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Research report

Amygdalar corticotropin-releasing factor mediates stress-induced anxiety

Ana Paula Ventura-Silva[a,b,1], Sónia Borges[a,b], Nuno Sousa[a,b], Ana João Rodrigues[a,b,*,2], José Miguel Pêgo[a,b,*,2]

[a] Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal
[b] ICVS/3B's-PT Government Associate Laboratory, Braga/Guimarães, Portugal

HIGHLIGHTS

- Knockdown of CRF in the CeA decreased stress-induced anxiety levels.
- Stress increases corticosterone levels and this effect is attenuated by CRF knockdown.
- Stress induces alterations in the expression of CRF receptors in the BNST.
- Amygdalar knockdown of CRF attenuates the stress-induced changes in the expression of CRF receptors.

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ABSTRACT

The extended amygdala, including the Central nucleus of the Amygdala (CeA) and the Bed Nucleus of the Stria Terminalis (BNST), is a complex structure that plays a pivotal role in emotional behavior. The CeA and the BNST are highly interconnected, being the amygdala traditionally more associated with fear and the BNST with anxiety. Yet, studies using excitotoxic lesions also show the involvement of the CeA in the development of stress-induced anxiety. Likewise, others have also highlighted the role of corticotropin-releasing factor (CRF), a neuropeptide highly expressed in CeA, as an anxiogenic factor and, consequently, important for in anxiety disorders.

Here, we used an inducible RNAi lentiviral system to assess the effects of reducing CRF expression in CeA in the development of anxiety-like behavior in a model of Chronic Unpredictable Stress. In addition, we evaluated CRF RNAi-mediated alterations in the stress-triggered molecular signature in the BNST.

Knockdown of CRF in the CeA decreased stress-induced anxiety levels. No differences were found in a fear-potentiated startle paradigm. Additionally, we observed that stress-induced alterations in the expression of CRF receptors within the BNST are attenuated by CRF knockdown in the CeA.

These results emphasize the importance of the role that amygdalar CRF plays in the modulation of anxiety-like behavior and in the molecular signature of stress in the BNST.

1. Introduction

Psychiatry disorders, such as anxiety, are one of the most prevalent disorders in modern society and create a heavy burden both to the well-being of individuals but also in health care systems. Anxiety is characterized by a persistent state of arousal/apprehension towards threatening cues (Koch, 1999; LeDoux, 2000) and its presence is often associated with stress exposure (Shin and Liberzon, 2010).

Brain areas involved in the stress network, such as the prefrontal cortex, hippocampus, bed nucleus of the stria terminalis (BNST) and the amygdala are often also associated with the development of anxiety-like behavior. In fact, using chronic unpredictable stress (CUS) as a model of anxiety, we have observed several morphological and neurochemical alterations in stress-related neuronal circuitries as well as an increase in anxiety-like behavior (Cerqueira et al., 2007; Ventura-Silva et al., 2012).

In particular, the extended amygdala, composed in part by the central and medial amygdala and the BNST (Alheid et al., 1998), is a cluster of complex structures that play an important role in emotional behavior functions, such as fear conditioning and anxiety (Lebow and Chen, 2016; LeDoux, 2000; Walker et al., 2003). The extended amygdala is, for this, particularly susceptible to chronic stress. We have

* Corresponding authors at: Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal.
E-mail addresses: ajrodrigues@med.uminho.pt (A.J. Rodrigues), jmpego@med.uminho.pt (J.M. Pêgo).
[1] Present address: APC Microbiome Institute, University College Cork, Cork, Ireland.
[2] These authors share seniority.

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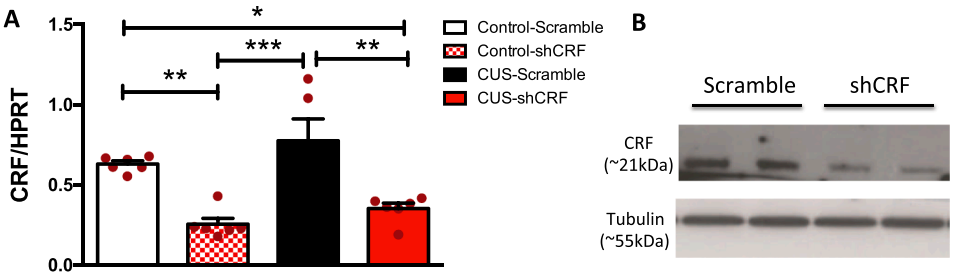

Fig. 1. Efficacy of the knockdown of CRF in the CeA using a lentiviral approach. Injection of shCRF in the CeA leads to decrease CRF levels of mRNA (A) and protein (B) in this amygdalar nucleus. Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p ≤ 0.001.

previously shown that CUS leads to an hypertrophy of the BNST, increased activation of the anterior BNST and also alterations in the expression of genes associated with the CRF, GABA and glutamatergic systems (Pego et al., 2008; Ventura-Silva et al., 2012). The amygdala and the BNST are particularly important in the development of fear and anxiety, with the amygdala modulating adaptive responses to imminent threats (phasic fear) and the BNST to more unspecific stimuli that lead to a long lasting state of apprehension (sustained fear or anxiety) (Walker et al., 2009). Although traditionally the amygdala has been more associated with phasic fear, this brain region is also involved in the modulation and development of anxiety (Adhikari et al., 2015; Davis et al., 2010; Walker et al., 2009). Importantly, we have shown that excitotoxic lesions in the central nucleus of the amygdala (CeA) attenuate the development of stress related anxiety (Ventura-Silva et al., 2013), specifically by attenuating anxiety-like behavior measured in the elevated plus maze (EPM) and stress-induced increase in corticosterone levels. Others also report that amygdalar lesions disrupt the hypothalamus-pituitary-adrenals (HPA) axis by decreasing ACTH/corticosterone secretion (Beaulieu et al., 1986).

Projections between the BNST and the central amygdala can modulate anxiety-like behavior (Yamauchi et al., 2018) and it his hypothesized that the BNST acts as a relay station between limbic areas such as the CeA and the PVN (Herman et al., 2005). This theory is reinforced by the strong projections existing between the CeA and the anterior BNST that, in turn, projects to the PVN (Dong et al., 2001).

Interestingly, the CeA is a major source of extra-hypothalamic corticotropin-releasing factor (CRF) in the brain. CRF is a small peptide that has been implicated in the neuroendocrine and behavioral responses to stress (for review (Deussing and Chen, 2018)). CRF and CRF-related peptides act through two different receptors, CRFR1 and CRFR2 receptors. CRFR1 is widely expressed throughout the brain while CRFR2 expression is more restricted but it is highly present in limbic areas (Perrin and Vale, 1999; Primus et al., 1997; Van Pett et al., 2000). CRFR1 chronic activation by stress-induced release of CRF has been associated with the development of anxiety disorders; whereas CRFR1 antagonists have been shown to reduce anxiety-like behavior (Holsboer, 1999; Muller et al., 2003; Refojo et al., 2011; Sahuque et al., 2006). On the other hand, CRFR2 are involved in the attenuation of the stress response (Henckens et al., 2016; Issler et al., 2014; Kishimoto et al., 2000). Evidence from our lab also points to alterations in the CRFergic system in a stress-model of anxiety (chronic unpredictable stress – CUS), specifically with a decrease in the expression of CRFR1 in nuclei located in the anterior BNST (Ventura-Silva et al., 2012). Moreover, it has been shown that stress can induce an increase in CRF levels in different brain areas, including the extended amygdala (Cook, 2004; Shepard et al., 2005).

