The role of corticotropin releasing factor in the development of anxiety disorders and stress vulnerability
Final research report (PD116589)
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Background and aims

Although severe traumatic stressors occur in the majority of general population, only a vulnerable subpopulation (~7-30%) develops maladaptive conditions, i.e. posttraumatic stress disorder (PTSD). Clinical studies documented several risk factors, indicating early-life adversities as the most significant predictors in the pathogenesis. However, underlying mechanisms are hardly understood how early-life stress shapes and shifts brain maturation towards a more vulnerable form. Corticotropin releasing factor (CRF) has been shown to regulate stress responses on endocrinological, autonomic, and behavioral levels with significant elevation in PTSD populations.

Our research aimed to test if CRF mediates early-life stress effects on anxiety-related circuitries, and hence, increase anxiety and stress vulnerability. We particularly focused on adolescent period that can be characterized by a second wave of plasticity and maturation in the brain, and importantly, this period is the average age of onset for most anxiety disorders. To test the impact of enhanced adolescent CRF signaling on long-term anxiety traits, we transiently induced CRF overexpression (CRFOEado) in the forebrain (extra-hypothalamically to exclude HPA-related changes) of double transgenic mice (gene induction limited to CRF neurons co-expressing CaMK2a: Michalon et al., 2005, Genesis 43(4): 205-212.) during adolescence/puberty (i.e. between postnatal days-PND 23-44, induced by doxycline administered in food chow) and tested long-term anxiety-related changes, including susceptibility for stress in adulthood. Since anxiety disorders and PTSD are twice as prevalent in females as in males, we have run both males and females and analyzed sex differences. Additionally, we contrasted our findings with additonal experiments run in pre-adolescent mice (CRFOE during PND 2-23) to identify age-specific effects of CRF.

At year 3, we started a series of casuality-oriented study focusing on local CRF effects in the bed nucleus of stria terminalis (BNST) based on our behavioral and gene expression findings.

Results

First, we confirmed that doxycycline reliably and transiently induces CRFOE in anxiety-related regions (Fig.1).

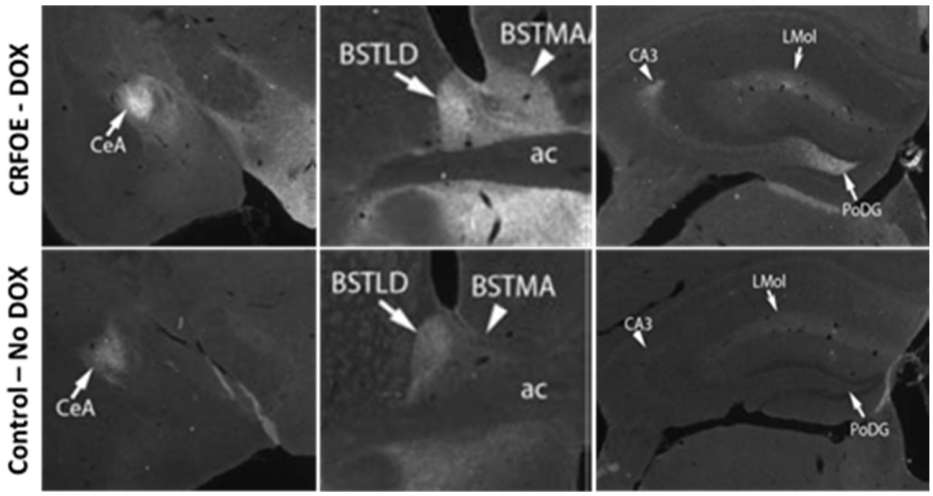

Fig.1. CRFOE induced by doxycycline (DOX) administration between PND23-44. CRFOE was apparent in major anxiety-related brain regions (i.e. central amygdala, BNST, hippocampus, monoaminergic nuclei (not illustrated here)). BSTLD, BSTMA-laterodorsal, anteromedial nuclei of bed nucleus of stria terminalis; ac-anterior commisure; CA3, Lmol, PoDG-subregions of hippocampus. DOX: doxycycline

In a second step, we tested if CRFOEado has long-term impact on anxiety-like phenotypes using the open field and light-dark box tests. We found anxiogenic effect in females indicated by reduced activity in the aversive/light part of the light-dark box ($F(1,10)=4.38$, $p<0.05$; Fig.2), suggesting that CRFOEado influence the maturation of anxiety-related circuitries in females, potentially contributing to their higher anxiety levels compared to males.

