/AgCl electrodes; one was placed on the orbicularis oculi muscle below the pupil and the other 1cm lateral to the first electrode. A common ground electrode was placed on the palm. Impedance levels were less than 6 kilo-ohms for each participant. The startle probe was a 108-dB(A)SPL, 40 ms burst of broadband noise delivered through headphones (Maico, TDH-39-P). The maximum amplitude of the eyeblink muscle contraction 20–200 ms after presentation of the startle probe was used as a measure of startle magnitude.

Heart-rate variability (HRV) data were acquired using the electrocardiogram (ECG) module of the Biopac system. ECG was recorded using two disposable Ag/AgCl electrodes pre-coated with electrolyte gel; one was placed on the right side of the upper torso, 1cm below the clavicle, and the second on the inside surface of the left wrist. Respiration was measured using a chest band transducer. HRV was quantified during one-minute intervals by spectral analysis of the time-sampled inter-beat interval series, according to the methods recommended by the Society for Psychophysiological Research Committee on Heart Rate Variability (Berntson et al., 1997). The LF/HF HRV ratio was derived from high-frequency HRV sampled from 0.12 to 0.40 Hz and low-frequency HRV sampled from 0.04 to 0.12 Hz.

The experimental paradigm began with a 2-minute acclimation period during which no startle probes were delivered, followed by a startle habituation segment, and a dark-enhanced startle segment that occurred without interruption. The startle habituation segment consisted of two blocks with four startle probes in each block, for a total of eight probes. Immediately following habituation, participants underwent the dark-enhanced segment consisting of two blocks each with eight startle probes. In each block, four startle probes were delivered in the dark phase and four were delivered in the light phase. The light and dark phases alternated, with each phase lasting one minute. The order of light and dark was counterbalanced across subjects. The lights in the startle booth were controlled

by a timer which was synchronized with the presentation of the startle probes. Across the entire experimental session, inter-trial intervals ranged from 9 to 22 seconds. The session duration was 8 minutes long (2 minutes acclimation, 2 minutes habituation, and 4 minutes dark-enhanced segment).

### *Statistical analyses*

The group variables in the analyses were derived from the abuse categories on the CTQ. For each type of abuse (physical, sexual, and emotional), subjects were divided into Low and High Maternal Abuse groups, according to the norms defined by Bernstein and Fink (1998). High Abuse included mothers who reported 'moderate to severe' levels of childhood abuse. If a mother reported high levels of abuse on more than one type of abuse, she was included in the High Abuse group for each category of abuse. The Low Abuse group included those with CTQ scale scores in the 'none to mild' range. We included the subjects with mild levels of abuse in the same category as those with no abuse due to the high prevalence of childhood trauma in this population.

Child demographic data such as age, and maternal clinical data such as PTSD and depression symptoms, were compared between the maternal abuse groups using analyses of variance (ANOVA). Baseline levels of startle magnitude and HRV during the habituation phase were compared between maternal abuse groups using 2-way mixed ANOVAs with Habituation Block (2 levels) as the within-subjects factor. The startle and HRV data during the dark-enhanced segment were analyzed in a 3-way mixed ANOVA, with Block (2 levels) and Phase (Light, Dark) as repeated measures factors and maternal Abuse (High, Low) as the between-groups factor. The dependent variables were startle magnitude and the LF/HF HRV ratio. Significant interactions were decomposed into respective univariate ANOVAs comparing diagnostic groups, with child sex and age used as covariates in the between-groups analyses of psychophysiological data in order to control for neurodevelopmental differences across age and between males and females (Gogtay et al., 2004). Finally, in order to examine the differential contributions of maternal abuse history and psychopathology to the dependent measures, we performed hierarchical regression analyses in which the child's age and sex, maternal symptoms of PTSD (PSS score) and depression (BDI score), and maternal childhood abuse levels (CTQ scores for Physical, Emotional, and Sexual Abuse) were added at each step to predict startle magnitude and HRV indices. Separate regression analyses were conducted for each dependent variable (LF/HF HRV ratio and Dark-enhanced startle).

In the repeated measures ANOVAs, we used the Sphericity Assumed statistic to correct for violations of the sphericity assumption. All analyses were performed in SPSS 18.0 for Windows (SPSS, Inc.) with an alpha level of 0.05.

## **Results**

### *Participant characteristics*

Thirty-six (18 male, 18 female) children and their mothers participated in the study. The age of the

children ranged from 6 to 13 years ( $M = 9.4$ ,  $SD = 2.1$ ). Of the 36 children, three stopped the session during the startle habituation phase, and another four children discontinued after the first dark-enhanced segment. The seven children that did not complete the startle sessions were younger than those that did complete ( $F(1,34) = 4.14$ ,  $p = 0.05$ ), but did not differ from the other 29 children on any other variables. Therefore, the final sample for the results of the habituation data and the first block of the dark-enhanced segment was 33, and for the second block of the dark-enhanced segment 29.

Three sets of analyses were performed – one for each type of abuse (Low vs. High Physical Abuse, Low vs. High Emotional Abuse, and Low vs. High Sexual Abuse). Table 1 shows descriptive and clinical data for each analysis. Across all abuse types, the children in the Low Abuse categories were younger than those in the High Abuse categories (Physical Abuse,  $F(1,32) = 5.78$ ,  $p = 0.02$ ; Emotional Abuse,  $F(1,32) = 7.96$ ,  $p < 0.01$ ; Sexual Abuse,  $F(1,32) = 6.41$ ,  $p = 0.02$ ). The distribution of boys and girls did not differ significantly between groups. As expected, the mothers in the High Abuse groups had significantly higher symptoms of PTSD and depression than mothers in the Low Abuse groups (Physical Abuse, PSS:  $F(1,32) = 5.79$ ,  $p = 0.02$ , BDI:  $F(1,32) = 6.69$ ,  $p = 0.02$ ; Emotional Abuse, PSS:  $F(1,32) = 6.28$ ,  $p = 0.02$ , BDI:  $F(1,32) = 13.06$ ,  $p = 0.001$ ; Sexual Abuse, PSS:  $F(1,32) = 15.89$ ,  $p < 0.001$ , BDI:  $F(1,32) = 11.13$ ,  $p = 0.002$ ). In order to account for possible moderating effects of these variables, we performed stepwise regression analyses (see below).

#### Physiological data: habituation phase

A repeated measures ANOVA of startle magnitude across the two Habituation Blocks and between High and Low Physical Abuse groups showed no effect of Block, no main effect of Abuse group, and no

interaction effect. The same was true for Emotional and Sexual Abuse groups. On the other hand, the analysis of LF/HF HRV ratio across the two Habituation Blocks revealed a significant interaction effect of maternal Physical Abuse and Block,  $F(1,31) = 6.26$ ,  $p = 0.02$ , but no main effects of Block or Abuse. High and Low Emotional Abuse also interacted significantly with Habituation Block on LF/HF HRV ratio,  $F(1,31) = 7.09$ ,  $p = 0.01$ . Maternal Sexual Abuse was not associated with LF/HF HRV ratio either as an interaction effect or as a main effect.

Follow-up univariate analyses of LF/HF HRV ratio within each Habituation Block demonstrated that children of mothers with High Physical Abuse tended to have greater ratios ( $M = 1.88$ ,  $SE = 0.48$ ) than children of mothers with Low Physical Abuse ( $M = 0.68$ ,  $SE = 0.33$ ) during the first Block,  $F(1,32) = 3.88$ ,  $p = 0.06$ . Similarly, children of mothers with High levels of Emotional Abuse had significantly higher ratios ( $M = 2.31$ ,  $SE = 0.53$ ) than those of mothers with Low levels of Emotional Abuse ( $M = 0.62$ ,  $SE = 0.31$ ) during the first Block,  $F(1,32) = 7.10$ ,  $p = 0.01$ . There were no group differences during the second Habituation Block. We repeated this analysis using a univariate ANCOVA with child demographics (age and sex), maternal psychopathology (PSS and BDI), as well as child trauma exposure on the TESI, as covariates, and the effect of maternal abuse remained significant,  $F(1, 26) = 5.29$ ,  $p = 0.03$ . These results suggest that children of mothers with a history of high levels of abuse have greater sympathetic nervous system activation relative to parasympathetic activation.

