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Overexpression of Forebrain CRH During Early Life Increases Trauma Susceptibility in Adulthood

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Although early-life stress is a significant risk factor for developing anxiety disorders, including posttraumatic stress disorder (PTSD), the underlying mechanisms are unclear. Corticotropin releasing hormone (CRH) is disrupted in individuals with PTSD and early-life stress and hence may mediate the effects of early-life stress on PTSD risk. We hypothesized that CRH hyper-signaling in the forebrain during early development is sufficient to increase response to trauma in adulthood. To test this hypothesis, we induced transient, forebrain-specific, CRH overexpression during early-life (pre-puberty, $CRHOE_{dev}$) in double-mutant mice (*Camk2a-rtta2* × *tetO-Crh*) and tested their behavioral and gene expression responses to the predator stress model of PTSD in adulthood. In one cohort of $CRHOE_{dev}$ exposed and unexposed mice, avoidance and arousal behaviors were examined 7–15 days after exposure to predator stress. In another cohort, gene expression changes in *Crhr1*, *Crhr2*, and *Fkbp51* in forebrain of $CRHOE_{dev}$ exposed and unexposed mice were examined 7 days after predator stress. $CRHOE_{dev}$ induced robust increases in startle reactivity and reductions in startle inhibition independently of predator stress in both male and female mice. Avoidance behaviors after predator stress were highly dependent on sex and $CRHOE_{dev}$ exposure. Whereas stressed females exhibited robust avoidance responses that were not altered by $CRHOE_{dev}$, males developed significant avoidance only when exposed to both $CRHOE_{dev}$ and stress. Quantitative real-time-PCR analysis indicated that $CRHOE_{dev}$ unexposed males exhibit significant changes in *Crhr2* expression in the amygdala and bed nucleus stria terminalis in response to stress, whereas males exposed to $CRHOE_{dev}$ did not. Similar to $CRHOE_{dev}$ males, females exhibited no significant *Crhr2* gene expression changes in response to stress. Cortical *Fkbp51* expression was also significantly reduced by stress and $CRHOE_{dev}$ exposure in males, but not in females. These findings indicate that forebrain CRH hyper-signaling in early-life is sufficient to increase enduring effects of adult trauma and attenuate *Crhr2* expression changes in response to stress in males. These data support growing evidence for significant sex differences in response to trauma, and support further study of CRHR2 as a candidate mechanism for PTSD risk.
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INTRODUCTION

The significant contrast between lifetime trauma incidence and the prevalence to develop PTSD (40–70% *vs* 7–10%, respectively; Kessler *et al*, 2010) supports the importance of identifying underlying mechanisms of stress vulnerability. Genetic studies have documented significant heritability of anxiety and stress vulnerability, implicating several genes as potential risk factors including CRH (Heim and Nemeroff, 2001; Skelton *et al*, 2012; Smoller *et al*, 2003). However, the causal role of these candidates and underlying mechanisms are still not clarified. By exhibiting high plasticity and intense maturation in limbic regions, developmental periods exhibit significant vulnerability for stress, and accordingly can lead to profound changes in the structure and function of these regions, eg, decreased volume of the hippocampus, and altered amygdala-prefrontal functions, which are considered significant risk factors for PTSD (Dannlowski *et al*, 2012; Heim and Nemeroff, 2001). Early-life stress may also induce latent alterations in brain development with functional consequences that are only precipitated by additional stress in later life (Hammen *et al*, 2000). Although multiple factors are likely involved in the mediation of early-life effects on neuropsychiatric risk, major coordinators of the stress response including HPA-axis elements such as glucocorticoid receptor, its binding protein FKBP5, and CRH signaling elements are primary neurobiological candidates in the pathogenesis of PTSD (Skelton *et al*, 2012).

Significant evidence suggests that CRH has a role in this process as the central coordinator of the stress response. For instance, CRH is elevated in the cerebrospinal fluid of patients diagnosed with PTSD and individuals with significant childhood trauma history (Bremner *et al*, 1997;

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Carpenter *et al*, 2004; Lee *et al*, 2005). Moreover, CRH receptor type 1 (*Crhr1*) polymorphisms moderate associations of childhood trauma with depression and anxiety (Bradley *et al*, 2008; Cicchetti *et al*, 2011). Rodent and primate studies also showed that early stress increases CRH concentration in the cerebrospinal fluid and limbic brain regions (Coplan *et al*, 1996; Plotsky *et al*, 2005) where CRH has been shown to modulate PTSD-related phenotypes (Radulovic *et al*, 1999; Regev *et al*, 2012).

Based on the above findings, we hypothesized that CRH hyper-signaling during development may be a critical driver of developmental stress effects on trauma response in adulthood. To test this hypothesis, we induced transient forebrain-specific CRHOE before puberty in double-mutant mice and exposed them to a single traumatic event in adulthood using a well-validated model of PTSD (Adamec *et al*, 2010; Bakshi *et al*, 2012). To determine the behavioral sequelae, we assessed PTSD-related symptom clusters, ie, startle reactivity, general and trauma-specific avoidance behaviors. To begin to understand potential mediators of $CRHOE_{dev}$ effects, we examined alterations in expression levels of *Crhr1*-, *Crhr2*-, and FK506-binding protein 5 gene (*Fkbp51*), molecules reported to have a role in childhood stress associations with PTSD risk (Binder, 2009; Bradley *et al*, 2008).

MATERIALS AND METHODS

Generation of Mice with Inducible Forebrain-Specific CRHOE

To induce CRHOE in spatio-temporally restricted manner, we used double-mutant mice carrying *CamkIIα* promoter-driven *rtta2* transgene (Michalon *et al*, 2005) and doxycycline (DOX)-regulated *tetO* promoter fused to the *Crh* gene (Vicentini *et al*, 2009) on a C57BL/6J background as previously described (Toth *et al*, 2014). The *Crh* transgene was turned on by DOX administration in breeder chow (Harlan Laboratories, Indianapolis, IN) to 'single-mutant' dams from postnatal day 2 for 3 weeks (PND2-PND23). Hence, CRHOE was induced only in double-mutant pups but not in dams. Typical litter sizes were four or five pups, producing one double-mutant male and one double-mutant female on average for testing. The DOX dose administered to the dam (6 mg/g food) induces forebrain-specific expression of *Crh* or *Lacz* reporter genes in the forebrain as early as PND0, with detectable levels after 4 days, reaching its maximum after 1 week and returning to baseline levels 14 days after DOX treatment is terminated (Michalon *et al*, 2005; Toth *et al*, 2014). We and others have previously established that DOX alone (administration between PND2-23) does not affect startle reactivity and avoidance behavior in wild-type mice (Kolber *et al*, 2010; Toth *et al*, 2014), therefore control subjects were double-mutant mice without DOX treatment.

