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DTI-identified microstructural changes in the gray matter of mice overexpressing CRF in the forebrain

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Abstract

Increased corticotropin releasing factor (CRF) contributes to brain circuit abnormalities associated with stress related disorders including posttraumatic stress disorder. However, the causal relationship between CRF hypersignaling and circuit abnormalities associated with stress disorders is unclear. We hypothesized that increased CRF exposure induces changes in limbic circuit morphology and functions. An inducible, forebrain specific overexpression of CRF (CRFOE) transgenic mouse line was used to longitudinally investigate its chronic effects on behaviors and microstructural integrity of several brain regions. Behavioral and diffusion tensor imaging studies were performed before treatment, after 3–4 wks of treatment, and again 3 mo after treatment ended to assess recovery. CRFOE was associated with increased perseverative movements only after 3 wks of treatment, as well as reduced fractional anisotropy at 3 wks in the medial prefrontal cortex and increased fractional anisotropy in the ventral hippocampus at 3 mo compared to the control group. In the dorsal hippocampus, mean diffusivity was lower in CRFOE mice both during and after treatment ended. Our data suggest differential response and recovery

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Declaration of Competing Interest

We can assure that all authors have contributed to the drafting or revision of the manuscript for important intellectual content. We can assure that: (i) the final manuscript has not been published before; (ii) it is not under consideration for publication anywhere else; (iii) its publication has been approved by all co-authors. We have no conflicts of interest to declare.

Compliance with ethical standards

Ethical approval: All experimental procedures in mice were in compliance with the Principles of Laboratory Animal Care, National Institutes of Health guidelines, as approved by the University of California San Diego Institutional Animal Care and Use Committee (protocol numbers S09179 and S15184).

Supplementary materials

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patterns of cortical and hippocampal subregions in response to CRFOE. Overall these findings support a causal relationship between CRF hypersignaling and microstructural changes in brain regions relevant to stress disorders.

Keywords

Corticotropin-releasing factor; Diffusion tensor imaging; Medial prefrontal cortex; Hippocampus; Spatial memory; Exploration

1. Introduction

CRF and its two receptors expressed in the central nervous system, CRF receptors type 1 (CRFR1) and type 2 (CRFR2), regulate neuroendocrine, immune and behavioral responses to stress (Bale and Vale, 2004; Dautzenberg and Hauger, 2002; Deussing and A. Chen, 2018). Chronic stress leads to the hyperactivation of the hypothalamicpituitary-adrenocortical (HPA) axis. In addition to the higher production and release of glucocorticoids, dysregulations in CRF signaling pathways have been associated with stress-related disorders, including posttraumatic stress disorder (PTSD) and depression, as well as neurodegenerative disorders, such as Alzheimer's disease (Jiang et al., 2019; Vandael and Gounko, 2019). In animals, overproduction of CRF induces anxiety-like behaviors (Risbrough and Stein, 2006; Stenzel-Poore et al., 1994) and increased enduring responses to traumatic stimuli (Toth et al., 2015). Acute and chronic activation of CRFR1 in limbic circuits including amygdala, hippocampus and medial prefrontal cortex (mPFC) in particular contributes to anxiety as well as hormonal responses to stress (Y. Chen et al., 2001; Jafari and Bhatnagar, 2007; Ketchesin et al., 2017; Van Pett et al., 2000). These preclinical data support a potential contribution of CRF signaling to symptom development in anxiety disorders. In humans, increased CRF levels in cerebrospinal fluid is reported in some individuals with trauma exposure and stress-related disorders (Baker et al., 1999; Bremner et al., 1997; Heim et al., 1997; Lee et al., 2005; Nemeroff et al., 2006; Sautter et al., 2003). Candidate gene studies suggest that CRFR1 gene polymorphisms are associated with enduring effects of childhood trauma (Bradley et al., 2008; Tyrka et al., 2009) and may predict onset of PTSD in traumatized pediatric patients (Amstadter et al., 2011). The largest GWAS of PTSD patients to date (N>165,000) recently reported that a mutation in the locus for the CRFR1 gene was significantly associated with PTSD symptoms (Gelernter et al., 2019). Hence evidence in PTSD patients supports CRF signaling as a potential contributor to PTSD risk and/or symptom development.

