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Title: Overexpression of forebrain CRH during early life increases trauma susceptibility in adulthood

Running title: CRH over-expression and trauma susceptibility

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Abstract

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, we induced transient forebrain-specific CRH over-expression during early-life (pre-puberty, $CRHOE_{dev}$) in double-mutant mice (*Camk2a-rtta2* x *tetO-Crh*), and behavioral responses in the predator stress model of PTSD, and related gene expression changes were examined in adulthood. $CRHOE_{dev}$ induced lasting increases in startle reactivity independent of predator stress. $CRHOE_{dev}$ effects on trauma-induced avoidance were dependent on both $CRHOE_{dev}$ and sex. Whereas females exhibited robust responses to stress that were not altered by $CRHOE_{dev}$, males only developed anxiety-like responses when exposed to both $CRHOE_{dev}$ and stress. Sex also modulated the reducing effect of $CRHOE_{dev}$ on *Crhr2* and *Fkbp51* gene expression in limbic and cortical areas, respectively. Moreover, $CRHOE_{dev}$ blocked stressed-induced modulation of *Crhr2* in the extended amygdala in males. These findings indicate that forebrain CRH hyper-signaling in early-life is sufficient to increase enduring effects of adult trauma in males with significant alterations in *Crhr2* expression. Our study provides evidence for important sex differences in the consequences of developmental CRH-exposure on stress responses in adulthood, supporting that developmental CRH-exposure may contribute to increased risk for PTSD in males exposed to early-life stress.

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, e.g. decreased volume of the hippocampus, and altered amygdala-prefrontal functions, which are considered significant risk factors for PTSD (Dannlowski *et al*, 2012; Heim *et al*, 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 plays a role in this process as the central coordinator of the stress response. For instance, CRH is elevated in the cerebrospinal fluid (CSF) of patients diagnosed with PTSD and individuals with significant childhood trauma history (Bremner *et al*, 1997; 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 CSF 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, i.e. 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 play a role in childhood stress associations with PTSD risk (Binder, 2009; Bradley *et al*, 2008).

Methods and Materials

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-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 doxycycline (DOX) administration in breeder chow (Harlan Laboratories, Indianapolis, IN) to 'single-mutant' dams from postnatal day 2 for three weeks (PND2-PND23). Hence, CRHOE was induced only in double-mutant pups but not in dams. Typical litter sizes were 4-5 pups, producing 1 double mutant male and 1 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 one 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 (same 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 hr light/dark cycle (lights off at 8:00 a.m.). 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 due to lower levels of baseline avoidance behaviors.

Experimental design

All testing occurred from 10:00 a.m. to 6:00 p.m. 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 (13^{th} 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 sacrified for gene expression analysis.

Predator exposure

Mice were presented to a cat (Liberty Research, Waverly-NY, USA) in a well-lit room (2.3 × 1.8 m; 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 (grabbing orally without biting) the mouse. None of these behaviors differed between groups ($CRHOE_{dev}$ vs. controls; Table S2) 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 × 40 × 40 cm; 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 × 25 cm), 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 × 60 cm 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 × 40 × 20 cm chambers joined by a 6 ×6 cm 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 sec. 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 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, one week prior to stress exposure, baseline startle was assessed over 3 consecutive days using Session 1, which presented ten 105 dB pulses over 50 dB background in dark chambers. One week after stress exposure, startle reactivity was re-assessed in two consecutive sessions (Session 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 prior to 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 prepulse inhibition (PPI), a second session was presented

immediately after the first (Session 2 with 65 dB background and lights on). This session included 5 blocks beginning with the delivery of 5 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 3 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 5 pulses of 120 dB (Block 5) to assess habituation (from Block1 to Block5). For more details see Supplemental Material.

Quantitative real-time polymerase chain reaction (qRT-PCR)

In two separate cohorts of mice, 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 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 sacrificed, 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 Supplemental Material. For each sample, expression of each gene of interest was compared to 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 was included as a within-subject factor (Systat, Chicago-IL, USA). Because of consistent main or interactional effects of sex across behavioral tests, comparisons within each sex were completed for each test (Table 1 and 2). If groups differed in activity measures, an additional covariate analysis was also presented to control for non-specific activity effects. qRT-PCR data were analyzed using covariance analysis and variance estimation/precison 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 and factor-weighted 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 Supplemental 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