Housing Conditions

All subjects were group housed (3–4 per cage) after weaning (PND28) in a temperature-controlled (21–22 °C) room under a reverse 12 h light/dark cycle (lights off at 0800 hours). As conducted previously, mice were isolated 1 week before predator stress and housed individually for the remainder of the experiment (Adamec *et al*, 2010), because pilot studies suggested that isolated mice exhibit stronger predator stress effects owing to lower levels of baseline avoidance behaviors.

Experimental Design

All testing occurred from 1000 hours to 1800 hours and was conducted in accordance with the *Principles of Laboratory Animal Care*, National Institutes of Health guidelines, as approved by the University of California San Diego. Before behavioral testing, subjects were brought into an adjacent room under a black cloth 60 min for habituation. For each test, equipment was cleaned thoroughly with water between testing sessions. One week before predator exposure (13th postnatal week), mice were handled for 1 min/day and completed a baseline startle assessment. Control and $CRHOE_{dev}$ mice were assigned to groups (predator exposure or handling, $N=74$, 8–11 per group per sex) after counterbalancing for baseline startle reactivity. Behavioral testing began 7 days after exposure with an open field test (AM) and behavioral pattern monitor (PM). The next day, mice were tested in the light–dark box test (AM) followed by startle assessment (PM). Fourteen and fifteen days after predator exposure, mice were tested in the 'trauma reminder' test. Two separate cohorts of mice with or without predator exposure and DOX administration (4 groups, 5–16 per group per sex, $N=91$ total) were killed for gene expression analysis.

Predator Exposure

Mice were presented to a cat (Liberty Research, Waverly, NY, USA) in a well-lit room ($2.3\times1.8\,m^2$; 150–200 lux) for 10 min. The mouse and cat could freely move within the room. The interaction was recorded and analyzed later by an experimenter blind to treatments. The intensity of stress exposure was quantified by the frequency and duration of the following variables: cat spent near the mouse (<1 ft), sniffing, pawing and mouthing (touching with the mouth without biting) the mouse. None of these behaviors differed between groups ($CRHOE_{dev}$ *vs* controls; Supplementary Table 2) and no physical injury occurred. After 10 min of free interaction, mice were returned to their home cages. Control subjects were exposed to handling for 1 min.

Open Field Test

Open field activity was assessed in an open arena ($40\times40\times40\,cm^3$; 800 lux) for 10 min and analyzed using Ethovision Tracking Software (Noldus, Leesburg, VA, USA). Total distance moved, entries into and duration of time exploring the center zone ($25\times25\,cm^2$), and latency of the first entry (mice were placed in the corner) were analyzed.

Open Field Test with Trauma-Reminder

Open field arena was used to assess avoidance of trauma-related cues: in a cross-over design, either clean mouse bedding or used cat litter (from the cat used for stress exposure; containing urine and fur) was placed into a 50-ml perforated conical tube and affixed to the floor in one corner

of the arena. The latency of first approach, number of approaches, and time spent within a 3-cm radius zone around the tubes was measured by Ethovision Tracking Software.

Behavioral Pattern Monitor

Locomotor and exploratory activity was measured in behavioral pattern monitor chambers (San Diego Instruments, San Diego-CA; Risbrough *et al*, 2006). Each chamber is a clear Plexiglas box containing a $30 \times 60\,cm^2$ holeboard floor. The location of the mouse is obtained from a grid of 12×24 photobeams 1 cm above the floor providing a resolution of 1.25 cm (+16 beams detecting rears). Mice were placed in the middle of the dark chamber and their activity was assessed by computing total distance moved, number of rears and hole-pokes over 30 min.

Light–Dark Box Test

The light–dark box consisted of two $20 \times 40 \times 20\,cm^3$ chambers joined by a $6 \times 6\,cm^2$ door. One was well-lit (950 lux), whereas the other was covered (<5 lux). Mice were placed in the dark chamber with closed door for 30 s. The test was started by opening the door and lasted 10 min. Latency of the first entry, the number of entries, and time spent in the light chamber were measured by Ethovision Tracking Software.

Acoustic Startle and Prepulse Inhibition (PPI) Assessment

Startle reactivity was assessed in Plexiglas chambers (San Diego Instruments, San Diego, CA) as previously described (Adamec *et al*, 2010; Toth *et al*, 2014). Briefly, 1 week before stress exposure, baseline startle was assessed over 3 consecutive days using Session 1, which presented 10 105 dB pulses over 50 dB background in dark chambers. One week after stress exposure, startle reactivity was re-assessed in two consecutive sessions (Sessions 1 and 2). Session 1 consisted the same parameters as in baseline assessment except that 10 additional pulses (in a pseudorandom order) were presented with houselights on for 2.95 s before the startle stimulus. This session replicated the acoustic startle session previously described for the mouse predator stress model of PTSD (Adamec *et al*, 2010). To further assess startle habituation and inhibition as measured by PPI, a second session was presented immediately after the first (Session 2 with 65 dB background and lights on). This session included five blocks beginning with the delivery of five each of 120 dB startle pulses (Block1) allowing startle to reach a stable level before specific testing. The second block tested response to 80, 90, 100, 110, and 120 dB stimulus intensities. The third block tested PPI using 120 dB startle pulses with three different prepulse intensities (69, 73, and 81 dB). The fourth block tested interstimulus interval effects on PPI: 73 dB prepulses preceding 120 dB pulses by 25, 50, 100, 200, or 500 ms. The session ended with five pulses of 120 dB (Block 5) to assess habituation (from Block1 to Block5). For more details, see Supplementary Material.

