Universidade do Minho
Escola de Ciências da Saúde

Ana Paula Ventura da Silva

Molecular and Functional Correlates of Stress-Related Anxiety

Outubro de 2012

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Tese de Doutoramento em Ciências da Saúde

Trabalho efetuado sob a orientação do
Professor Doutor José Miguel Pêgo

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DECLARAÇÃO

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Aos meus pais e irmão

Para ser grande, sê inteiro: nada
Teu exagera ou exclui.
Sê todo em cada coisa. Põe quanto és
No mínimo que fazes.
Assim em cada lago a lua toda
Brilha, porque alta vive

Ricardo Reis

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Molecular and Functional Correlates of Stress-Related Anxiety

Abstract

Mood disorders, including anxiety or depression, are one of the most prevalent disorders in the modern society. Underlying the etiology of these disorders is, among other factors, a chronic exposure to stress. Stress can be defined as a challenge to the organism's homeostasis that prepares the organism to a "fight or flight" situation. However, when the stressor is too intense or too prolonged in time, there is a maladaptation of the organism that is associated with a series of morphological and molecular alterations in the central nervous system.

Among the brain areas associated with the response of the organism to stressful stimuli, the bed nucleus of the stria terminalis (BNST) is one of the most important for stress-related anxiety. The BNST plays a critical role in the integration and modulation of other limbic inputs; namely from the Infralimbic Cortex (ILCx), Medial and Central Amygdala (CeA) or hippocampus; and in turn it sends projections to the paraventricular nucleus of the hypothalamus, a key region in the activation of the hypothalamic-pituitary-adrenal (HPA) axis that leads to the activation of the hormonal response to stress. Additionally, the BNST has a critical role in activation of stress-related behaviors and is particularly associated with the development of stress-related anxiety.

Previous work from our lab has shown that when rats are presented with a stress-model of anxiety (CUS: chronic unpredictable stress), the BNST of these animals suffers a series of morphological alterations. In the present work we further characterized the alterations induced by CUS in the BNST, particularly at the molecular and electrophysiological level. We have shown that stress induces alterations in several neurotransmitter systems important for the stress response: decrease of the corticotrophin-releasing factor receptor 1 (CRFR1) while increasing the mRNA levels of receptors for glutamate (NR2B), GABA (GABAA receptor) and corticotrophin-releasing factor receptor 2 (CRFR2). At the functional level, we attempted to add new insights to how the neuronal circuit between ILCx and BNST and CeA and BNST was affected by stress exposure. We observed that stress induces a hyperactivation of neurons of the BNST by increasing the excitatory inputs from ILCx while decreasing the inhibitory inputs from CeA.

Additionally, we further explored the CeA-BSNT pathway in the development of anxiety behavior by performing excitotoxic lesions in CeA in animals that were later submitted to CUS. CeA is the major extra-hypothalamic source of corticotrophin-releasing factor (CRF), a key player in the

stress response and particularly in anxiety-behavior with several authors showing that the activation of CRFR1 is able to induce anxiety-like behavior. For this, we hypothesized that a lesion in CeA would lead to reduced CRF innervation in the BNST and consequently decreased anxiety-like behavior. Our observations show that the lesions allowed, indeed, an attenuation of the stress-related anxiety, showing that an intact innervation between CeA and the BNST is essential for a full manifestation of anxiety associated with stress.

Together, our findings allowed us to better understand the role of the BNST in the response to stress, showing that stress triggers a hyperactivation of the BNST while altering the mRNA expression of several important receptors. In particular, the decrease of CRFR1 in the BNST can be the result of increased CRF inputs arriving in the BNST from the CeA and that are essential for the manifestation of stress-related anxiety. The observation that a lesion in CeA, and putatively a decrease in CRF input into the BNST, can attenuate the development of anxiety-like behavior supports the essential role of CRF in the expression of stress-induced anxiety.

Ansiedade e Stress: Correlatos Moleculares e Funcionais

Resumo

Perturbações do humor, como ansiedade e depressão, são bastante prevalentes na sociedade moderna. Na génese destas perturbações estão, entre outros fatores, uma exposição crónica ao *stress*. O *stress* pode ser definido como um desafio à homeostasia do organismo, que o prepara para uma situação de "fuga ou luta". No entanto, quando um estímulo stressante é demasiado intenso ou demasiado prolongado no tempo, existe uma inadequada adaptação do organismo que está associada a uma série de alterações morfológicas e moleculares no sistema nervoso central.

Dentro das áreas cerebrais associadas com a resposta do organismo a um estímulo stressante, o núcleo da estria terminal (do inglês: BNST) é uma das mais importantes para ansiedade relacionada com *stress.* O BNST tem um papel fundamental na integração e modulação de estímulos provenientes do sistema límbico: nomeadamente, do córtex infralímbico (ILCx), da amígdala central (CeA) e medial ou do hipocampo; e por sua vez envia informação para o núcleo paraventricular (PVN) do hipotálamo, uma região central na ativação do eixo hipotálamo-pituitária-suprarenal (HPA) que leva à ativação da resposta hormonal ao *stress*. Adicionalmente, o BNST tem um papel essencial na ativação de comportamentos relacionados com *stress*, particularmente o desenvolvimento de comportamento ansioso.

Experiências anteriores do nosso laboratório mostraram que, quando os ratos são expostos com um modelo de ansiedade induzida por *stress* (CUS*: stress* crónico imprevisível), o BNST destes animais sofre uma série de alterações morfológicas. Neste trabalho, tentámos caracterizar mais pormenorizadamente as alterações induzidas por *stress* crónico, particularmente ao nível molecular e eletrofisiológico. Mostrámos que o *stress* induz alterações em diversos sistemas de neurotransmissores importantes na resposta ao *stress*: diminuição do recetor do fator libertador de corticotrofina do tipo 1 (CRFR1) ao mesmo tempo que aumenta os níveis do RNA mensageiro do recetor do fator libertador de corticotrofina do tipo 2 (CRFR2), de um recetor para glutamato (NR2B) e para GABA (recetores GABAA). A nível funcional, tentámos providenciar novas informações à forma como os circuitos neuronais entre o ILCx e o BNST e a CeA e o BNST são afetados por exposição a *stress*. Observámos que o *stress* induz uma hiperactivação dos

neurónios do BNST uma vez que aumenta as projeções excitatórias do ILCx ao mesmo tempo que diminui as projeções inibitórias da CeA.

Adicionalmente, explorámos em mais detalhe a via CeA-BNST no desenvolvimento de comportamento ansioso e para isso induzimos uma lesão excitotóxica na CeA em animais que em seguida foram submetidos a um protocolo de *stress* crónico. A CeA é, a seguir ao hipotálamo, a segunda maior fonte de fator libertador de corticotrofina (CRF), um componente essencial na resposta ao *stress* e em particular no comportamento ansioso. De facto, vários autores demonstraram que a ativação de CRFR1 por CRF é indutora de comportamento ansioso. Por isto, colocámos a hipótese de que uma lesão na CeA levaria a uma redução da inervação de CRF no BNST e consequentemente a diminuição do comportamento ansioso. As nossas observações mostraram que a lesão levou realmente a uma atenuação do comportamento ansioso associado a *stress,* mostrando que uma via intacta entre a CeA e o BNST é essencial para uma manifestação total deste comportamento.

Em conjunto, as nossas observações permitiram-nos compreender melhor qual é o papel do BNST na resposta ao *stress* e mostrando que o stress induz uma hiperactivação do BNST ao mesmo tempo que altera a expressão de RNA mensageiro de vários recetores importantes. Em particular, a diminuição de CRFR1 no BNST pode ser o resultado de um aumento de CRF vindo da CeA e que é essencial na manifestação de comportamento ansioso. A observação que uma lesão na CeA e, consequentemente, uma diminuição de inervação CRF para o BNST, pode atenuar o desenvolvimento de comportamento ansioso dá suporte ao papel essencial do CRF na expressão de ansiedade induzida por *stress*.

Abbreviation List

ACTH – Adrenocorticotropic Hormone

AVP – Arginine-vasopressiin

BDNF – Brain-derived neurotrophic factor

BLA – Basolateral amygdala

BNST – Bed Nucleus of the Stria Terminalis

BNSTdm – dorsomedial nucleus of the bed nucleus of the stria terminalis

BNSTfu – fusiform nucleus of the bed nucleus of the stria terminalis

BNSTL – Lateral Bed nucleus of the stria terminalis

BNSTM – Medial bed nucleus of the stria terminalis

BNSTpr – principal nucleus of the bed nucleus of the stria terminalis

CeA – Central Nucleus of the Amygdala

Cont - Control

CRF – Corticotropin-Releasing Factor

CRFR1 – Corticotropin-Releasing Factor receptor 1

CRFR2 – Corticotropin-Releasing Factor receptor 2

CS – Conditioned stimulus

CUS – Chronic Unpredictable Stress

EPM – Elevated plus maze

FPS – Fear-potentiated startle

GR – Glucocorticoid receptor

HIPP - Hippocampus

HPA – Hypothalamic-Pituitary-Adrenal

ILCx – Infralimbic Cortex

MeA – Medial Amygdala

MR - Mineralocorticoid receptor

mPFC – media Prefrontal Cortex

PACAP – Pituitary adenylate cyclase-activating polypeptides

PBS - phosphate-buffered solution

PTSD – Posttraumatic stress disorder

PSTH – Peristimulus Time Histogram

PVN – Paraventricular Nucleus of the Hypothalamus

SD – Standard Deviation

SEM – standard error of the mean

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Chapter 1

Introduction

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Chapter

AN INTEGRATED PERSPECTIVE ON THE ROLE OF THE AMYGDALA AND BED NUCLEUS OF THE STRIA TERMINALIS IN THE REGULATION OF STRESS RESPONSES

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ABSTRACT

Stress is defined as a challenge to the homeostatic equilibrium of the organism. In stressful situations, a cascade of hormonal and behavioral changes is generated in an attempt to maintain homeostasis. However, maladaptation to stress is considered to be an etiological factor in the emergence of mood disorders like depression and anxiety, which are associated with a series of morphological and neurochemical alterations in the brain, in particular within limbic structures.

The bed nucleus of the stria terminalis (BST) and the centromedial amygdala share many similarities, but they seem to have different roles in the regulation of emotional behavior. Both regions are important for anxiety-related behaviors, but the amygdala is

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essential for conditioned fear responses, whereas the BST seems to be involved mainly in unconditioned fear and anxiety. In addition, the amygdala, which plays a critical role in the activation of the hypothalamic-pituitary-adrenal (HPA) axis during stress, can exert both an excitatory and inhibitory influence over the anteromedial division of the BST that will, in turn, activate the hypothalamus through direct activation or disinhibition. At the same time, the BST plays a crucial role in stress responses by serving as a relay station for inputs from other parts of the limbic system (namely, from the prefrontal cortex, hippocampus) to the hypothalamus.

Amongst other mediators, corticotropin-releasing factor (CRF) exerts major regulatory control over endocrine responses to stress, while also determining emotional behavior. In the hypothalamus, CRF is involved in the control of the activity of the HPA axis, whereas in the amygdala, it is strongly implicated in the control of various types of emotional behaviors, as well as modulatory action on the activity of the BST. In this chapter, therefore, we review the morphological and neurochemical changes in the amygdala and BST after exposure to stress, as well as the interaction between these two areas during stress responses.

1. INTRODUCTION

Mood disorders, namely anxiety and depression, are highly prevalent and are expected to grow in incidence as individuals attempt to meet the ever-growing demands of our modern society. Chronic exposure to stress is considered to be a key triggering factor for these disorders, and the limbic system, which plays a fundamental role in the regulation of emotional behavior and endocrine responses to stressful events, is widely believed to be a central target and executor of (mal)adaptive responses to stress. Limbic areas, in turn, coordinate endocrine and behavioral stress responses through downstream projections originating in the central amygdala (CeA) and bed nucleus of the stria terminalis (BST), which innervate various hypothalamic and brainstem areas (Walker et al., 2003). Both the CeA and BST receive glutamatergic projections from the medial prefrontal cortex (mPFC; mainly infralimbic division), ventral subiculum of the hippocampus and basolateral amygdala (Pitkänen, 2000; Dong et al., 2001; Vertes, 2004; Herman et al., 2005). However, there is some indication that the CeA regulates the output of the BST, whereas the BST drives neuroendocrine responses to stress (Cullinan et al., 1993; Spencer et al., 2005). Hence the CeA provides GABAergic inputs to the BST (indicated by predominance of GABAergic projection neurons; see Cassell et al., 1999) and widespread peptidergic projections, including CRFergic fibers, the latter known to be important for stress responses (Sakanaka et al., 1986; Gray, 1993).