Specific manipulations of CRF levels in the CeA have led to conflicting results regarding the effects of this neuropeptide in anxiety; while overexpression of CRF seems to lead to increase in anxiety-behavior and an hyperactivity of the HPA axis (Kalin et al., 2016; Kunugi et al., 2006; Natividad et al., 2017; Paretkar and Dimitrov, 2018), the effects of a downregulation of this peptide in the CeA in anxiety behavior are not clear. Different studies have used knockdown of CRF in CeA followed by acute stress approaches and have shown either an attenuation of anxiety following acute stress (Regev et al., 2012) or no effects in this behavior (Callahan et al., 2013). Considering our previous work showing the importance of the CeA in the development of anxiety following chronic stress (Ventura-Silva et al., 2013), it is important to understand the impact of manipulation of CRF levels in a model of chronic stress. For this, we have used CUS as a model of anxiety and we evaluated the effects of knocking down CRF expression in the CeA in the development of stress-related anxiety. As we have shown previously that chronic stress has a tremendous impact in the expression of CRF related genes in the BNST and in the activation of this brain area, we have also evaluated the effects of this CeA CRF silencing in the stress-induced molecular signature and activation of the BNST.

2. Results

2.1. CRF knockdown in the CeA attenuates the effects of chronic stress protocol

First, we evaluated if the RNAi protocol was functioning properly *in vivo* by measuring both mRNA (Experiment 1a) and protein (Experiment 1b) levels of CRF in the CeA. For mRNA levels, we observed a significant decrease of CRF in both Cont-shCRF and CUS-shCRF when comparing with Cont-Scramble and CUS-Scramble groups (shCRF effect: $F_{(1,19)} = 37.5$, $p < 0.001$; Cont-Scramble vs Cont-shCRF: $p = 0.002$; CUS-Scramble vs CUS-shCRF: $p = 0.013$) (Fig. 1A). Regarding protein levels, we also observed a decrease in CRF protein levels in animals injected with shCRF when compared with controls (Fig. 1B).

Chronic stress protocol induced a significant decrease in body-weight gain in CUS-Scramble group when comparing with control group ($F_{(1,58)} = 5.01$; $p = 0,003$; Cont-Scramble vs CUS-Scramble $p = 0.006$). However, CUS protocol had no effect in body eight in CUS-shCRF group (Cont-Scramble vs CUS-shCRF: $p = 0.141$; Cont-shCRF vs

Table 1
Biometric markers revealed that the CUS protocol decreased body-weight gain and increases adrenal weight.

	Control-Scramble	Control-shCRF	CUS-Scramble	CUS-shCRF
Body weight gain (g)	64.1 ± 2.5	62.3 ± 4.6	*47.5 ± 3.8***	*54.5 ± 2.9*#
Thymus weight (gr/BW)	0.24 ± 0.01	0.25 ± 0.02	0.21 ± 0.02	0.22 ± 0.01
Adrenal weight (gr/BW)	0.019 ± 0.001	0.020 ± 0.001	*0.024 ± 0.001**	0.023 ± 0.001

*vs Control-Scramble; #vs Control-shCRF. *p < 0.05; **p < 0.01. Data presented as Mean ± SEM.

CUS-shCRF: p = 0.030. No differences were observed between the two CUS groups (CUS-Scramble vs CUS-shCRF: p = 0.364) (Table 1).

Adrenal weight is significantly altered by the treatment ($F_{(1,53)}$ = 4.05; p = 0.014), with the adrenal weight of CUS-Scramble animals being significantly higher than Cont-Scramble animals (Cont-Scramble vs CUS-Scramble: p = 0.014), no differences were found among the remaining groups. There are no significant differences for thymus weight between groups ($F_{(1,58)}$ = 0.94; p = 0.432) (Table 1).

CORT levels were also used as a measure of the efficacy of the stress protocol with the treatment inducing an alteration in CORT levels in the inactive period of the animals (daytime) (Interaction: $F_{1,50}$ = 16.44; p < 0.001). The group CUS-Scramble presented higher levels of CORT when compared with controls (Cont-Scramble vs CUS-Scramble: p < 0.001; Cont-shCRF vs CUS-Scramble: p < 0.001). Interestingly, CUS-shCRF CORT levels are significantly lower than CUS-Scramble (CUS-Scramble vs CUS-shCRF: p < 0.001) but are not significant different from control groups (Cont-Scramble vs CUS-shCRF: p = 0.846; Cont-shCRF vs CUS-shCRF: p = 0.992) showing that the amygdalar decrease in CRF attenuates the stress-induced increase in CORT. For CORT levels measured in the active period (night time), we observe a significant increase in CORT in the CUS-Scramble group when comparing with Cont-Scramble (Cont-Scramble vs CUS-Scramble: p = 0.005) but no differences between the other groups (Fig. 2).

2.2. CRF knockdown in the CeA reduces stress-induced anxious behavior

To evaluate anxiety-behavior, we used the elevated plus maze (EPM) and Light-Dark box (DLB) tests. In the EPM, we observed that there is no effect of the interaction between experimental groups regarding the time spent in open arms ($F_{1,49}$ = 2.219; p = 0.1427), but there is significant stress – induced behavioral alterations ($F_{1,49}$ = 12.44; p < 0.001) and also significant differences comparing Scramble and shCRF groups ($F_{1,49}$ = 26.64; P < 0.001). Specifically, we observed that CUS-Scramble animals spend significantly less time in the open arms than the control groups (Cont-Scramble vs CUS-Scramble: p < 0.001 Cont-shCRF vs CUS-Scramble: p < 0.001), indicative of an anxious phenotype (Fig. 3A). Of relevance, CUS-shCRF group does not present this anxious trait, being similar to control group (CUS-Scramble vs CUS-shCRF: p < 0.001). This indicates that the CRF-knockdown in the CeA prevents stress-induced anxiety. Regarding the number of entrances in Open Arms no significant differences were found (interaction: $F_{1,49}$ = 2.46; p = 0.130) although CUS-Scramble animals show a tendency for a decreased number of entries in the open arms (Cont-Scramble vs CUS-Scramble: p = 0.127) (Fig. 3B). There are also no significant differences in the number of entrances in Closed Arms (interaction: $F_{1,49}$ = 0.604; p = 0.445) (Fig. 3B).

In the DLB test, we observed an effect of stress in the performance in this test ($F_{1,56}$ = 9.967; p = 0.0024) but not in the interaction between groups ($F_{1,56}$ = 2.551; p = 0.1150). CUS-Scramble animals spent significant more time in the dark area of the test when compared with control animals (Cont-Scramble vs CUS-Scramble: p < 0.001; Cont-shCRF vs CUS-Scramble: p = 0.005) (Fig. 3C). Similar to what we observed in the EPM, CUS-shCRF animals present a significant reduction in anxiety-like behaviour when compared with CUS-Scramble animals (CUS-Scramble vs CUS-shCRF: p = 0.007).

To rule out possible confounding effects, we also evaluated locomotor activity in the Open Field test. No differences were observed in the total distance that animals travelled in the arena (interaction: $F_{1,53}$ = 0.33; p = 0.570) (Fig. 3E). Likewise, there are no significant differences in time spent in center/periphery, when comparing Control and CUS animals ($F_{1,53}$ = 0.30; p = 0.865) (Fig. 3D).