Mice exposed to predator stress showed increased avoidance of the center (all frequency, duration and latency measures: $F_{stress}(1,66)>8.08$, $p<0.01$; Fig.1; Table 1 and 2) with an overall decrease in exploration as measured by total distance moved ($F_{stress}(1,66)=13.25$, $p<0.001$; Table 1 and 2). Reduced center activity was independent from locomotor activity change as center time 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 $CRHOE_{dev}$ exposure on latency to enter the center was significantly modulated by sex and stress ($F_{sex\ x\ stress\ x\ CRHOE}(1,66)=5.23$, $p<0.05$), with increased latency in male mice exposed to both stress and $CRHOE_{dev}$ (compared to all other male groups: $0.017<p<0.085$; Table 1).

Avoidance in the light-dark box

$CRHOE_{dev}$ alone did not affect avoidance behavior but $CRHOE_{dev}$ females exhibited a trend for increased avoidance in non-stressed groups (duration: $F_{stress\ x\ CRHOE}(1,32)=3.49$, $p=0.071$, post hoc: $p=0.098$; Table 2) as described previously (Toth *et al*, 2014). 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; Table 1 and 2) in a sex- and $CRHOE_{dev}$-dependent manner (frequency, duration and latency measures: $F_{sex\ x\ stress\ x\ CRHOE}(1,66)=7.15$, $p<0.01$; $F_{sex\ x\ stress\ x\ CRHOE}(1,66)=5.62$, $p<0.05$; $F_{sex\ x\ stress\ x\ CRHOE}(1,66)=2.91$, $p=0.092$, respectively). Posthoc analysis revealed that male mice exposed to both $CRHOE_{dev}$ and stress exhibited higher avoidance (frequency and duration: $p<0.05$ and

p=0.063, respectively compared to handled $CRHOE_{dev}$; Fig.1 and Table 1). In contrast, females exhibited main effect of stress regardless of $CRHOE_{dev}$ exposure (Fig.1 and Table 2).

Avoidance of trauma-associated cue

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). In contrast, 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), and its effect on latency in males was driven by increased total distance moved ($F_{CRHOE}(1,66)=4.40$; $p<0.05$).

Avoidance across testing paradigms: combined avoidance score

The average z-score of three avoidance tests confirmed the highly significant effect of stress on avoidance (all measures: $F_{stress}(1,66)>11.08$, $0.001<p<0.002$; duration shown in Fig.1) which showed strong interaction with $CRHOE_{dev}$ in a sex-dependent manner (frequency and duration: $F_{sex\ x\ stress\ x\ CRHOE}(1,66)>7.82$, $0.002<p<0.007$). Post hoc analysis confirmed our finding in individual tests: predator stress increased avoidance only in male mice previously exposed to $CRHOE_{dev}$ as compared to handled $CRHOE_{dev}$ controls (frequency and duration: $p<0.05$ and $p<0.01$, respectively), whereas male stressed non- $CRHOE_{dev}$ did not show change of avoidance (Fig.1; Table1 and 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; $F_{sex\ x\ stress\ x\ CRHOE}(1,66)=10.19$, $p<0.01$;: post hoc: handled vs. stressed $CRHOE_{dev}$ $p<0.01$). 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; Fig.1 and Table 2).

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). 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 ($F_{stress}(1,66)=8.20$, $p<0.01$; Table S1).

Startle reactivity and PPI

Both prior to and following stress exposure, male and female $CRHOE_{dev}$ mice showed higher startle magnitude during Session 2 and 1 ($F_{CRHOE}(1,66)=4.61$, $p<0.05$; $F_{CRHOE}(1,66)=9.52$, $p<0.01$, respectively; Fig.2A and Fig.S1). $CRHOE_{dev}$ robustly reduced startle habituation in both sexes ($F_{block\ x\ CRHOE}(4,264)=2.72$, $p<0.05$; block effects: $p<0.05$ in controls, $p>0.3$ in $CRHOE_{dev}$ mice; Fig.2B). Similarly, PPI was significantly reduced by $CRHOE_{dev}$ ($F_{CRHOE}(1,66)=9.43$, $p<0.01$), although this effect was stronger in males (Fig.2C). When startle magnitude was added as a covariate, the $CRHOE_{dev}$ effect on PPI remained significant ($F_{stress}(1,65)=15.84$, $p<0.001$). Predator stress alone had no effect on any startle measures ($F_{stress}<2.08$, ns; $F_{stress\ x\ CRHOE}<1.22$, ns; Fig.2).