Changes in the brain microstructure have been investigated using diffusion tensor imaging (DTI) following psychological trauma. DTI allows the measure of fractional anisotropy (FA) and mean diffusivity (MD). FA describes the directional coherence of water diffusion in the brain, reflecting the axonal or dendritic projections. MD, on the other hand, indicates the rotationally invariant magnitude of water diffusion within the brain tissue, and reflects the degree of tissue density. Whereas reduced FA in brain tissue can result from decreased axonal number, increased MD signals less tissue density (Alexander et al., 2007). Moreover, increased FA and reduced MD in brain have been associated with glial activation and axonal

swelling. Gulf War veterans with PTSD exhibit higher FA and lower MD in the cingulum compared to veterans without PTSD (Bierer et al., 2015). Another study found decreased FA and increased MD in the ventromedial PFC (vmPFC) in trauma-exposed victims with PTSD, compared to the same trauma-exposed individuals without PTSD (Sun et al., 2013). However, the causal relationship between stress and circuit abnormalities associated with stress disorders are unclear. One potential mechanism is through hypersignaling of the corticotropin releasing factor (CRF) pathway (Risbrough and Stein, 2006). As a potential link between stress and brain structural alteration, animal studies showed the implication of CRF overexpression (CRFOE) and hypersignaling in the loss of dendritic spines, simplification of dendritic trees and atrophy of apical dendrites in both the hippocampus and mPFC following stress induction (Leuner and Shors, 2013; Maras and Baram, 2012). Activation of CRFR1 and CRFR2 has been linked to altered amygdalar-mPFC and hippocampal function (Ivy et al., 2010; Rainnie et al., 2004; Risbrough and Stein, 2006) that are commonly observed in patients with PTSD (Vasterling et al., 2009). Central or peripheral injection of a CRFR1 antagonist prevents dendritic atrophy in hippocampus in chronically stressed rats (Ivy et al., 2010). Together, these findings suggest that CRF hypersignaling contributes to the brain structural impairments associated with stress.

Altogether these findings suggest that trauma history is associated with CRF hypersignaling and altered hippocampal and amygdala-prefrontal functions, which are also significant risk factors for stress-related disorders (Deslauriers et al., 2017a). However, the causal relationship between excess CRF signaling and microstructural changes in the brain remains unclear. DTI provides information about cellular structure (O'Donnell et al., 2012) and, thus, may be especially sensitive to the microscopic loss of dendritic spines and subtle retraction of apical dendrites hypothesized to cause stress disorders (Gorman and Docherty, 2010). Human DTI studies generally involve cross-sectional comparisons of non-randomized groups, raising the possibility that premorbid or comorbid group differences might have caused the group differences in DTI signals rather than differences in the central response to stress. Identifying microstructural changes induced by transient forebrain-specific CRFOE may help to elucidate CRF-related mechanisms underlying the pathophysiology of stress-related disorders, including PTSD. By using DTI, a non-invasive methodology translatable across species, we tested the hypothesis that forebrain-specific CRFOE induces specific microstructural changes in the mPFC, dorsal hippocampus (dHP), ventral hippocampus (vHP) and amygdala, in correlation with memory impairment.

2. Material and Methods

2.1. Generation of mice with inducible forebrain-specific CRFOE and experimental timeline

Male and female mice with inducible forebrain-specific CRFOE were generated by crossing two genetically modified mouse lines carrying a CaMKII α promoter-driven rtTA2 transgene and a doxycycline (DOX)regulated tetO promoter fused to the CRF gene to produce double mutant mice (Michalon et al., 2005; Toth et al., 2014; Vicentini et al., 2009). Genotyping was carried out by quantitative polymerase chain reaction (Transnetyx, Memphis, Tennessee, USA). CRFOE was induced with DOX administration in mouse chow at a dose of 3.5 mg/g