The CeA, medial amygdala (MeA) and BST share several neurochemical and morphological similarities and display reciprocal connections in the form of bidirectional projections along the stria terminalis (Alheid and Heimer, 1988; Dong et al., 2001). This led to the concept of the extended amygdala, which includes the CeA and medial nucleus of the amygdala, the BST and sublenticular regions in the basal forebrain (Alheid and Heimer, 1988). Moreover, the amygdala (including the basolateral nucleus of the amygdala and CeA) and BST are strongly implicated in fear and anxiety-related behaviors. However, the BST seems to play an important role in anxiety, unconditioned fear and long-lasting fear or stress responses, while the CeA is essential for acute fear responses to dangerous or threatening stimuli and various types of conditioned responses (Koch, 1999; Walker et al., 2003).

Therefore, this chapter will focus on the involvement of the CeA and BST in stress responses and will discuss how chronic exposure to stress modulates their structure and function.

2. ACTIVATION OF STRESS RESPONSES

Stress can be defined as a challenge to the organism's homeostasis. Exposure to physical insults or changes in the environment, as well as emotional challenges, elicits a series of adaptive responses that are aimed at maintaining homeostatic equilibrium. These responses are primarily hormonal (the most prominent being the release of corticosteroids by the adrenal glands) and behavioral. Normally, these mechanisms enable the organism to cope with stress by making appropriate adjustments. However, the organism's ability to terminate stress responses can fail under certain conditions, e.g., when the exposure to stressors is prolonged or intense. This failure can induce pathophysiological changes in the brain, as well as immune, cardiovascular and metabolic systems. Such alterations in the brain associated with uncontrollable stress lead to pathological anxiety and depression, as well as cognitive impairment (Porsolt, 1997; Drevets, 2000; Herman et al., 2005).

The activation of the hypothalamic-pituitary-adrenal (HPA) axis is one of the key features of the endocrine stress response, as it determines the release of corticosteroids by the adrenal glands. The HPA axis is activated by stress, probably through corticotropin-releasing factor (CRF)-containing fiber systems originating in various hypothalamic nuclei (perifornical and dorsomedial nuclei, dorsolateral hypothalamic area), the BST, Barrington's nucleus and dorsal raphe that innervate the paraventricular nucleus of the hypothalamus (PVN) (Bloom et al, 1982; Moga and Saper, 1994; Champagne et al., 1998). During stress, parvocellular neurons of the hypothalamic PVN secrete neuroregulatory peptides, such as CRF and arginine-vasopressin (AVP), at the medial eminence into the bloodstream, which then reach the anterior pituitary through the portal circulation. The anterior pituitary, in turn, releases adrenocorticotropic hormone (ACTH) into the circulatory system, which, upon reaching the adrenal cortex, increases the synthesis and secretion of corticosteroids (Antoni, 1986; Whitnall, 1993; Herman et al., 2005).

The PVN is a hypothalamic region that mediates both autonomic and endocrine responses to stress, and responds to stimuli of both central and peripheral origin (for review: Pyner, 2009; Kc and Dick, 2010; Nunn et al., 2011). Its magnocellular part is responsible for neuroendocrine secretion of AVP and oxytocin in the posterior pituitary (Frank and Landgraf, 2008; Bealer et al., 2010). In contrast, the parvocellular PVN projects to the medial eminence for the release of CRF and AVP into the portal circulation, providing the positive drive to the pituitary-adrenal unit, and it innervates areas controlling autonomic functions in the brainstem (dorsal vagus-solitary tract complex, nucleus ambiguus, central gray, pedunculopontine tegmental nucleus, locus coeruleus, parabrachial nuclei, rostral ventrolateral medulla) and spinal cord (preganglionic sympathetic neurons in the intermediolateral column, also called lateral horn), the latter neurons containing AVP (Aguilera and Rabadan-Diehl, 2000; Pyner, 2009; Kc and Dick, 2010; Nunn et al., 2011). Furthermore, stressors such as hemorrhage, systemic inflammation or pain (Ericsson et al., 1997; Palkovits et al., 1999) can directly activate the PVN, bypassing cortical and limbic regulation. However, at the central level, the limbic system is a major regulatory component of the HPA axis. The mPFC, hippocampus

and amygdala (Herman et al., 2005), together with the hypothalamus (Figure1), keep the secretion of corticosteroids under control by mediating negative feedback through reduced synthesis of CRF and AVP (de Kloet, 2004); the pituitary gland (ACTH synthesis) and hippocampus are also sites of corticosteroid negative feedback regulation (Sapolsky et al., 1984; de Kloet, 2000). In the CeA, however, corticosteroids were reported to increase CRF mRNA levels (Shepard et al., 2000).

3. MOLECULAR MEDIATORS OF STRESS

3.1. Corticotropin-Releasing Factor (CRF)

As already mentioned, CRF is an important mediator of stress responses in the brain. CRF is a 41-amino-acid peptide that is produced in several brain areas, the PVN, amygdala and BST being of special relevance to this chapter. CRF binds to two different G protein-coupled receptors: the CRF receptor 1 (CRFR1) and CRF receptor 2 (CRFR2).

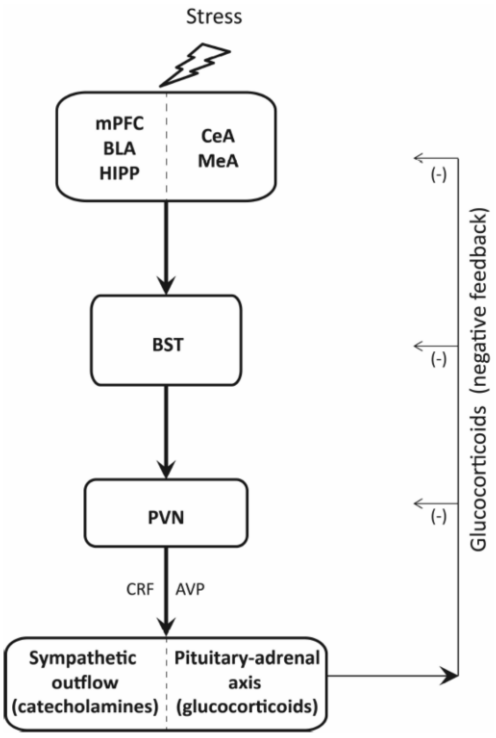

Figure 1. Overview of limbic brain regions involved in the activation of the hypothalamic-pituitary-adrenal (HPA) axis and regulating sympathetic outflow. The bed nucleus of the stria terminalis (BST) is as a relay station between the limbic system and paraventricular nucleus of hypothalamus (PVN). It receives input from the medial prefrontal cortex (mPFC) and various amygdaloid nuclei, namely the basolateral (BLA), central (CeA) and medial amygdaloid nuclei (MeA) and hippocampus (HIPP). The negative glucocorticoid feedback loop is well documented for the HIPP, PVN and pituitary gland, but the effect on the remaining regions is hypothetical. AVP arginine vasopressin; CRF corticotropin releasing factor.

Both receptors are expressed in the limbic system, including the amygdala, but the CRFR1 is most abundant in frontoparietal and temporal cortical regions and the cerebellum, whereas CRFR2 expression seems to be more restricted to subcortical areas and peripheral tissues (De Souza and Van Loon, 1983; Primus et al., 1997; Perrin and Vale, 1999). In addition, several CRF-like peptides have been identified, namely urocortin 1, 2 and 3 (Vaughan et al., 1995; Reyes et al., 2001; Lewis et al., 2001). CRF and urocortin 1 bind with high affinity to the CRFR1, but only urocortin 2 and 3 bind with high affinity to the CRFR2 (Vaughan et al., 1995).

The effect of CRF in stress-related behaviors has been widely studied. CRF-containing axons form a widespread network in brain regions involved in autonomic stress responses that spans from the CeA, BST, paraventricular thalamic nucleus, PVN, lateral hypothalamus, midbrain central gray, raphe nuclei, parabrachial region, Barrington's nucleus and dorsal motor vagus nucleus to the nucleus of the solitary tract (Swanson et al., 1983; Veening et al., 1984; Sakanaka et al., 1986; Moga et al., 1989; Moga and Gray, 1985; Gray and Magnuson, 1992; Gray, 1993; Moga and Saper, 1994; Otake and Nakamura, 1995; Lechner and Valentino, 1999; Van Bockstaele et al., 2001; Rodaros et al., 2007; Panguluri et al., 2009). Intracerebral injections of CRF produce a behavioral phenotype that mimics some of the behavioral effects of stress (Sherman and Kalin, 1988; de Pedro et al., 1993; Liang et al., 1992). In fact, several studies have associated the activation of the CRFR1 with anxiogenesis; for example, knockout mice for CRFR1 showed decreased anxiety-like behavior (Smith, 1998). In contrast, the role of the CRFR2 is still unclear, although a recent study suggested that, unlike the CRFR1, the CRFR2 may be responsible for anxiolysis (Bale, 2000). In addition to these effects, it is also widely accepted that CRF plays an important role in the behavior of animals that were submitted to early life stress, such as maternal separation. In the latter paradigm, alterations in the expression of CRFRs were reported in various brain regions of adult animals that had been exposed to early life stress. Among those regions, decreased levels of CRFR1 and increased levels of CRFR2 were observed in the amygdala (Ladd et al., 2000; Plotsky et al., 2005). These findings attest to the important role of CRF in determining behavioral aspects of stress responses, in addition to its role in regulating hormonal responses to stress.

3.2. Corticosteroids

Corticosteroids are stress hormones released by the adrenal cortex under the regulatory control of the HPA axis. The actions of corticosteroids are mediated by two receptors: mineralocorticoid receptors (MRs) and glucocorticoid receptors (GRs). These receptors differ in their pharmacological properties; MRs bind corticosteroids with slightly higher affinity than GRs (Reul and de Kloet, 1985). As a result, GRs are only fully occupied when corticosteroid levels exceed a certain threshold, such as during stress (de Kloet, 2000). These receptors also display a differential distribution in the brain; whereas GRs are ubiquitously distributed in neurons and glia, MRs are present mainly in the hippocampus, septal nucleus and some cortical areas (Kwak, 1993). Both MRs and GRs are so-called “nuclear receptors,” i.e., ligand-activated transcription factors. Upon activation, they bind to the promoters of responsive genes via their hormone-responsive elements and regulate the expression or repression of their target genes. In addition to this classical mechanism of action, it is

becoming increasingly apparent that corticosteroids may also trigger rapid responses by interacting with putative receptors in the plasma membranes of neurons and other cell types. Thus, corticosteroids can influence fear, stress and anxiety responses by regulating the activity of limbic areas and the HPA axis through rapid non-genomic transmitter-like activities, as well as nuclear receptors altering gene expression (Groeneweg et al., 2011).

3.3. Other Factors

The peptide AVP mentioned above and the pituitary adenylate cyclase-activating polypeptide (PACAP) also contribute to the activation of the HPA axis. They are expressed in both fibers innervating the median eminence, where they are released into the hypothalamo-hypophysial portal system, and in magnocellular PVN neurons (Aguilera and Rabadan-Diehl, 2000; Badoer, 2010; Hashimoto et al., 2011). Oxytocin, somatostatin and angiotensin II are other peptides involved in the regulation of stress responses, e.g., by modifying the function of the HPA axis and/or their direct release into systemic circulation, or through projections to autonomic regions in the brainstem and spinal cord (Kc and Dick, 2010; Nunn et al., 2011). Vasoinhibins are peptides generated by the proteolytic cleavage of prolactin, which may be local regulators of vascularization and hormone release in the anterior and posterior pituitary glands (Méndez et al., 2010).

In addition, humoral delivery of cytokines, such as interleukin 1, can contribute to the stress-related activation of neuroendocrine circuitries, which often act through prostaglandin-dependent mechanisms (Ericsson et al., 1997).

The function of the PVN can also be modulated by a variety of neuropeptidergic fibers, many of which originate in other hypothalamic areas; i.e., enkephalinergic fibers originate in the lateral septal nucleus (ventral part) and anterior hypothalamic area (lateral anterior nucleus), somatostatinergic fibers in the lateral hypothalamic area (ventral part) and arcuate nucleus, neurotensinergic in the anteroventral periventricular nucleus and retrochiasmatic area, and natiuretic peptide-positive fibers in the tuberomammillary nucleus. Moreover, CRF-containing fibers originate in the BST, and natriuretic peptide-containing neurons innervating the PVN are also located in the pedunculopontine and laterodorsal tegmental nuclei (Moga and Saper, 1994). Among ascending fiber systems regulating the activity of the hypothalamic PVN, the role of the noradrenergic system is well documented (e.g., Palkovits et al., 1999; Fernandes et al., 2007).