In order to examine the differential contributions of maternal abuse and psychopathology on the above effects, we entered child demographics, maternal psychopathology, and maternal abuse history as predictors in a stepwise hierarchical regression model. In the first analysis, the dependent variable was the LF/HF HRV ratio during the first block of

**Table 1** Demographic and clinical data for the sample, divided between childhood abuse groups

Maternal Abuse Group	Child demographics		Maternal psychopathology	
	Age	Sex	PTSD	Depression
Physical Childhood Abuse	<i>M ± SD</i>	<i>% female</i>	<i>M ± SD</i>	<i>M ± SD</i>
Low ( $n = 22$ )	8.9±2.0	54.5	11.5±11.0	17.3±10.0
High ( $n = 11$ )	10.7±2.0*	45.5	22.3±13.9*	28.5±14.7*
Emotional Childhood Abuse	<i>M ± SD</i>	<i>% female</i>	<i>M ± SD</i>	<i>M ± SD</i>
Low ( $n = 24$ )	8.9±2.1	54.2	11.9±10.7	16.7±9.7
High ( $n = 9$ )	11.1±1.4*	44.4	23.7±15.0*	32.2±13.6**
Sexual Childhood Abuse	<i>M ± SD</i>	<i>% female</i>	<i>M ± SD</i>	<i>M ± SD</i>
Low ( $n = 17$ )	8.6±2.2	52.9	7.7±7.9	14.8±8.5
High ( $n = 16$ )	10.4±1.8*	50.0	22.7±12.8**	27.7±13.3**

The High and Low Maternal Abuse groups differ significantly in age of the child and the degree of maternal psychopathology. Childhood trauma was assessed using the Childhood Trauma Questionnaire (CTQ); PTSD symptoms were assessed using the PTSD Symptom Scale (PSS); Depression symptoms were assessed using the Beck Depression Inventory (BDI).

\* $p < 0.05$ ; \*\* $p < 0.01$ .

**Table 2** Regression analysis of predictors of increased sympathovagal balance

Habituation LF/HF HRV ratio Model	$R^2$	$R^2$ change	$F$ Change	$p$
1. Child's demographics	0.02	0.02	0.34	0.71
2. Maternal psychopathology	0.20	0.18	2.95	0.07
3. Maternal childhood physical abuse	0.25	0.05	1.80	0.19
4. Maternal childhood emotional abuse	<b>0.46</b>	<b>0.21</b>	<b>9.48</b>	<b>0.005</b>
5. Maternal childhood sexual abuse	0.46	0.00	0.02	0.90

Hierarchical regression analyses examining the contributions of child demographics (age, sex), degree of maternal psychopathology (PTSD, MDD), and level of each type of maternal childhood abuse on LF/HF HRV ratio in children during the first block of the experiment. Abbreviations: PTSD: posttraumatic stress disorder; MDD: major depression disorder; LF/HF HRV: low frequency to high frequency heart-rate variability.

Habituation. Table 2 shows the results of the regression analysis, which showed that after controlling for other variables, maternal Emotional Abuse accounted for 20.6% of the variance in HRV,  $F_{\text{change}}(1,25) = 9.48$ ,  $p = 0.005$ , while neither Physical nor Sexual Abuse had significant effects. In order to examine the effect of the child's own trauma exposure, we repeated the regression analyses and added maternal report of the child's exposure to trauma (TESI) in the final step. This analysis revealed an even stronger relationship between maternal childhood Emotional Abuse and increased sympathetic activation after controlling for the child's trauma  $F_{\text{change}}(1,20) = 14.88$ ,  $p = 0.001$ .

#### Physiological data: dark vs. light phase

A 3-way repeated measures ANOVA of startle magnitude across the two Dark-enhanced Blocks (2 levels) as well as Phase (Dark vs. Light), between High and Low Physical Abuse groups, showed a trend level effect of Block,  $F(1,27) = 3.32$ ,  $p = 0.08$ , and a significant main effect of Phase,  $F(1,27) = 14.19$ ,  $p = 0.001$ , as well as a significant interaction effect of Phase and Physical Abuse,  $F(1,27) = 4.86$ ,  $p = 0.04$ , see Table 3. For the other two types of abuse, there was also a significant effect of Phase, but no interaction of maternal Abuse group and Phase. On the

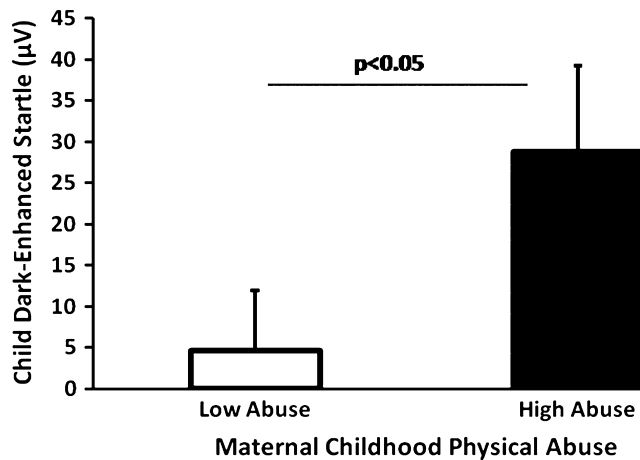
other hand, the analysis of LF/HF HRV ratio across the two Dark-enhanced Blocks and Phase revealed a significant 3-way interaction effect of maternal Physical Abuse and Phase and Block,  $F(1,26) = 4.13$ ,  $p = 0.05$ , but no main effects of Block, Phase, or Abuse. High and Low Emotional Abuse also showed a 3-way interaction on LF/HF HRV ratio,  $F(1,26) = 4.03$ ,  $p = 0.05$ . Maternal Sexual Abuse was not associated with LF/HF HRV ratio either as an interaction effect or as a main effect. The 3-way analyses were followed up by 2-way ANOVAs within each Block, given that four children discontinued the session after the first block.

A 2-way analysis of Phase (Dark vs. Light) and maternal Physical Abuse during the first Block revealed a significant interaction effect,  $F(1,29) = 5.80$ ,  $p = 0.02$ , with children of High Abuse mothers showing a greater increase in startle during the Dark Phase compared to children of mothers with Low levels of abuse. There were no group or interaction effects in the second block. The 2-way interaction was followed up by examining the relative increase during the Dark vs. Light phase in each group. In order to compare the change in startle magnitude between groups and control for individual differences in startle response, we calculated a difference score (startle during Dark Phase minus startle during Light Phase) for the first block. A univariate

**Table 3** Child startle results across experimental conditions (Dark vs. Light Phase) in the first block and groups (High vs. Low Maternal Abuse)

Maternal Abuse Group	Child startle magnitude ( $\mu V$ )				Effect		
	Light		Dark		Phase	Abuse	Phase $\times$ Abuse
Physical Childhood Abuse	$M$	SE	$M$	SE	$p = 0.01$	ns	$p = 0.02$
Low ( $n = 22$ )	40.7	9.0	45.4	8.7			
High ( $n = 11$ )	46.3	12.7	75.1	12.4			
Emotional Childhood Abuse	$M$	SE	$M$	SE	$p = 0.01$	ns	$p = 0.04$
Low ( $n = 24$ )	43.6	8.6	50.3	8.7			
High ( $n = 9$ )	39.8	14.0	68.7	14.2			
Sexual Childhood Abuse	$M$	SE	$M$	SE	$p = 0.05$	ns	ns
Low ( $n = 17$ )	44.4	10.2	51.3	10.5			
High ( $n = 16$ )	40.6	10.5	59.6	10.8			

Startle magnitude was greater in the dark phase; for physical and emotional abuse, there was a significant Phase  $\times$  Abuse group interaction.