body weight/day from postnatal day ~90–120, after which DOX was removed. In the same CRFOE mouse model, we have previously shown significant increases in CRF protein levels in the cortex, amygdala and hippocampus 3 wks after start of treatment, which returned to normal 3 mo after treatment had ended (Toth et al., 2014). Double mutant littermates without DOX treatment (therefore not overexpressing CRF) were used as CRFOE negative control subjects in all experiments. Twenty-two mice (11 males and 11 females) were randomized to DOX treatment and twenty (9 males and 11 females) to control treatment (no DOX) and underwent all imaging and behavioral sessions (see Figure 1 for experimental design and timeline). 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 7:00 AM). All imaging sessions and behavioral tests were performed from 9:00 AM to 6:00 PM and conducted in accordance with the *Principles of Laboratory Animal Care*, National Institutes of Health guidelines, as approved by the University of California San Diego Institutional Animal Care and Use Committee. Imaging and behavioral assessments were performed at three time points in each animal: baseline (before DOX treatment; T_1), ~3 weeks of DOX treatment (T_2), and again ~3 months after DOX treatment was ended (T_3) (Figure 1). Mean age in months of mice at each time point was matched for the two groups (DOX: $T_1 = 3.08 \pm 0.50$, $T_2 = 4.73 \pm 0.61$, $T_3 = 7.70 \pm 1.01$; No DOX: $T_1 = 3.00 \pm 0.51$, $T_2 = 4.50 \pm 1.03$, $T_3 = 7.50 \pm 1.03$). We limited the behavioral assessments to spatial memory (object location task) and exploration (behavioral pattern monitor; BPM) to avoid stressful paradigms that could affect the brain morphology.

2.2. Object location task

The object location task provides a measure of hippocampus-dependent spatial memory. This test takes advantage of a rodent's natural preference for novelty without external reinforcement (Vogel-Ciernia and Wood, 2014). As previously described (Denninger et al., 2018), the task was performed in an open arena (58 × 58 × 30 cm). Two out of the four inner walls had either horizontal or vertical stripes design. Before each behavioral assessment, the mice were habituated to the testing room for 60 min. For three consecutive days, mice were habituated to the arena for 10 min. The fourth day, two trials were performed. During the first trial, the mice were allowed to investigate 2 Erlenmeyer flask filled with water for 10 min. The mice were then removed from the arena and the second trial started after an inter-trial interval (ITI) of 5 min. One of the two objects was moved to another corner, and the mice were placed in an empty corner. The time spent within a 2-cm radius zone around the objects was measured for 10 min using Ethovision Tracking Software (Noldus, Leesburg, VA, USA). The preference for the novel location in percent was calculated as 100 multiplied by the time spent within the novel location zone (t_N) divided by the sum of the time spent within the novel location and familial zones ($t_N + t_F$): $100 \times [t_N / (t_N + t_F)]$.

2.3. Behavioral Pattern Monitor

Exploratory and locomotor activities were assessed in BPM chambers (San Diego Instruments, San Diego, CA, USA) (Deslauriers et al., 2017b; Risbrough et al., 2006). Each BPM chamber is a clear Plexiglas box containing a 30 × 60 cm holeboard floor. A grid of 12 × 24 photobeams 1 cm above the holeboard floor providing a resolution of 1.25 cm (+16 beams detecting rears) allows us to locate the mouse. Each chamber contains eight wall

holes (1.25 cm diameter, 1.9 cm above floor) and three floor holes. Holepoking is detected with an infrared beam in each hole. Mice were placed in the BPM chambers (under light), and number of transitions (locomotor activity) and holepokes (exploratory behavior) were recorded for 60 min.