2.3. CRF knockdown in the CeA does not impact fear behavior

Next, we evaluated fear response using the acoustic startle and the fear-potentiated startle paradigm. In the acoustic startle response, there is an increase of responsiveness for stressed animals with data showing a significant effect of the treatment (Fig. 3F; $F_{1,49}$ = 2.739; p = 0.039). Specifically, at 120 Db, stress induced a significant increase in startle amplitude in both stress groups ($F_{1,49}$ = 9.743; p = 0.004). The CRF-shCRF in the CeA does not induce any significant effects, with CUS-Scramble and CUS-shCRF presenting no differences (Fig. 3F).

Regarding the fear-potentiated startle paradigm, no significant differences were induced by either stress ($F_{1,54}$ = 0.205; p = 0.655) or shCRF injections ($F_{1,54}$ = 0.027; p = 0.871) (Fig. 3G).

2.4. Stress-induced BNST molecular signature is altered by CeA CRF knockdown

We knew from our previous work that exposure to chronic stress leaves a distinct molecular fingerprint on the BNST (Ventura-Silva et al., 2012). So, in order to understand how downregulation of CRF in CeA is impacting the BNST at a molecular level, we assessed the expression of several genes of interest in BNST subdivisions (anterior and posterior). Specifically, we looked into the CRFergic system by assessing the expression of *Crfr1* and *Crfr2* and *Crf*, and based on our previous work assessing the impact of chronic stress in gene expression in the BNST, we have also analyzed the expression of the GABA receptor *Gabaar* and glutamatergic with *Nr2b* receptor (Ventura-Silva et al., 2012).

In the anterior BNST, the expression of *Crf* was downregulated in both stressed groups (Fig. 4A, $F_{1,18}$ = 16.76; p < 0.001), although the effect is only statistically significant in the CUS-Scramble group (Cont-Scramble vs CUS-Scramble: p = 0.027; Cont-shCRF vs CUS-Scramble: p = 0.021) there is a clear trend for decreased *Crf* expression also in the CUS-shCRF group (Cont-Scramble vs CUS-shCRF: p = 0.080; Cont-shCRF vs CUS-shCRF: p = 0.069). In opposition, we saw no effect from either stress or amygdalar knockdown of CRF in the expression of this peptide in the posterior BNST (Fig. 4D).

Regarding the expression of *Crfr1*, we observed that stress decreases the expression of this receptor in the anterior division of the BNST for CUS animals (Fig. 4B) ($F_{1,18}$ = 27.80; p < 0.0001: Cont-Scramble vs CUS-Scramble: p = 0.015; Cont-shCRF, CUS-Scramble: p = 0.0012) but the knockdown of CRF significantly attenuates this effect ($F_{1,18}$ = 9.41; p = 0.0058 CUS-Scramble vs CUS-shCRF: p = 0.018). For the posterior division of the BNST, no differences were found in the expression of *Crfr1* (Fig. 4E).

On the other hand, we found significant differences in the expression of *Crfr2* in the posterior division (Fig. 4F) but not in the anterior division (Fig. 4C). For the posterior BNST, although there is no

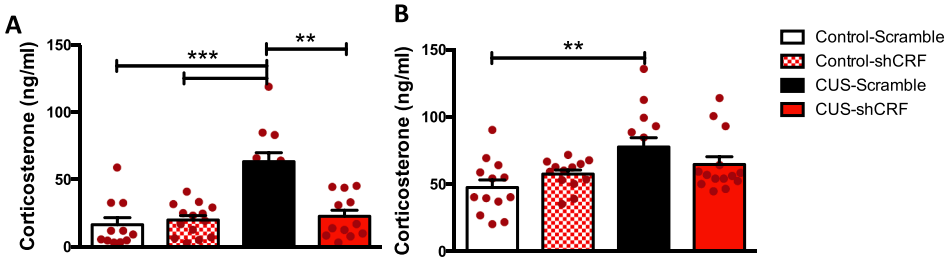

Fig. 2. Corticosterone (CORT) concentration in serum. CUS leads to an increase in CORT levels that is rescued by shCRF in the inactive period ("lights on") of the animals (A). During the active period of the animals ("lights off") the levels of CORT are higher in CUS-Scramble animals (B). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p ≤ 0.001.

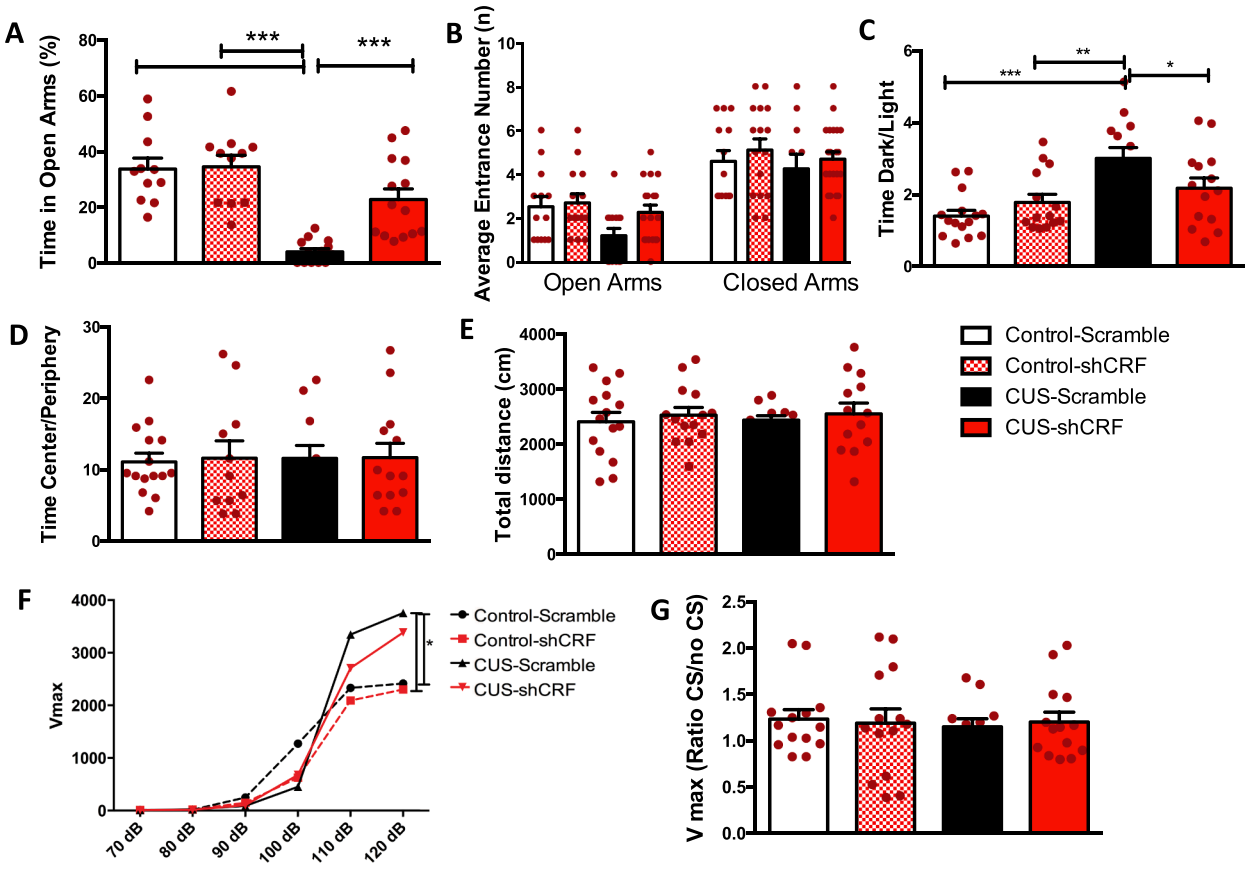

Fig. 3. Behavioral Analysis. CUS induces an increase in anxiety-like behaviour that is decreased in animals injected with shCRF (A–C). The percentage of time spent in the open arms of the Elevated Plus Maze is increased in CUS-scramble group but not in CUS-shCRF (A) and no differences were found in the number of entrances in Closed and Open Arms of the EPM (B) CUS-scramble animals spent more time in the dark compartment of the Dark-Light Box test (C). In the open field test, no differences were found between the time spent in the periphery/center areas (D) or between total locomotion (E). In the acoustic startle, stress increases the response of the animals, independently of shCRF injection (F). Neither stress or shCRF injections altered fear-potentiated startle (G). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p ≤ 0.001.