Quantitative Real-Time PCR (qRT-PCR)

We assessed expression levels of four CRH-related genes in order to identify CRH-induced changes, which may mediate increased vulnerability to traumatic stress. We assessed *Crhr1* and *Crhr2* expression in three brain regions: amygdala, bed nucleus of stria terminalis (BNST), and lateral septum, which are areas of relatively high expression for at least one of these genes (Van Pett *et al*, 2000). We also assessed *Fkbp51* in the hippocampus and neocortex, areas of moderate to high expression for these genes (Scharf *et al*, 2011). Briefly, male/female DOX-treated/untreated and handled/predator stressed double-mutant mice were killed 7 days after predator stress exposure, regions of interest were dissected on ice-cold platform immediately after brain extraction and were placed in 1.5 ml tubes containing 500 µl of RNA Later (Life Technologies, Carlsbad, CA). Taqman qRT-PCR was run following RNA extraction and cDNA synthesis using commercially available kits. For more details, see Supplementary Material. For each sample, expression of each gene of interest was compared with the housekeeping gene *Gapdh*. Fold differences *vs* control (no DOX) were calculated for each sex. Because of technical reasons sample sizes varied across regions.

Statistical Analysis

Behavioral and qRT-PCR data were analyzed using factorial ANOVA tests with sex, stress, and $CRHOE_{dev}$ as between-subject factors for all tests and in the case of startle habituation block or intensity was included as a within-subject factor (Systat, Chicago, IL, USA). In the case of main effects or interactions with sex, data were then analyzed separately within each sex. If groups differed in activity measures, an additional covariate analysis was also presented to control for non-specific activity effects. qRT-PCR data were also analyzed using covariance analysis and variance estimation/precision model to test if there was difference between cohorts: significant changes are shown only if latter indicated no cohort-effect. Data were logarithmic or square-root transformed where necessary. When appropriate, Fisher's LSD *post hoc* comparisons were also conducted. However, given that multiple tests were used to measure a similar behavioral construct (avoidance) with relatively lenient statistical cutoffs, we also created a composite avoidance score (average *z*-score of time in the aversive area in each avoidance test: center of open field; light compartment of light–dark box; near the tube filled with cat litter), which is common in clinical research when multiple measures of a similar construct are conducted (for more details, see Supplementary Material). This approach enables a more accurate determination of consistent changes in avoidance behavior across multiple tests, calculates overall effect size, and reduces family-wise error due to multiple testing.

RESULTS

Avoidance in the Open Field

Predator stress exposure increased avoidance of the center (frequency, duration and latency measures: $F_{stress}(1,66) > 8.08$, $p < 0.01$; Figure 1; Tables 1 and 2) and decreased

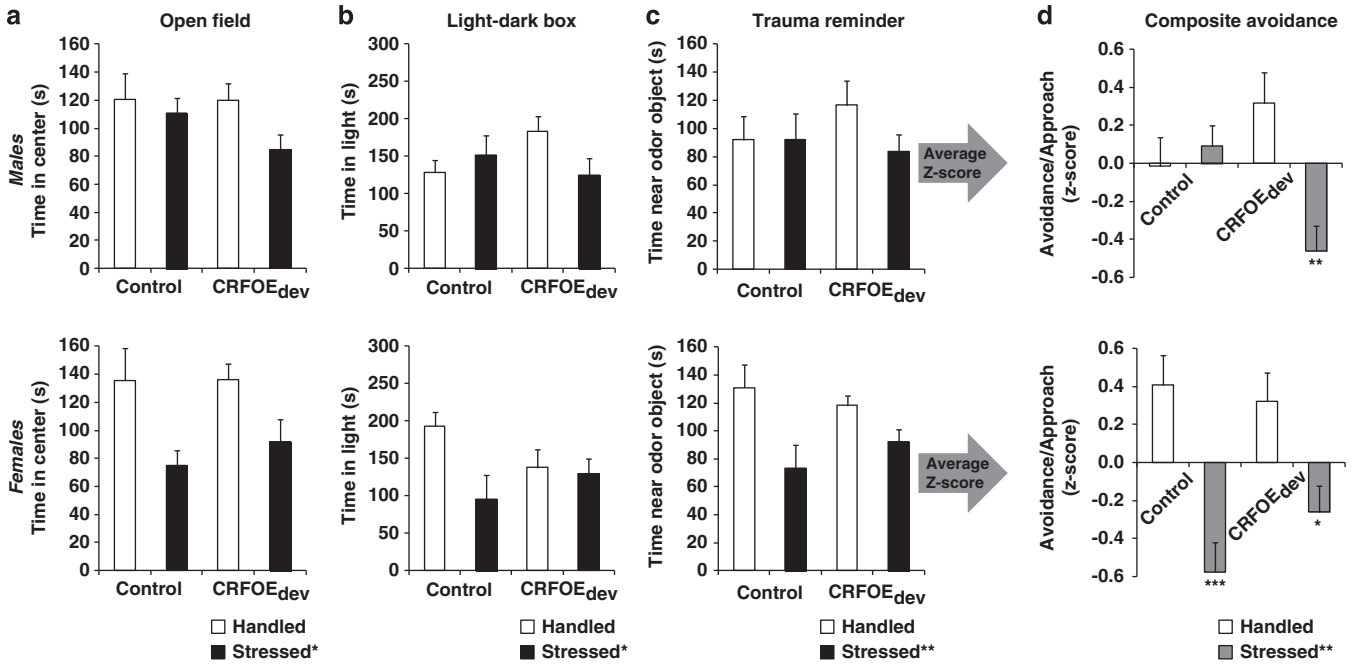

Figure 1 Avoidance behavior in open field (a), light–dark box (b), and trauma reminder (c) tests, and their composite avoidance score (d) indexed by average *z*-scores of the three tests. All graphs indicate time spent in the aversive arenas (ie, center of the open field, light compartment of the light–dark box, and zone around the tube filled with cat litter). Lower/negative scores indicate increased avoidance of the aversive zone. Upper and lower panels show data from males and females, respectively. Data are presented as mean ± SEM. Asterisks in legends indicate significant main effect of stress (*$p<0.05$; **$p<0.01$; ***$p<0.001$; indicated by three-way ANOVA); whereas asterisks above bars indicate significant interactions followed by *post hoc* comparisons *vs* handled group with the same CRF background. *CRHOE$_{dev}$*, transitional CRH overexpression before puberty.