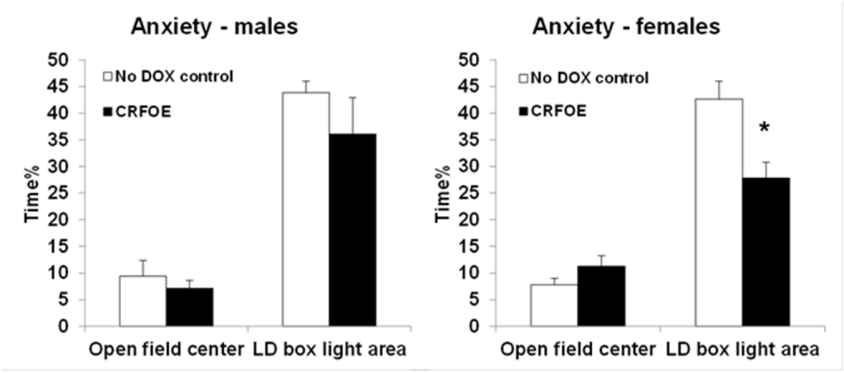

Fig.2. Long-term (adult) anxiety induced by adolescent CRFOE. Females exhibited increased anxiety indicated by reduced time spent in the aversive/light part of the light-dark (LD) box. Data are presented as mean ± SEM. #trend: $p<0.10$; DOX: doxycycline

Next, we tested if CRFOEado leads to altered fear learning in a Pavlovian conditioned fear paradigm by means of electric footshock pairings (7x 0.7mA) in a specific context. CRFOEado subjects of both sexes showed unaltered fear acquisition and contextual fear recall compared to controls as indicated by their freezing behavior (Fig.3; fear acquisition: $F(1,23)<1$, $p>0.75$; fear recall: $F(1,23)<1$, $p>0.45$).

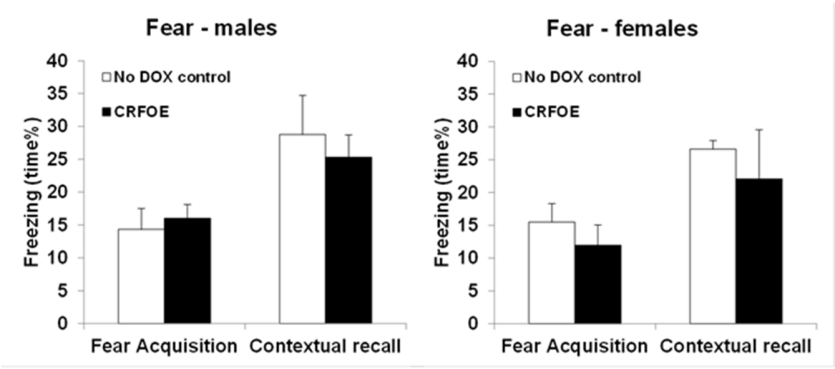

Fig.3. Adolescent CRFOE did not alter fear conditioning and recall. Fear aquisition was unaltered indicated by time spent with freezing during and between shock parings. Similarly, CRFOEado did not alter levels of freezing when exposed to shock-assaciated context 2 days later ('contextual recall'). Data are presented as mean ± SEM. DOX: doxycycline

We also tested if contextual learning/recognition under low-stress conditions is affected by CRFOEado by means of Y place recognition task, where preference of a novel (unexplored) arm over a familiar (previously explored for 10 min) arm of a test box indicates contextual/spatial recognition performance. Similar to fear learning, we found no difference between groups ($F(1,42)<1$, $p>0.45$), suggesting intact contextual learning/memory following CRFOEado under both stressful/aversive and low-stress conditions (although significant hippocampal CRFOE occurs following CRFOEado: see Fig.1). Noteworthy, these negative findings are in line with effects found in pre-pubertal CRFOE mice, i.e. no effect on conditioned fear characteristics (Toth et al., Neuropsychopharmacology, 2014, 39(6): 1409-1419).