2.4. Imaging acquisition

We acquired in vivo MRI scans at the University of California San Diego Center for Functional MRI using a Bruker 7.0T/20cm horizontal magnet with Avance II hardware (Bruker, Billerica, MA, USA). Mice were anesthetized with an isoflurane-oxygen mixture (2% with an oxygen flow of 1.2–1.4 l/min) in order to minimize motion throughout the session. We positioned the mice in an animal holder with foam pads on each side of the head, front teeth were hooked onto a bite bar, and the shoulders secured with tape to further minimize head movement. A rectal thermometer monitored body temperature, which was kept at $\sim 37^{\circ}\text{C}$ core temperature using warm airflow during the entire imaging session. T2-weighted images were acquired using a Rapid Acquisition with Relaxation Enhancement (RARE) protocol in order to define regions of interest (ROIs) (TR/TE=9247/35ms, RARE factor=8, bandwidth=35,714Hz, field of view (FOV) $21.0 \times 16.1 \text{ mm}^2$, matrix 140×107 , in-plane resolution $150\mu\text{m} \times 150\mu\text{m}$, 80 slices, thickness=150 μm , averages=16, flip angle 85° , time=24min). Diffusion images were acquired using a navigated, 30 direction, 4-shot, spin echo DTI-echo planar images (EPI) protocol generating 5 images $b=0\text{s/mm}^2$, 30 diffusion directions, with $b=1000\text{s/mm}^2$, partial k-space and zero filling in the read direction. The flip angle was 90° , TR/TE 7500/23.34ms, bandwidth=250kHz, FOV $26.4 \times 12.0\text{mm}^2$, reconstructed matrix 176×80 , in-plane resolution $150\mu\text{m} \times 150\mu\text{m}$, 30 slices, thickness=600 μm , 1 average, time=17.5min. We acquired images using a two channel local receive coil combined with a 72 mm ID birdcage volume transmitter.

2.5. Image processing

We used Paravision software (Niendorf et al., 2015) to calculate diffusion tensors with eddy current correction and used Analysis of Functional NeuroImaging (AFNI) (Cox, 1996) and UCSD-developed software to process the resulting eigenvector maps. We calculated fractional anisotropy (FA) using the standard formula (Basser and Jones, 2002) and calculated mean, axial, and radial diffusivity from the eigenvalue maps (MD: average of the 3 eigenvalues λ_1 , λ_2 , and λ_3 ; AD: axial diffusivity (λ_1); RD: average of λ_2 and λ_3). The T2-weighted images were intensity bias corrected using the minimum contrast images to correct for field inhomogeneity. Skull and extraneous tissue were removed from the images using the AFNI 3dSkullStrip tool with manual editing when needed. Images were warped into the Waxholm Space (WHS) Atlas (Johnson et al., 2010) of the C57BL/6 mouse brain using a 12 parameter affine transformation with the absolute value of the local Pearson correlation as the cost function. The skull-stripped S_0 DTI image was then aligned to the WHS-transformed T2 images from the same session, using the alignment protocol described above, and the realignment parameters were applied to the remaining diffusion images (FA, MD, AD, RD).

2.6. Region of Interest Definition (ROIs)

We used DTI to investigate tissue integrity in four ROIs: the mPFC, amygdala, dorsal (dHP) hippocampus and ventral hippocampus (vHP). Although DTI metrics are often used to study the microstructural integrity of white matter, DTI measurements of gray matter also convey information about tissue integrity. In many gray matter regions, the primary diffusion direction is perpendicular to the cortical surface or to a plane normal to the cortical surface (Miller et al., 2013; McNab et al., 2011), perhaps reflecting the preferred orientation of cortical pyramidal cells (Briggs, 2010; Leguey et al., 2019). Other regions, such as the motor cortex (M1), show radial diffusion patterns (McNab et al., 2011). Regional variation in diffusion measurements might, therefore, be informative about regional variation in the cellular architecture of the cortex (McNab et al., 2011).

Two raters drew the hippocampus on T2 images of six mice warped to the WHS (see alignment protocol above). We calculated the Dice index of agreement for the hippocampal tracings to identify one male and one female mouse with good inter-rater agreement (Dice Female = .96, Dice Male = .98). To separate dHP from vHP, the hippocampal tracings of these two mice were divided at about Bregma -2.5, where the ventral extension of the hippocampal tracings began to appear (Franklin and Paxinos, 2008). The amygdala mask was drawn using Amira (Thermo Fisher Scientific, Waltham, MA, USA). The mPFC was defined on the WHS cortical ROI to encompass the frontal association cortex and extended to about Bregma 1.5, while avoiding the olfactory bulb. Although masks and DTI images had all been warped to the WHS atlas, the overlay of each ROI mask onto the FA and MD images was visually inspected for each mouse and manually nudged if a mask was misaligned a DTI image.