exploration (total distance moved; $F_{stress}(1,66)=13.25$, $p<0.001$; Tables 1 and 2). Reduced center exploration was independent of overall locomotor activity changes as center duration and latency to enter the center remained significantly lower in the stressed groups when total distance moved was considered as a covariate ($F_{stress}(1,65)>5.68$, $p<0.05$). The impact of stress on latency to enter the center was significantly modulated by sex and CRHOE$_{dev}$ exposure ($F_{sex\times stress\times CRHOE}(1,66)=5.23$, $p<0.05$), with increased latency to enter reduced in male mice exposed to both stress and CRHOE$_{dev}$ compared with all other male groups ($0.017<p<0.085$; Table 1). Females exposed to predator stress showed significant reductions in center duration and number of entries regardless of CRHOE$_{dev}$ exposure (Figure 1 and Table 2).

Avoidance in the Light–Dark Box

Mice exposed to predator stress exhibited increased avoidance of the light chamber (frequency: $F_{stress}(1,66)=5.16$, $p<0.05$; duration: $F_{stress}(1,66)=4.87$, $p<0.05$; latency: $F_{stress}(1,66)=2.21$, ns; Tables 1 and 2) in a sex- and CRHOE$_{dev}$-dependent manner (frequency, duration, and latency measures: $F_{sex\times stress\times CRHOE}(1,66)=7.15$, $p<0.01$; $F_{sex\times stress\times CRHOE}(1,66)=5.62$, $p<0.05$; $F_{sex\times stress\times CRHOE}(1,66)=2.91$, $p=0.092$, respectively). Male mice exposed to both CRHOE$_{dev}$ and stress exhibited higher avoidance (frequency and duration: $p<0.05$ and $p=0.063$, respectively compared with handled CRHOE$_{dev}$; Figure 1 and Table 1). In contrast, predator stress increased avoidance of the light chamber in females regardless of CRHOE$_{dev}$ exposure (Figure 1 and Table 2). CRHOE$_{dev}$ females also exhibited a trend for increased avoidance in non-stressed groups (duration: $F_{stress\times CRHOE}(1,32)=3.49$, $p=0.071$, *post hoc*: $p=0.098$; Table 2) as described previously (Toth *et al*, 2014).

Avoidance of Trauma-Associated Cue

Overall, predator stress increased avoidance of the trauma reminder as indexed by decreased exploration of the tube containing cat litter (frequency: $F_{stress}(1,66)=4.38$, $p<0.05$; duration: $F_{stress}(1,66)=7.78$, $p<0.01$; latency: $F_{stress}(1,66)<1$, ns), which was independent of sex and CRHOE$_{dev}$. Exploration of the neutral tube was not affected by predator stress (all measures: $F_{stress}(1,66)<2.63$, ns). CRHOE$_{dev}$ alone had no effect on avoidance of either tube (duration and frequency: $F_{CRHOE}(1,66)<1$, ns), but did increase latency to explore the litter tube as well as increased total distance moved ($F_{CRHOE}(1,66)>4.40$; $ps<0.05$).

Avoidance Across Testing Paradigms: Combined Avoidance Score

To better quantify the 'overall' avoidance profile of mice exposed to CRHOE$_{dev}$ and predator stress, we utilized a composite score approach on the combined *z*-scores of the avoidance tests. This approach highlights where an individual falls in the overall distribution of each test most consistently. The average *z*-score of three avoidance tests confirmed the highly significant effect of stress on avoidance

Table 1 Avoidance Behavior in Males Exhibited in the Open Field, Light–Dark Box, and Modified Open Field with Trauma-Reminder

CRH	Stress	Number of entries	Latency of first approach	Distance traveled (cm)
Open field				
Control	Handled	79.0 ± 6.8	10.8 ± 2.9	5724 ± 458
	Stressed	74.8 ± 5.0	14.6 ± 4.8	5298 ± 401
$CRHOE_{dev}$	Handled	87.6 ± 7.1	9.3 ± 2.8	5851 ± 281
	Stressed	**59.4 ± 7.5***	**59.8 ± 19.8***	4471 ± 308
$CRHOE_{dev}$:		F(1,34) < 1, NS	F(1,34) < 1, NS	F(1,34) < 1, NS
Stress:		F(1,34) = 5.65, $p < 0.05$	F(1,34) = 6.17, $p < 0.05$	F(1,34) = 7.54, $p < 0.05$
Stress × $CRHOE_{dev}$:		F(1,34) = 3.40, $p = 0.073$	F(1,34) = 3.90, $p = 0.057$	F(1,34) = 1.72, NS
Light–dark box				
Control	Handled	15.6 ± 2.2	15.5 ± 6.6	NA
	Stressed	19.7 ± 3.8	103.3 ± 66.0	NA
$CRHOE_{dev}$	Handled	23.0 ± 3.8	40.4 ± 19.3	NA
	Stressed	**13.2 ± 2.3***	86.5 ± 64.3	NA
$CRHOE_{dev}$:		F(1,34) < 1, NS	F(1,34) < 1, NS	
Stress:		F(1,34) < 1, NS	F(1,34) < 1, NS	NA
Stress × $CRHOE_{dev}$:		F(1,34) = 4.62, $p < 0.05$	F(1,34) < 1, NS	
Open field with trauma reminder				
Control	Handled	33.8 ± 4.3	12.3 ± 4.6	6017 ± 590
	Stressed	27.9 ± 5.5	24.6 ± 16.5	5609 ± 470
$CRHOE_{dev}$	Handled	47.3 ± 7.1	7.1 ± 3.2	7052 ± 702
	Stressed	31.8 ± 5.6	2.1 ± 0.8	5548 ± 492
$CRHOE_{dev}$:		NA	NA	NA
Stress:				
Stress × $CRHOE_{dev}$:				
Composite (z-)scores				
Control	Handled	0.04 ± 0.19	0.19 ± 0.06	0.13 ± 0.33
	Stressed	− 0.02 ± 0.21	− 0.15 ± 0.30	− 0.16 ± 0.26
$CRHOE_{dev}$	Handled	0.37 ± 0.22	0.10 ± 0.14	0.30 ± 0.25
	Stressed	**− 0.39 ± 0.26***	− 0.16 ± 0.19	− 0.39 ± 0.21
$CRHOE_{dev}$:		F(1,34) < 1, NS	F(1,34) < 1, NS	F(1,34) < 1, NS
Stress:		F(1,34) = 3.44, $p = 0.072$	F(1,34) = 2.47, NS	F(1,34) = 3.17, $p = 0.087$
Stress × $CRHOE_{dev}$:		F(1,34) = 2.87, $p = 0.098$	F(1,34) < 1, NS	F(1,34) < 1, NS