**Figure 1** Mean dark-enhanced startle (difference score between Dark and Light Phases) during the first block in children of mothers with High and Low Physical Abuse

analysis of the difference score between Abuse groups found a significantly greater difference between Dark and Light in the High Abuse group compared to the Low Abuse group,  $F(1,32) = 6.00$ ,  $p = 0.02$  (see Figure 1). We repeated this analysis using a univariate ANCOVA with child demographics (age and sex), maternal psychopathology (PSS and BDI), as well as child trauma exposure on the TESI, as covariates, and the effect of maternal abuse remained significant,  $F(1, 26) = 6.61$ ,  $p = 0.02$ . Two-way ANOVAs of Phase and Abuse on LF/HF HRV ratio separately within each Block of the Dark-enhanced segment found no significant interaction or main effects of either Physical or Emotional Abuse.

A regression analysis using the same predictors as above and the difference score between the Dark and Light phase as the dependent variable was performed. Table 4 shows the results of this analysis, with maternal Physical Abuse alone accounting for 16% of the variance in Dark-enhanced startle,  $F_{\text{change}}(1,26) = 5.55$ ,  $p = 0.03$ , while neither Emotional nor Sexual Abuse had significant effects. Again, the child's trauma exposure strengthened the association between maternal trauma and the child's Dark-enhanced startle,  $F_{\text{change}}(1,20) = 8.81$ ,  $p = 0.008$ . Since controlling for the child's trauma

did not reduce or eliminate the effect on child's startle, these data suggest that maternal, rather than child, trauma is accounting for the observed effect.

## Discussion

In this study of children growing up in low-income urban environments at high risk for trauma exposure and subsequent psychopathology, maternal childhood physical abuse was related to increased dark-enhanced startle in their offspring. In addition, childhood emotional abuse of the mother predicted increased sympathetic nervous system activation to the startle probe in her children. On the other hand, maternal sexual abuse was not associated with psychophysiological markers in the children. Importantly, the relationship between maternal abuse and child psychophysiology was not accounted for by either maternal psychopathology or the child's own trauma exposure.

A growing body of literature suggests familial aggregation of anxiety disorders (e.g., (Cooper, Fearn, Willetts, Seabrook, & Parkinson, 2006; Last, Hersen, Kazdin, Orvaschel, & Perrin, 1991), as well a 'vicious cycle' of abuse from one generation to the next (Egeland, Jacobvitz, & Sroufe, 1988). Intergenerational transmission of psychopathology has been found in several studies examining the effects of maternal PTSD (Yehuda et al., 2001a, 2001b) and depression on the presence of anxiety symptoms in first and even second generation offspring (Warner, Wickramaratne, & Weissman, 2008). This transmission likely involves both behavioral and biological mechanisms; for example, mothers with a history of child abuse show alterations in their responses to their own children (Möhler et al., 2009) as well as child-relevant stimuli (Casanova, Domanic, McCanne, & Milner, 1994). Such disruptions in the interactions between abused mothers and their children could result in heightened child anxiety. On the other hand, biological mechanisms are implicated in studies showing that trauma exposure in mothers alters HPA axis function in their children (Brand et al., 2010; Yehuda & Bierer, 2008; Yehuda et al., 2005), which has been found to be a risk factor for developing PTSD (Yehuda, 2002). Aside from this

**Table 4** Regression analysis of predictors of increased dark-enhanced startle

Dark-enhanced startle MODEL	$R^2$	$R^2$ change	$F$ change	$p$
1. Child's demographics	0.03	0.03	0.52	0.60
2. Maternal psychopathology	0.09	0.06	0.85	0.44
3. Maternal childhood physical abuse	<b>0.25</b>	<b>0.16</b>	<b>5.55</b>	<b>0.03</b>
4. Maternal childhood emotional abuse	0.25	0.00	0.04	0.84
5. Maternal childhood sexual abuse	0.32	0.06	2.20	0.15

Hierarchical regression analyses of potential predictors of increased dark-enhanced startle, examining the contributions of child demographics (age, sex), degree of maternal psychopathology (PTSD, MDD), and level of each type of maternal childhood abuse on dark-enhanced startle in children during the first block of the experimental session. Abbreviations: PTSD: posttraumatic stress disorder; MDD: major depression disorder.

small number of neuroendocrine findings, no other research studies that we are aware of have found effects of maternal trauma on physiological markers of anxiety in their children.

The physiological markers that were affected by maternal trauma in this study, namely, dark-enhanced startle and LF/HF HRV ratio, have been associated with arousal and anxiety. Dark-enhanced startle is the human analogue of light-enhanced startle in rodents, which is dependent on nuclei in the limbic system (Walker & Davis, 1996). This startle index is elevated in adults with PTSD and trauma survivors without PTSD (Grillon, Morgan, Davis, & Southwick, 1998) as well as children at risk for anxiety disorders (Grillon, Dierker et al., 1997). LF/HF HRV ratio is related to the balance between the sympathetic and parasympathetic components of the autonomic nervous system – in addition to being an acute measure of arousal, this is also an index of cardiovascular health (Monk et al., 2001). It is possible that maternal trauma increases risk of negative mental and physical health outcomes in their children. A recent meta-analysis of HRV studies suggests that sympathetic drive is higher than parasympathetic tone in anxiety disorders (Cohen & Benjamin, 2006), a finding that is consistent with heightened LH/HF HRV ratio as a marker of anxiety in our study of children of abused mothers. We found that this measure was specifically associated with high levels of emotional abuse experienced by the mother in her childhood, while dark-enhanced startle was related to physical maternal abuse. There is an emerging body of research on the ways in which specific types of childhood abuse demonstrate differential effects on neurobiology (Cicchetti et al., 2010). For example, Carpenter and colleagues (Carpenter et al., 2009) found that emotional abuse and age at time of abuse predicted reactivity to cortisol in adulthood. In our previous study of adults with a history of childhood abuse, we found that sexual and physical abuse, but not emotional abuse, increased startle responses (Jovanovic et al., 2009).

Dark-enhanced startle was significantly elevated in offspring of abused mothers during the first block of the session, but not the second block. The discrepancy between Block 1 and Block 2 could be due to a tendency for the startle response to habituate from one block to the next; although the average startle magnitude in the dark was still greater than the light in the second block, overall all startle responses were reduced. It is possible that the initial impact of darkness has the greatest effect on startle. Notably, the four children who discontinued the session did not account for the effect of darkness in the first block. The effect remained even after the four children were removed from the analysis of the first block. The advantage of the main effects being present in the initial phase of the experiment is that the session could be potentially shortened, thereby making it even more tolerable to children.

It is important to note that maternal PTSD and depression symptoms, while increased in the mothers with high compared to low levels of childhood abuse, did not account for the increased physiological activation in their children. After controlling for maternal psychopathology, it was maternal trauma that predicted the children's responses. Furthermore, the effects were also not accounted for by the child's own trauma exposure. Given that mothers with childhood trauma may raise their children in environments that increase risk of trauma exposure, this would be a likely candidate for the effects of intergenerational transmission of psychopathology. However, the maternally reported trauma exposure of the child did not contribute to the child's physiological responses. Clearly, a limitation of the study is reliance of maternal report for the measure of child trauma levels. Specifically, it is possible that mothers withheld information about overly harsh or abusive parenting. Therefore, one possible explanation for our data is that maternal history of abuse may have led to increased levels of harsh verbal and/or physical punishment towards the child resulting in increased stress reactivity (as indicated by increased dark-enhanced startle and increased sympathetic nervous system activation to the startle probe). Our future research will include interviews with the children in the absence of the mothers, to get a more direct measure of child trauma exposure.

A limitation of the study is the use of a retrospective self-report measure of child abuse. While this instrument has been validated in previous studies (Bernstein et al., 2003; Bradley et al., 2008), there may be inherent reporting bias in adult assessments of childhood trauma history. Given that we do not have longitudinal, objective measures of child abuse we are relying on the subjects' perception of their abuse history. However, given the high rates of trauma exposure, substance abuse, and history of incarceration in this low-income population, it is likely that the reported abuse is accurate. The CTQ has shown very good convergence with other measures of child abuse, indicating that it is a valid instrument (Bernstein et al., 1994). Furthermore, this subjective perception may in fact be related to individual sensitivity, which in itself may be a risk factor for psychopathology. In order to tease these contributing factors apart, future studies may incorporate a more direct measure of child abuse and utilize prospective approaches.