Next, we were interested if latent changes (i.e. without manifested behavioral phenotype) were induced by CRFOEado that can be precipitated by subsequent stressors (i.e. 'stress vulnerability'). We applied a strong ecologically valid stressor, i.e. predator exposure, and tested subsequent anxiogenic consequences. We found significant interaction between CRFOEado and predator stress in females (but not males), i.e. CRFOEado enhanced the anxiogenic profile of predator stress in females only, that was most obvious when combined anxiety scores were calculated (Fig.4D-H; CRFHOE: $F(1,54)=11.31$, $p<0.01$, stress: $F(1,54)=4.06$, $p<0.05$). Latter

quantification aimed to reduce family-wise error and provide trait-like anxiety variables across tests as we previously found that multiple testing provide more reliable 'trait-like' anxiety scores whereas single testing is highly state-dependent. Interestingly, latter open field test detected anxiogenic effect induced by CRFOEado in both sexes (Fig.4A-E; CRFOE: $F(1,107)>6.26$, $p<0.05$), and also reproduced the anxiogenic effect in females found above (i.e. light-dark box, Fig.4F; CRFOE: $F(1,54)=5.42$, $p<0.05$).

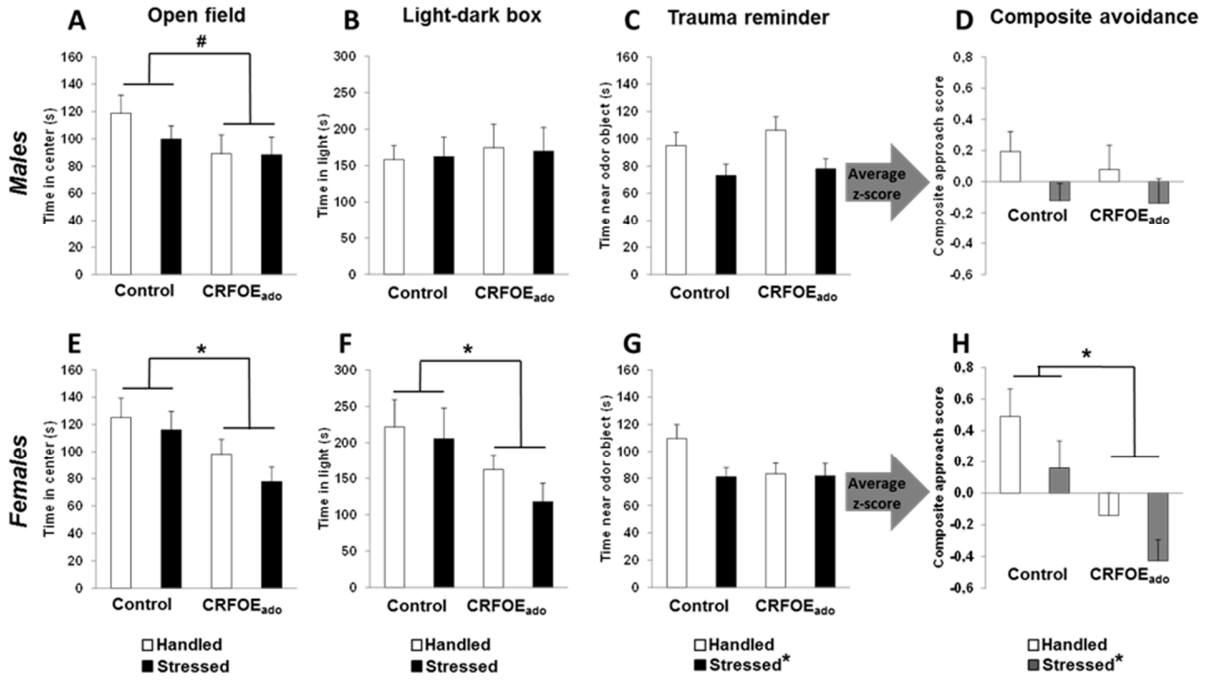

Fig.4. **Adolescent CRFOE increases avoidance behaviors in females that enhances predator stress-induced anxiety.** Anxiogenic effect was detected in males in the open field (A). Trauma reminder took place in the open field areana, where cat litter was presented in a corner and its avoidnace was measure as an index of anxiety. Composite avoidance scores were calculated as average z-score of time spent in the aversive arenas of the three tests (i.e. center of the open field, light compartment of the light-dark box, and zone