2.7. Statistical analysis

All diffusion tensor images processing and analyses, as well as behavioral analyses (novel location preference, transitions, holepokes) were conducted blinded to treatment. Missing data were mean imputed within CRFOE group and sex at each time point. Analyses comparing baseline assessments in control and CRFOE mice at baseline (before the start of DOX treatment) revealed no significant differences between control and CRFOE mice (Supplemental Table 1). With baseline value as a covariate, assessments at the 3wk and 3-mo time points were analyzed using repeated measures analysis of variances (ANOVAs) with time as within-subject factor, CRFOE and sex as between-subject factors. The inclusion of the baseline covariate in the statistical model, in order to control for between mouse variation at baseline, created regressed change scores as the primary outcome measures at 3 wks and 3 mo (Cohen, Cohen, West, Aiken, 2003). The main effect of CRFOE vs. control reflected group differences in a study variable averaged over the 3-wk and 3-mo follow-up periods, whereas the interaction of CRFOE with time represented the differential impact of CRFOE at the two follow-up time points. All ANOVAs were followed by Sidak post hoc comparisons. FA and MD were the primary DTI outcomes reported, with AD and RD results described when they helped to interpret the primary DTI outcomes.

3. Results

With baseline value as a covariate, repeated measures ANOVAs (time $\times$ CRFOE $\times$ sex) on the assessments at the 3-wk and 3-mo time points showed that sex did not interact with CRFOE in all parameters. Thus, the data have been collapsed across sexes, and repeated measures ANOVAs with time as within-subject factor, CRFOE as between-subject factor, and baseline assessment as covariate were conducted.

3.1. Forebrain-specific CRFOE increased holepokes, but did not affect spatial memory performance and locomotor activity

In the object location task, no effect of time, CRFOE or their interaction was found on preference for novel object location (Figure 2a), with no difference in spatial memory found across time between control and CRFOE mice. In the behavioral pattern monitor, no effect of time, CRFOE or their interaction was found on locomotor activity (transitions) (Figure 2b). CRFOE had a main effect on the number of holepokes ($F_{1,34} = 11.41$; $p < 0.01$; $\eta_v^2 = 0.32$), with increased number of holepokes in CRFOE mice compared to control mice 3 wks after the start of DOX treatment ($p < 0.001$ following post hoc test) (Figure 2c). This increase in CRFOE mice was reduced at 3 mo after the end of DOX treatment, with no significant difference between CRFOE and control groups (Figure 2c).

3.2. Forebrain-specific CRFOE induce changes in the gray matter structure

In the mPFC, repeated measures ANOVA of FA revealed a main effect of CRFOE ($F_{1,36} = 11.14$; $p < 0.01$; $\eta_v^2 = 0.25$) and interaction with time ($F_{1,36} = 10.92$; $p < 0.01$; $\eta_v^2 = 0.24$). Post hoc comparisons revealed that CRFOE mice exhibited lower FA compared to control mice ($p < 0.001$) 3 wks after the start of DOX treatment, but similar FA levels were found between CRFOE and control mice 3 mo after the termination of the treatment (Figure 3a). Figure 4 shows that the largest CRFOE effect at 3 wks occurred within the prelimbic and infralimbic regions of the mPFC. As Figure 3a suggests, the apparent normalization of FA at 3 mo was due in part to a reduction in FA from 3 wks to three months among control mice ($F_{1,19} = 4.93$, $p = .039$, $\eta_v^2 = 0.21$). Moreover, the effect of CRFOE on FA at 3 wks appears to be primarily due to reduced AD in CRFOE mice compared to control mice ($p < 0.05$, followed post hoc test), because no significant effects of CRFOE were observed in RD or MD (Table 1, Figure 3b). Interestingly AD continued to be lower in the CRFOE mice at 3 mo after termination of treatment (Table 1).