Gene expression changes induced by $CRHOE_{dev}$

Crhr1 in the amygdala showed a trend for decreased expression induced by stress ($F_{stress}(1,86)=3.14$, $p=0.080$), which was also present in the BNST in female mice ($F_{sex}(1,85)=4.39$, $p<0.05$; $F_{stress\ x\ CRFOE}(1,40)=4.60$; $p<0.05$; $p=0.098$ handled controls vs. stressed controls; Fig.3). The impact of stress on *Crhr2* expression was significant in all three regions investigated, but highly dependent on sex and/or $CRHOE_{dev}$ exposure. In the amygdala, *Crhr2* was reduced in 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: $F_{stress\ x\ CRFOE}(1,40)=3.35$; $p=0.074$; Fig.3). In the BNST, stress increased *Crhr2* level in control males only ($F_{sex}(1,76)=8.01$, $p<0.01$; males: $F_{stress\ x\ CRFOE}(1,35)=14.33$, $p<0.001$; posthoc: $p<0.001$ compared to all other groups), while it was reduced by $CRHOE_{dev}$ in females ($F_{CRFOE}(1,36)=5.69$, $p<0.05$). In the lateral septum, *Crhr2* showed significant reduction in both sexes induced by $CRHOE_{dev}$ ($F_{CRFOE}(1,74)=7.81$, $p<0.05$; Fig.3). Similarly, stress-inudced alterations of *FKBP51* expression were modulated by sex and $CRHOE_{dev}$.: stress and $CRHOE_{dev}$ alone (but not double exposure) decreased cortical expression in males ($F_{sex}(1,86)=3.60$, $p=0.066$; males: $F_{stress\ x\ CRFOE}(1,16)=5.38$, $p<0.05$; posthoc: $p<0.05$ handled $CRHOE_{dev}$ and stressed controls vs. handled controls; Fig.3), whereas stress marginally increased hippocampal 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 suggest that consequences of excess CRH signaling during development on stress pathways are dependent on sex, which 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 $CRHOE_{dev}$ in the present study may be due to differential CRH receptor expression in males and females (Weathington *et al*, 2014), and the reduced ability of females to desensitize CRH receptors (Bangasser *et al*, 2010). The present study shows that $CRHOE_{dev}$ produced long-term changes of *Crhr2* expression in adulthood, also leading to altered expression changes under stressful conditions. Additionally,

males, but not females, exposed to $CRHOE_{dev}$ exhibited reduced expression and stress-reactivity 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). Plasma *Fkbp5* is reduced in PTSD, and is negatively correlated with symptom severity (Sarapas *et al*, 2011; Yehuda *et al,* 2009). Recent prospective studies also indicate that reduced *Fkbp5* expression *before trauma* is a risk factor for development of PTSD (van Zuiden *et al*, 2012). Hence, reduced *Fkbp51* expression found in males is a plausible candidate mechanism for $CRHOE_{dev}$ effects on response to trauma, and is suggestive that forebrain CRH hyper-signaling during development is sufficient to induce an enduring shift in this pathway. The next step will be to utilize treatments that normalize *Fkbp51* expression in $CRHOE_{dev}$ mice to determine if this pathway is causally related to the increased susceptibility to stress.

Unlike in males, $CRHOE_{dev}$ exposure in females did not alter predator-stress response. A potential limitation is a ceiling effect of predator stress on anxiety-like responses which could compromise our ability to detect an increase in the $CRHOE_{dev}$ group. However, the overall composite score (Fig.1B) in female mice suggests that the combination of $CRHOE_{dev}$ and predator exposure tended to produce less avoidance than predator exposure alone. In females, $CRHOE_{dev}$ induced significant reductions of *Crhr2* expression in the BNST and lateral septum, and marginally increased stress-reactivity in the amygdala. It has been shown previously that reduction in *Crhr2* expression in the BNST reduces PTSD-like susceptibility in mice (Elharrar *et al,* 2013), but opposite effects were also shown (Lebow *et al*, 2012). In the lateral septum, *Crhr2* receptor expressing neurons mediate anxiogenic effects (Anthony *et al,* 2014). Therefore, it is possible that *Crhr2* expression changes induced competing anxiogenic-anxiolytic effects across regions, and moderated the effects of predator stress in female $CRHOE_{dev}$ mice. An alternative

explanation is that unlike males, female $CRHOE_{dev}$ mice did not develop reduced *Fkbp51* expression which may mediate $CRHOE_{dev}$ exacerbation of predator stress response. Importantly, the differential pattern of *Crhr2* vs. *Fkbp51* expression changes in female and male mice supports the hypothesis that sex significantly modulates adaptive responses to 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, while CRHR2 receptor stimulation increased PPI (Risbrough *et al*, 2003; Risbrough *et al*, 2004). 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).