Additional limitations of the study are the small sample size, and the lack of inclusion of clinical anxiety measures for the children. Follow-up studies will collect these measures to see if they correlate with the physiological indices. Of interest will also be to collect measures of sleep quality in the children to see whether darkness has relevance in their daily functioning. Here we present our preliminary and highly novel results of the association between

maternal trauma and child physiological markers that have been previously associated with anxiety (Dierckx et al., 2009; Grillon, Dierker, and Merikangas, 1997a; Monk et al., 2001; Waters et al., 2008). The low-income African American population under investigation is at high risk for both trauma exposure and anxiety disorders. Anxiety disorders begin to develop in children as young as 5 years old (Paus, Keshavan, & Giedd, 2008) – investigating early biomarkers of these disorders is a high research priority in the field of psychiatry.

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## Correspondence to

Tanja Jovanovic, Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, 49 Jesse Hill Jr Dr, Atlanta, GA 30303, USA; Tel: +1 404 778-1485; Email: tjovano@emory.edu

## Key points

- Prior studies have found that heightened dark-enhanced startle responses and dysregulated sympathovagal balance are associated with anxiety in adults and children.
- The current study found that maternal childhood physical abuse was associated with heightened dark-enhanced startle in their children during the initial phase of the experiment.
- The current study found maternal childhood emotional abuse was associated with heightened sympathetic nervous system activity in their children.
- The effects of maternal childhood trauma were not accounted for by maternal PTSD or depression, or the child's own trauma exposure.
- This is the first study to find transgenerational effects of maternal trauma on psychophysiological markers of anxiety in children.

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# Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor

Kerry J. Ressler<sup>1,2,4</sup>, Kristina B. Mercer<sup>1</sup>, Bekh Bradley<sup>2,3</sup>, Tanja Jovanovic<sup>2</sup>, Amy Mahan<sup>4</sup>, Kimberly Kerley<sup>1</sup>, Seth D. Norrholm<sup>2,3</sup>, Varun Kilaru<sup>2</sup>, Alicia K. Smith<sup>2</sup>, Amanda J. Myers<sup>5</sup>, Manuel Ramirez<sup>5</sup>, Anzhelika Engel<sup>5</sup>, Sayamwong E. Hammack<sup>6</sup>, Donna Toufexis<sup>4,6</sup>, Karen M. Braas<sup>7</sup>, Elisabeth B. Binder<sup>2,8</sup> & Victor May<sup>7</sup>

Pituitary adenylate cyclase-activating polypeptide (PACAP) is known to broadly regulate the cellular stress response. In contrast, it is unclear if the PACAP–PAC1 receptor pathway has a role in human psychological stress responses, such as post-traumatic stress disorder (PTSD). Here we find, in heavily traumatized subjects, a sex-specific association of PACAP blood levels with fear physiology, PTSD diagnosis and symptoms in females. We examined 44 single nucleotide polymorphisms (SNPs) spanning the PACAP (encoded by *ADCYAPI*) and PAC1 (encoded by *ADCYAP1R1*) genes, demonstrating a sex-specific association with PTSD. A single SNP in a putative oestrogen response element within *ADCYAP1R1*, rs2267735, predicts PTSD diagnosis and symptoms in females only. This SNP also associates with fear discrimination and with *ADCYAP1R1* messenger RNA expression in human brain. Methylation of *ADCYAP1R1* in peripheral blood is also associated with PTSD. Complementing these human data, *ADCYAP1R1* mRNA is induced with fear conditioning or oestrogen replacement in rodent models. These data suggest that perturbations in the PACAP–PAC1 pathway are involved in abnormal stress responses underlying PTSD. These sex-specific effects may occur via oestrogen regulation of *ADCYAP1R1*. PACAP levels and *ADCYAP1R1* SNPs may serve as useful biomarkers to further our mechanistic understanding of PTSD.

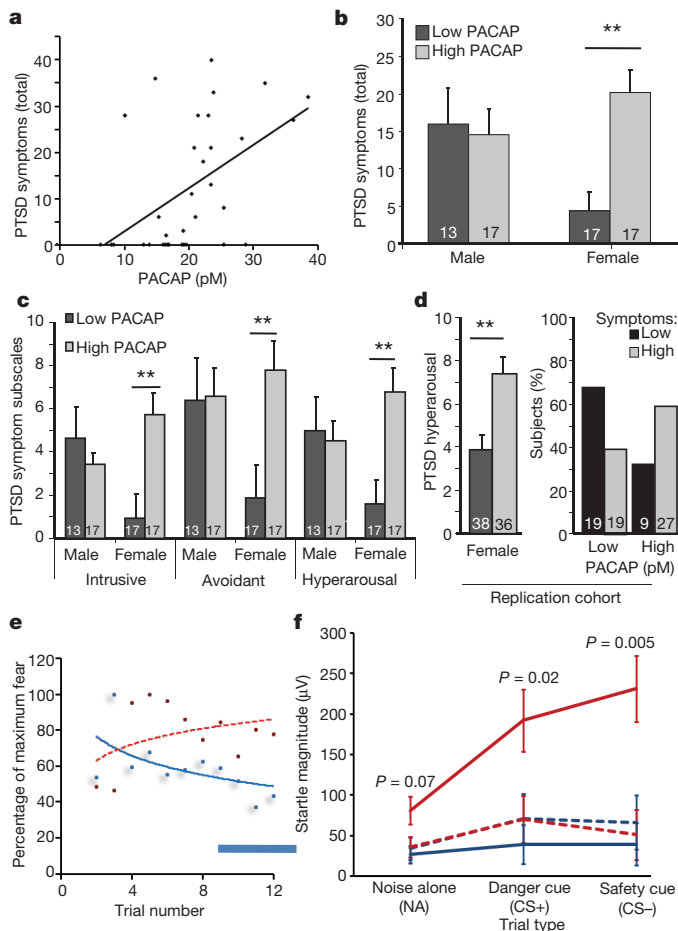
PACAP was first isolated from ovine hypothalamic extracts based on its ability to stimulate cyclic AMP production in anterior pituitary cells<sup>1</sup>. It is a highly conserved member of the VIP/secretin/glucagon peptide family, with pleiotropic functions in development, cell signalling, metabolism, homeostasis and cell protection<sup>2–6</sup>. Among these myriad functions, studies have demonstrated (1) high expression of PACAP peptide and its selective PAC1 receptor in hypothalamic and limbic structures, (2) PACAP regulation of corticotropin releasing hormone and autonomic function, (3) actions of PACAP in stress-related behaviour, (4) reduced anxiety-like phenotypes in PACAP and PAC1 receptor null mice, and (5) blunted corticosterone response in knockout animals after emotional stressors. Thus, PACAP–PAC1 receptor signalling is integrally involved in stress mechanisms<sup>7,8</sup>. We hypothesized that PACAPergic systems may be important mediators of abnormal stress responses following psychological trauma contributing to PTSD, which is an extreme maladaptive and debilitating psychiatric disorder affecting up to 40% of individuals over lifetime exposure to traumatic events<sup>9,10</sup>.

Little is known about the biological processes regulating PTSD and other stress-related responses. To examine whether the PACAP–PAC1 pathway is associated with PTSD in a high risk, heavily traumatized population, we analysed blood levels of PACAP, and genetic variation and methylation of the PACAP (*ADCYAPI*) and PAC1 receptor (*ADCYAP1R1*) genes, in a cohort of more than 1,200 highly traumatized subjects with and without PTSD (see Supplementary Tables 1 and 2 for demographic information).