In the dHP, there were no significant effects of CRFOE, time, and their interaction on FA (Figure 3c). MD however, was significantly lower among CRFOE mice both 3 wks after the start of treatment and 3 mo after its completion (main effect of CRFOE only: $F_{1,36} = 4.32$; $p < 0.05$; $\eta_v^2 = 0.11$, Figure 3d). In contrast to the dHP, in the vHP CRFOE mice had significantly larger FA values at the 3-wk and 3-mo time points (main effect of CRFOE: $F_{1,36} = 4.40$; $p < 0.05$; $\eta_v^2 = 0.11$). There was also an overall effect of time, with lower FA observed across groups at the 3 mo after treatment completion compared with the 3-wk treatment point (main effect of time: $F_{1,36} = 7.29$; $p < 0.01$; $\eta_v^2 = 0.17$). Although the interaction of time with treatment was not significant, there was a significant linear decrease in FA from 3 wks of treatment to 3 mo post-treatment among control mice ($p < 0.05$),

leading to significantly higher FA in the CRFOE group at 3 mo ($p < 0.05$ compared to control group, following post hoc test) (Figure 3e). No effect of time, CRFOE or their interaction was found on MD in the vHP (Figure 3f and Table 1). In the amygdala, CRFOE, time or their interaction did not significantly affect FA or MD (Figure 3g–h and Table 1).

4. Discussion

The present study aimed to identify brain microstructural changes induced by forebrain-specific CRF hypersignaling. Forebrain-specific CRFOE induced alterations in both cortical and hippocampal circuits but showed different patterns of recovery. CRFOE mice exhibited reduced FA in mPFC after 3 wks of treatment that was recovered 3 mo after treatment ended. In the dHP, MD was lower in CRFOE mice both after 3 wks of treatment and 3 mo after treatment ended, suggesting more enduring effects of CRFOE in this hippocampal subregion. Finally, FA in the vHP was marginally higher after 3 wks of treatment in CRFOE mice compared to controls, but was significantly higher than controls after the 3 mo recovery period. These data suggest differential response patterns of cortical and hippocampal subregions to CRFOE and differential recovery after DOX treatment cessation.

CRF has been suggested to play a role in memory impairments and cognition (Bangasser and Kawasumi, 2015), and constitutive CRFOE in both brain and pituitary induces spatial learning impairments (Heinrichs et al., 1996). When CRFOE was limited to forebrain regions in adulthood however we found no changes in spatial learning, similar to other more limited CRFOE manipulations (Groenink et al., 2003). Here we limited the behavioral assessments to relatively simple spatial memory task and exploration to avoid stressful paradigms that could affect the brain morphology (e.g. water maze), thus we cannot conclude that forebrain CRFOE has no effect on other forms of spatial learning or memory. However spatial memory as assessed here does not seem to be related to the CRFOE effects observed on limbic morphology discussed below. CRFOE mice also exhibited a transient increase in holepokes, indicative of perseverative movements (Henry et al., 2013). This increase in perseveration in parallel with microstructural changes in the mPFC of CRFOE mice are in line with a previous study showing increased perseverative responding following mPFC lesions in prelimbic and infralimbic cortex (Passetti et al., 2002).

Interpretation of the cortical effects of CRF overexpression must account for naturally occurring maturational changes. We examined changes of brain microstructure in mice at 4 and 7 mo of age, with decreased FA over time in the mPFC and vHP of control mice. During this period cortical cells proliferate at a diminished rate in mice (Bordiuk et al., 2014). This reduction is largely based on reduced proliferation of oligodendrocyte precursor cells in the cortex (Colangelo et al., 2019; Hong et al., 2016; Ryu et al., 2016; Spalding et al., 2013) which might have diminished the concentration of myelinated pyramidal cells in the mPFC across time during the study. High-throughput electron microscopy of single pyramidal cells of the mouse neocortex has revealed a longitudinal distribution of their myelinated segments (Tomassy et al., 2014). Consequently, changes in the pattern of these longitudinal myelinated segments could alter the anisotropy of water diffusion, leading to decreased FA. Moreover, excitatory pyramidal neurons in the vHP send long-range projections to pyramidal cells in layer 5 of the prelimbic and infralimbic regions of the mPFC (Dégénétais

et al., 2003; Liu and Carter, 2018; Phillips et al., 2019). Our findings indicate that cortical maturation in young adult mice is associated with microstructural changes in the terminal nodes of this vHP-mPFC pathway. Overall, decreased FA in the vHP-mPFC pathway over time is in line with demyelination and decreased oligodendrocyte numbers and functionality associated with aging (Peters, 2002).