Taken together, our 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 its postsynaptic machinery (CRHR2) or the glucocorticoid feedback (Fkbp5) during development. Indeed, early-life CRH hyper-signaling results in

hippocampal deficits (Chen *et al*, 2004), and its anxiogenic and despair-like effects cannot be reproduced by adult-onset CRHOE (Kolber *et al*, 2010; Toth *et al*, 2014). Importantly, these early-life stress effects are markedly modulated by sex, potentially via sex-specific compensatory mechanisms in response to CRH hyper-signaling. Consistently, accumulating evidence suggests the importance of sex differences in the neurobiological consequences of stress pathway activation during development (De Bellis and Keshavan, 2003; Everaerd *et al*, 2012).

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26 Mate Toth

Table 1. Avoidance behavior in males exhibited in the open field, light-dark box and modified open field with trauma-reminder. Data (presented as mean ± SEM) show the number of entries into the aversive arena (i.e. center, light compartment and zone around the tube filled with cat litter), the latency of the first approach, and distance travelled (total locomotor activity). Latter could not be quantified in the light-dark box as the dark compartment is fully covered (N/A). *p<0.05 compared to handled controls with the same CRH condition (post hoc); *$CRHOE_{dev}$*: transitional CRH over-expression before puberty.

CRH	***Stress***	***Number of entries***	***Latency of first approach***	***Distance travelled (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
Main effect of $CRHOE_{dev}$: Main effect of stress: Stress x $CRHOE_{dev}$:		F(1,34)<1, ns **F(1,34)=5.65, p<0.05** F(1,34)=3.40, p=0.073	F(1,34)<1, ns **F(1,34)=6.17, p<0.05** F(1,34)=3.90, p=0.057	F(1,34)<1, ns **F(1,34)=7.54, p<0.05** F(1,34)=1.72, ns
Light-dark box				
Control	Handled	15.6 ± 2.2	15.5 ± 6.6	N/A
	Stressed	19.7 ± 3.8	103.3 ± 66.0	N/A
$CRHOE_{dev}$	Handled	23.0 ± 3.8	40.4 ± 19.3	N/A
	Stressed	**13.2 ± 2.3***	86.5 ± 64.3	N/A
Main effect of $CRHOE_{dev}$: Main effect of Stress: Stress x $CRHOE_{dev}$:		F(1,34)<1, ns F(1,34)<1, ns **F(1,34)=4.62, p<0.05**	F(1,34)<1, ns F(1,34)<1, ns F(1,34)<1, ns	N/A
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
Main effect of $CRHOE_{dev}$: Main effect of stress: Stress x $CRHOE_{dev}$:		F(1,34)=2.14, ns **F(1,34)=3.92, p=0.056** F(1,34)<1, ns	**F(1,34)=4.72, p<0.05** F(1,34)<1, ns F(1,34)<1, ns	F(1,34)<1, ns F(1,34)=2.59, ns F(1,34)<1, ns
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

Main effect of $CRHOE_{dev}$:	F(1,34)<1, ns	F(1,34)<1, ns	F(1,34)<1, ns
Main effect of stress:	F(1,34)=3.44, p=0.072	F(1,34)=2.47, ns	F(1,34)=3.17,p=0.087
Stress x $CRHOE_{dev}$:	F(1,34)=2.87, p=0.098	F(1,34)<1, ns	F(1,34)<1, ns

Table 2. Avoidance behavior in females exhibited in the open field, light-dark box and modified open field with trauma-reminder. Data (presented as mean ± SEM) show the number of entries into the aversive arena (i.e. center, light compartment and zone around the tube filled with cat litter), and the latency of the first approach and distance travelled (total locomotor activity). Latter could not be quantified in the light-dark box as the dark compartment is fully covered (N/A). *p<0.05 compared to handled controls with the same CRH condition (post hoc); *$CRHOE_{dev}$*: transitional CRH over-expression before puberty.