4. FEAR AND ANXIETY

A large proportion of the world population is affected by anxiety disorders. In fact, the World Health Organization (WHO) estimates that, by 2020, emotional disorders (including anxiety) will be the second leading global burden of illness. Anxiety disorders include phobias, panic disorder, posttraumatic stress disorder (PTSD) and generalized anxiety disorder. Fear and anxiety share many phenotypical similarities; however, while fear is a response to a specific and imminent threat, anxiety is characterized by a sensation of discomfort and apprehension in response to unconditioned diffuse cues (Koch, 1999;

LeDoux, 2000). Fear is a primary protective reaction to potentially harmful challenges. It is, therefore, an important evolutionary landmark that contributes significantly to the survival of the individual. Importantly, fear responses start abruptly and end when the threat is removed, while anxiety can be long-lasting and persist to pathological states.

Fear/anxiety behavior can be studied using specific behavioral paradigms. The gold standard used for studying anxiety in rodents is the elevated plus maze (EPM) test. The EPM consists of two facing open arms and two closed arms that create a conflict between the rodent's innate tendency to explore novel environments and their fear of bright light and open spaces. A decreased ratio between time spent on open and closed arms is an indicator of anxiety (Carobrez and Bertoglio, 2005). Other tests commonly used to examine anxiety are the open field, operant conflict, light-dark box, shock probe avoidance and ultrasonic vocalization tests (Davis, 1997; Engin and Treit, 2007). Alternatively, tests measuring changes in reflex responses to aversive stimuli can be employed. Startle-based tests like CRF-enhanced startle or the light-enhanced startle can be used to examine brain areas involved in anxiety-related behavior.

In fact, it was shown that CRF infusions into the brain increase the amplitude of the acoustic startle response in rats; conversely, anxiolytic drugs were able to reduce the startle amplitude in CRF-enhanced startle (Swerdlow, 1986), suggesting that CRF systems are specifically implicated in anxiety behavior. In contrast, the fear-potentiated startle (FPS) response is a form of Pavlovian fear-conditioning. In the FPS model, a conditioned stimulus (CS; e.g., light) is associated with an aversive unconditioned stimulus (footshock). After conditioning training, animals are presented with a series of startle-eliciting noises in the presence or absence of the conditioned stimuli. Higher startle amplitude upon presentation of the CS (the FPS response) is indicative of fear-conditioning, whereas the increase in the startle response is blocked by amygdala lesions (Walker et al., 2003).

Another difference between fear and anxiety is that the brain circuitries leading to these behaviors overlap only partially; acute fear responses and conditioned fear seem to be specifically dependent on the amygdala, including the FPS response (Walker et al., 2003), whereas innate and unconditioned anxiety responses are mediated by the amygdala and BST, as well as the hippocampus. Numerous studies based on local infusion of drugs using the open field, EPM, operant conflict, light-dark box, passive shock probe avoidance or ultrasonic vocalization paradigms have shown that the amygdala, BST and hippocampus are involved in anxiety behavior (Davis, 1997; Lee and Davis, 1997; Cecchi et al., 2002; Pardon et al., 2002; Engin and Treit, 2007).

Beyond these findings, the BST seems to be involved selectively in the modulation of unconditioned fear responses (e.g., light-enhanced startle or fox odor-induced freezing) and conditioned stimuli that are of long duration and/or related to context or overtraining in rodents (Walker et al., 2003; Burow et al., 2005; Fendt et al., 2005; Waddell et al., 2006; Poulos et al., 2010). Thus, there is a double dissociation between the involvement of the BST and CeA in startle increases produced by conditioned versus unconditioned fear (Walker and Davis, 1997). Moreover, there is ample evidence that a dysfunction and/or chronic activation of the HPA axis plays a critical role in the development of anxiety disorders and depression (Drevets, 2000; Kasckow et al., 2001; Young et al., 2008). Although the amygdala and BST seem to regulate the activity of the HPA (Herman et al., 1994; Dayas et al., 1999; Forray and Gysling, 2004), the mechanisms through which this activation occurs are less clear.

5. CONCEPT OF EXTENDED AMYGDALA

The amygdala, sometimes referred to as the amygdaloid complex, is a heterogenous region located in the temporal lobe. It is composed of several nuclei that can be distinguished on the basis of cyto- and chemo-architectural characteristics, which also differ in their connectivity. It was first described in the nineteenth century, but its major nuclei known today were identified in various species in the early twentieth century (Völsch, 1906), and the nomenclature of amygdala nuclei commonly used today was proposed by Johnston (1923). Johnston divided the amygdala into a group of nuclei that are evolutionarily associated with the olfactory system (central, medial and cortical nuclei) and a more recently evolved deep group, comprising the lateral and basal nuclei (Johnston, 1923).

Later, it became clear that the BST and centromedial amygdala containing the central (CeA) and medial amygdaloid nuclei (MeA), as well as the caudal substantia innominata, can be considered a single morphological unit, often summarized as the extended amygdala (Alheid and Heimer, 1988; de Olmos and Heimer, 1999). These nuclei share many similarities in terms of cell types, peptide content and neural inputs and outputs (Alheid et al., 1998). The components of the extended amygdala all influence autonomic and neuroendocrine functions and are reciprocally interconnected. Communication between the centromedial amygdala and BST mostly occurs through the stria terminalis (dorsal pathway) and the ansa peduncularis (ventral pathway). The CeA projects densely to the anterior region of the BST, in particular its lateral part (BSTL). Moreover, the concept of a single extended amygdalar unit gains further currency when one considers that the projections of subnuclei in the CeA and BSTL, as well as MeA and medial BST (BSTM), are quite similar, i.e., they project to specific regions in the forebrain, hypothalamus and brainstem, including ascending monoaminergic and cholinergic groups of neurons (e.g., locus coeruleus, substantia nigra, ventral tegmental area or nucleus basalis) (Pitkänen, 2000; Davis and Whalen, 2001). Therefore, an integrative view of these various nuclei and subnuclei will undoubtedly provide better insights into the contribution of these subcortical areas to the modulation of stress and stress-related behaviors (Figure 2).

6. ROLE OF BED NUCLEUS OF THE STRIA TERMINALIS IN STRESS

The BST is a cerebral gray matter embedded in and surrounding the stria terminalis, which is a fiber tract connecting the basal forebrain with the amygdala (for review: Völsh, 1906; Johnson, 1923). On the basis of similarities of neuronal cell types and their neurochemical characteristics, as well as connections (hodology) with nuclei in the amygdala, it can be divided into a BSTM (medial BST) resembling the MeA, and a BSTL (lateral BST) resembling the CeA (Ju and Swanson, 1989; Ju et al., 1989; Moga et al., 1989). Considering that the MeA and CeA each have at least three subnuclei (Cassell et al., 1999, Pitkänen, 2000; Choi et al., 2005), it is not surprising that the BST has a rather complex cyto- and chemo-architecture, although it is a relatively small nucleus. The nomenclature of the BST is further complicated by the fact that many nuclei are named based on topographical rather than hodological criteria, leading to a further increase in the number of distinguishable nuclei. Swanson (1998) described 15 different cell groups or nuclei in the BST.

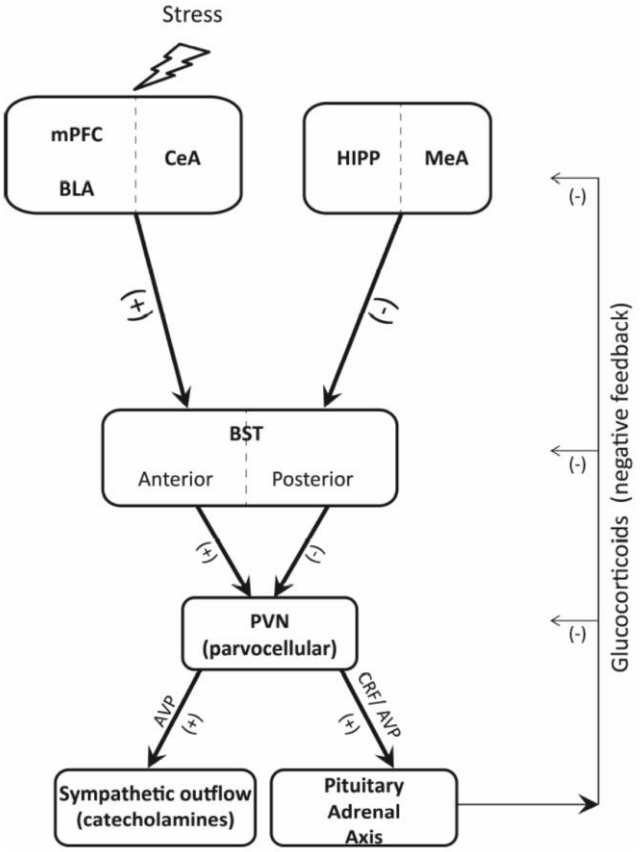

Figure 2. The anterior and posterior parts of the BST receive segregated input from the mPFC and specific amygdala nuclei, and probably have opposing roles in the activation of the HPA. Various groups of parvocellular neurons in the PVN form a high order sympathetic center or control the output of the HPA axis.

In functional studies, often only an anterior and posterior division were distinguished in the BST, as discussed below. In their review, Dong and coauthors (2001) indicated that especially the lateral group in the anterior BST (anterolateral BSTL or BSTLa) and the medial group in the posterior BST (BSTMp) have non-overlapping input from specific amygdala nuclei, whereas the input to the anteromedial BST (their medial group or BSTMa) from various amygdaloid nuclei shows considerable overlap.

Since the morphological complexity of the BST is also reflected at a functional level, its different divisions were implicated in different functions. The anterolateral BST (BSTLa) is involved in the modulation of autonomic responses (Veening *et al.*, 1984; Casada and Dafny, 1991; Dunn and Williams, 1995). Accordingly, the anterolateral group receives bidirectional inputs from the CeA and cortex-like amygdaloid nuclei, but not the MeA (Dong et al., 2001), and neurons in the BSTLa project heavily to the hypothalamus and lower brainstem nuclei associated with the control of autonomic responses (Dong and Swanson, 2004a; Dong and Swanson, 2006a). The anteromedial nuclei (BSTMa) are also innervated by the CeA, but in addition receive strong inputs from the MeA and other subnuclei of the amygdala (Dong et al., 2001). In turn, the BSTMa projects densely to parvo- and magnocellular neuroendocrine

subdivisions of the PVN (Dong and Swanson, 2006b), although the BSTLa also contributes to the innervation of neuroendocrine hypothalamic nuclei (Dong and Swanson, 2004a). In addition, the BST can influence the release of posterior pituitary hormones through direct projections to the magnocellular neurosecretory neurons of the PVN (Sawchenko and Swanson, 1983). Here it must be noted that it is the BSTLa where the majority of CRF-immunoreactive neurons are located (Ju et al., 1989), but CRF immunoreactive neurons projecting to the PVN were found mainly in the ventral lateral part of the anterior BST (Moga and Saper, 1994). In contrast, the BSTMp (posterior medial group) has been implicated in the control of reproductive (Gu et al., 2003) and defensive behaviors (fight or flight). It does not receive any projections from the CeA, but rather dense inputs from the MeA and cortex-like caudal amygdala nuclei (Dong and Swanson, 2004b). The connections between the amygdala and BSTMp are bidirectional, with the BSTMp projecting to the MeA, as well. The BSTMp also projects to the lateral septal nucleus and medial hypothalamus. Like most of the BST, the posterior division is rich in GABAergic neurons (Cullinan et al., 1993; Bowers et al., 1998).