interaction effect, there is an effect from both stress ($F_{1,22}$ = 7.68; p = 0.0011) and the knockdown of CRF in the amygdala ($F_{1,22}$ = 6.17; p = 0.0021). CUS-Scramble shows a higher level of *Crfr2* when compared to the other groups (Cont-Scramble vs CUS-Scramble: p = 0.025; Cont-shCRF vs Cus-Scramble: p = 0.008; CUS-Scramble vs CUS-shCRF p = 0.024). Again, in the CUS-shCRF group we do not see any significant differences when compared with control groups, showing that the CRF-shCRF in the CeA precludes the stress-induced alterations in this receptor.

We found no significant differences in the expression of GABAA receptor either in the anterior or posterior divisions of the BNST (data not shown).

Finally, we analyzed the expression of *Nr2b* in the posterior division (Fig. 4G) and we also observed an effect in this receptor induced by both the manipulation of CRF in the amygdala ($F_{1,22}$ = 11.43; p = 0.0036) and stress ($F_{1,22}$ = 5.45; p = 0.032; Cont-Scramble vs CUS-Scramble: p = 0.053; Cont-shCRF vs CUS-Scramble: p = 0.005) that is absent in stressed animals with the decreased CRF expression in CeA (CUS-Scramble vs CUS-shCRF: p = 0.008). The level of expression of this receptor in the anterior division was below detectable levels.

2.5. Effects of CRF knockdown in amygdala and PVN recruitment

To evaluate the effects of the decreased levels of CRF in CeA in downstream regions such as the BNST and the PVN, we also performed immunohistochemistry to analyse the presence of the immediate early gene C-FOS in BNST subnuclei and the PVN after exposure to an anxiogenic stimuli (acoustic startle paradigm).

We observed no differences in the number of C-FOS+ cells in the anterodorsal and dorsomedial nuclei (Fig. 5C and 5D, respectively). However, when looking at the fusiform nucleus of the BNST (Fig. 5E) we found an individual effect of both stress ($F_{1,17}$ = 11.83; p = 0.0037) and CRF knockdown ($F_{1,17}$ = 10.77; p = 0.0051) as well as an effect of the interaction of these two factors ($F_{1,17}$ = 5.86; p = 0.027). More specifically, there is a significant increase of C-FOS+ cells in the fusiform nuclei of the CUS-Scramble group when compared with controls and with the CUS-shCRF group. (Cont-Scramble vs CUS-Scramble: p = 0.0054; Cont-shCRF vs CUS-Scramble: p < 0.001; CUS-Scramble vs CUS-shCRF: p = 0.0042). For the posterior division (Fig. 5F), we observed a decrease in C-FOS+ cells induced by stress but no differences induced by CRF knockdown (stress effect: $F_{1,16}$ = 11.03; p = 0.0043; Cont-Scramble vs CUS-Scramble: p = 0.028; Cont-Scramble vs CUS-shCRF: p = 0.027).

On the other hand, in the PVN (Fig. 5G) we observed an effect of stress ($F_{1,17}$ = 29.91; p < 0.001) with an increase in C-FOS+ cells that is induced by stress but also no differences between Scramble and groups with CRF-shCRF in the CeA (Cont-Scramble vs CUS-Scramble: p = 0.018; Cont-shCRF vs CUS-Scramble: p = 0.004; Cont-shCRF vs CUS-shCRF: p = 0.002).

3. Discussion

The extended amygdala has been associated for a long time with fear and anxiety disorders. While traditionally, the amygdala has been

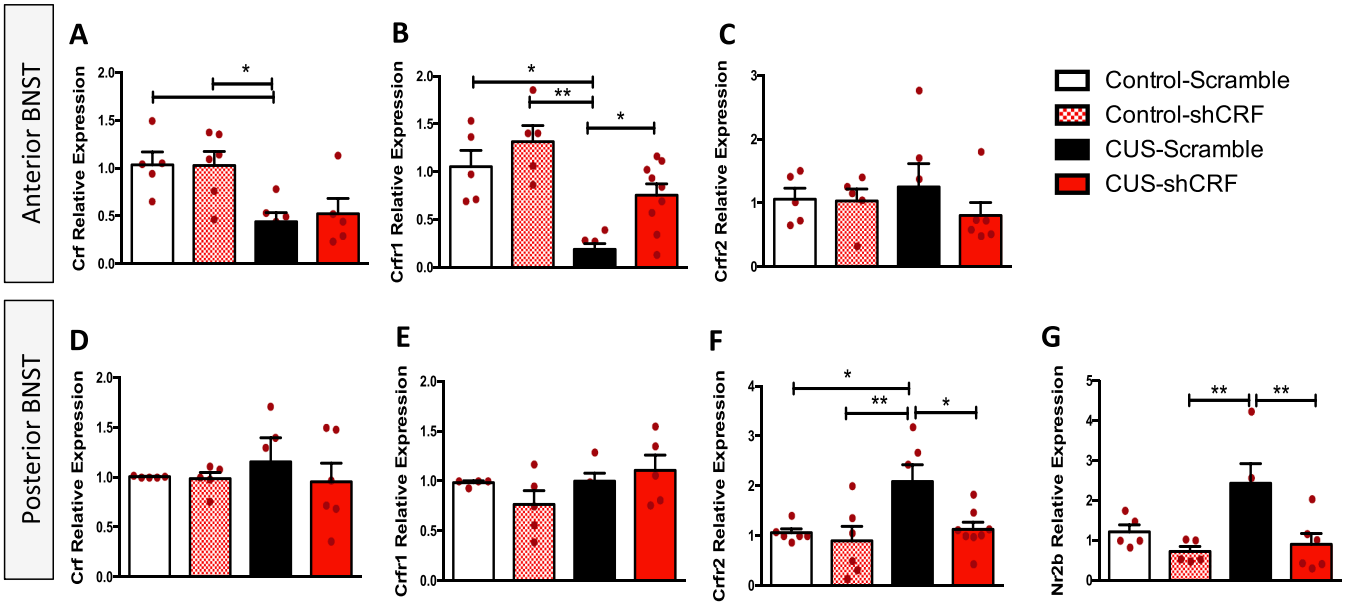

Fig. 4. Gene expression analysis in the anterior and posterior BNST. Expression in the BNST Anterior (A-C) and posterior (D-G) of the following genes: A and D – *Crf*, B and E – *Crfr1*, C and F – *Crfr2* and G – *Nr2B*. CUS decreases the expression of *Crf* (A) and *Crfr1* (B) in the anterior BNST. The expression of *Crfr1* but not of *Crf* is partially rescued in CUS-shCRF animals. In the anterior BNST there are no changes in the expression of *Crfr2* (C). In the posterior BNST there are no alterations in the levels of *Crf* (D) and *Crfr1* (E). Expression of *Crfr2* is increased in the posterior BNST in CUS-Scramble group but not in CUS-shCRF (F). CUS also leads to an increase in the expression of *Nr2b* in the posterior BNST that is rescued by shCRF injection in the CeA (G). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p ≤ 0.001.