Data (presented as mean ± SEM) show the number of entries into the aversive arena (ie, center, light compartment, and zone around the tube filled with cat litter), the latency of the first approach to the aversive arena, and total distance traveled. Distance traveled is not available in the light–dark box as the dark compartment is covered. *$p < 0.05$ *post hoc* test compared with handled controls with the same CRH condition (indicated in bold); *$CRHOE_{dev}$*: transitional CRH overexpression before puberty. Note: only overall effects of predator stress were detected in the trauma reminder test, thus separate analyses across sexes are not presented, see Results.

(all measures: $F_{stress}(1,66) > 11.08$, $0.001 < p < 0.002$; duration shown in Figure 1), which showed strong interaction with $CRHOE_{dev}$ in a sex-dependent manner (frequency and duration: $F_{sex \times stress \times CRHOE}(1,66) > 7.82$, $0.002 < p < 0.007$). *Post hoc* analysis confirmed the general pattern of findings in the individual tests: with predator stress increasing avoidance only in male mice previously exposed to $CRHOE_{dev}$ (Figure 1; Tables 1 and 2; effect of stress on frequency and duration in $CRHOE_{dev}$ groups: $p < 0.05$ and $p < 0.01$, respectively; no effect of stress in male non-$CRHOE_{dev}$ mice). However, there was a trend for increased approach in handled $CRHOE_{dev}$ male mice compared to handled controls ($p = 0.076$). Consistently with individual tests, *post hoc* analysis in females showed a robust effect of predator stress on avoidance in both $CRHOE_{dev}$ and non-$CRHOE_{dev}$ groups ($p < 0.05$ and $p < 0.001$, respectively; Figure 1 and Table 2). Factor loading-weighted *z*-scores showed highly similar results (duration in open field, ligh–dark box and odor test loadings: 0.73, 0.68, and 0.49, respectively; $F_{sex \times stress \times CRHOE}$ $(1,66) = 10.19$, $p < 0.01$; *post hoc*: handled *vs* stressed $CRHOE_{dev}$ $p < 0.01$).

Locomotor and Exploratory Activity

$CRHOE_{dev}$ increased the total distance moved in the behavioral pattern monitor but did not affect the number of rears and hole-pokes ($F_{CRHOE}(1,66) = 5.50$, $p < 0.05$; $F_{CRHOE}(1,66) < 1$, ns; $F_{CRHOE}(1,66) < 1$, ns, respectively).

Table 2 Avoidance Behavior in Females Exhibited in the Open Field, Light–Dark Box, and Modified Open Field with Trauma-Reminder

CRH	Stress	Number of entries	Latency of first approach	Distance traveled (cm)
Open field				
Control	Handled	114.8 ± 21.4	5.2 ± 1.6	8158 ± 1258
	Stressed	60.6 ± 13.9	25.2 ± 7.1	4966 ± 739
$CRHOE_{dev}$	Handled	99.6 ± 13.6	13.6 ± 3.5	6878 ± 1011
	Stressed	73.0 ± 15.5	29.9 ± 16.1	5305 ± 726
$CRHOE_{dev}$:		$F(1,32) < 1$, NS	$F(1,32) < 1$, NS	$F(1,32) < 1$, NS
Stress:		$F(1,32) = 7.43$, $p < 0.01$	$F(1,32) = 2.50$, NS	$F(1,32) = 5.70$, $p < 0.05$
Stress × $CRHOE_{dev}$:		$F(1,32) < 1$, NS	$F(1,32) = 2.09$, NS	$F(1,32) < 1$, NS
Light-dark box				
Control	Handled	23.0 ± 2.3	30.7 ± 18.9	NA
	Stressed	11.8 ± 3.0	189.0 ± 92.5	NA
$CRHOE_{dev}$	Handled	19.1 ± 3.9	91.0 ± 66.1	NA
	Stressed	15.4 ± 3.3	119.7 ± 50.3	NA
$CRHOE_{dev}$:		$F(1,32) < 1$, NS	$F(1,32) < 1$, NS	NA
Stress:		$F(1,32) = 5.56$, $p < 0.05$	$F(1,32) = 5.92$, $p < 0.05$	
Stress × $CRHOE_{dev}$::		$F(1,32) = 1.44$, NS	$F(1,32) < 1$, NS	
Open field with trauma reminder				
Control	Handled	53.7 ± 6.3	1.7 ± 0.7	8564 ± 1074
	Stressed	35.6 ± 6.5	25.2 ± 15.3	6504 ± 1238
$CRHOE_{dev}$	Handled	61.5 ± 9.3	6.4 ± 2.9	9031 ± 1526
	Stressed	65.8 ± 12.0	3.2 ± 1.6	10 306 ± 1625
$CRHOE_{dev}$:		NA	NA	NA
Stress:				
Stress × $CRHOE_{dev}$:				
Composite (z-)scores				
Control	Handled	0.38 ± 0.19	0.34 ± 0.06	0.27 ± 0.31
	Stressed	− 0.60 ± 0.11	− 0.29 ± 0.22	− 0.49 ± 0.26
$CRHOE_{dev}$	Handled	0.16 ± 0.23	0.18 ± 0.09	0.12 ± 0.33
	Stressed	− 0.26 ± 0.21	− 0.29 ± 0.25	− 0.19 ±0.22
Main effect of $CRHOE_{dev}$:		$F(1,32) < 1$, NS	$F(1,32) < 1$, NS	$F(1,32) < 1$, NS
Main effect of stress:		$F(1,32) = 12.70$, $p < 0.01$	$F(1,32) = 10.82$, $p < 0.01$	$F(1,32) = 3.31$, $p = 0.078$
stress × $CRHOE_{dev}$:		$F(1,32) = 2.01$, NS	$F(1,32) < 1$, NS	$F(1,32) < 1$, NS

Data (presented as mean ± SEM) show the number of entries into the aversive arena (ie, center, light compartment, and zone around the tube filled with cat litter), and the latency of the first approach and total distance traveled. Distance traveled is not available in the light–dark box as the dark compartment is covered. *$p < 0.05$ compared with handled controls with the same CRH condition (*post hoc*); *$CRHOE_{dev}$*: transitional CRH overexpression before puberty. Note: only overall effects of predator stress were detected in the trauma reminder test, thus separate analyses across sexes are not presented, see Results.