## PACAP levels associated with PTSD in females

Using radioimmunoassay, we first examined PACAP peptide levels in peripheral blood samples from a previously described, highly traumatized, at risk population<sup>11–13</sup> that had been matched on age, sex, and trauma histories ( $n = 64$ , see Supplementary Tables 1–3 for demographics). We found that PTSD symptoms (PTSD symptom scale<sup>14</sup>) were significantly correlated with PACAP38 (PACAP peptide containing 38 residues) blood levels in females ( $P < 0.005$ ,  $r = 0.497$ , Fig. 1a), but not in males ( $P > 0.5$ ). Also in females, PTSD diagnosis was associated with PACAP38 levels ( $P \leq 0.001$ ), with higher PACAP38 found in the PTSD cohort. Furthermore, PACAP levels (median split, low versus high) were differentially associated with PTSD symptoms in females (Fig. 1b). PACAP38 levels also predicted differential response on all three symptom clusters necessary to fulfil diagnostic criteria for PTSD (intrusive re-experiencing (for example, trauma flashbacks), avoidance (for example, avoidance of trauma reminders) and hyperarousal (for example, increased startle response)) in females but not males (Fig. 1c). These analyses were repeated in a second, all female cohort ( $N = 74$ ) with similar findings (Fig. 1d; high versus low PACAP38 levels, controlling for age, substance abuse and total trauma exposure, one-tailed  $t$ -tests: total symptoms,  $P \leq 0.05$ , hyperarousal symptoms,  $P \leq 0.001$ ; and percentage with clinically significant symptoms,  $\chi^2 = 4.9$ ,  $P < 0.05$ ). These observations were especially notable, as females may be at twice the risk for PTSD as compared to males<sup>9,11</sup>, implicating roles for sex hormones, especially oestrogen, in the disorder<sup>15–17</sup>. When we controlled for

<sup>1</sup>Howard Hughes Medical Institute, Chevy Chase, Maryland 20815, USA. <sup>2</sup>Department of Psychiatry and Behavioral Sciences, Emory University School of Medicine, Atlanta, Georgia 30322, USA. <sup>3</sup>Atlanta VA Medical Center, Atlanta, Georgia 30033, USA. <sup>4</sup>Yerkes National Primate Research Center, Atlanta, Georgia 30329, USA. <sup>5</sup>University of Miami, Miller School of Medicine, Miami, Florida 33136, USA. <sup>6</sup>Department of Psychology, University of Vermont, Burlington, Vermont 05401, USA. <sup>7</sup>Departments of Anatomy and Neurobiology and Pharmacology, University of Vermont College of Medicine, Burlington, Vermont 05401, USA. <sup>8</sup>Max Planck Institute of Psychiatry, Munich 80804, Germany.



**Figure 1 | PACAP blood levels predict PTSD symptoms in females.** **a**, PTSD symptoms (scale range 0–51), relative to plasma PACAP38 blood levels (pM); ( $N = 34$  females;  $r = 0.497$ ,  $P \leq 0.005$ ). **b**, Total PTSD symptoms plotted relative to sex and levels of plasma PACAP38 ( $N = 64$ , low:  $<20$  pM, high:  $>20$  pM); females with high PACAP blood levels have increased symptoms (\*\* $P < 0.0005$ ). **c**, PACAP levels (low versus high) are also differentially associated with PTSD intrusive, avoidance and hyperarousal symptoms in females ( $N = 64$ , \*\* $P < 0.005$ ). **d**, PACAP levels (low versus high) were examined in a replication sample of highly traumatized women, with differential association in hyperarousal symptoms (left,  $N = 74$ , \*\* $P = 0.002$ ) and in the percentage of subjects with significant symptoms (right,  $\chi^2 = 4.9$ ,  $P < 0.05$ ). **e**, Acoustic startle reflex (EMG) relative to the fear conditioning trial in subjects without PTSD (blue) versus with PTSD (red). Habituation is seen in non-PTSD subjects during late acquisition (bar). **f**, Startle magnitude during the late acquisition period versus trial type (noise alone, CS+ and CS–), showing that females with high PACAP levels show enhanced startle responses to both fear cues (CS+,  $P = 0.02$ ) and safety cues (CS–,  $P = 0.005$ ) ( $N = 27$ ; 16 male, 11 female). Dashed lines, low PACAP; solid lines, high PACAP; blue, male; red, female. Bars represent mean  $\pm$  s.e.m.,  $N$  values for each group at bottom of bar graphs.

common stress-related phenomena (depression and history of substance abuse), the effect of PACAP level on PTSD remained ( $P < 0.05$ ). In contrast, there was no effect of PACAP level on depression symptoms or substance abuse when controlling for PTSD.

In addition to the psychological symptoms that define the syndrome, subjects with PTSD have been found to have abnormally high conditioned fear responses. This high level of fear may result from a combination of an inability to habituate to aversive stimuli, a decreased ability to extinguish (learn to inhibit) fear memories, and possibly an over-consolidation of the original fear memory<sup>18–22</sup>. Hence, we determined the physiological (electromyographic) levels of conditioned fear for 27 participants (16 male, 11 female) with PACAP blood levels. Fear potentiation was determined by measuring

the acoustic startle reflex in the presence of startle cues alone, or startle cues combined with stimuli paired (conditioned stimulus, CS+) or unpaired (CS–) with an aversive airblast. Female (but not male) subjects with high PACAP38 levels demonstrated markedly increased startle reflex responses to both CS+ ( $P = 0.02$ ) and CS– ( $P = 0.005$ ) cues. This was particularly pronounced during the late acquisition phase when normal subjects had habituated to the fearful stimuli (Fig. 1e, f). In aggregate, these data suggest that PACAP38 peptide is strongly associated with the psychological and physiological symptoms of PTSD in women with a history of trauma.

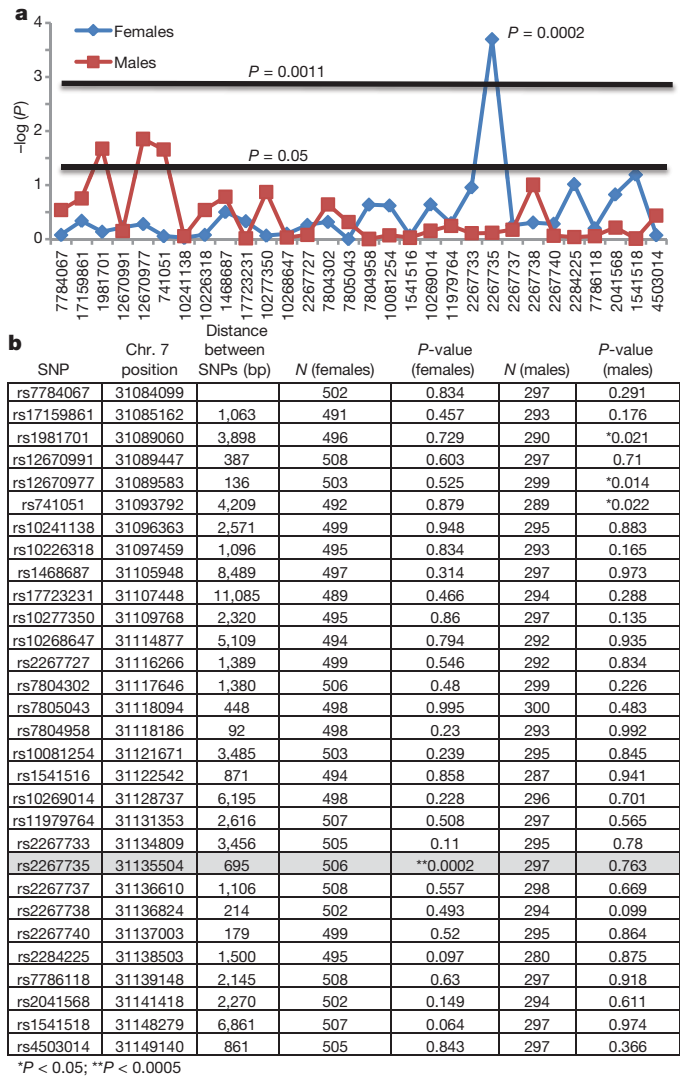
### ADCYAP1R1 associated with PTSD in females