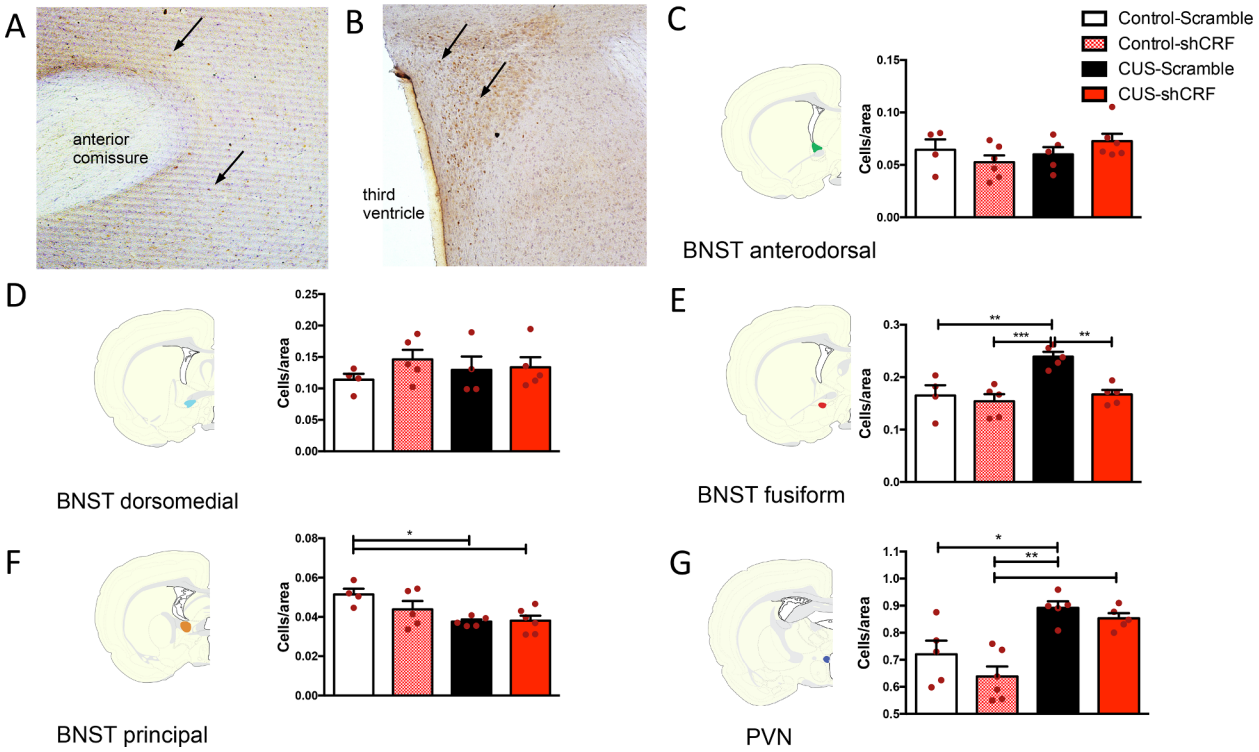

Fig. 5. Immunohistochemistry analysis of C-FOS positive cells in BNST subnuclei and the Hypothalamus. Data represented as total number of cells per area*1000. CUS and shCRF differentially regulate the levels of C-FOS in these brain regions. (A) and (B) are representative images of C-FOS staining in the BNST (A) and PVN (B). Arrows indicate examples of C-FOS + cells. No differences in C-FOS levels were found in the anterodorsal (C) and dorsomedial (D) nuclei of the BNST. In the fusiform subnucleus of the BNST (E), CUS-scramble animals show higher levels of C-FOS and this effect is reversed in CUS-shCRF. CUS also induces a decrease in C-FOS in the principal nucleus of the BNST (F) and increase in C-FOS in the paraventricular nucleus of the hypothalamus (G). Data presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p ≤ 0.001.

more associated with fear-behavior and the BNST with anxiety, we present evidence that supports a role for CeA CRF in the development of stress-related anxiety and present new information regarding the effects of amygdalar CRF in downstream areas.

Despise the more traditional division between the roles of the CeA and the BNST, the lines that separate the involvement of the different nuclei in anxiety are not well defined. Authors have demonstrated that the activation of the CeA by behavioral anxiogenic stimuli exposure activates CRF neurons in PVN and subsequently the HPA axis (McEwen, 2003; Risbrough and Stein, 2006). In contrast, lesions of the CeA have shown an attenuation of anxiety-like behavior (Ventura-Silva et al., 2013).

Previous studies have been conflicting in regards to the specific effects of a downregulation of CRF in the CeA: while all the reports show disruptions of the HPA axis activity, and consequently alterations in corticosterone levels (Callahan et al., 2013; Regev et al., 2012), following a knockdown of CeA CRF levels, the specific effects, both on CORT levels and in anxiety-like behavior induced by this manipulation are not clear. Regev et al. (2012) showed an attenuation of anxiety-like behaviour following an acute stress in mice that had a knockdown of CRF levels in the CeA and an increase in CORT in basal conditions. On the other hand, Callahan et al. (2013) found that silencing CRF in the CeA did not alter anxiety-like behaviour in basal condition or after exposure to an acute stress but attenuated CORT levels following stress. On the other hand, specific activation using DREADDs shows that activation of CRF neurons in the amygdala increases anxiety, whereas inactivation of these neurons induce the opposite effect (Paretkar and Dimitrov, 2018). Using CUS as an established model of anxiety-like behaviour, we offer supporting evidence that, while a downregulation of CRF in CeA does not alter anxiety-behavior in a basal situation, it will hinder the development of stress-related anxiety. Additionally, we also observed a disruption of the HPA axis activity following decreased expression of CRF, with a decrease in CORT levels when compared with stressed-Scramble animals. Of relevance, while stressed animals with CRF knockdown show attenuation in anxiety-like behavior, no differences were observed when comparing the two groups of control animals. This shows us that, while CRF signaling integrity is necessary for the development of stress-induced anxious behavior, stress seems to be required for this influence to be present.

Interestingly, an overexpression of CRF in the CeA also seems to lead to conflicting data, with authors reporting either a deregulation of the hypothalamic–pituitaryadrenal (HPA) axis and alterations in the baseline response to acoustic stimuli or attenuation of stress-induced anxiety (Kalin et al., 2016; Keen-Rhinehart et al., 2009; Natividad et al., 2017; Paretkar and Dimitrov, 2018; Regev et al., 2012).