around the tube filled with cat litter). For composite avoidance, positive and negative values indicate increased approach and avoidance, respectively, compared to the average of the same-sex experimental population. Upper and lower panels show data from males and females, respectively. Data are presented as mean ± SEM. Asterisks in legends indicate significant ($p<0.05$) stress effect compared to handled controls, whereas asterisks ($p<0.05$) and hash signs ($p<0.10$) above lines indicate significant or trend-like effects of CRHOEado.

Latter difference in the open field may be traced back to different anxiogenic settings of the test since we needed to change testing environment slightly between experiments (different rooms with moderately different light conditions, i.e. 600 lux previously vs. 900 lux in the latter experiment). This hypothesis is also supported by the fact that predator stress manifested more apparent anxiety-like differences between CRFOEado and control female mice, which again suggest that latent changes indeed occured following adolescent CRFOE.

In contrast to adolescent CRFOE, pre-adolescent CRFOE resulted in a 'double-hit' effect in males, i.e. males exposed to pre-adolescent CRFOE showed a markedly increased anxiety only when they were exposed to predator stress in adulthood (Fig.5; Stress: $F(1,34)=4.42$, $p<0.05$; Stress x CRFOE interaction: $F(1,34)=1.61$ $p<0.05$). These contrasting findings between pre-adolescent and adolescent CRFOEs point out the crucial role of timing including sex-specific effects as pre-adolescent CRF affected males only by enhancing stress sensitivity, whereas adolescent CRFOE affected females only by enhancing anxiety more generally with and without stress.