CRH	***Stress***	***Number of entries***	***Latency of first approach***	***Distance travelled (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
Main effect of $CRHOE_{dev}$: Main effect of stress: Stress x $CRHOE_{dev}$:		F(1,32)<1, ns **F(1,32)=7.43, p<0.01** F(1,32)<1, ns	F(1,32)<1, ns F(1,32)=2.50, ns F(1,32)=2.09, ns	F(1,32)<1, ns **F(1,32)=5.70, p<0.05** F(1,32)<1, ns
Light-dark box				
Control	Handled	23.0 ± 2.3	30.7 ± 18.9	N/A
	Stressed	11.8 ± 3.0	189.0 ± 92.5	N/A
$CRHOE_{dev}$	Handled	19.1 ± 3.9	91.0 ± 66.1	N/A
	Stressed	15.4 ± 3.3	119.7 ± 50.3	N/A
Main effect of $CRHOE_{dev}$: Main effect of stress: Stress x $CRHOE_{dev}$:		F(1,32)<1, ns **F(1,32)=5.56, p<0.05** F(1,32)=1.44, ns	F(1,32)<1, ns **F(1,32)=5.92, p<0.05** F(1,32)<1, ns	N/A
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	10306 ± 1625
Main effect of $CRHOE_{dev}$: Main effect of stress: Stress x $CRHOE_{dev}$:		F(1,32)=2.82, ns F(1,32)=1.12, ns F(1,32)=2.24, ns	F(1,32)<1, ns F(1,32)=1.90, ns **F(1,32)=6.65, p<0.05**	F(1,32)=2.38, ns F(1,32)<1, ns F(1,32)=1.45, ns
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 x $CRHOE_{dev}$:		F(1,32)=2.01, ns	F(1,32)< 1, ns	F(1,32)< 1, ns

Figure legends

Fig.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 above tests. All graphs indicate time spent in the aversive arenas (i.e. center of the open field, light compartment of the light-dark box, and zone around the tube filled with cat litter). Accordingly, higher/positive scores indicate increased approach of the arenas, lower/negative scores indicate increased avoidance. 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 vs. 'no-DOX' controls (*p<0.05; **p<0.01; ***p<0.001; indicated by 3-way ANOVA); whereas asterisks above bars indicate significant interaction effects with additional significant posthoc comparison vs. handled group with the same CRF background. $CRHOE_{dev}$: transitional CRH over-expression before puberty.

Fig.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 x $CRHOE_{dev}$ interaction; indicated by repeated measure ANOVA). $CRHOE_{dev}$: transitional CRH over-expression before puberty.

Fig.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 to no DOX controls. Asterisks and hash signs (*p<0.05; **p<0.01; $^{\#}0.05<p<0.05$; 3-way ANOVA) indicate either main effects of stress (*Crhr1*-Amygdala, *Fkbp51*-Hippocampus), main effect of $CRHOE_{dev}$ (*Crhr2*-Lateral Septum, *Crhr1*-BNST in females), or significant posthoc comparison to

respective groups as indicated by lines between groups. *BNST*: bed nucleus of stria terminalis; $CRHOE_{dev}$: transitional CRH over-expression before puberty; *Crhr1/Crhr2*: CRH receptor type 1 and type 2; *Fkbp51*: FK506 binding protein of the glucocorticoid receptor.

Fig.1.