Predator stress did not alter total distance moved or number of rears ($F_{stress}(1,66) < 1$, ns) but decreased the number of hole-pokes ($F_{stress}(1,66) = 8.20$, $p < 0.01$; Supplementary Table 1).

Startle Reactivity and PPI

Both before and following stress exposure, $CRHOE_{dev}$ exposed mice showed higher startle magnitude regardless of sex ($F_{CRHOE}(1,66) = 4.61$, $p < 0.05$; $F_{CRHOE}(1,66) = 9.52$, $p < 0.01$, respectively; Figure 2a and Supplementary Figure 1, $F_{CRHOE \times Stress \times Sex}(1,66) < 1$, ns). $CRHOE_{dev}$ also robustly reduced startle habituation independently of sex or stress exposure ($F_{block \times CRHOE}(4,264) = 2.72$, $p < 0.05$; *Post hoc* main effect of block $p < 0.05$ in controls, $p > 0.3$ in $CRHOE_{dev}$ mice; Figure 2b). Similarly, PPI was significantly reduced by $CRHOE_{dev}$ ($F_{CRHOE}(1,66) = 9.43$, $p < 0.01$), although this effect appeared to be stronger in males (Figure 2c). When startle magnitude was added as a covariate, the $CRHOE_{dev}$ effect on PPI remained significant ($F_{stress}(1,65) = 15.84$, $p < 0.001$), suggesting the PPI effect was independent of effects on startle. Predator stress alone had no effect on any startle measures ($F_{stress} < 2.08$, ns; $F_{stress \times CRHOE} < 1.22$, ns; Figure 2).

Gene Expression Changes Induced by $CRHOE_{dev}$

Crhr1 expression was slightly reduced in the amygdala in stressed mice regardless of sex or $CRHOE_{dev}$ exposure (Figure 3a; $F_{stress}(1,86) = 3.14$, $p = 0.080$). *Crhr1* expression

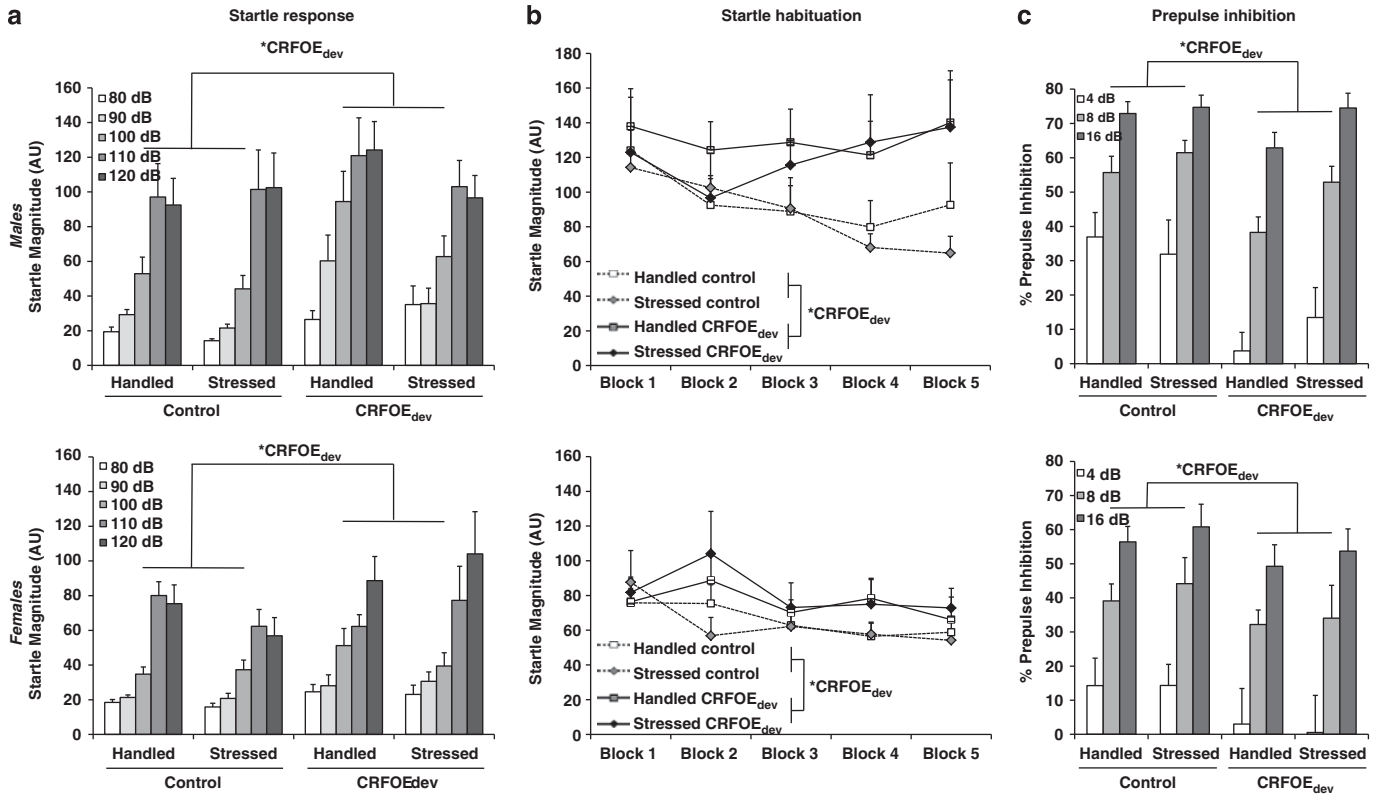

Figure 2 The magnitude (a), habituation (b), and prepulse inhibition (c) of the startle response. Upper and lower panels show data from males and females, respectively. Data are presented as mean ± SEM. Asterisks indicate significant (*$p < 0.05$) main effect of CRHOE$_{dev}$ (or in the case of habituation, block × CRHOE$_{dev}$ interaction; indicated by repeated measure ANOVA). *CRHOE$_{dev}$*, transitional CRH overexpression before puberty.