Due to the privileged location of the BNST as an intermediate relay station between the amygdala and the hypothalamus, we hypothesized that manipulations of the amygdala may be modulated downstream by the BNST. The BNST, particularly the anterior division, is richly innervated by CeA projections (Dong et al., 2001), which are rich in GABA and CRFergic neurons (Champagne et al., 1998; Sun and Cassell, 1993). We have previously demonstrated that the BNST is strongly affected by chronic stress and we have tried to identify molecular correlates of stress in the BNST as well as different activation patterns in BNST subnuclei (Ventura-Silva et al., 2012). Our observations show that stress induces alterations in the expression of different receptors in the BNST and, of relevance, we observed a decrease in the expression of *Crfr1* in nuclei in the anterior division of the BNST and an increase in *Crfr2* in nuclei in the posterior division of the BNST. CRF binds with high affinity to CRFR1 and it has been show that the activation of this receptor can lead to an increase in anxiogenesis (Holsboer, 1999; Muller et al., 2003; Paretkar and Dimitrov, 2018; Refojo et al., 2011; Sahuque et al., 2006). Our results show that exposure to chronic stress leads to a decrease in the expression of *Crf*, in both CUS-Scramble and CUS-shCRF groups, suggesting that it is not CRF that is being expressed in the BNST that is responsible for the differential effect of knockdown of CRF in the expression of *Crfr1* in this brain region. In fact, we hypothesize that stress induces an increase in amygdalar CRF that, in turn, leads to an increase in CRF projections to the anterior BNST that are compensated by a decrease in *Crfr1* levels in these nuclei. With the knockdown of CRF in CeA preventing increased CRF projections from CeA to the BNST, the expression of *Crfr1* is kept at similar levels than control animals. The activation of CRFR2, contrary to CRFR1, has been shown to lead to a reduced anxiety in different paradigms (Henckens et al., 2017; Issler et al., 2014; Kishimoto et al., 2000).

Interestingly, while we do not see an alteration in the global C-FOS levels in the anterior division of the BNST in CRF CeA knockdown animals, but we do observe a difference in the fusiform nucleus of the anterior division, in which stress induces and increase in the number of C-FOS+ cells that is attenuated by the downregulation of CRF in the CeA. This shows us that, while the BNST seems to be an important mediator in the regulation of amygdalar inputs, different subnuclei of the BNST may respond differently to amygdalar inputs leading to different regional activation within the BNST.

Additionally, the mediation of the stress response is complex and different neurotransmitter systems and brain regions will also be involved in the regulation of both anxiety and the HPA axis activity. Both the CeA and the BNST are rich in GABAergic neurons and these are known to be an important factor for anxiety. Importantly, we have seen no differences in GABAA receptors in the BNST following CRF downregulation in the CeA. We observed alterations in the glutamate receptor *Nr2b*. As far as we known, there are no strong glutamatergic between the CeA and the anterior BNST so this is likely being mediated by other afferents of the BNST that are being indirectly affected by reduced CRF release in this region. Of relevance, the CeA has strong connections with the basolateral amygdala (BLA) and the infralimbic cortex (McDonald et al., 1999; Reppucci and Petrovich, 2016), which in turn also project to the BNST. Both the BLA and the infralimbic cortex have an elevated number of glutamatergic neurons and are potential mediators of glutamatergic transmission into the BNST. These two areas are highly interconnected and are involved in the mediation of different behaviors including fear expression (Senn et al., 2014). Sensory inputs that can lead to anxiety or fear responses flow from cortical structures to the BLA are processed in this nucleus before being transmitted to the central amygdala or further downstream areas such as the BNST (Babaev et al., 2018; Calhoon and Tye, 2015; Tovote et al., 2015). Interestingly, intra-amygdala projections from the BLA to the CeA are able to specifically modulate anxiety-like behavior with specific inhibition of this projection leading to increased anxiety (Tye et al., 2011).

Curiously, and despise the well-known role that the amygdala plays in fear behavior (Davis, 1997; Walker et al., 2009), we did not observe any differences in the fear potentiated startle in animals with decreased levels of CRF in CeA. This observation goes in line with previous reports showing that manipulations of CRF, specifically injections of CRF in the CeA did not alter this specific behavior (Isogawa et al., 2013) although CRF seems to be involved in fear memory (Paretkar and Dimitrov, 2018) in other paradigms. Additionally, recent studies suggest that different intra-amygdala circuits may be involved in the expression of fear and anxiety (Cai et al., 2014; Janak and Tye, 2015).

One limitation of this study is that the lentiviral system used to decrease CRF expression in the CeA is inducible by doxycycline and for this, all animals received doxycycline in drinking water throughout the duration of the experiment. Doxycycline is a broad-spectrum antibiotic that may alter the composition of the gut microbiota, leading to alterations in the gut-brain-axis (Cryan et al., 2019). In addition, doxycycline is able to cross the blood-brain barrier, leading to an anti-inflammatory effect in the brain (Yrjänheikki et al., 1998), which can impact behaviour (Mello et al., 2013). As stress is known to increase inflammation in the brain (Wohleb et al., 2014), we cannot fully exclude a potential confounding effect induced by doxycycline. Nevertheless, since all animals received the antibiotic, this suggests that the

differences observed between groups were due to the effects of both stress and CRF expression levels and not solely induced by doxycycline.

In conclusion, the stress response and the development of anxiety after exposure to stress is a complex mechanism involving different neurotransmitters and brain regions. In this work, we have demonstrated that CRF levels in the CeA can affect the BNST and disrupt the HPA axis response to stress, with a remarkable impact in stress-induced anxiety but not fear behavior.

4. Experimental procedures

4.1. Animals

Animal experiments were conducted in accordance with the European Communities Council Directive (86/609/EEC). All experiments were approved by the Animal Ethics Committee of the Portuguese National Veterinary Directorate.

Adult male Wistar rats (Charles Rivers Laboratories, Barcelona, Spain) were housed in groups of 2 per cage under standard laboratory conditions (temperature 22 °C; artificial light/dark cycle of 12/12 h; lights on at 8 a.m) and with ad libitum access to commercial chow and water.

4.2. Stereotaxic surgery

Eighty-eight male rats (8 weeks old) were submitted to stereotaxic surgery under ketamine/medetomidine anaesthesia. The animals were randomly distributed to one of two groups: Scramble-expressing and short-hairpin CRF-expressing virus (shCRF). Scramble animals were injected with GFP-Scramble (rLV-tTs-ZsGreen-rScramble ShRNA, CD BioSciences Inc, Shirley, NY, USA) (n = 44). ShCRF animals (n = 44) were injected with a lentivirus containing rLV-tTS-ZsGreen-rCrh2 shRNA (CD BioSciences Inc). Injections were performed in the central amygdala (-2.2 mm from bregma, 4.2 mm from midline, 7.0 mm from brain surface). The total volume of the injection was 1 μL (~10^8 TU/mL), injected at a rate of 0.1 μL/min.

After the surgery animals started being treated with doxycycline in drinking water (200 mg/l; Sigma) and the treatment lasted until the end of behavioral tests. Animals were given one week to rest, post-surgery, and then were subdivided into 4 groups: Control-Scramble (Cont-Scramble; n = 22 randomly chosen from animals injected with the scrambled vector); control-CRF knockdown (Cont-shCRF; n = 22 randomly chosen from animals that were injected with shCRF lentivirus); CUS-Sc (n = 22 randomly chosen from animals injected with scrambled vector) and CUS-CRF knockdown (CUS-shCRF; n = 22 from the animals injected with shCRF lentivirus).