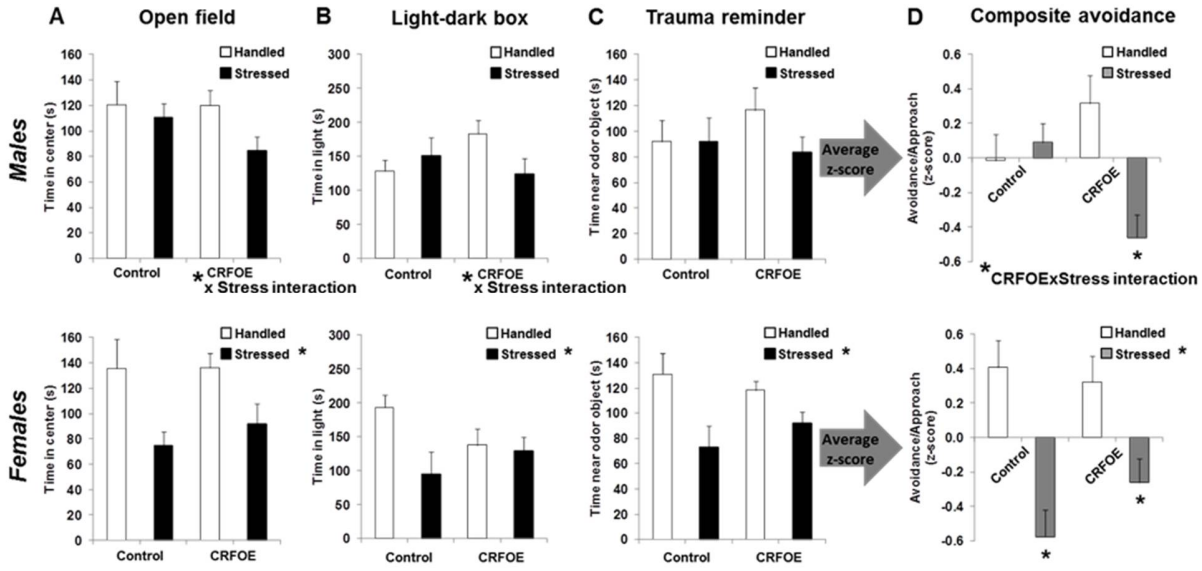

Fig.5. **Pre-adolescent CRFOE resulted in enhanced sensitivity for predator stress effect in males.** Stress increased anxiety in females without CRFOE effects. In contrast males exhibited no stress-induced anxiogenic effects alone (controls), but they exhibited increased anxiety when they were exposed to both pre-adolescent CRFOE and stress. This effect was most apparent in composite scores, which were calculated as average z-score of time spent in the aversive arenas of the three tests (i.e. center of the open field, light compartment of the light-dark box, and zone around the tube filled with cat litter). For composite avoidance, positive and negative values indicate increased approach and avoidance, respectively, compared to the average of the same-sex experimental population. Upper and lower panels show data from males and females, respectively. Data are presented as mean ± SEM. Asterisks in legends indicate significant ($p<0.05$) stress effect compared to handled controls, whereas asterisks ($p<0.05$) in males indicate significant interaction between stress and CRFOE.

This effect of time was also apparent in startle reactivity. Adolescent CRFOE decreased startle reaction in males (CRFOE: $F(1,54)=5.47$, $p<0.05$) whereas pre-adolescent CRFOE enhanced startle reactivity in both sexes (CRHOE: $F(1,66)=9.52$, $p<0.01$) (Fig.6), again underlying the importance of developmental stages with differential maturation and plasticity changes, and marked changes in CRF receptor expression (Weathington and Cooke, Endocrinology, 2012, 153(12): 5701-5705).

Noteworthy, reduced startle reaction in males exposed to adolescent CRFOE is surprising since CRF has been repeatedly shown to enhance startle reactivity using either systemic or local manipulation in the extended amygdala (Gresack and Risbrough, Int J Neuropsychopharmacol, 2011, 14(9):1179-94.; Toth et al., Psychopharmacology, 2013, 229(4):579-89). This unexpected outcome may be resolved by the fact that CRFOEado exerted a more chronic stimulation compared to acute pharmacological manipulations, and potential changes in CRF receptors can lead to different outcomes in pre-adolescent and adolescent subjects.