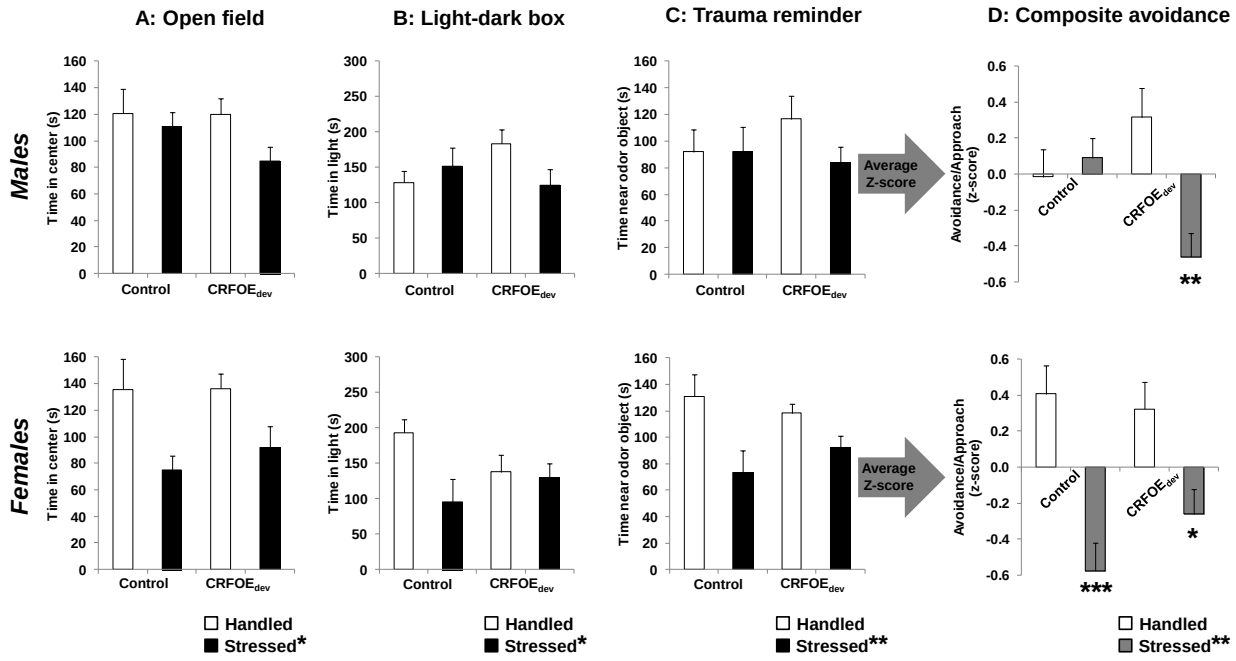

A: Open field
B: Light-dark box
C: Trauma reminder
D: Composite avoidance
Males
Females
Time in center (s)
Time in light (s)
Time near odor object (s)
Avoidance/Approach (z-score)
Average Z-score
Control
CRFOE$_{dev}$
Handled
Stressed*
Stressed**

Fig.2.