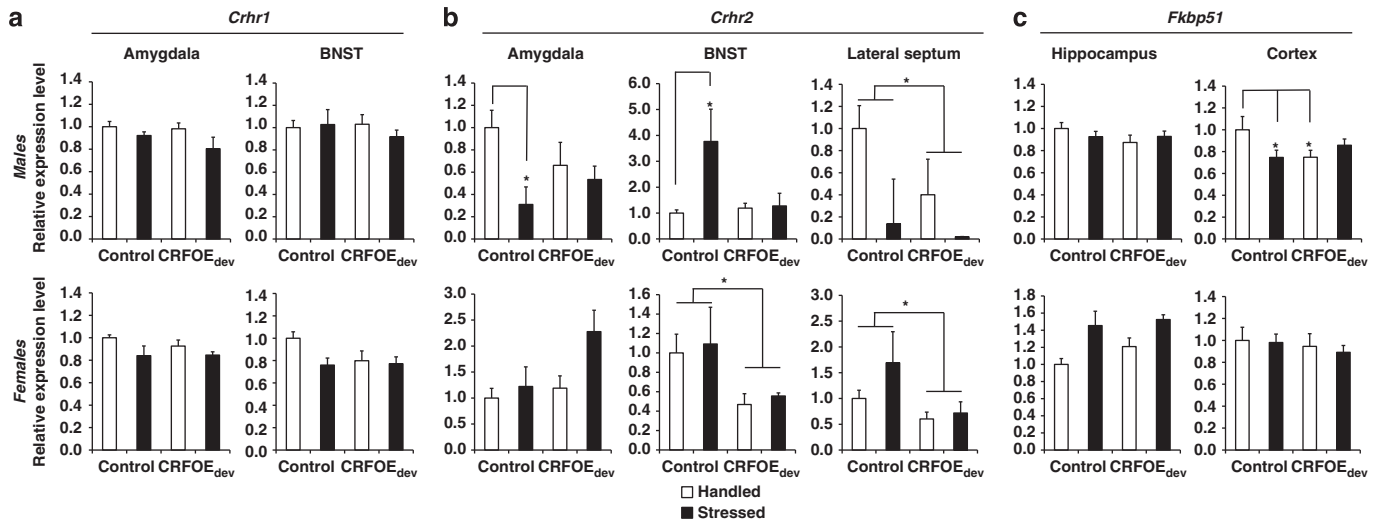

Figure 3 Long-term expression changes of *Crhr1* (a), *Crhr2* (b), and *Fkbp51* (c) in regions of interest. Data are presented as mean ± SEM of fold changes compared with handled 'no DOX' controls (normalized to housekeeping gene *Gapdh*). *$p < 0.05$; **$p < 0.01$ main effect of *CRHOE$_{dev}$* (*Crhr2*-Lateral Septum), or significant *post hoc* comparison to respective groups as indicated by lines. For full description of all main effects and trends, see Results. *BNST*, bed nucleus of stria terminalis; *CRHOE$_{dev}$*, transitional CRH overexpression before puberty; *Crhr1*/*Crhr2*, CRH receptor type 1 and type 2; *Fkbp51*, FK506-binding protein of the glucocorticoid receptor.

was also slightly decreased in BNST in female mice exposed to stress compared with handled controls (Figure 3a; $F_{sex}(1,85) = 4.39$, $p < 0.05$; $F_{stress \times CRHOE}(1,40) = 4.60$, $p < 0.05$; Tukey's *post hoc* test $p = 0.098$ handled controls *vs* stressed controls; Figure 3). The impact of stress on *Crhr2* expression depended on sex and CRHOE$_{dev}$ exposure. In the amygdala, *Crhr2* was reduced in stressed males, and marginally increased in females exposed to both CRHOE$_{dev}$ and stress

($F_{sex}(1,79) = 15.66$, $p < 0.001$; males: $F_{stress}(1,32) = 5.17$; $p < 0.05$; females: $F_{stress \times CRHOE}(1,40) = 3.35$; $p = 0.074$; Figure 3). In the BNST, stress increased *Crhr2* in non-CRHOE exposed males only ($F_{sex}(1,76) = 8.01$, $p < 0.01$; males: $F_{stress \times CRHOE}(1,35) = 14.33$, $p < 0.001$; *post hoc*: $p < 0.001$ compared to all other groups), whereas stress had no effect on *Crhr2* levels in females. $CRHOE_{dev}$ exposure, however, significantly reduced *Crhr2* levels in females regardless of stress ($F_{CRHOE}(1,36) = 5.69$, $p < 0.05$). In the lateral septum, *Crhr2* was significantly reduced by $CRHOE_{dev}$ in both sexes ($F_{CRHOE}(1,74) = 7.81$, $p < 0.05$; Figure 3) with no significant effects of stress. Stress-induced alterations of *Fkbp51* expression was also modulated by sex and $CRHOE_{dev}$. In males, predator exposure or $CRHOE_{dev}$ reduced *Fkbp51* expression compared with handled controls ($F_{stress \times CRHOE}(1,16) = 5.38$, $p < 0.05$; *post hoc*: $p < 0.05$ handled $CRHOE_{dev}$ and stressed controls *vs* handled controls; Figure 3). Stress marginally increased hippocampal *Fkbp51* expression in females ($F_{sex}(1,86) = 17.39$, $p < 0.001$; females: $F_{stress}(1,41) = 3.12$, $p = 0.084$).