Through its dense interconnections with limbic structures and the PVN, the BST is in a strong position to regulate the HPA axis; briefly, it has been hypothesized that the BST acts as a relay station between limbic structures and the PVN (Figure 1). Higher limbic structures, such as the mPFC, hippocampus and amygdala, have an established and often stressor-specific role in the regulation of the HPA axis (e.g., Herman et al., 2005; Radley and Sawchenko, 2011; Flandreau et al., 2012). However, the mPFC (Floyd et al., 2001; Vertes, 2004) and hippocampus (Risold et al., 1997; Petrovich et al., 2001), and possibly also the CeA (Prewitt and Herman, 1998), have only a few direct projections to the PVN. Although a previous report on the connections of the CeA contradicts the latter observation (Gray et al., 1989), the direct CeA-PVN projection probably does not contain CRF-positive neurons (Gray, 1990; Moga and Saper, 1994). Another amygdaloid candidate for regulating the activity of the PVN is the MeA, which innervates both the BSTMp (Herman et al., 2005) and PVN, but does not have widespread brainstem projections like the CeA (Pitkänen, 2000); in particular, it lacks direct connections to catecholaminergic cell groups known to play an important role in stress responses (Dayas et al., 1999; Ziegler et al., 1999). Therefore, it has been suggested that the BST relays the information coming from the mPFC, hippocampus and amygdala to the PVN for the regulation of the HPA axis and autonomic stress responses (Cullinan et al., 1993; Spencer et al., 2005), which is under the control of noradrenergic stimulation (Forray and Gysling, 2004). Nonetheless, subregions of the mPFC seem to influence the output of the BST differentially, at least in acute stress, as indicated by lesion studies; the prelimbic field seems to suppress c-fos and CRF expression in neurosecretory PVN neurons, whereas the infralimbic field activates the neurosecretory compartment of the PVN, but inhibits regions of the PVN involved in central autonomic control (Radley et al., 2006). This effect is probably mediated through the BST that is interposed between the mPFC and PNV, but from an anatomical point of view, the infralimbic mPFC provides a much stronger excitatory input to the BST, especially to its anterior division, than the prelimbic mPFC (Hurley et al., 1991; Takagishi and Chiba, 1991; Floyd et al., 2001; Vertes, 2004; Radley et al., 2009). For the hippocampus, it has been shown that the ventral subiculum, but not other hippocampal fields, inhibit the activation of the HPA axis (Herman et al., 1995). The morphological substrate of this effect may be the direct glutamatergic projection from the ventral subiculum to the BSTMp (Herman et al., 2005). The innervation of the BST by the amygdala is more complex. While the cortex-like nuclei (posterior basolateral nucleus,

anterior basomedial nucleus) provide a strong and most likely glutamatergic input to the anterior BST, the CeA and MeA generate heavy projections to the BST that are topographically organized as described above (Dong et al., 2001), and most likely originate in GABAergic neurons coexpressing peptides (Veinante et al., 1997).

The BST, in turn, receives direct input from the three mentioned limbic areas and projects heavily to the hypothalamus and, specifically, to the PVN. Studies involving stimulation of the BST have shown that, depending on targeted regions, there may be either an activation or inhibition of the HPA axis (Casada and Dafny, 1991; Zhu et al., 2001). Notably, anterior lesions (Crane et al., 2003) lead to a decreased response to stress and lower CRF mRNA levels in the PVN, while posterior lesions result in increased CRF mRNA in the PVN (Herman et al., 1994). The anterior BST, which is richly endowed with CRFergic neurons, is thus intimately involved in the activation of the HPA axis. Nevertheless, specifically the GABAergic neurons in the anterior BST may be an inhibitory relay station for prelimbic afferents known to suppress the HPA axis (Radley et al., 2009). On the contrary, the principal nucleus of the BSTMp is mainly comprised of GABAergic neurons, and seems to be involved in inhibition of hormonal responses to stress. Choi and colleagues provided further evidence for this by making sub-nuclei specific lesions in the BST, specifically in the dorsomedial/fusiform nuclei in anterior BST and principal nucleus in the posterior BST. They showed that lesions in the dorsomedial/fusiform nuclei attenuated corticosterone secretion without altering the expression of CRF and AVP in the PVN. However, lesions of the principal nucleus led to an increase in corticosterone secretion and CRF and AVP expression within the PVN (Choi et al., 2007). This information on the distinct effects of lesions in different areas of the BST highlights the importance of considering the subnuclei of the BST individually rather than the BST as a whole, especially in studies aimed at understanding how information is modulated at the level of the BST (Figure 3).

The heterogeneity of the BST makes the understanding of the overall role of the BST in mood disorders a challenging task. We only started to understand the role of the subnuclei of the BST in specific stress-related behaviors, but our understanding of the importance of this nucleus in mood disorders is still in its infancy.

Some studies report that lesions in the BST help to prevent the development of anxiety-like behavior in rodents (Hammack et al., 2004). In accordance, some studies demonstrated that local infusion of CRF into the BST elicits anxiety-like behavior (Lee et al., 2008; Sahuque et al., 2006). The modulation of anxiety behavior by other neuropeptides like galanin within the BST has also been reported (Khoshbouei et al., 2002). Importantly, the role of BST seems to be specifically to mediate long-lasting responses, whereas lesions of the BST did not have an effect on the fear-potentiated startle response (Walker et al., 2003). Again, these studies highlight the greater involvement of the BST in anxiety rather than in conditioned fear.

Although only few studies have actually addressed this issue, there seems to be consensus on structural changes in the BST related to chronic stress. Vyas et al. (2003) reported that after chronic restraint stress exposure, there were significant alterations in the morphology and length of dendrites of BST neurons; in particular, an increased number of branches in the dendritic arborisation were found. Subsequent studies from our lab confirmed that chronic unpredictable stress leads to an increase in BST volume, as well as an increase in dendritic length and number of spines (Pêgo et al., 2008). These structural alterations observed after chronic stress are paralleled by changes in the expression of a number of genes. For example,

different stress paradigms induced an increase in CRF expression in the BST (Kalin et al., 1994; Stout et al., 2000; Makino et al., 1994; Santibañez et al., 2006). Interestingly, injections of corticosterone into the amygdala mimicked these stress-induced changes in CRF expression in the BST (Shepard et al., 2006; Makimo et al., 1994).

Obviously, CRF is not the only neurotransmitter/modulator target of stress; for example, increases in the expression of pituitary adenylate cyclase-activating polypeptides (PACAP) and PAC1 receptor (pituitary adenylate cyclase-activating polypeptide type I receptor**)**, as well as brain-derived neurotrophic factor (BDNF), were observed in the BST of chronically stressed animals (Hammack et al., 2009). Nur77, a transcription factor that was identified as a marker of stressful stimuli (Katunar et al., 2010), was also increased in the BST after a single exposure to immobilization stress or repeated exposure to the same stressor (Campos-Melo et al., 2011).

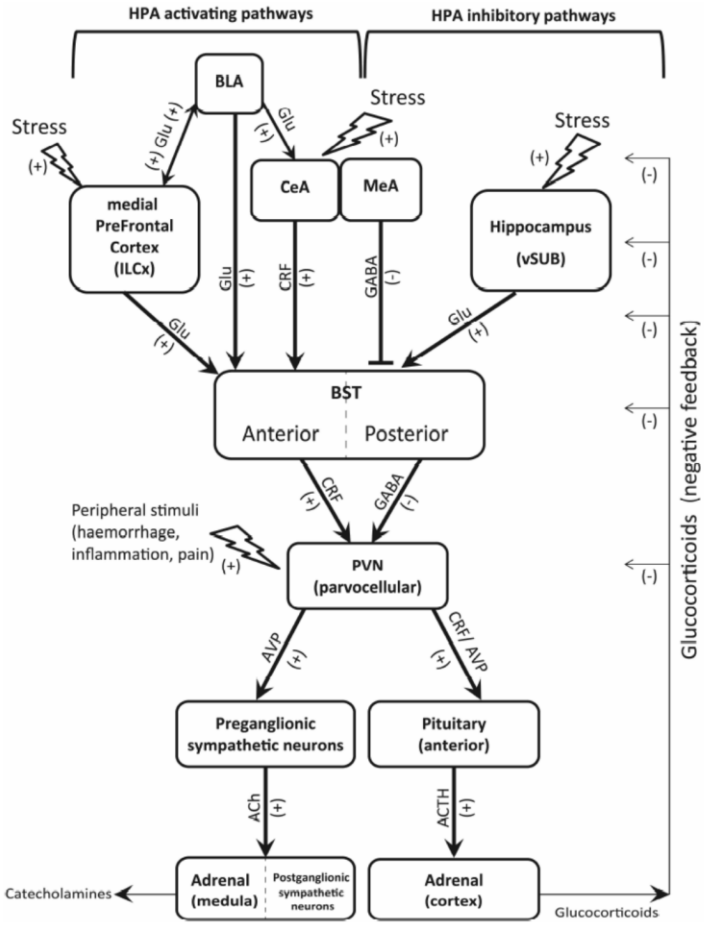

Figure 3. Diagram showing the role of the BST as a relay station between the limbic system and hypothalamus in more detail. The infralimbic field (ILCx) of the mPFC, and the BLA and CeA project to the anterior BST, a division involved in the activation of the HPA (hypothalamic-pituitary-adrenal) axis and innervates preganglionic sympathatetic neurons. In contrast, the MeA and ventral subicular field (vSUB) of the hippocampus project to the posterior BST that has an inhibitory role over the HPA axis. Ach acetylcholine, ACTH adrenocorticotropic hormone, AVP arginine vasopressin, CRF corticotropin releasing factor, GABA gamma-aminobutyric acid, Glu glutamate.

7. ROLE OF AMYGDALA IN STRESS

The amygdala is a nuclear complex composed of deep (lateral, basolateral and basomedial nuclei) and superficial cortex-like nuclei (cortical nuclei) and non-cortex-like nuclei (CeA, MeA, intercalated nuclei). The lateral amygdala is the major receptive field of the amygdala, receiving inputs from distinct sensory systems, whereas the CeA is the major output region of the amygdala, with projections to the hypothalamus and brainstem. The CeA also projects to the BST (for review: Pitkänen, 2000), which innervates, as mentioned before, the autonomic and magno-/parvocellular neurosecretory divisions of the PVN (Sawchenko and Swanson, 1983; Dong and Swanson, 2004a, 2006b). The CeA receives feedback projections from the hypothalamus but is driven mainly by other amygdaloid nuclei, namely the basolateral nucleus (BLA). In fact, the amygdala nuclei display extensive internuclear connections, which are beyond the focus of this chapter (Pitkänen et al., 1997; Swanson and Petrovich, 1998; Sah et al., 2003). Sensory information, for example, enters the amygdala mainly through the lateral and, to some extent, other cortex-like nuclei (e.g., BLA and cortical nuclei). It is first processed locally and then conveyed to the CeA. The CeA, in turn, transmits information to specific nuclei of the BST and downstream brain areas. The CeA and MeA contain a large number of GABAergic neurons, suggesting that projections from these nuclei are inhibitory (Veinante et al., 1997; Asan, 1998; Saha et al., 2000). Of particular relevance to the regulation of stress responses is the fact that the CeA, after the hypothalamus, is the second richest source of CRF in the brain, and it is noteworthy to mention that CRF is often localized in GABAergic neurons (Veinante et al., 1997; Day et al., 1999; Sah et al., 2003).

The amygdala has a central role in emotional processing, particularly in fear and fear conditioning (Davis, 1997; LeDoux, 2000; Sah et al., 2003). The responses to fear are characterized by freezing behavior, release of stress hormones and alterations in heart rate and blood pressure. Electrical stimulation of the amygdala was shown to elicit fear responses, while lesions of the amygdala had the opposite effect – they block some types of unconditioned fear. The amygdala is also involved in various types of conditioned fear responses, and its lesioning disrupts the acquisition of such responses. Although Pavlovian fear-conditioning is among the paradigms most commonly used to understand the circuitry in the amygdala, the emotional processing of conditioned stimuli in the amygdala is not confined to processing of fear stimuli.

In fact, the CeA also modulates conditioning to other stimuli (e.g., appetitive stimuli, like food, sex and drugs), and is involved in a variety of anxiety responses (Davis, 1997; Walker and Davis, 2003). Both the basolateral complex and CeA are known to play critical roles in acquisition and expression of fear-related behaviors. The association of conditioned stimuli with unconditioned stimuli occurs at the level of the lateral amygdala, with the information being sent to the CeA (Sah et al., 2003). It is the activation of the CeA that leads to the stimulation of the hypothalamic nuclei, periaquaductal gray and other brainstem areas that are, directly or indirectly, responsible for fear- and stress-related autonomic, hormonal and behavioral responses (Walker et al., 2003).

Because the CeA is particularly rich in CRFergic neurons, and in light of the relevance of CRF in stress responses and fear-related behaviors, several authors have proposed that CRF neurons in the amygdala are involved in anxiety-like and fear behaviors in response to stressful stimuli (Davis, 1992; Menzaghi et al., 1993; Makino et al., 1995, 1999). Lesions of

the CeA dramatically reduce ACTH responses to immobilization stress, which is associated with increased noradrenergic but reduced dopaminergic activity in the CeA (Beaulieu et al., 1987). CRF neurons in the CeA also play a critical role in the activation of the dorsal raphe, which in turn modulates serotonin release in the mPFC (Forster et al., 2008). Lentiviral overexpression of CRF in the CeA results in dysregulation of the HPA axis, an increase in the baseline acoustic startle response (mimicking fear-induced elevation) and depressive-like behavior in the forced swim test (Keen-Rinehart et al., 2009; Flandreau et al., 2012).