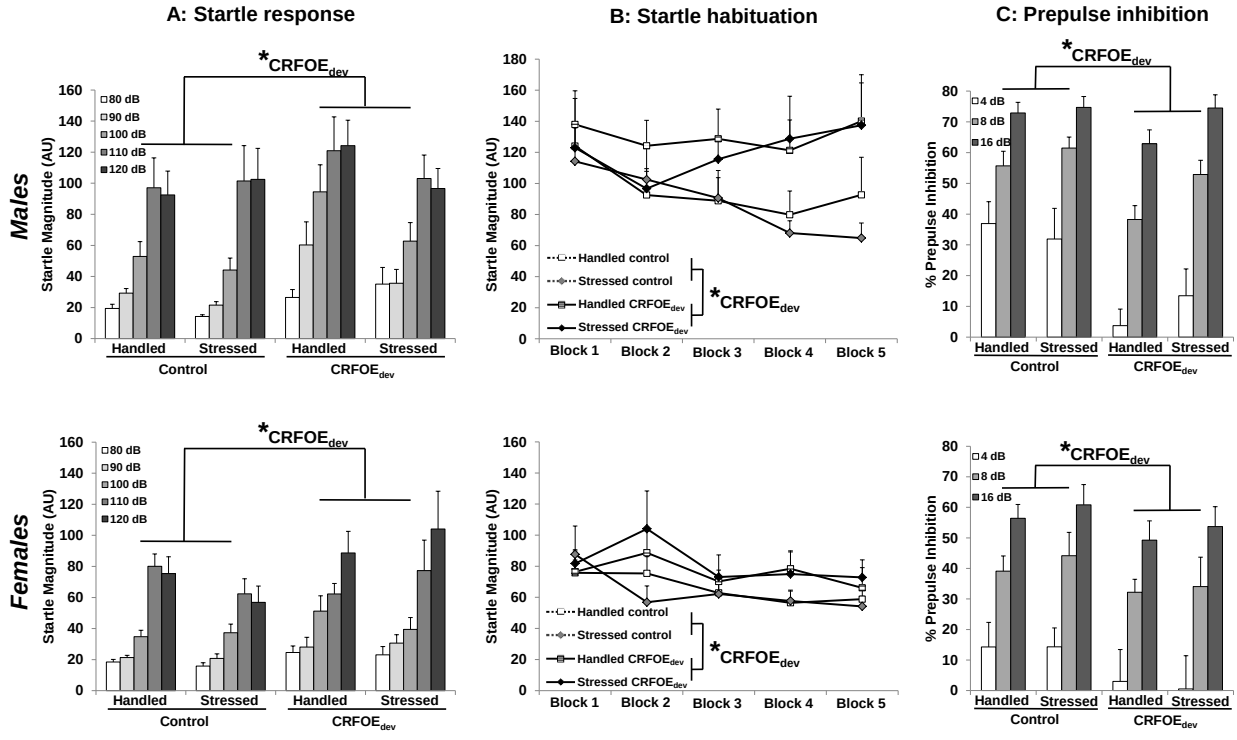
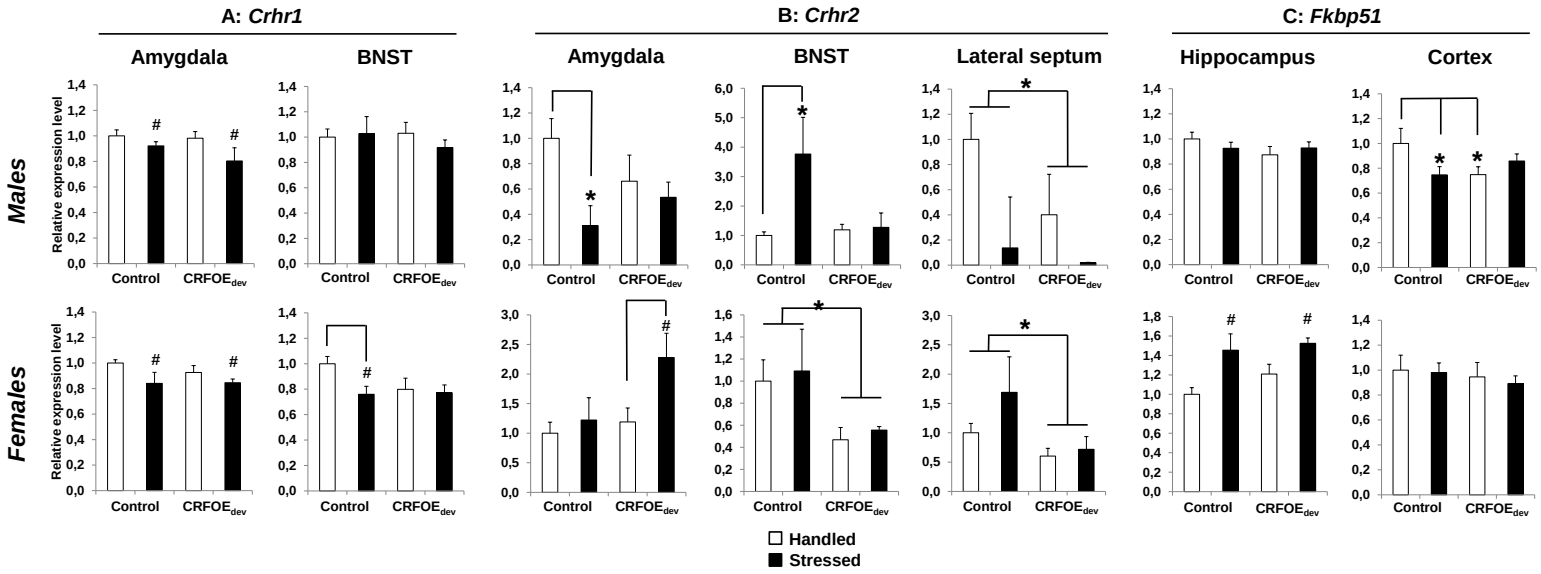

A: Startle response
B: Startle habituation
C: Prepulse inhibition
Males
Females
*CRFOE$_{dev}$
80 dB
90 dB
100 dB
110 dB
120 dB
Startle Magnitude (AU)
Handled
Stressed
Control
CRFOE$_{dev}$
Handled control
Stressed control
Handled CRFOE$_{dev}$
Stressed CRFOE$_{dev}$
Block 1
Block 2
Block 3
Block 4
Block 5
4 dB
8 dB
16 dB
% Prepulse Inhibition

Fig.3.


A: Crhr1
B: Crhr2
C: Fkbp51
Amygdala
BNST
Amygdala
BNST
Lateral septum
Hippocampus
Cortex
Males
Females
Relative expression level
Control
CRFOE dev
Handled
Stressed