DISCUSSION

Here we show that a single 'traumatic stress' event induced significant avoidance behavior that was modulated by forebrain-specific CRHOE during early-life in a sex-dependent manner. In female mice, trauma-induced avoidance was pronounced, but was not significantly influenced by early-life CRHOE. In contrast, male mice exhibited significant trauma-induced avoidance only when they had been exposed to early-life CRHOE. Hence, in males, forebrain CRH signaling during development may be sufficient to induce the 'double hit' phenomenon, in which early-life stress interacts with adult trauma to induce PTSD-like symptoms. Moreover, early-life CRHOE led to lasting increases of arousal indexed by startle reactivity in both sexes. Sex-specific alterations of *Fkbp51* and *Crhr2* expression in response to stress and/or $CRHOE_{dev}$ suggest that consequences of excess CRH signaling during development on stress pathways are dependent on sex and may explain the sexually dimorphic behavioral outcomes.

That predator stress significantly impacted avoidance in control females, but not in control males, suggests that this model may be predictive for mechanisms related to clinical findings reporting higher risk for women to develop stress disorders, including PTSD (Kessler *et al*, 2010; Koenen and Widom, 2009; Tolin and Foa, 2006). Moreover, it was only with the additional manipulation of CRHOE during early-life that males exhibited a response to predator stress. Accumulating evidence indicates that CRH-related mechanisms contribute to sex differences in stress reactivity and anxiety. For instance, sexes differ in CRH receptor and *Fkbp5* expression during early development, particularly following early-life stress (Bourke *et al*, 2013; Weathington *et al*, 2014). Moreover, enhanced CRH neurotransmission during early-life induces sex-specific alterations in monoaminergic systems (Curtis *et al*, 2006; Howerton *et al*, 2014; McEuen *et al*, 2009). The sex-dependent effects of predator stress in the present study may also be due to the reduced ability of females to desensitize CRH receptors (Bangasser *et al*, 2010). In the present study, males that were not exposed to $CRHOE_{dev}$ showed robust expression changes in *Crhr2* in response to predator stress, although the direction of change was different across brain regions in keeping with the differential effects of CRHR2 signaling on behavior across these regions (Hauger *et al*, 2009). Conversely, males exposed to $CRHOE_{dev}$ and female mice show no significant changes in *Crhr2* expression in response to stress. For example, males exhibited a >3-fold increase in *Crhr2* in BNST in response to stress, whereas females and males exposed to $CRHOE_{dev}$ showed no significant change in response to stress (Figure 3). These findings suggest that the *Crhr2* expression changes observed in males is a candidate mechanism for resiliency against enduring effects of trauma, and that early-life exposure to CRH could attenuate this adaptive response. Supporting the former suggestion, lentivirus-mediated increases in *Crhr2* expression in the BNST reduce PTSD-like susceptibility in male rats (Elharrar *et al*, 2013) but see Lebow *et al* (2012). 'PTSD-responsive' mice also show less overall transcriptional change in response to stress compared with 'PTSD-resilient' mice (Lebow *et al*, 2012), suggesting that enduring anxiety after trauma may be in part related to *attenuated* adaptation of stress systems. The present study also observed that males, but not females, exposed to $CRHOE_{dev}$ or stress exhibited reduced cortical expression of *Fkbp51*, a protein that curbs excess glucocorticoid signaling and modulates the association between early-life stress and PTSD (Binder, 2009; Yehuda *et al*, 2009; Sarapas *et al*, 2011; Yehuda *et al*, 2009). Recent prospective studies indicate that reduced *Fkbp5* expression *before trauma* is a risk factor for the development of PTSD (van Zuiden *et al*, 2012). Hence, reduced *Fkbp51* expression found in males is another candidate mechanism for $CRHOE_{dev}$ effects on anxiety. These data must be interpreted with caution, however, as specific manipulation of *Fkbp51* and *Crhr2* expression is required to confirm any causal relationship between expression changes and PTSD-like phenotypes observed. Overall, the differential pattern of *Crhr2 vs Fkbp51* expression changes in female and male mice supports the hypothesis that sex significantly modulates adaptive responses in CRH signaling during development (Bale *et al*, 2002; Bangasser *et al*, 2010).

Our present findings also support the conclusion that early-life CRH signaling modulates development of startle circuitry. These data are consistent with our and others' previous reports showing reduced PPI and habituation following developmental or lifetime CRHOE (Dirks *et al*, 2002; Groenink *et al*, 2008; Toth *et al*, 2014). Pharmacological and genetic manipulation studies reported increased startle and reduced PPI following CRHR1 receptor hypersignaling, whereas CRHR2 receptor stimulation increased PPI (Risbrough *et al*, 2003, 2004). Given that CRHR2 receptor stimulation increases PPI, $CRHOE_{dev}$-induced deficits in PPI may be due to reductions in expression of *Crhr2* in the lateral septum observed across sexes (Figure 3). In the lateral septum, *Crhr2* receptor expressing neurons mediate anxiogenic effects (Anthony *et al*, 2014), however, the effects of septal CRHR2 signaling on sensorimotor gating are unknown. In the present study, predator stress had no further impact on startle, despite previous reports that predator stress increases startle magnitude (Adamec *et al*, 2010). These data indicate that the predator stress model may be most consistent in modeling the avoidance-like components

of PTSD rather than full PTSD-syndrome. It is important to consider that reports of increased baseline startle, reduced habituation, and PPI are inconsistent in PTSD patients (Acheson *et al*, 2014). Indeed, PTSD is more robustly associated with increased startle reactivity in response to specific threat, not under baseline conditions as was assessed here (Grillon and Baas, 2003; Orr *et al*, 2002; Acheson *et al*, 2014).

Taken together, these data support the suggestion that early-life CRH hyper-signaling in the forebrain is sufficient to increase enduring effects of adulthood trauma in males. CRH may exert these effects via altering signaling (CRHR2) or the glucocorticoid feedback (Fkbp5) during development. Importantly, early-life CRH hyper-signaling results in structural deficits (Chen *et al*, 2004), anxiogenic, and despair-like effects, which cannot be reproduced by adult-onset CRHOE (Kolber *et al*, 2010; Toth *et al*, 2014). Here we show that these early-life stress effects are markedly modulated by sex, potentially via sex-specific compensatory mechanisms in response to CRH hyper-signaling. Accumulating evidence supports the importance of sex differences in the neurobiological consequences of stress pathway activation during development and response to trauma (De Bellis and Keshavan, 2003; Everaerd *et al*, 2012).

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