To assess whether there may be a genetic association of PTSD with polymorphisms in either the PACAP (*ADCYAP1*) or PAC1 receptor (*ADCYAP1R1*) locus, we performed a tag-SNP analysis ( $r^2 = 0.8$ ; minor allele frequency (MAF) = 0.1) across both genes with a total of 44 SNPs (14 *ADCYAP1* and 30 *ADCYAP1R1* SNPs). Using logistic regression, we examined whether each SNP was associated with PTSD diagnosis in this cohort of highly traumatized urban civilian subjects ( $n = 798$ )<sup>11,12,23</sup>, in total, or stratified by gender (females:  $n = 503$ ; males:  $n = 295$ ). Only the *ADCYAP1R1* receptor SNP rs2267735 ( $P = 0.0002$  in females; NS in males) remained significant after experiment-wide multiple correction for sex and 44 independent tests (Fig. 2a and b, Supplementary Fig. 1). No SNPs in the peptide *ADCYAP1* gene met experiment-wide criteria for association (Supplementary Fig. 2). Given these striking gender differences and recent data demonstrating that *ADCYAP1R1* gene expression may be dynamically modulated by oestrogen<sup>24</sup>, the distribution of oestrogen response elements (EREs) within the *ADCYAP1R1* gene was examined (Supplementary Table 4). We found that rs2267735 was within a predicted ERE (Fig. 2c, Genomatix; matrix similarity = 0.877, core similarity = 1.0). Because rs2267735 is positioned within the central variable region of the consensus sequence, *in silico* analyses do not currently allow us to predict how the ‘C’ versus ‘G’ allele may differentially alter ERE function, and further *in vitro* analyses are warranted.

We next determined if the association between rs2267735 and PTSD diagnosis could be replicated in an additional 439 subjects. These subjects were from the same overall study, but were interviewed and had DNA collected after the original discovery population. Thus they served as a replication source from the same population but distinct in time and with different interviewing staff. The table in Fig. 3a shows the logistic regression results for males and females separately in the initial population described in the tag-SNP analysis, the replication sample from the same population, and the combined sample of 1,237 individuals. The main effect of the SNP on PTSD diagnosis could be replicated in women ( $P < 0.05$ ) and combining both samples increased the significance of the association ( $N = 763$ ,  $P < 0.00002$ ). As in the discovery sample, no effects were observed in males (male combined sample  $N = 474$ ,  $P = 0.7$ ).

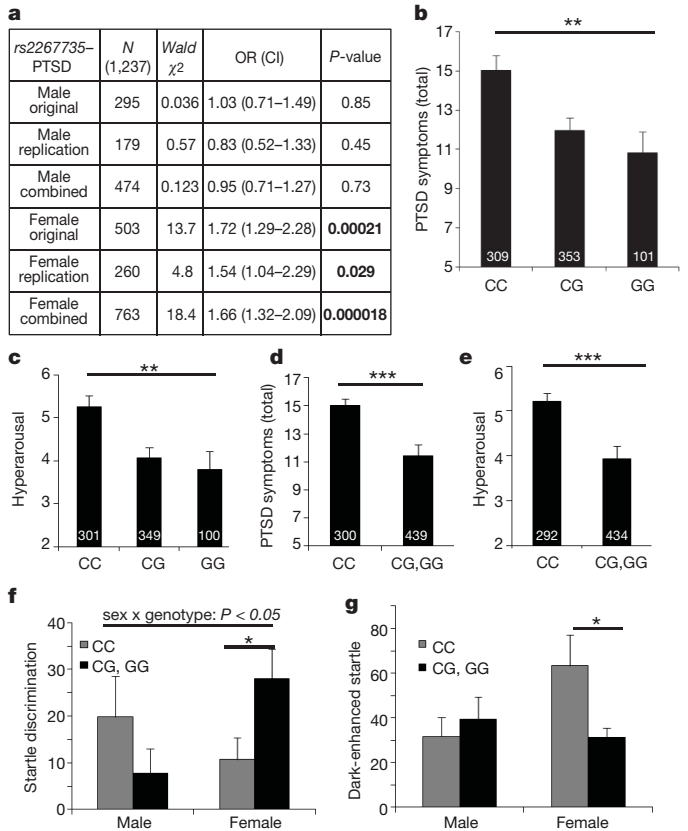
To further examine *ADCYAP1R1* rs2267735 SNP associations with continuous PTSD symptom levels in females, we analysed both an additive and a dominant model with total PTSD symptoms and symptom subscales using the combined samples (Fig. 3b–e). The ‘CC’ genotype was most robustly associated with total PTSD symptoms, and among subscales, hyperarousal symptoms were the most strongly associated with rs2267735. Notably, even after controlling for childhood trauma history and adult trauma, age and race (which slightly reduces total  $N$  owing to missing data), the rs2267735 ‘CC’ genotype was associated with higher levels of PTSD hyperarousal symptoms compared to ‘G’ carriers in women ( $P = 0.0008$ , Fig. 3e), but not men ( $P = 0.51$ ).

We repeated the above analyses with Beck depression inventory (BDI) symptoms and history of life-time substance abuse, and found no associations with these measures and rs2267735 (Supplementary Fig. 3), suggesting that this association may be relatively specific to PTSD. To address whether rs2267735 might be associated with other severe psychiatric illnesses, we performed analyses using bipolar



**Figure 2 | Genetic association of PAC1 receptor (*ADCYAP1R1*) with PTSD.** **a**, 30 SNPs spanning the *ADCYAP1R1* gene (*x* axis), with the  $-\log(P)$ -value of logistic regression for each SNP association with PTSD (diagnosis based on DSM-IV criteria from PTSD Symptom Scale). Subjects were analysed with logistic regression in females only ( $N = 503$ ) or males only ( $N = 295$ ). Horizontal lines represent the nominal  $P = 0.05$  or the corrected  $P$ -value,  $P = 0.0011$  (44 SNPs, correcting for 30 *ADCYAP1R1* SNPs and 14 *ADCYAP1* SNPs; Supplementary Fig. 1). rs2267735 is the only SNP remaining significant after multiple corrections ( $P = 0.0002$ ). **b**, Table of  $P$ -values resulting from the association of each genotyped, *ADCYAP1R1* SNP with PTSD diagnosis (by gender). The location on chromosome 7 for each SNP including the distance (bp) between the SNPs is given. The average distance between SNPs is 2.5 kb. SNP rs2267735 is located in an intron of *ADCYAP1R1*, and is not in linkage disequilibrium with other SNPs (for African Americans in our population, data not shown). **c**, rs2267735 (C/G), in red, is located within a canonical oestrogen response element (ERE) binding site (capital letters, conserved canonical ERE nucleotides; blue letters, mismatches with the *ADCYAP1R1* gene and canonical ERE; reverse strand shown).

disorder, schizophrenia, and Alzheimer's disease samples. From the data of the Genetic Association Information Network (GAIN) publicly accessible database (<http://www.genome.gov/19518664>), we analysed the association of rs2267735 (included on the Affymetrix 6.0 SNP array) with bipolar disorder as well as schizophrenia. We did not observe a significant association of this SNP with these two disorders in subjects with African American (954 cases, 1,195 controls) or



**Figure 3 | Association of *ADCYAP1R1* rs2267735 with PTSD symptoms and physiological fear responses.** **a**, Table demonstrating the  $N$ , Wald  $\chi^2$ , odds ratio (OR) for C as risk allele and  $P$ -value, in males and females, in the original, replication and combined samples for logistic regression of rs2267735 with PTSD diagnosis. CI, confidence interval. **b**, Total PTSD symptoms are differentially associated with rs2267735 genotype in females ( $P \leq 0.001$ ). **c**, Hyperarousal is the most robustly associated symptom with rs2267735 genotype ( $P = 0.0009$ ). **d**, In a dominant/recessive model, even after controlling for childhood trauma, adult trauma and age, genotype predicts total PTSD symptoms ( $P \leq 0.001$ ) and **e**, hyperarousal symptoms ( $P \leq 0.0001$ ). **f**, Fear discrimination, measured with potentiated startle (CS+ startle minus CS- startle) is impaired in females with rs2267735 'CC' genotype. **g**, Dark-enhanced startle (startle<sup>dark</sup> - startle<sup>light</sup>) is significantly increased in females with rs2267735 'CC' genotype.  $N$  values are shown at base of each bar, bars represent mean  $\pm$  s.e.m.  $N$  values are slightly different across analyses owing to differences in number of subjects across measures. \* $P < 0.05$ ; \*\* $P < 0.001$ ; \*\*\* $P < 0.0002$ .