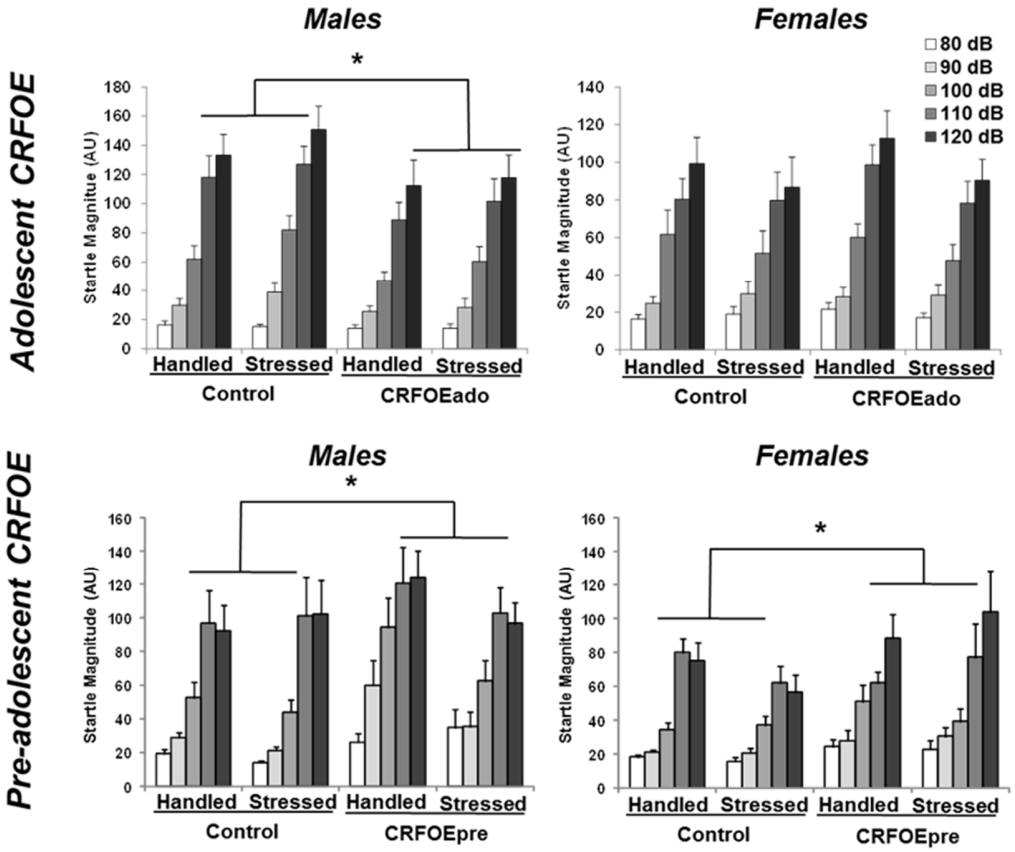

Fig.6. Startle response is reduced in males following adolescent CRFOE, whereas pre-adolescent CRFOE enhanced startle reactivity in both sexes. Data are presented as mean ± SEM. Asterisk indicate significant ($p<0.05$) main effect of CRFOE.

In order to identify CRF signaling related alterations in anxiety-relevant regions induced by CRFOE, we have run gene expression analysis for CRF receptors (type 1 and 2) in the amygdala, prefrontal cortex, hippocampus and BNST in both pre-adolescent and adolescent CRFOE mice. Interestingly, we found a single hit: significant decrease of CRF receptor type 2 in the BNST in females exposed to pre-adolescent CRFOE (Fig.7; CRFOE: $F(1,36)=5.69$, $p<0.05$).

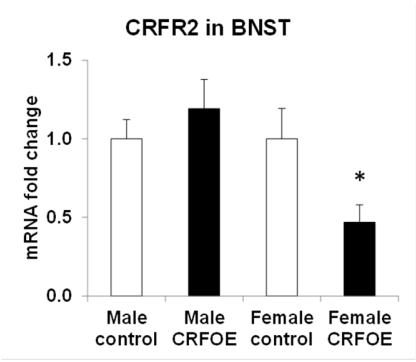

Fig.7. **CRF receptor type 2 expression is significantly reduced in females by pre-adolescent CRFOE.** Data are presented as mean ± SEM. Asterisk indicate significant ($p<0.05$) effect of CRFOE in females compared to all other groups.

Consistent negative findings on CRF-related genes following adolescent CRFOE led us to a decision point to turn to rather causal testing of CRF signaling in the BNST using chemogenetic manipulations of CRF+ neurons to identifiy specific circuitries potentially mediating anxiogenic effects, instead of further exploration of multiple set of candidate genes following CRFOE.

To provide tonic stimulation of CRF+ neurons of BNST, we expressed stimulatory and inhibitory 'designer receptor exclusively activated by designer drugs' (DREADD) receptors (AAV8-hSyn-DIO-hM3D-mCherry) or its control (fluorophore containing no active DREADD receptor: AAV8-hSyn-DIO-mCherry) in transgenic vGAT-ires-cre (vGAT: vesicular GABA transporter specific to GABAergic cells) and CRF-ires-cre male mice to manipulate BNST globally and CRF-specific manner, respectively. After 4 weeks of virus expression, we tested mice in three paradigms mentioned above: anxiety test, fear conditioning, and fear induced by predator stress exposure. We activated/inhibited BNST neurons by acute injection of the synthetic ligand of DREADD receptors, clozapine-N-oxyde (CNO) (intraperitoneal, 1mg/kg dose, 40 min before testing).