In this study we found that CRF hypersignaling altered naturally occurring maturational microstructural changes in the hippocampus of young adult mice. Compared to controls, CRFOE mice exhibited lower MD in the dHP and higher FA in the vHP both after 3 wks of treatment and after 3 mo recovery period. Interestingly, these microstructural changes persisted over time despite the normalization of CRF protein levels in the hippocampus 3 mo after the end of DOX treatment as previously shown (Toth et al., 2014). These findings are also in line with a preclinical study that has associated lower MD in the dHP with stress susceptibility in a chronic stress paradigm (Liu et al., 2018). This latest study altogether with our data support the contribution of CRF in stress-induced microstructural changes. A limitation of our study was that double-mutant mice not treated with DOX were used as negative controls instead of wild-type mice. However, we chose this control group in order to ensure littermate controls (e.g. similar genotype). Moreover, DOX alone has been previously shown to not exert functional effects in wild-type animals (Toth et al., 2014).

Chronic exposure to excessive levels of CRF in the hippocampus results in dendritic spine retraction and atrophy of dendrites (Chen et al., 2012). Either as a direct effect of CRF activation or as a result of their contribution to dendritic injury, stress hormones also activate glial cells and mast cells in brain (Kempuraj et al., 2017; Skaper et al., 2017). In mouse models of mild head injury, increased glial activation is linked to higher levels of FA and decreased MD in the hippocampus (Braeckman et al., 2019; Budde et al., 2011). The increased FA we observed in the vHP of CRFOE mice in the vHP at 3 mo and the reduction of MD we observed in the dHP at both time periods might have been due to reactive astrocytic activation. Alternatively, increases in anisotropy and decreases in diffusivity parameters may also be interpreted as axonal swelling (Bazarian et al., 2012; Mayer et al., 2010). Whether the DTI changes we observed in the hippocampus were due to neuroinflammation or to damage-induced axonal swelling will require further studies involving histological analyses of cell populations and their morphology.

CRFOE mice also exhibited reduced FA in mPFC after 3 wks of treatment that was recovered 3 mo after treatment ended. These observations are temporally in line with CRF expression in our model, in which we have previously shown increased CRF protein levels in the cortex 3 wks after start of treatment, that normalized 3 mo after treatment had ended (Toth et al., 2014). Blockade of CRFR1 prevents stress-induced apical dendritic retraction and spine loss in mPFC layer 5 pyramidal neurons (Yang et al., 2015). Damage to longitudinal pyramidal cells, particularly in layer 5, could lead to the lower levels of FA we observed in the mPFC after 3 wks of treatment in CRFOE mice. Inflammation and infiltration of microglia (Guglielmetti et al., 2016; Qin et al., 2018) may further alter diffusion of water molecules. Overall, the reduced FA in CRFOE mice suggests a mPFC dysfunction (Hupalo et al., 2019), in line with the elevated perseverative movements observed in the same mice (Passetti et al., 2002).

It is to be noted that in hippocampal and cortical regions CRF is primarily expressed in gamma-aminobutyric acid (GABA)-ergic interneurons (Gunn et al., 2019; Ketchesin et al., 2017) whereas our transgenic model expresses CRF in all forebrain neurons. In rodents, overall CRFOE in the forebrain induces behaviors relevant to stress induced activation of the endogenous CRF pathway (Lu et al., 2008; Toth et al., 2014), but forebrain-restricted CRFOE in GABAergic interneurons does not affect stress-coping behaviors (Lu et al., 2008). Altogether these findings suggest that CRFOE in the entire forebrain system, not specific neuronal populations, is critical to induce effects relevant to hyperactivation of the CRF system in response to stress. Still, it remains to be determined whether CRFOE specific to GABA interneurons induce distinct microstructural changes vs. those described in this study.