European (1,378 cases, 1,351 controls) ancestry. Specifically, we found that all pre-computed  $P$ -values for associations of rs2267735 with schizophrenia or bipolar disorder were higher than  $P$  uncorrected = 0.01, indicating no major contribution of this variant.

Additionally, we examined the association of rs2267735 and Alzheimer's in a previously characterized Alzheimer's disease sample<sup>25</sup>. In this cohort of 342 subjects, we found no association with rs226735 and Alzheimer's disease diagnosis using either the additive genetic model ( $P = 0.19$ ) or the dominant/recessive model ( $P = 0.89$ ). These data suggest that we find robust associations with rs2267735 in women, but not men, with PTSD. In contrast, we find no association with depression symptoms, substance abuse, Alzheimer's disease, bipolar disorder, or schizophrenia across different samples. Note that for all of these negative results, owing to the limited sample sizes, we cannot rule out the possibility that rs2267735 may be associated with PTSD in men or with other disorders with a smaller effect size than we see with PTSD in women.

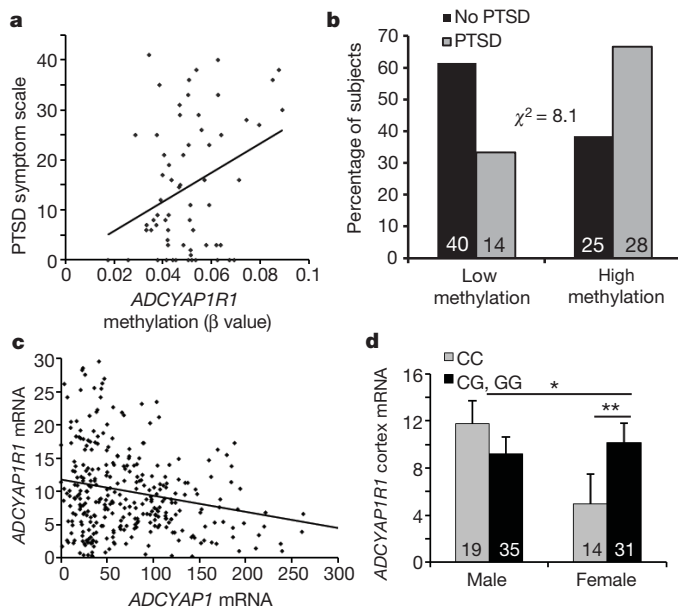
To parallel our results with plasma PACAP38 levels, we next examined whether physiological measures of fear are differentially associated with the *ADCYAP1R1* rs2267735 SNP. In PTSD, but not depression<sup>18</sup>, fear response to an inhibitory CS-, or 'safety signal', is

exaggerated. The discrimination between CS+ and CS− improves across the training procedure in controls, but not in those with PTSD. We examined whether rs2267735 was associated with impaired fear discrimination late in conditioned acquisition, during the same period noted in Fig. 1e. Notably, females with the ‘CC’ genotype were significantly less able to discriminate CS+ from CS− signals (Fig. 3f, sex × genotype interaction,  $P < 0.05$ , and ‘CC’ versus ‘G’ carriers in females,  $P < 0.05$ ).

We next examined whether a difference in dark-enhanced startle, a measure of increased anxiety in humans that is similar to light-enhanced startle in rodents<sup>26–28</sup>, was differentially associated with rs2267735. Again, we found that females, but not males, with the ‘CC’ genotype showed significantly more startle in the dark compared to the light (Fig. 3g, males,  $N = 35$ ,  $P = 0.71$ ; females,  $N = 53$ ,  $P = 0.02$ ). Together, these data suggest that the *ADCYAP1* rs2267735 SNP may be relatively specific in its association with PTSD psychological and physiological phenotypes. Further, the robust association of rs2267735 with hyperarousal symptoms suggests that the role of PACAP–PAC1 may be specifically involved in the normalization of the stress response, a process which is particularly dysregulated in PTSD.

### *ADCYAP1* methylation and mRNA expression

Environmental, genetic and epigenetic mechanisms are likely to moderate the long-term effects of trauma exposure. Using the Illumina HumanMethylation27 BeadChip, we interrogated methylation in DNA extracted from peripheral blood at the first site within the *ADCYAP1* CpG island (Supplementary Fig. 2). Methylation at this site was significantly associated with total PTSD symptoms (Fig. 4a,  $N = 94$ ,  $r = 0.354$ ,  $P < 0.0005$ ) in a sex-independent manner. Further, CpG methylation level (median split) was associated with PTSD



**Figure 4 | *ADCYAP1* methylation and mRNA expression.** **a**, Methylation within the first CpG island of *ADCYAP1* (β value, Illumina cg27076139) is positively correlated with total PTSD symptoms (both sexes;  $N = 107$ ;  $r = 0.354$ ,  $P < 0.0005$ ). **b**, Subjects with PTSD have higher levels of *ADCYAP1* methylation (median split,  $N = 107$ ;  $\chi^2$  analyses,  $P < 0.005$ ). **c**, *ADCYAP1* mRNA levels are inversely correlated with *ADCYAP1* mRNA levels in cortex (from prior data set<sup>13</sup>) ( $r = -0.219$ ;  $P < 0.001$ ). **d**, *ADCYAP1* mRNA levels are differentially expressed in females compared to males based on imputed *ADCYAP1* rs2267735 genotype (from prior data set<sup>13</sup>) (\* $P < 0.05$  male versus female CC carriers, \*\* $P < 0.05$ , one-tailed, CC versus G-carriers). Bars represent mean  $\pm$  s.e.m.  $N$  values for each group at bottom of each graph.

diagnosis (Fig. 4b,  $\chi^2 = 8.1$ ,  $P < 0.005$ ), but not depression ( $P > 0.05$ , Supplementary Fig. 3e). There was no significant association between methylation of *ADCYAP1* and PTSD symptoms. These data suggest that *ADCYAP1* is regulated, in part, through epigenetic mechanisms that contribute to differential function of the PAC1 receptor in PTSD.

To examine the potential relationship of genotype and brain mRNA expression as described previously<sup>29</sup>, we used a brain mRNA expression data set<sup>30</sup> to test whether *ADCYAP1* rs2267735 is associated with differential gene expression. We first examined whether cortical *ADCYAP1* and *ADCYAP1* mRNA levels were correlated. As shown in Fig. 4c, these mRNA levels were significantly inversely correlated ( $r = -0.219$ ,  $P < 0.001$ , including males and females), suggesting that brain levels of PACAP peptide and PAC1 mRNA are tightly regulated.