These behavioral changes are paralleled by increases in neuronal activation in the CeA, as indicated by elevated CRF levels after both adrenalectomy and lesions of the PVN, suggesting a deficit in negative feedback mechanisms (Palkovits et al., 1998).

In addition, MeA contributes to the activation of the HPA axis, because lesions of the MeA greatly reduce restraint-induced activation of the medial PVN-containing CRF-positive cells at the apex of the HPA axis (Dayas et al., 1999). Reports on the direct innervation of the PVN by the CeA are controversial, but the MeA seems to have direct projections to this hypothalamic nucleus (Silverman et al., 1981; Tribollet and Dreifuss, 1981; Sawchenko and Swanson, 1983; Gray et al., 1989; Prewitt and Herman, 1998).

The neurochemical alterations in the amygdala that follow exposure to stress can also be correlated with structural reorganization. Importantly, however, the structural changes appear to be stimulus-specific. While repeated restraint stress was found to lead to an increase in dendritic length and dendritic spine density in BLA neurons, such changes are not seen when animals are subjected to a chronic unpredictable stress (CUS) protocol (Vyas et al., 2002; Vyas et al., 2003); the latter was confirmed by experiments in our own laboratory (Pêgo et al., 2008). In rodents, stress is not the only stimulus that can alter amygdala morphology. For example, we have shown that chronic neuropathic pain, a condition associated with depressive-like behavior, also induces structural alterations in the amygdala. Specifically, chronic neuropathic pain results in an increase in the volumes of the BLA and CeA; the addition of new neurons (neurogenesis) in these nuclei accounts, at least partly, for these volumetric changes (Gonçalves et al., 2008). However, it may be not surprising that the BLA shows signs of plasticity after stress, because the BLA has strong connections with the CeA and receives input from the mPFC, an area known to be involved in stress responses.

Similar to the situation in rodents, patients with mood disorders are known to display structural alterations in the amygdala. For example, fMRI studies in depressed subjects have demonstrated an increased activation (Sheline et al., 2001) as well as volumetric increase in the amygdala (Drevets, 2000; Frodl et al., 2002; Tebartz van Elst et al., 2000).

Other studies in humans have shown that a reduction in perceived stress is accompanied by a significant decrease in gray matter density in the BLA (Hozel et al., 2010). Further, military veterans suffering from PTSD display increased activation of the amygdala upon exposure to combat sounds (Dykman et al., 1997).

8. CONCLUSIONS

There is indisputable evidence that the BST and amygdala, two densely interconnected areas, play important roles in the endocrine and behavioral responses to stress. Stress-induced structural and functional alterations in these areas appear to be critical to the pathology of

mood disorders. The BST is crucially involved in the regulation of anxiety-like behavior under stressful conditions, whereas the amygdala is essential for the elicitation of fear behavior to specific stimuli that represent a threat to the organism, and is also activated in anxiety. Despite their differential role in regulating various aspects of emotional behavior, the functional and structural similarities and intimate interconnections of the two structures argue in favor of considering them as a single unit when attempting to understand the biology and pathology of stress.

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N.G.

Chapter 1.9

Context of the Work and Aims

Context of the work and Aims

Historically, the study of stress-related pathways has focused largely in the hypothalamic-pituitary-adrenal axis function and how it is regulated by higher-order areas such as the hippocampus and, more recently the bed nucleus of the stria terminalis (BNST) (Herman et al., 2005; Choi et al., 2007). The location and connections of the BNST lend it a pivotal role in the modulation of the stress response. By receiving projections from limbic structures like the amygdala, the hippocampus or the prefrontal cortex and in turn, sending heavy projections to the paraventricular nucleus of the hypothalamus (PVN), the BNST can be considered a relay point of the brain's response to stressful stimuli (Cullinan et al., 1993; Dong et al., 2001, Radley & Sawchenko, 2011). Coherently, it is not surprising that the BNST is also important for stress-induced behavior, particularly for stress-related anxiety with several authors pinpointing the role of the BNST in the expression of anxiety-like behavior in rodents (Durvaci et al., 2009; Pêgo et al., 2008; Hammack et al., 2009, 2010; Walker et al., 2009). Therefore, the BNST is an ideal candidate to study when trying to better understand what are the molecular and functional changes occurring in the brain after stress exposure.

The study of the BNST in our lab had started (Pêgo et al., 2008) with the demonstration that chronic unpredictable stress (CUS) exposure induces anxiety-like behavior that can be correlated with morphological changes in the BNST, particularly, an increase in spine density and dendritic length in neurons of the anterolateral BNST. In this work, we aimed to further understand the alterations induced in the BNST by stress, particularly at the molecular and electrophysiological level. Of relevance, we focused on individual subnuclei and not on the BNST as a whole since different BNST divisions display distinct biological roles.

When studying the BNST it is not easy to completely dissociate this area from the amygdala. Even though these two regions play different roles in the modulation of anxiety and fear behavior (Pêgo et al., 2008; Walker & Davis, 2008), they share many similarities (Alheid et al., 1998). The amygdala and in particular the central nucleus (CeA), the main output nuclei of the amygdala, send heavy projections to the anterior BNST (Dong et al., 2001). The CeA is rich in GABAergic neurons but also has an elevated expression of neuropeptides like dynorphin, encephalin and, with particular relevance for this work, corticotrophin releasing factor (CRF) (Veinante et al., 1997; Day et al., 1999). CRF is described as one of the most important modulators of the response to stress and it has a very important role in the expression of anxiety-like behavior

(Swerdlow et al., 1986; Smith et al., 1998). Specifically, injections of CRF in the BNST have been shown to induce anxiety-behavior in rodents (Walker et al., 2009). Since the CeA is the second most important source of CRF in the brain (after the hypothalamus) and with the strong projections from CeA to the BNST, we hypothesized that CRF projections from CeA to the BNST are implicated in the expression of stress-induced anxiety. Thus, in the first part of this Thesis we have explored the stress-induced changes in the different subdivisions of the BNST and how an excitatory lesion of the CeA would affect the function of the BNST.

One aspect that is important to consider when studying the modulation of the stress-response is the several inputs that are being integrated in a particular area. Therefore, we needed to extend our study to a system level by the use of in vivo electrophysiology. This technique allowed us to investigate the role of limbic inputs into the activity of BNST neurons in control and animals submitted to CUS. In that part of our work, we have focused on the role of the CeA but also of the ILCx inputs in the activity of anterior BNST neurons. As mentioned before, CeA sends strong projections to the BNST but as far as we know, until now there were no studies trying to understand what is the effect of CeA stimulation upon the activity of BNST neurons. The ILCx, together with CeA, is another important source of inputs to the anterior BNST and it was known before that ILCx stimulation was able to drive an excitation of BNST neurons (Massi et al., 2008) in control animals; however it was not known how stress was able to modulate this response. Thus, in this Thesis, we have tried to understand how both ILCx and CeA inputs are acting upon BNST neurons after stress exposure.

In summary, the main goals of this Thesis were:

- Identify a molecular signature of stress-induced anxiety in BNST subnuclei
- Evaluate the effects of an excitotoxic lesion in CeA in the development of anxiety induced by chronic stress exposure
- Assess how the electrophysiological proprieties of BNST neurons are affected by stimulation of the CeA and ILCx and also how chronic stress alters these networks.

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Chapter 2

Experimental Work

Chapter 2.1

Methodological Considerations

Methodological Considerations

Animal Model

The stress protocol used to assess the effects of stress in the BNST was a model of Chronic Unpredictable Stress (CUS). The animals were submitted to one stressor per day (with duration of approximately 1h) in a random order each day. The stressors consisted of exposure to cold water, restraining, shaking, exposure to a hot stream of air and overcrowding. The use of this unpredictable model as opposite to other chronic stress paradigms (e.g. chronic restraining) prevents the habituation of animals to the stressor and leads to elevated levels of corticosteroids through the duration of the treatment. The use of physical and psychological stressors allows a better representation of the variability of stressors in real life situations. The unpredictable component of the stress induces a state of permanent alert to keep the organism ready for a "fight or flight" response with the HPA axis being continuously activated which explains the increase in basal levels of corticosteroids. The duration of stress is also an important aspect to consider, acute and chronic stress models can show differences in behavior and effects of stress at the brain level. The use of a chronic model allows for the development of persistent alterations induced by stress that cannot be seen when using an acute model. Several biomarkers of stress (decreased weight gain, increase in adrenals weight or poor quality of the fur) start developing only after an exposure to stress longer than 2 weeks. Similarly, a phenotype of state anxiety develops after a chronic stress exposure.

CUS exposure induces several alterations in limbic structures that are, in many cases, the result of the elevated levels of corticosteroids. Specifically, CUS induces structural alteration in the prefrontal cortex (PFC), in particular, volumetric reductions (Cerqueira et al., 2005) and remodeling of pyramidal cells (Cerqueira et al., 2007; Radley et al., 2005, 2009). CUS also leads to morphological alterations in the BNST, in specific, increased volume of the anteromedial division of the BNST and dendritic remodeling in this division (increase in dendritic length and in the number of spines) (Pêgo et al., 2008).

Apart from the morphological changes, CUS also induces an alteration in the animal behavior by creating an anxious phenotype measured in the Elevated Plus Maze (EPM) or the acoustic startle (Pêgo et al., 2008).

Ibotenic Acid Lesions

Ibotenic Acid is a toxin acting on NMDA receptors and that causes excitotoxic neuronal death. The use of neurotoxins provide some advantages over the use of conventional techniques for lesioning the brain (aspiration, electrolytic) since it allows for a more restricted lesion site and also without damaging fibers of passage. The lesions were performed in the central amygdala to evaluate the importance of projections originating in this nucleus to the development of anxiety behavior after CUS exposure.

Behavioral Tests

The BNST has been associated with the development of anxiety behavior as opposed to the amygdala that plays a role in fear behavior (Pêgo et al., 2008, Walker & Davis, 2008; Walker et al., 2009). Anxiety can be characterized by a sensation of discomfort and apprehension in response to unconditioned diffuse cues (Koch et al., 1999; LeDoux, 2000). Anxiety can be measured in several different paradigms with the gold-standard test for anxiety being the EPM. The EPM consists of a plus-shaped maze, elevated 60 to 90cm from the ground with two facing open arms and two closed arms. This test creates a conflict between the animals desire to explore new environment and their fear of open and bright areas. Animals with an anxious phenotype show a decreased ratio between the time spent in open arms and the time spent in closed arms. The number of entries and explorations in each arm can also be used as an indicator of the exploratory activity of the animals.

Another way to measure anxiety-behavior is by using startle based paradigms as the acoustic startle reflex (AS). The AS apparatus consists of a ventilated and sound attenuated chamber with a plexiglass cylinder inside (diameter 8.8cm; length 22.2cm) mounted in a plexiglass platform. The animals are presented with a series of acoustic stimuli of different intensities and the amplitude of the reflex startle movement is measured. Opposing to the EPM, startle based tests can also be applied in animals with decreased basal exploratory activity or locomotion deficits as it is dependent on a natural reflex and not on the will/capacity of the animal to explore a novel environment.

One important component of tests based on the exploratory activity such as the EPM is to assess the locomotor activity of the animal. Animals with locomotor deficits may perform worse due to their deficits and not due to their real phenotype, for that, it is important to assess the locomotor activity of the animals in a test as the open field. The open field consists of an illuminated open area that the animals are allowed to explore. Total distance is used as indicator of locomotor activity. The open field can also be used as an indicator of anxiety-behavior by the measure the ratio of the time spent and distance travelled in the central area by the total time/distance.

Molecular analysis

Since the BNST is a highly complex area constituted by several nuclei with different characteristics and functions we found relevant to perform a molecular analysis of specific divisions/nuclei.