The findings here provided useful insights on the effects of maturation and CRF hypersignaling on brain microstructural changes. First, in this type of longitudinal imaging studies DTI measures cannot be assumed to be static over time, and interpretation of the outcomes must account for naturally occurring maturational changes. Second, CRFOE-induced effects on the cortical and hippocampal microstructure suggest a relationship between CRF hypersignaling and brain changes relevant to the pathophysiology of stress-related disorders. Individuals with PTSD display altered increased CRF levels (Baker et al., 1999; Bremner et al., 1997; Heim et al., 1997; Lee et al., 2005; Nemeroff et al., 2006; Sautter et al., 2003), and the largest GWAS of PTSD patients to date reported that a CRFR1 gene mutation was significantly associated with PTSD symptoms (Gelernter et al., 2019). Altogether these findings suggest that variance in CRF signaling pathways may play a role in PTSD risk and symptom maintenance. In line with our study showing CRFOE-induced microstructural changes in the mPFC, dHP and vHP, alterations in mPFC and hippocampal microstructure have been correlated with PTSD severity in children and adults following a traumatic event (Li et al., 2016; 2014; Niu et al., 2018). Our findings support a causal relationship between CRF hypersignaling and microstructural changes in brain regions relevant to the pathophysiology of stress-related disorders, and support interventions targeting CRF signaling pathways may be effective therapeutic strategies for disorders associated with CRF hypersignaling such as PTSD, depression, and Alzheimer's disease (Hauger et al., 2012; Vandael and Gounko, 2019).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Outline of the longitudinal design.

CRF overexpression was induced for one month with doxycycline (DOX) administration in mouse chow at a dose of 3.5 mg/g body weight/day starting at postnatal day ~90. Behavioral tasks and imaging sessions were conducted a month before (baseline) and 3–4 weeks after the start of DOX treatment, as well as 3 months after the end of DOX treatment (see material and methods for details).

Fig. 2. Forebrain-specific CRF overexpression increased exploratory behavior, but did not affect spatial memory and locomotor activity.

Spatial memory, as well as locomotor (transitions) and exploratory (holepokes) activities were assessed in control and CRF-overexpressing mice 3 weeks after the start and 3 months after the end of DOX treatment. Preference for the novel location (a), and the number of transitions (b) and holepokes (c) are presented as mean $\pm$ SEM in both male and female mice. Values were adjusted for baseline assessment in the statistical analysis model. *** $p < 0.001$ vs. control group (n=19–22 per group)

Fig. 3. Forebrain-specific CRF-overexpression induced microstructural changes in the gray matter structure.

Fractional anisotropy and mean diffusivity were assessed in control and CRF-overexpressing mice 3 weeks after the start and 3 months after the end of DOX treatment. Data are presented as mean±SEM for the medial prefrontal cortex (mPFC) (a-b), dorsal (c-d) and ventral (e-f) hippocampus, and amygdala (g-h) in both male and female mice. Values were adjusted for baseline assessment in the statistical analysis model. *p < 0.05 and ***p < 0.001 vs. control group (n=20–22 per group).

Fig. 4. Comparison of DOX-treated (CRFOE) mice to no DOX control mice.

Images are coronal slices in radiological view (left side of brain is the right hemisphere). Fractional anisotropy (FA) parametric maps are demonstrating Hedges' g effect sizes at 3–4 wks after start of DOX treatment (A) or 3 mo after the treatments ends (B) vs. baseline (before DOX treatment). Positive values reflect lower FA in DOX-treated mice (CRFOE) compared to control mice and negative values represent higher FA in the DOX-treated (CRFOE) group.

Table 1

Forebrain-specific CRF-overexpression induced microstructural changes in gray matter structures.

	Control 3 wks DOX	CRFOE	
		3 months post-DOX	3 months post-DOX
mPFC	AD	0.954 ± 0.007	0.946 ± 0.006
	RD	0.642 ± 0.008	0.658 ± 0.008
Dorsal Hippocampus	AD	1.030 ± 0.020	1.094 ± 0.029
	RD	0.692 ± 0.015	0.723 ± 0.017
Ventral Hippocampus	AD	0.993 ± 0.012	0.979 ± 0.015
	RD	0.653 ± 0.006	0.668 ± 0.011
Amygdala	AD	1.172 ± 0.021	1.212 ± 0.021
	RD	0.523 ± 0.013	0.523 ± 0.021

Axial (AD) and radial (RD) diffusivities were assessed in control and CRF-overexpressing mice 3 weeks after the start and 3 months after the end of DOX treatment. Data are presented as mean ± SEM for the medial prefrontal cortex (mPFC), dorsal and ventral hippocampus, and amygdala.

* $p < 0.05$ vs. control group at the same time point ($n = 20-22$ per group).