Chemogenetic activation of vGAT+ neurons had significant anxiogenic effect in the open field (Fig.8A; $F(1,25)=6.56$, $p<0.05$), whereas activation of CRF+ neurons had an opposite anxiolytic trend (Fig.8C; $F(1,25)=3.03$, $p=0.08$). In contrast to effects in the open field (low-stress conditions), chemogenetic manipulation of vGAT or CRF neurons did not result changes in predator odor avoidance (innate fear response: Fig.8B-D; $F<1$, $p>0.72$).

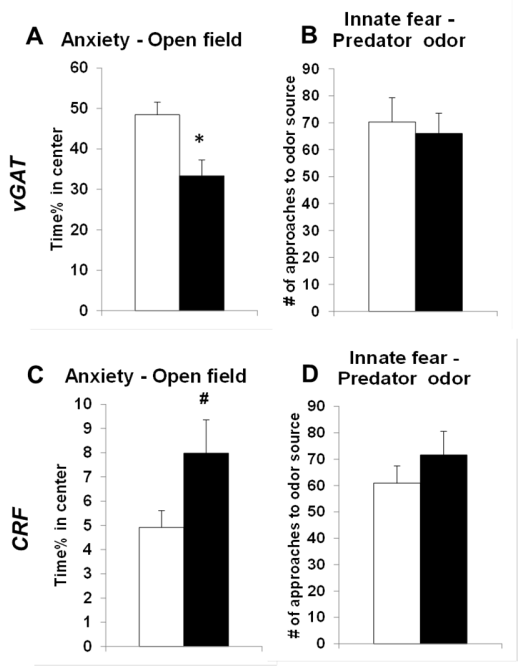

Fig.8. **Chemogenetic activation of GABAergic and CRF+ neurons of the BNST resulted in singificant anxiogenic and anxiolytic effect in the open field, respectively (A-C).** In contrast, avoidance of predator odor was not altered by chemogenetic manipulations (B-D). Data are presented as mean ± SEM. Asterisk indicate significant ($p<0.05$) of chemogenetic activation.

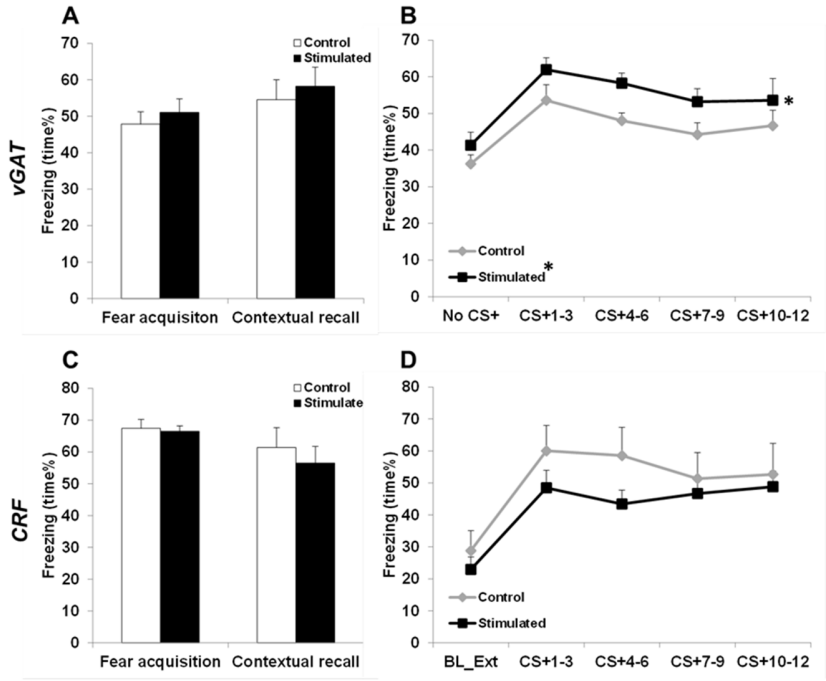

Fig.9. **Chemogenetic activation of BNST CRF+ neurons during fear acquisition results in enhanced cue-dependent fear recall** (B) without effecting fear acquisiton or contextual fear recall (A). Manipulation of CRF+ neurons had no significant effect on fear learning cparameters. Data are presented as mean ± SEM. Asterisk indicate significant ($p<0.05$) main effect of chemogenetic activation. CS+: conditioned stimulus paired with footshock.