To assess the expression of genes with RT-PCR we used laser capture microdissection (LCM) to be able to extract RNA samples from specific subnuclei of the BNST. Using LCM it is possible to perform dissection of small groups of cells with almost no contamination from surrounding tissue; this allows for a more specific analysis of small nuclei or groups of cells. This is particularly important in a region like the BNST that is constituted by several subnuclei playing different biological roles (Choi et al., 2007). Using LCM, we were able to dissect the dorsomedial, fusiform and principal nuclei of the BNST. The choice of these nuclei was based on studies of cfos activation and also due to the different role they play in the activation of the HPA axis (Choi et al., 2007) with dorsomedial/fusiform nuclei having an excitatory role over the axis and principal nuclei being mostly inhibitory. LCM of BNST nuclei provided several technical challenges that were overcome with the optimization of the protocol in our lab. The reduced dimensions of the microdissected tissue led to a reduced quantity of RNA being extracted, which made the maintenance of the quality of this biological material critical. Several methods were used to assure that the end product being used for gene expression was from the highest quality possible, namely by using a lab-on-a-chip system to measure the quality and quantity of RNA in each sample. We also performed an amplification of the RNA to provide us with higher quantity of material to work with.

We performed a molecular analysis of several genes of interest related with some of the neurotransmitter systems of relevance in the BNST. More specifically, we analyzed the following projections from limbic areas to the BNST: glutamatergic (with neuronal projections from prefrontal cortex and hippocampus) and CRFergic and GABAergic (with these neurotransmitters being involved in projections coming from the amygdala to the BNST). For that, we determined the expression levels of the two CRF receptors (CRFR1 and CRFR1) and also the level of CRF in the BNST and Central Amygdala. CRF is one important mediator in the stress response, being expressed, among others, in the hypothalamus, central amygdala and BNST. CRF receptors have also been reported to have anxiogenic (CRFR1) and anxiolytic role (CRFR2) (Radulovic et al., 1999; Risbrough et al., 2004; Smith, 1998; Bale, 2000; Pelleymounter et al., 2002; 2004). For the GABAergic system, we analyzed the gene expression of the subunit alpha 3 of GABAA receptor and also the gene expression of *Gad2* and *Gad1*. In the last years several authors have pointed out evidences for a role of subunit alpha3 in mediating the anxiolytic effects of some drugs (benzodiazepines: Dias et al, 2005; a3IA: Atack et al, 2005), also on the interaction between serotonin and GABA receptors in mediating an anxiolytic response (Vinkers et al., 2010). As for the GAD2 and GAD1, in control conditions there is a predominance of the expression of GAD1 but it was reported that in the BNST, stress exposure induces a shift in the expression pattern of this protein, with an increase in the ratio between GAD2/GAD1 (Herman et al., 2001). This ratio can be used as a marker of stress within the BNST. Concerning the glutamatergic system we evaluated the expression of NR2B receptor, a receptor that has been widely associated with the stress response (Kash et al., 2009).

Electrophysiology

We used single-cell recording to evaluate the electrical activity of neurons in the anterior BNST. Considering the relatively small size of the BNST and the fact that it presents several subnuclei with different properties, as well as a vast number of different types of neurons that are still not fully characterized, the use of single-cell recordings provide the advantage of being able to let us discriminate between individual neurons and the individual contribution of each neuron.

The neuronal activity recorded was either the spontaneous activity of BNST neurons or their activity in response to an electrical stimulation in either the infralimbic cortex or the central

amygdala. This technique is important to provide an insight to the way the ILCx-BNST and CeA-BNST networks are altered in a stress condition. Glass micropippetes filled with pontamine-sky blue in acetic acid were used to perform the single-cell recordings upon stimulation of each area. The micropippetes had a tip of 1-2μm allowing for a better recording of the BNST neurons. Tungsten stimulating electrodes were lowered into the ILCx and CeA and electrical stimulation consisted of 100 pulses at 0.5Hz with different intensities of stimulation (0.1-0.5mA for CeA and 0.2-1.0mA for ILCx). We also performed pharmacological modulation of the BNST neurons while performing stimulation in CeA. For that we used double-barrel micropipettes, these pipettes consist of an injection pipette together with a recording one (Akaoka et al., 1991). The 2 pipette are prepared separately and then brought together using precision micromanipulators. The pipettes are assembled so that the tips are parallel and the recording microelectrode tip extends 140μm beyond the injection. The use of the double-barrel pipettes allow us to perform pharmacological manipulation in the close proximity of the neuron being recorded, this manipulation allow us to better discriminate the possible neurotransmitters involved in the cellular activity being recorded. In particular in this study we focused on GABA and CRF systems by injecting a GABAA antagonist (picrotoxin) and a CRFR1 antagonist (antalarmin) in the BNST.

The electrophysiology technique used herein, while providing exciting results, presents several technical limitations and considerations. One of the most important aspects when working with live animals is to consider the anesthesia being used and the dose. All the electrophysiological experiments were performed using volatile air anesthesia (isoflurane) at a dose between 1-1,5%.

Another technical aspect to consider is the wide variability of neurons in the BNST. The spontaneous activity recordings allowed us to identify neurons of at least 3 different rages of activity: neurons with a low basal activity (less than 1spike/min), neurons with high neuronal activity (more than 10 spikes/min) and neurons with intermediate levels of activity. In the present studies, we have analyzed neurons with an average activity of 7.6-10Hz. It is important to note that the majority of the neurons analyzed responded in a similar way to each of the stimulations. Of relevance, the majority of the data was recorded for the same neuron after ILCx and CeA stimulation since BNST neurons often presented an alteration in activity after each stimuli. This would allow us a more insightful analysis of the neurons being recorded. As mentioned before, another issue concerning the BNST is the wide number of subnuclei in the BNST that present different characteristics. In the electrophysiological studies we have focused our analysis in the

anterior BNST, with more relevance to nuclei belonging to the anterolateral division. The location of each recording was assessed by performing an iontophoretic injection of pontamine sky-blue at the end of each session.

In conclusion, we believe that the combined use of these techniques, allowed us to have a more integrated perspective of the stress-induced effects on the BNST.

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Chapter 2.2

Ventura-Silva AP, Pêgo JM, Sousa JC, Marques AR, Rodrigues AJ, Marques F, Cerqueira JJ, Almeida OFX, Sousa N.

Stress shifts the response of the bed nucleus of the stria terminalis to an anxiogenic mode

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Stress shifts the response of the bed nucleus of the stria terminalis to an anxiogenic mode

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Keywords: anxiety, bed nucleus of the stria terminalis, microdissection, rat, stereology

Abstract

The bed nucleus of the stria terminalis (BNST) is critically implicated in anxiety behavior and control of the hypothalamus–pituitary–adrenal axis. Having previously shown that chronic stress triggers dendritic/synaptic remodeling in specific nuclei of the BNST, we characterised the pattern of activation of neurons within different regions of the BNST under basal conditions and after an anxiogenic stimulus in control and stressed rats. Under basal conditions, stressed, but not control, animals displayed increased cFOS expression in the dorsomedial nucleus and decreased activation of the principal nucleus. This pattern resembled that observed in controls that had been exposed to the anxiogenic stimulus. Subsequent analysis of various BNST subnuclei revealed differential patterns of gene expression in controls and stressed animals. We found decreased levels of corticotropin-releasing hormone 1 receptor mRNA expression in the dorsomedial and fusiform nuclei, and a global increase in the levels of corticotropin-releasing hormone 2 receptor in the principal nucleus. In addition, we found subnuclei-specific increases in $GABA_A$ and NR2B receptors in stressed animals, which suggest changes in the GABAergic and glutamergic innervation of the BNST. Importantly, these findings were associated with increased anxiety-like behavior and impaired control of the hypothalamus–pituitary–adrenal axis in stressed animals. In summary, these data reveal that chronic stress shifts the pattern of response of the BNST to an anxiogenic mode and provide new information on the underlying mechanisms of the stress-induced hypercorticalism and hyperanxious status.

Introduction

Stress is a causal factor in several psychiatric disorders, including anxiety (Arborelius *et al.*, 1999). In rodents, stress exposure, as well as exogenous corticosteroids, induces anxiety (File, 1996; Vyas *et al.*, 2002; Anisman & Matheson, 2005; Pêgo *et al.*, 2008; Conrad & Winder, 2010; Conrad *et al.*, 2011) and elicits neurostructural changes in the bed nucleus of the stria terminalis (BNST) (Pêgo *et al.*, 2008), amygdala (Vyas *et al.*, 2002, 2003), prefrontal cortex (PFC) (Cerqueira *et al.*, 2005b, 2007; Dias-Ferreira *et al.*, 2009) and hippocampus (Bessa *et al.*, 2009). Importantly, these areas regulate emotional behavior and control the stress response.

The BNST is a complex and heterogeneous structure (Ju & Swanson, 1989; Ju *et al.*, 1989; Choi *et al.*, 2007) implicated in anxiety behavior in both rodents (Davis *et al.*, 1997, 2010) and humans (Somerville *et al.*, 2010). Initial descriptions, based on gross structural landmarks, described the BNST as being comprised of three main divisions (anteromedial, anterolateral and posterior) (Ju & Swanson, 1989; Dong *et al.*, 2001). Recently, using stringent structure–function criteria, a parcellation of the BNST into various functional compartments was proposed (Choi *et al.*, 2007). Considering this structure–function delineation, we thought it of interest to establish the pattern of BNST subnuclei activation in response to an anxiogenic stimulus and in an animal model of anxiety. For this, we assessed neural activation by tracking the expression of the immediate early gene cFOS in controls and stressed animals exposed to a startle stimulus.

The activity of the BNST is determined by distinct inputs. It receives an important projection from the amygdala, most likely originated in neurons co-expressing GABA and corticotropin-releasing hormone (CRH) (Veinante *et al.*, 1997; Day *et al.*, 1999). CRH1 receptors (CRHR1) in the BNST are strongly implicated in stress-related behaviors (Smith *et al.*, 1998; Bale & Vale, 2004) and the modulation of anxiety (Davis *et al.*, 1997). In contrast, CRH2 receptors (CRHR2) are critical for the termination of the endocrine response to stress (Bale *et al.*, 2000; Reul & Holsboer, 2002). Importantly, mice deficient in CRHR2 show increased anxious-like behavior (Kishimoto *et al.*, 2000), which is consistent with the known inverse regulation of CRHR1 and CRHR2 by CRH (Korosi *et al.*,

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2006). Glutamatergic (especially the NR2B subunit of the *N*-methyl-D-aspartate receptor) and GABAergic transmission have been implicated in the regulation of anxiety (Sajdyk *et al.*, 2008; Riaza Bermudo-Soriano *et al.*, 2012). $GABA_A$ receptors are widely distributed in BNST neurons receiving inhibitory projections from subcortical regions such as the amygdala (Li *et al.*, 2012), whereas the glutamatergic inputs to the BNST arise mainly from the PFC and hippocampus. In addition to this heterogeneous neurochemical innervation of the BNST, a complex topographical innervation pattern determines a distinct role for each subnuclei within the BNST. This must be considered in order to understand the role of this brain region in the control of the hypothalamus–pituitary–adrenal (HPA) axis and anxiety. In an attempt to identify a molecular signature of stress-induced anxiety we undertook an analysis of the receptors for these neurotransmitters in distinct subdivisions/subnuclei of the BNST.

Materials and methods

Animals and treatments

All experiments were conducted in accordance with local regulations (European Union Directive 2010/663/EU on the protection of animals used for scientific purposes) and NIH guidelines on animal care and experimentation. All experiments were approved by the Animal Ethics Committee of the Portuguese National Veterinary Directorate.

Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) were housed in groups of two under standard laboratory conditions with an artificial light/dark cycle of 12/12 h (lights on at 08:00 h) and room temperature of 22 °C, and were provided with food and water *ad libitum*. Treatment protocols were initiated when the animals were 8 weeks old and continued over a period of 4 weeks.

To assess the influence of chronic stress on BNST activation and neurochemistry, rats were exposed to a chronic unpredictable stress (CUS) protocol (Cerqueira *et al.*, 2007). Animals were exposed to one of the following stressors: cold water (18 °C), restraint, overcrowding, exposure to a hot air stream and vibration. Stressful stimuli were scheduled in a random order, with a different stressor being applied daily (1 h/day) between 09:00 and 16:00 h for the duration of treatment (4 weeks). Controls (Cont) were handled daily during the same period. The paradigm included psychological and physical elements in order to reduce the chances of adaptation and to better mimic the variability of stressors encountered in daily life (Sousa *et al.*, 1998; Joëls *et al.*, 2004). This protocol was previously shown to result in a state of chronic hypercorticalism, characterised by increased adrenal weight and serum corticosterone levels, reduced thymus weight, and reduced body weight gain (Sousa *et al.*, 2000; Cerqueira *et al.*, 2005a). In the present study, the efficacy of the protocol was also verified (Table 1) by weekly body weight recordings and post-mortem thymus and adrenal weights. Serum corticosterone levels were measured (blood collections between 09:00 and 10:00 h) by radioimmunoassay (MP Biomedicals, Costa Mesa, CA, USA).