4.3. Virus construction and production

We have selected 4 target sequences of CRF gene (Gene ID: 81648; shRNA Targeting sequences: shRNA1: GCCGTTGAATTTCTTGCAACC; shRNA2: GCATGGGTGAAGAATACTTCC; shRNA3: GAGGGAAGTCTTG GAAATGGC; shRNA4: GCAGTTAGCTCAGCAAGCTCA), to create shRNAs. Sequences were amplified using the primers in supplementary Table 1. Sequences were cloned into vector pLVX-vector using PstI and BamHI cloning sites. Of these, the shRNA2-showed the greatest knockdown efficacy in PC12 Cells (Sup. Fig. 1; primers for RT-PCR in Sup Table 1) and was selected for further studies. Lentivirus containing pLVX-CMV-tTs-Ubc_ZsGreen-rCrh2 vector were produced by Creative Biogene Company. Lentivirus particles had a final titer of 1 × 10^8 TU/ml (assessed in Hek293 cells).

4.4. Stress protocol

Stress protocol started when the animals were 9 weeks of age, and it lasted 28 days. Animals were exposed to one different stressor per day (30 min/d) of one of the following aversive stimuli: immersion in cold water (18 °C), vibration of the home cage, restraining, overcrowding and exposure to a hot air stream (Cerqueira et al., 2007). The stressors were scheduled in a random order for the duration of the experiment. Control animals were handled on a daily basis over the 4 weeks. Weekly body weights and post-mortem weight of adrenals and thymus were recorded as indicator of the efficacy of the stress protocol (Table 1). At the end of the stress protocol, animals were divided in two subsets to perform two different experiments. For experiment 1, a subset of animals (Cont-Scramble: n = 16; Cont-shCRF: n = 16; CUS-Scamble: n = 16; CUS-shCRF: n = 16) was subjected to different behavioral tests to assess anxiety, locomotor and fear-behavior and their brains were later used for molecular studies. In Experiment 2, a second subset of animals (Cont-Scramble: n = 6; Cont-shCRF: n = 6; CUS-Scramble: n = 6; CUS-shCRF: n = 6) was subjected to an anxiogenic stimulus after the end of the stress protocol to later access c-fos activation in the brains of these animals.

4.5. Behavioral tests

After the end of the stress exposure, animals from experiment 1 were used to assess anxiety-like behavior (elevated plus maze (EPM), dark-light box and acoustic startle), locomotor activity (open field) and fear conditioning (fear-potentiated startle). Behavioral tests were performed in the following order to minimize the effects each test could have in the following test: EPM, dark-light box, open field, acoustic startle and fear-potentiated startle. The behavioral tests started 24 h after the last stressor was applied and each test was performed with a 24 h interval between tests except for the fear-potentiated startle that was performed one week after the acoustic startle in which the animals were still submitted to the chronic stress protocol.

4.5.1. Elevated plus maze

Animals were tested over 5 min in a black polypropylene “plus”-shaped maze (ENV-560, MedAssociates Inc, St. Albans, VT 05478) at a height of 72 cm above the floor. The maze consisted of two facing open arms (50.8 × 10.2 cm) and two closed arms (50.8 × 10.2 × 40.6 cm). Testing was performed under bright white light. The time spent in the open arms, junction area and closed arms, as well as the number of entrances and explorations in each section were recorded using a system of infrared photobeams, the crossings of which were monitored by computer. The times spent in each of the compartments of the EPM are presented as percentage of the total duration of the trial.

4.5.2. Dark-light box

Animals were tested for 10 min in an arena (43.2 × 43.2 cm) where half of the arena was covered by black acrylic walls (dark compartment) and the other half had transparent acrylic walls and was illuminated (light compartment). The dark and light compartments were connected by a small opening in the middle (Med Associates Inc., St. Albans, Vermont, USA). Each subject was initially placed in the center of the light compartment and the time spent in the light and dark compartments was recorded.

4.5.3. Open field

Animals were individually tested for 5 min each in an open field arena (43.2 × 43.2 cm) that had transparent acrylic walls and a white floor (model ENV-515, MedAssociates Inc, St. Albans, VT 05478). Each subject was initially placed in the centre of the arena and horizontal activity and instant position were registered, using a system of two 16-beam infrared arrays connected to a computer. Total distances were used as indicators of locomotor activity. Times and distances in the predefined central and peripheral areas were recorded and used to calculate the ratio of time spent in the central area over total time of the trial, and distance travelled in the central as a function of total area. Number and duration of rearings were recorded. The test room was illuminated with bright white light.

4.5.4. Acoustic startle

Startle reflex was measured in startle response apparatuses (SR-LAB, San Diego Instruments, San Diego, CA, USA), each consisting of a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber. Animals were habituated to the apparatus (5 min daily) for 2 days before actual testing. Cylinder movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system (San Diego Instruments, San Diego, CA, USA) was used to ensure comparable startle magnitudes. Startle stimuli were presented through a high frequency speaker located 33 cm above the startle chambers. Animals were presented with 60 startle stimuli each lasting 50 ms but with different intensities, from 70 to 120db applied in a random order. The startle stimuli were presented with a random duration between each startle: from 5 to 20 s. Startle magnitudes were sampled every millisecond (ms) over a period of 200 ms, beginning with the onset of the startle stimulus. A startle response is defined as the peak response during 200 ms recording period.

4.5.5. Fear-potentiated startle

Rats were placed in the first test chamber, a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), the floor of which consisted of a stainless-steel grid through which a software-controlled electric current could be passed. Animals were rehabituated to the startle chamber for 5 min and 5 baseline trials were administered (50-ms pulse of white noise at 120 dB) at an interstimulus interval of 30 s. The purpose of these baseline trials was to familiarize the animal with the startle stimulus in order to facilitate more accurate measurement of the animal's overall startle amplitude. Next, animals were presented with 20 light-shock pairings, at 30 s intervals. The shock (0.6 mA) was presented during the last 500 ms of the 5 s light pulse. The light stimulus was delivered via a 3-watt incandescent light bulb fastened to the inside wall of the startle chamber. After completion of the conditioning trials, animals were returned to their home cages. The same testing procedure was applied on the following day, except that 20, rather than 5, baseline trials were administered before testing. Additionally, startle measurements were made in the same grid holder that was used to condition the animals. After delivery of the final baseline trial, animals were randomly presented 10 startle stimuli, each with an intensity of 120 dB and duration of 50 ms. In half of the trials, the startle stimulus was presented concomitantly with the conditioned stimulus (CS light). Startle stimuli paired with the CS were delivered during the last 50 ms of the 5 s light presentation. The magnitude of the difference between the startle response at 120db (Vmax) in the presence or absence of the CS will be a reflection of fear-behavior (Walker et al., 2003).

4.5.6. Acoustic startle as an anxiogenic stimulus

An acute state of anxiety was induced by the acoustic startle reflex paradigm as previously reported (Pego et al., 2008). Animals were habituated to the apparatus (5 min daily) for 2 days before the actual trial. Rats were placed in the startle chamber and allowed to acclimate to the chamber for 5 min. They were then presented with 20 anxiogenic startle stimuli (50 ms pulse of white noise at 120 dB) at a randomly assigned interstimulus interval ranging from 10 to 20 s. The procedure lasted 15 min in total. Animals were then returned to a resting cage for 90 min before being sacrificed. Trials on individual animals were conducted sequentially. Between tests, the chambers and acrylic holders were thoroughly cleaned (10% ethanol) to eliminate residual olfactory cues.