Chemogenetic activation GABAergic neurons of BNST during fear acquisition (shock-cue conditioning) did not change either fear acquisition or contextual fear recall indexed by freezing behavior (Fig.9A; $F<1$, $p>0.84$), but enhanced cue-dependent fear recall (Fig.9B; $F(1,15)=6.77$, $p<0.05$). In contrast, stimulation of CRF+ neurons had no significant effect on cue-dependent fear recall (similarly to CRFOE effects) (Fig.9D; $F(1,21)=1.14$, $p>0.29$), with no alteration in acquisiton and contextual recall (Fig.9C; $F(1,21)<1$, $p>0.57$).

Summary

In the present research, we showed that early-life CRF hyper-signaling (a major neurochemical component of early-life stress centrally) has a significant impact on anxiety-like characteristics on the long-term. Importantly, present CRF manipulations were limited to early-life periods and excluded endocrine effects by using a forebrain/extra-hypothalamic transgenic model. In this model, we showed that timing of CRF signaling changes is crucial which/how behavioral domains are affected, and these effects are sex-dependent. Pre-adolescent CRF hyper-signaling resulted startle hyper-reactivity in both sexes with additional enhanced stress sensitivity in males. In contrast, adolescent CRF hyper-signaling affected females only implying that the emergence of sex differences in anxiety (higher in females) may manifest during this period (by puberty and second wave of plasticity). Interestingly, these behavioral effects were specific to anxiety-like traits (avoidance, startle reactivity) and did not involve fear learning characteristics. Downstream mechanisms are still to be elucidated since we could not detect marked CRF receptor-related expression changes in anxiety-relevant brain regions. Our chemogenetic studies targeting the BNST pointed out that competing effects may occur in the CRF system indicated by anxiolytic effect by chemogenetic stimulation of CRF neurons whereas anxiogenesis was elicited by global BNST stimulation.

Dissemination

Our findings were presented as several poster presentations at the IBRO Workshops, annual meetings of International Behavioral Neuroscience Society, FENS Regional Meeting, FENS, Society for Neuroscience (2019), Munich Winter Conference on Stress (2019), European Brain and Behaviour Society (2019). Major findings of the grant were published in two first-author papers (Toth et al., 2016; Mikics, Toth et al., 2017), in one co-author paper (Deslauiers et al.,

2019), and an invited review and a book chapter focusing on stress vulnerability and PTSD models (Deslauiers et al., 2018; Flandreau and Toth, 2017), whereas one manuscripts it to re-submission to Psychoneuroendocrinology, and a further manuscript is in preparation with some additional experiments to complete (submission planned this year).

Published articles based on this research:

1. Deslauriers J, Toth M, Zhou X, Risbrough VB. (2019) Heritable Differences in Catecholamine Signaling Modulate Susceptibility to Trauma and Response to Methylphenidate Treatment: Relevance for PTSD. Front Behav Neurosci, 13:111.
2. Deslauriers J, Toth M, Der-Avakian A, Risbrough VB. (2018) Current Status of Animal Models of Posttraumatic Stress Disorder: Behavioral and Biological Phenotypes, and Future Challenges in Improving Translation. Biol Psychiatry, 83(10): 895-907.
3. Flandreau EI, Toth M. (2018) Animal Models of PTSD: A Critical Review. Curr Top Behav Neurosci, 38: 47-68.
4. *Mikics E, *Toth M, Biro L, Bruzsik B, Nagy B, Haller J. (2017) The role of GluN2B-containing NMDA receptors in short- and long-term fear recall. Physiol Behav, 177: 44-48. (*same contribution first authorship)
5. Toth M, Flandreau EI, Deslauriers J, Geyer MA, Mansuy IM, Merlo Pich E, Risbrough VB. (2016) Overexpression of Forebrain CRH During Early Life Increases Trauma Susceptibility in Adulthood. Neuropsychopharmacology, 41(6):1681-90.