In Experiment 1 we determined the activation pattern of the BNST and paraventricular nucleus of the hypothalamus (PVN) in basal conditions and after exposure to an anxiogenic stimulus. At 24 h after the last exposure to stress or handling, some of the animals ($n = 4$/group) were killed, whereas the remainder ($n = 4$) were submitted to one session of acoustic startle (anxiogenic stimuli) and killed 30 min later. Animals were previously habituated to the startle apparatus for 5 days to decrease the effect of novelty. For killing, animals were deeply anesthetised with pentobarbital (0.5 mg/kg) 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 cFOS immunohistochemistry and morphological analysis.

A second set of animals was used to identify morphological correlates of the differences in functional activation (Experiment 1) of the BNST subnuclei and neurochemical changes (Experiment 2). For this, we coupled high-precision laser microdissection with quantitative real-time PCR (qRT-PCR) to examine the gene expression profiles (CRHR1, CRHR2, $GABA_A$ and NR2B receptors) in specific subnuclei of the BNST. Rats were randomly assigned to Cont or CUS treatment groups ($n = 7$/group). At the end of the treatment, animals were transcardially perfused with RNAase-free saline under pentobarbital anesthesia. Brains were collected, covered in Optimal Cutting-Temperature compound (Leica) and deep frozen in liquid nitrogen for laser microdissection and qRT-PCR analysis.

Anxiety behavior

At the end of 4 weeks of treatment, animals were tested for anxiety behavior in the elevated plus maze and in the acoustic startle as reported previously (Pêgo *et al.*, 2008).

Elevated plus maze

At the end of 4 weeks of treatment (Cont or CUS), animals were tested over 5 min in a black polypropylene 'plus'-shaped maze (ENV-560, MedAssociates Inc., St Albans, VT, USA) as previously reported (Pêgo *et al.*, 2008). The times 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 elevated plus maze are presented as a percentage of the total duration of the trial.

TABLE 1. Biometric markers revealed that the CUS protocol used here decreased body-weight gain and thymus weight

	Cont	CUS	Cont+AxStim	CUS+AxStim		Significance
Body weight gain (g)	96.1 ± 3.1	79.3 ± 2.4			$t = 4.26$	$P < 0.001$
Thymus weight (g/100 g)	0.54 ± 0.02	0.38 ± 0.02			$t = 5.03$	$P < 0.001$
Adrenal weight (g/100 g)	0.88 ± 0.01	0.94 ± 0.01			$t = -0.86$	$P < 0.41$
Plasma corticosterone levels (ng/mL)	55.9 ± 3.9	91.7 ± 9.6	62.3 ± 6.0	117 ± 11.1	$F = 10.384$	$P < 0.001$

Although CUS caused only a slight increase in adrenal weight, the reduced thymic weights attest to the higher levels of corticosterone in the CUS-treated group. Exposure to chronic stress resulted in persistently raised plasma corticosterone levels, which were still significantly higher than those found in controls at 12 h after the last stress exposure. Data presented as mean ± SEM.

Acoustic startle as an anxiogenic stimulus

An acute state of anxiety was induced by the acoustic startle reflex paradigm, using a startle response apparatus (SR-LAB, San Diego Instruments, San Diego, CA, USA) as previously reported (Pêgo *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 30 min before killing. Trials on individual animals were conducted sequentially. Between tests, the chambers and acrylic holders were thoroughly cleaned (10% ethanol) to eliminate residual olfactory cues.

Regional boundaries

In the BNST, three main divisions [anteromedial ($BNST_{am}$), anterolateral ($BNST_{al}$), posterior ($BNST_p$)] can be grossly recognised on the basis of structural landmarks. However, when using such parcellation there is some degree of functional overlap. For example, an important GABAergic projection from the $BNST_{am}$ exerts an inhibitory influence over the PVN but an excitatory projection originating in the same division of the BNST serves to activate the HPA axis (Choi *et al.*, 2007). Interestingly, both projections are under the control of an excitatory glutamatergic input from the PFC and hippocampus (Cullinan *et al.*, 1993) and of an inhibitory GABAergic input from the central and medial nuclei of the amygdala (Prewitt & Herman, 1998, Herman *et al.*, 2004). These apparent discordances can only be resolved by using parcellations that subdivide the major divisions into subnuclei so as to combine structural with stochastic and functional data.

In order to compare the changes in morphology with previous reports (Pêgo *et al.*, 2008), volumes of the three main BNST divisions were outlined in cFOS-immunostained sections using stereological tools. The $BNST_{am}$, $BNST_{al}$ and $BNST_p$ regions were outlined according to anatomical references and recognised on the basis of clear cytoarchitectural differences: density of cells, size of the perikarya and relative position (Swanson, 1998; Pêgo *et al.*, 2008) (Fig. 1).

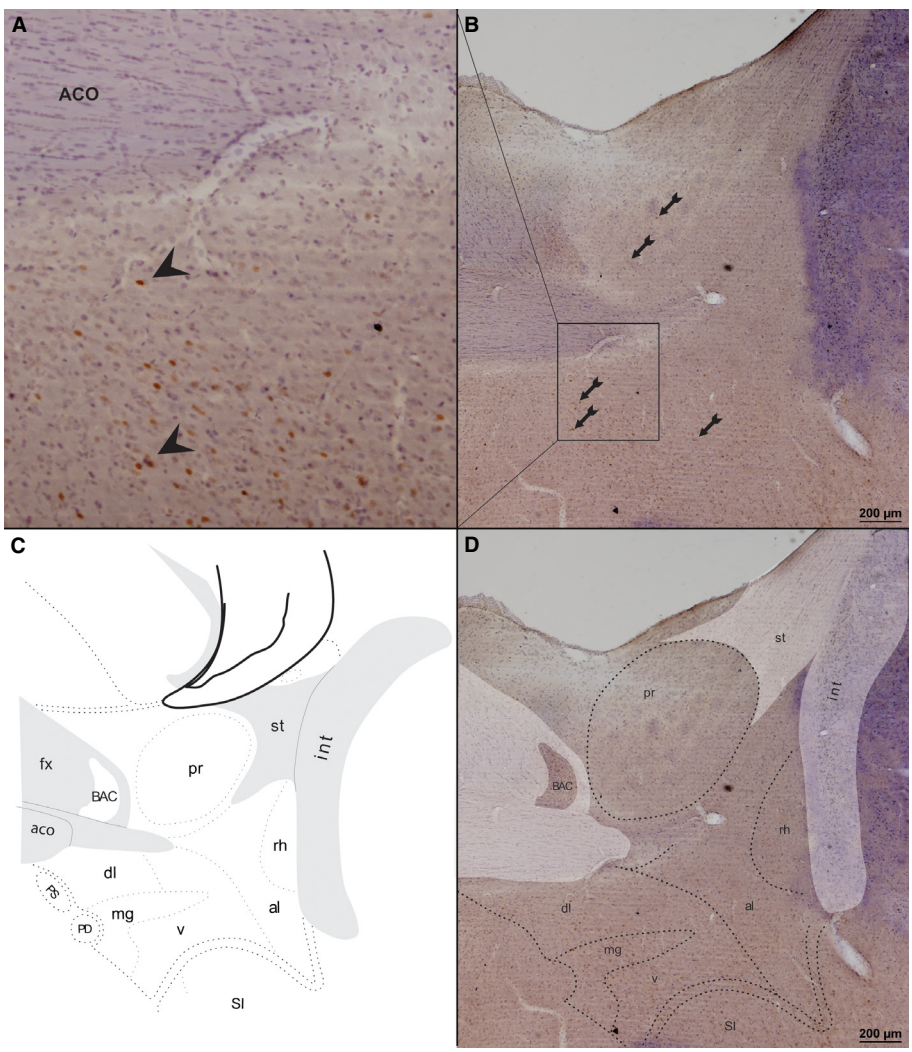

FIG. 1. Representative micrographs of immunostained sections for cFOS of the BNST and delineation of its subnuclei. (A) Detail of cFOS-immunoreactive (Fos-IR) neurons (arrowheads). (B) Overview of the BNST showing distribution of Fos-IR neurons (arrows). (C) Atlas drawing corresponding to the section (Swanson, 1998) (Bregma −0.51 mm) shown in B. (D) Overlay of atlas drawing on the section. aco, anterior commissure; al, anterolateral nucleus; BAC, bed nucleus of anterior commissure; dl, dorsolateral nucleus; fx, fornix; int, internal capsule; mg, magnocellular nucleus; PO, preoptic nucleus; pr, principal nucleus; PS, parastrial nucleus; rh, rhomboid nucleus; SI, substantia innominata; st, stria terminalis; v, ventral nucleus.

In order to compare the changes in the activation of the HPA axis we also assessed how stress and anxiogenic stimuli affected the activation of parvocellular neurons in the PVN in cFOS-immunostained sections. This region was outlined according to anatomical references (Swanson, 1998).

Immunohistochemistry and quantification procedures

Coronal sections (50 μm thick), were serially collected in PBS. Alternate sections were immunostained for cFOS by overnight incubation with rabbit anti-cFOS polyclonal antibody (1 : 10 000; Calbiochem, Darmstadt, Germany) after blocking (2 h) in PBS containing 0.3% Triton X-100, 0.1 M glycine and 10% normal fetal bovine serum. Following washes in PBS containing 0.3% Triton X-100, 0.1 M glycine and 10% normal fetal bovine serum, sections were incubated in biotinylated goat anti-rabbit antibody (Dako, Glostrup, Denmark) followed by an Avidin/Biotin Complex (ABC solution; Vectorstain Elite, Burlingame, CA, USA) and finally visualised with diaminobenzidine. Sections were counterstained with hematoxylin to help to delimit regional boundaries before mounting and coverslipping.

The cFOS-immunoreactive neurons were marked by a dark brown diaminobenzidine precipitate (Fig. 1). The number of cFOS-immunoreactive neurons in each of the main divisions of the BNST and PVN was counted and the number per area was calculated to establish comparisons. Additionally, cFOS-immunoreactive neurons were mapped out onto drawings (Swanson, 1998) to depict their relative distribution in the subnuclei making up each of the main divisions. Comparisons were made between the effects of anxiogenic stimuli in Cont vs. CUS-treated animals.

Laser microdissection and quantitative real-time polymerase chain reaction analysis

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 laser microdissection (Microdissector CellCut, Olympus) of three subnuclei of the BNST [dorsomedial ($BNST_{dm}$), fusiform ($BNST_{fu}$) and principal ($BNST_{pr}$) nucleus], based on data obtained from Experiment 1 (Fig. 2).

RNA from the microdissected samples was extracted using the RNeasy Plus Micro Kit (Qiagen) and frozen at −80 °C until use. One RNA aliquot was used to assess the quantity and quality of the RNA (Experion RNA HighSense Analysis kit, Bio-Rad) (Table 2). RNA was amplified with the SuperScript RNA Amplification System (Invitrogen) following the manufacturer's instructions, and 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, TTTTCCTAGTGCTGCGGAGT; Rw, AGCCTTCCACAAACATCCAG), CRH (*Crh*; Fw, GCTAACTT TTTCCGCGTGTT; Rw, GGTGGAAGGTGAGATCCAGA), GA-BA_A receptor, subunit alpha 3 (*Gabra3*; Fw, TGGTCATGTTG TTGGGACAG; Rw, TGGCAAGTAGGTCTGGATGA), glutamic acid decarboxylase 1 (*Gad1*; Fw, TCGGTTTTTCAACCAGCTCT; Rw, AACAAACACGGGTGCAATTT), glutamic acid decarboxylase 2 (*Gad2*; Fw, GCCAACTCTGTGACATGGAA; Rw, GGTAGGAA GCATGCATCTGG), 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 CTTTGCTTTCCTTGG; 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).

Data analysis

All results are expressed as group means ± SE. One-way ANOVA or Student's *t*-test were used, as appropriate, to compare means. *Post-hoc* analysis was performed using the Tukey test. Spearman's correlation was performed when appropriate. Statistical significance was accepted for a probability level below 0.05.