4.6. Corticosterone measurement

At the end of the stress protocol blood was collected for corticosterone (CORT) assessment. Collection was performed at different time-points: one 1 h after "lights on" and the other 1 h after "lights off". The blood was rapidly collected after a small incision in the tail of the animals. After collection, blood was centrifuged at 13000 rpm for 10 min. Serum (supernatant) was removed and stored at −80 °C until further analysis. CORT levels were measured by ELISA using a commercial kit (Enzo Life Sciences) and according to the manufacturer's instructions. Briefly, serum samples were diluted (1:50) with ELISA assay buffer. After dilution, 100 µL of each serum sample was added to an ELISA plate. To each sample, 50 µL of Corticosterone ELISA conjugated was added, immediately followed by addition of 50 µL of Corticosterone ELISA antibody. Samples were incubated at room temperature for 2 h. After incubation, the plate was washed three times with wash buffer and then 200 µL of p-Npp substrate was added to every well. After, the plate was incubated again for 1 h at RT. At the end of the incubation, 50 µL of stop solution was added to every well and CORT concentration was determined by measuring the optical density at 405 nm in a microplate reader. As a measure of precision for this method, the intra-assay coefficient of variation was calculated by dividing the standard deviation by the mean of each duplicate and multiplied by 100. The average intra-assay coefficient of variation measured was of 2.1%.

4.7. Histological procedures

For experiment 1, following behavioral tests the animals were deeply anaesthetized with pentobarbital and perfused transcardiacally with saline. Each group was further divided in 2 subgroups: Experiment 1a (n = 8 per group) and Experiment 1b (n = 8 per group). For experiment 1a, brains were collected, involved in Optimal Cutting-Temperature compound (Leica Biosystems, Wetzlar, Germany) and frozen. The brains were kept at −20 °C until histological processed for laser microdissection of CeA that was performed to assess CRF levels in the CeA. For experiment 1b, we performed macrodissection to dissect several different areas: CeA (for western blot for CRF), BNST anterior and BNST posterior (for real-time PCR of different genes of interest). The areas were collected in 1,5 ml tubes and frozen at −80 °C until further analysis.

For experiment 2, 90 min after exposed to the acoustic startle as an anxiogenic stimulus, animals were deeply anaesthetized with pentobarbital and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 m phosphate-buffered solution (PBS). Brains were dissected, post-fixed (4% paraformaldehyde) for 4 h, immersed in 8% sucrose in 0.1 m PBS (2 days at 4 °C) and then further processed for C-FOS immunohistochemistry and morphological analysis.

4.8. Laser microdissection

Coronal cryostat sections (20 µm) were mounted on Molecular Machines & Industries membrane-coated slides (Olympus), immersed in 70% isopropanol (1 min), rinsed in Diethylpyrocarbonate-treated water, and stained with hematoxylin, before final immersion in 100% isopropanol (2 min). After air-drying, sections were ready for lasermicrodissection (Microdissector CellCut, Olympus) of the CeA.

4.9. RNA extraction and qRT-PCR

From the CeA tissue collected in laser microdissection and the anterior BNST and posterior BNST tissue collected in Experiment 1b, we extracted RNA using a commercial kit (RNesasy Micro Kit), following the manufacturer's instructions. cDNA was subsequently synthesised using the SuperScript First Strand Synthesis for RT-PCR kit (Invitrogen). qRT-PCR analysis was used to measure the levels of mRNA encoding the following proteins: CRHR1 (Crhr1; Fw, CCTTAGGGCTTCTTTGTG; Rw, GGACTGCTTGATGCTGT GAA), CRHR2 (Crhr2; Fw, TTTTCCTAGTGC TGCGGAGT; Rw, AGCCTTCCACAAACATCCAG), CRH (Crh; Fw, GCTA ACTT TTTCCGCGTGTT; Rw, GGTGGAAGGTGAGATCCAGA), GABAA receptor, subunit alpha 3 (Gabra3; Fw, TGGTCATGTTG TTGGGACAG; Rw, TGGCAAGTAGGTCTGGATGA) and N-methyl-d-aspartate receptor

subunit 2B (Nr2b; Fw, GCATGCCTACATGGGAAAGT; Rw, GTTGAGCA CAGCTGCATCAT). Levels of the house-keeping gene hypoxanthine guanine phosphoribosyl transferase mRNA (Hprt; Fw, GCAGA CTTTG CTTTCCTTGG; Rw, TCCACTTTCGCTGATGACAC) were also monitored and used for normalisation.

The qRT-PCR was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad), using the QuantiTect SYBR Green RT-PCR reagent kit (Qiagen).

4.10. Western blot analysis

Briefly, NP-40 lysis buffer (150 mM NaCl, 50 Mm Tris pH = 8, 2 mM EDTA, 1% NP-40, 1 mM PMSF, protease inhibitors (Roche, Basel, Switzerland)) was added to frozen samples before homogenization by repeated passages through a 25G syringe. Samples were incubated on ice for 30 min with occasional shaking and then centrifuged (10,000 g, 10 min, 4 ˚C). Protein content of the supernatant was measured using the Bradford assay and lysates (50 ug total protein) were separated in 15% SDS-PAGE gels and transferred to nitrocellulose membranes using a Bio-Rad transblot apparatus. After blocking with 5% non-fat milk/PBS-0.5% Tween, membranes were incubated with the primary antibody (1:500 Anti-CRF; Novus Biologicals) in 2.5% milk/PBS-0.5% Tween (overnight, 4 ˚C).

Anti-Goat secondary antibody (1:10,000, Santa Cruz Biotechnology) was incubated for 1 h at room temperature. Detection was performed by chemiluminescence (Santa Cruz Biotechnology) and optical densities of bands were quantified with ImageJ software (NIH). Alpha-tubulin was used to monitor house-keeping proteins. Each western blot was replicated at least twice.

4.11. Immunohistochemistry

Coronal sections (50 μm thick) were serially collected in PBS. Alternate sections were immunostained for C-FOS by overnight incubation with rabbit anti-C-FOS polyclonal antibody (1:1000; rabbit anti-C-FOS Ab-5 polyclonal antibody, Calbiochem) after blocking (120 min) in a PBS solution, containing 0.3% Triton X-100 and 2.5% fetal bovine serum. Following washes in PBST, sections were incubated for 1 h in biotinylated swine anti-rabbit antibody (1:200, Dako) followed by an ABC solution (Vectorstain Elite), and finally visualized with diaminobenzidine (DAB). Sections were counterstained with hematoxilin to help delimit regional boundaries before mounting and coverslipping. C-FOS-immunoreactive (C-FOS+) neurons were marked by a dark brown DAB precipitate. The number of C-FOS+ neurons in the regions of interest was counted (6 sections for each region were counted) and the number of C-FOS+ cells per area was calculated. Total area analysed for each region was on average: BNST anterodorsal – 699.883 μm^2; BNST dorsomedial – 275.464 μm^2; BNST fusiform – 104.123 μm^2; BNST principal – 791.098 μm^2; PVN – 439.927 μm^2.

4.12. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using repeated measures test or two-way ANOVA to compare means between groups where appropriate. Post-hoc analysis was performed using Tukey test. Statistical significance was accepted when $p < 0.05$.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Contributors

APVS performed research, analysed and interpreted data and wrote the manuscript. SB performed research and analysed data. NS designed the experiment and interpreted data. AJR and JMP designed the experiment, interpreted data and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.brainres.2019.146622.

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