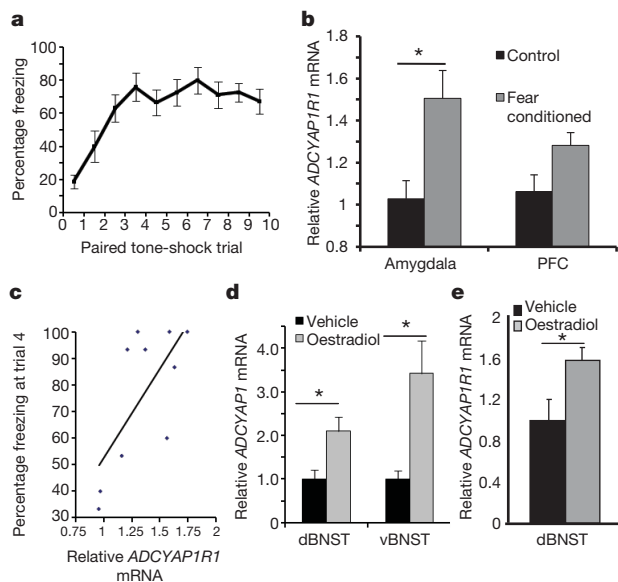
We next used a previously analysed data set with combined genome-wide association and brain mRNA expression data<sup>30</sup> to examine whether *ADCYAP1* rs2267735 imputed genotypes were associated with differential *ADCYAP1* expression in brain. We found a sex × genotype effect (Fig. 4d,  $F(3,99) = 4.3$ ,  $P < 0.05$ ) with females with the ‘CC’ genotype expressing significantly less *ADCYAP1* mRNA than males ( $F(1,33) = 5.5$ ,  $P < 0.05$ ) or than females who are ‘G’ carriers (one-tailed,  $F(1, 45) = 2.87$ ,  $P < 0.05$ ). Thus, mRNA encoding the PACAP peptide and PAC1 receptor appeared to be tightly regulated within the human cortex, and *ADCYAP1* mRNA levels were associated with the *ADCYAP1* rs2267735 SNP.

### Fear induces *Adcyap1r* in mouse amygdala

Despite prior studies examining PACAP–PAC1 receptor function in central/peripheral nervous system development, endocrine homeostasis, metabolism, cellular protection/regeneration and chronic stress responses<sup>2,3,6,31–34</sup>, a role for PACAP signalling in fear conditioning has not been evaluated. Given our data implicating PACAP in PTSD, we wondered if *Adcyap1r* mRNA was differentially regulated in mice using Pavlovian fear conditioning<sup>35–40</sup>, a means of studying acute fear and trauma responses that has been proposed to model PTSD<sup>19,22</sup>. We performed classical fear conditioning experiments using male mice, in which a previously neutral tone CS (6 kHz) was paired with 10 foot-shocks (1 mA, 0.5 s; Fig. 5a). This conditioning paradigm consistently provides robust fear learning in mice leading to changes in gene expression within the amygdala, a region critical for fear learning and expression. Quantitative PCR analyses shows that amygdala *Adcyap1r* mRNA increased ~1.5-fold during the consolidation of fear (Fig. 5b,  $P < 0.05$ ), with a similar trend within the medial prefrontal cortex (mPFC). When peak freezing was compared with brain mRNA levels, we find a significant correlation between fear learning and *Adcyap1r* mRNA (Fig. 5c,  $r^2 = 0.49$ ,  $P < 0.05$ ).

### Oestrogen induces *Adcyap1r* in rat BNST

To further establish the relationship between PACAP–PAC1 receptors and oestrogen in a validated model of sex hormone regulation, we examined oestrogen-induced changes in *Adcyap1* and *Adcyap1r* transcripts in the bed nucleus of stria terminalis (BNST) in female rats. The BNST is a component of the extended amygdala that is subject to significant gonadal hormonal control<sup>7,27,28</sup>. In rodents, it is critical for emotional behaviour, mediating stress responses and the light-enhanced startle response. We examined gene expression in the BNST in ovariectomized female rats following 21-day implantation of continuous release oestrogen pellets. Compared to control implants, oestradiol increased *Adcyap1* transcripts in the dorsal and ventral BNST 2.1- and 3.4-fold, respectively ( $P \leq 0.01$ , Fig. 5d). Additionally, oestradiol increased *Adcyap1r* mRNA 1.5-fold in the dorsal BNST samples ( $P < 0.05$ , Fig. 5e), and future studies should also examine oestradiol sensitivity of these genes in amygdala. While these rodent studies are complex and have differing experimental designs, our data clearly illustrate dynamic PACAP–PAC1 receptor regulation within central areas mediating fear, stress and oestrogen responsiveness.



**Figure 5 | Regulation of *Adcyap1r1* and *Adcyap1* mRNA in rodent models.** **a**, Percentage of time freezing, in mice, to the conditioned tone (CS+) following tone-shock pairings during the conditioned fear trials. **b**, RT-PCR analyses of mRNA levels within mouse amygdala and mPFC 2 h after fear conditioning or in control handling conditions, showing a significant increase in amygdala *Adcyap1r1* mRNA ( $N = 15$ , 1.47 fold,  $P < 0.05$ ) and a non-significant trend in mPFC (1.19 fold change). **c**, Correlation between average amygdala and PFC *Adcyap1r1* mRNA and percentage freezing at trial 4, demonstrating an association between *Adcyap1r1* mRNA with rate of fear learning ( $r^2 = 0.49$ ,  $P < 0.05$ ). **d**, *Adcyap1* mRNA in rat BNST in female rats ( $N = 12$  per group) following ovariectomy and oestradiol implant versus vehicle replacements. *Adcyap1* mRNA is increased in both dorsal BNST (dBNST, 2.1-fold) and ventral BNST (vBNST, 3.4-fold) after oestradiol implantation. **e**, *Adcyap1r1* transcripts are also increased in dorsal BNST (1.6-fold,  $N = 4$  per group). Bars represent mean  $\pm$  s.e.m. \* $P < 0.05$ .

## Discussion

Since its identification more than 20 years ago, PACAP has been increasingly implicated in diverse cellular stress response pathways and neurotrophic function. However, the organizational role of the PACAP system in orchestrating behavioural stress responses has yet to be clarified. Our data suggest that PACAP–PAC1 receptor expression and signalling may be integrally involved in regulating the psychological and physiological responses to traumatic stress. Further, we report an association of an ERE-embedded *ADCYAP1R1* SNP with PTSD, and we demonstrate fear- and oestrogen-dependent regulation of PACAP systems within stress-responsive regions of the brain. These data may begin to explain sex-specific differences in PTSD diagnosis, symptoms and fear physiology. Future work targeting the PACAP–PAC1 receptor system may lead to novel and robust biomarkers; it may also further our understanding of the neural mechanisms underlying pathological responses to stress, and help identify potential therapeutic targets for the prevalent and debilitating syndrome of PTSD.

## METHODS SUMMARY

This highly traumatized, civilian, cross-sectional cohort has been previously described in candidate gene-association studies of PTSD and depression<sup>11–13</sup>. Research interviews, salivary DNA and blood samples were collected from patients receiving services in the primary care clinics at Grady Memorial Hospital (Atlanta, Georgia, USA). All study procedures have been reviewed and approved by the Emory Institutional Review Board and the Grady Hospital Research Oversight Committee. PTSD measures in this manuscript are based on the PTSD symptom scale<sup>14</sup>, which has been validated within this population using the Clinician Administered PTSD Scale. PACAP38 radioimmunoassay (1:30,000, Peninsula Laboratories) was performed at University of Vermont, using double antibody immunoprecipitation as described<sup>41</sup>. For genotyping, pairwise tagging ( $r^2 > 0.8$ ,

MAF > 0.1) was used to choose tag-SNPs for both *ADCYAP1* and *ADCYAP1R1*. The coordinates were chr. 18 885000–906000 and chr. 7 31048667–31117836 for *ADCYAP1* and *ADCYAP1R1*, respectively (NCBI B36), which includes approximately 10 kilobases (kb) upstream and 5 kb downstream of the coding regions for both genes. Genotypes for the tag-SNPs were generated using Sequenom iPLEX with follow-up analyses using Taqman. For methylation analyses, bisulphite-converted DNA was whole-genome amplified, fragmented, and hybridized to the HumanMethylation27 BeadChip (Illumina). Individual samples were stratified to separate BeadChips according to PTSD status to limit bias. The BeadChips were scanned using a BeadStation 500GX, and the methylation level ( $\beta$  value) was calculated using the Methylation Module of the BeadStudio software. The eyeblink component of the acoustic startle response was measured by electromyographic recordings of the right orbicularis oculi muscle with two 5-mm Ag/AgCl electrodes filled with electrolyte gel, as described<sup>18,19</sup>. The mouse fear conditioning and rat oestrogen replacement studies are described in detail in Supplementary Methods.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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