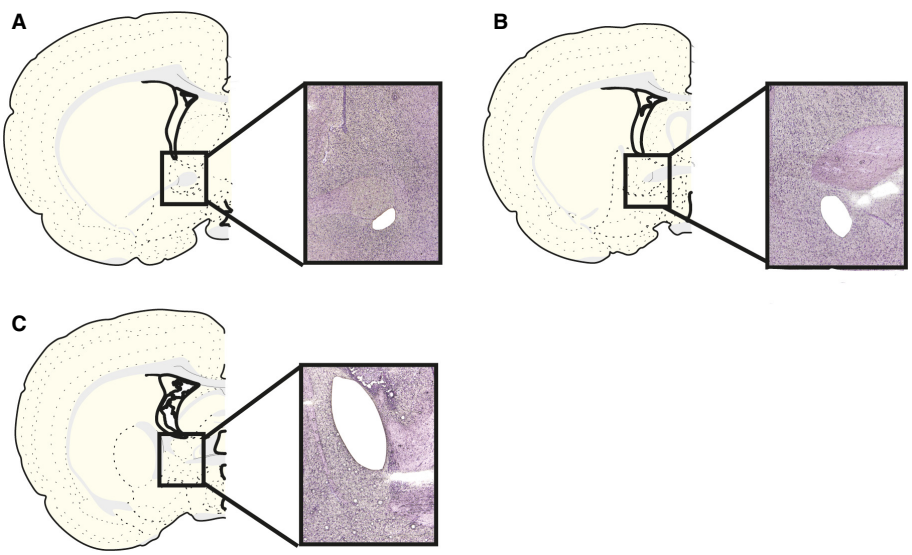

FIG. 2. Schematic representation of sections according to the atlas of Swanson (1998) and representative pictures of the microdissected nuclei of the BNST. (A) $BNST_{dm}$; (B) $BNST_{fu}$; (C) $BNST_{pr}$.

TABLE 2. RNA quality indicator (RQI) for each RNA sample extracted after laser capture microdissection

Dorsomedial nucleus				Fusiform nucleus				Principal nucleus			
Control		CUS		Control		CUS		Control		CUS	
Sample	RQI	Sample	RQI	Sample	RQI	Sample	RQI	Sample	RQI	Sample	RQI
1	6.3	1	5.7	1	8.1	1	8.7	1	8.6	1	6.6
2	6.5	2	6	2	7	2	6	2	8	2	7.7
3	7.3	3	7.2	3	6.8	3	7	3	6.3	3	6.4
4	6.7	4	6.3	4	7.1	4	7.1	4	7.2	4	7.1
5	6.3	5	6.6	5	6.4	5	7.1	5	7.6	5	7.7
6	5.8	6	6.6	6	6.5	6	6.6	6	6.5	6	8.3
7	5.9	7	6.2	7	6.7	7	7.8	7	7.1	7	7.1

Samples presented similar quality values within each nucleus of the BNST dissected with no significant difference between Cont and CUS groups.

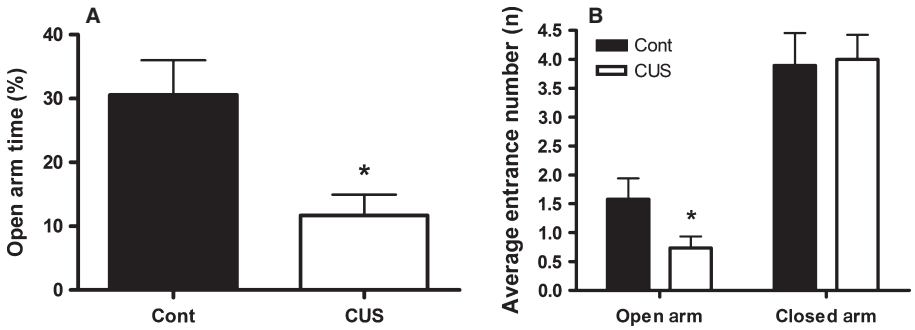

FIG. 3. Results from the elevated plus maze test. (A) Time spent in the open arms given as percentage of total time. (B) Number of entries in the open and closed arms. Results are presented as mean SEM. *$P < 0.05$.

Results

Stress triggers anxiety-like behavior and impaired shut-off of the hypothalamus–pituitary–adrenal axis that are associated with structural changes in the bed nucleus of the stria terminalis

Chronic stress induced an anxiety-like phenotype insofar that, as compared with Cont animals, CUS rats spent significantly less time in ($t = 2.99$, $P < 0.005$) and made fewer entries into ($t = 2.04$, $P < 0.05$) the open arms of the elevated plus maze. Although the ratio of open/closed-arm times was significantly smaller in CUS animals ($t = 2.50$, $P < 0.02$), the number of closed-arm entries did not differ significantly between the groups ($t = -0.15$, $P = 0.88$), indicating that exploratory/locomotory activity was preserved (Fig. 3).

Importantly, for the assessment of the HPA axis activity we determined the plasmatic levels of corticosterone in controls and stressed animals in basal conditions and after 12 h of exposure to an anxiogenic stressor. The data show that exposure to chronic stress resulted in persistently raised plasma corticosterone levels, which were significantly higher than those found in controls at 12 h after the last stress exposure (Table 1). This hypercortisolemic status was associated with a significant increase in cFOS-positive neurons in the parvocellular component of the PVN of stressed animals ($F = 3.50$, $P = 0.04$; Fig. 4).

Stereological estimates of BNST areas showed that the total area of the anteromedial division was increased in CUS animals by 28% when compared with Cont animals (Cont, 0.55 ± 0.02 mm^3; CUS, 0.70 ± 0.03 mm^3; $t = -3.74$, $P < 0.01$). Neither the $BNST_{al}$ (Cont, 0.34 ± 0.02 mm^3; CUS, 0.41 ± 0.02 mm^3; $t = -2.02$, $P = 0.13$) nor $BNST_p$ (Cont, 0.41 ± 0.03 mm^3; CUS, 0.49 ± 0.01 mm^3; $t = -2.06$, $P = 0.09$) divisions of Cont and CUS animals differed significantly in terms of volume. The observed changes are consistent with those of previous studies (Pêgo *et al.*, 2008), namely, that corticosteroid levels correlate ($R^2 = 0.435$, $P = 0.001$) with enlargement of the anteromedial ($BNST_{am}$) division of the BNST.

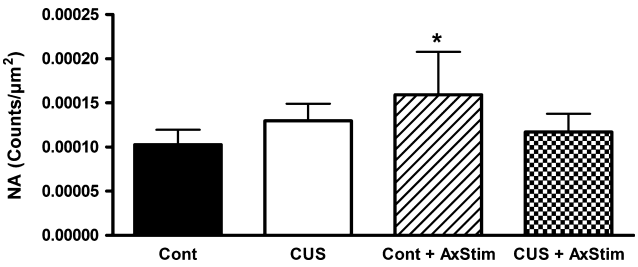

FIG. 4. cFOS expression in the PVN. Stress induced a significant effect on cFOS expression in the PVN. Cont+AxStim, control rats submitted to anxiogenic stimuli; CUS+AxStim, CUS rats submitted to anxiogenic stimuli. NA, number of cells per area. Results are presented as mean SEM. *$P < 0.006$ Cont vs. Cont+AxStim.

The activational pattern of the bed nucleus of the stria terminalis after stress reflects an anxiogenic status

To establish the importance of each BNST subnucleus for the behavioral response to stress, comparisons of the cFOS activation pattern of the BNST under basal conditions (Cont) and following exposure of Cont animals to an acoustic anxiogenic stimulus were made (Cont + AxStim). This revealed an effect of treatment in the activation of the dorsomedial BNST as measured by one-way ANOVA ($F = 4.129$, $P = 0.045$) in response to stressful stimuli (chronic stress or anxiogenic stimuli) (Fig. 5, Table 3). *Post-hoc* analysis revealed a trend for altered

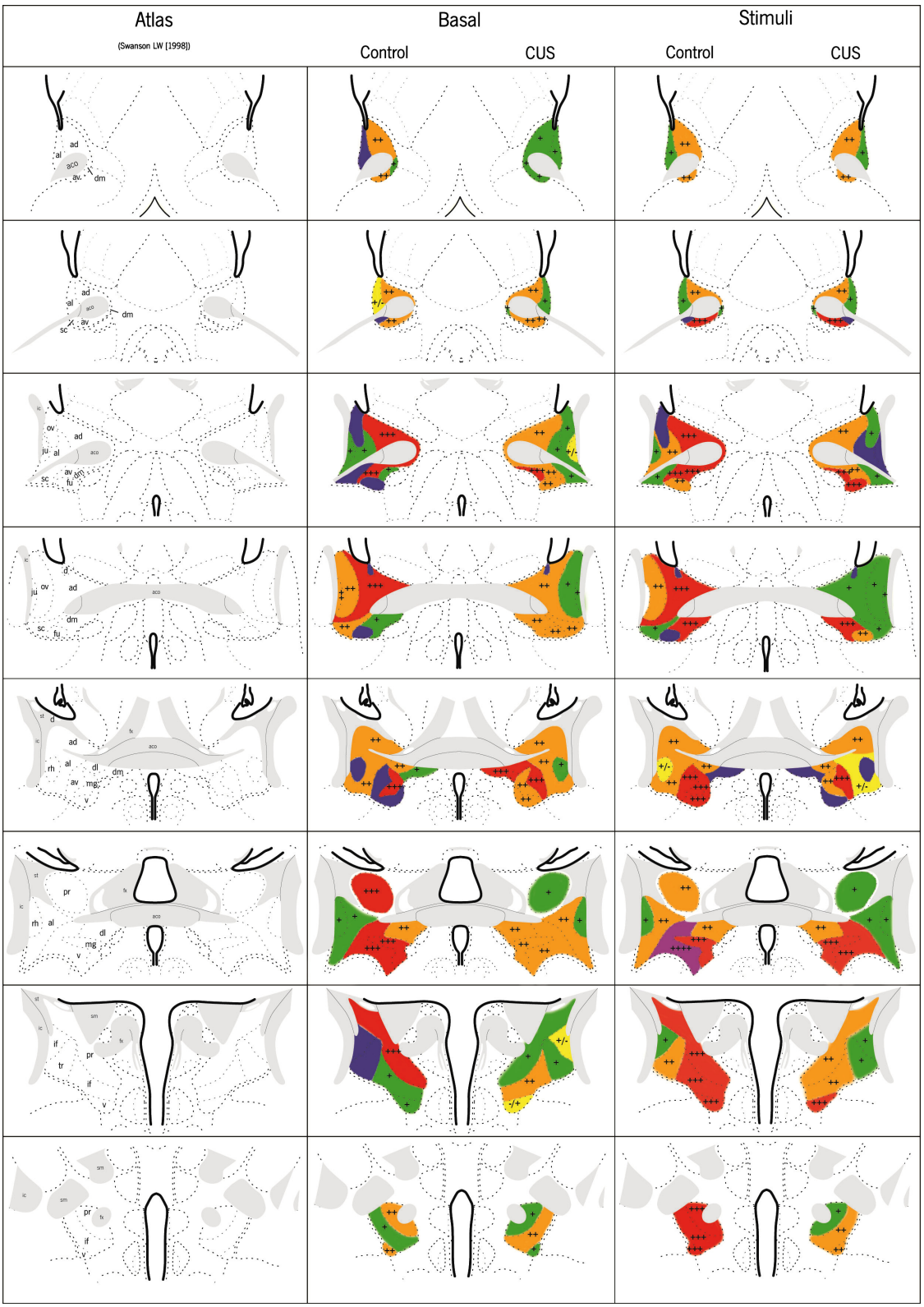

FIG. 5. Schematic representation of the relative density of cFOS-immunoreactive (Fos-IR) neurons in the BNST of the rat in control and CUS animals, in basal conditions and after exposure to anxiogenic stimuli. Left panels: schematic representation of the sections according to the atlas of Swanson (1998). From top to bottom, drawings represent coronal sections of the rat brain relative to bregma: +0.10, +0.00, −0.11, −0.26, −0.46, −0.51, −0.60 and −1.08 mm. Middle panels: basal conditions. Right panels: after anxiogenic stimuli. Crosses indicate relative densities of Fos-IR neurons in qualitative terms: -, absent/rare; +, light; ++, moderate; +++, dense; ++++, very dense. Colors are coded for relative densities; in the right panel only those showing differences from baseline are signaled. aco, anterior commissure; ad, anterodorsal; al, anterolateral; av, anteroventral; d, dorsal; dl, dorsolateral; dm, dorsomedial; fu, fusiform; fx, fornix; ic, internal capsula; if, interfascicular; ju, juxtacapsular; mg, magnocellular; ov, oval; pr, principal; rh, rhomboid; sc, subcommissural zone; sm, stria medularis; st, stria terminalis; tr, transverse; v, ventral.

TABLE 3. Distribution of cFOS-positive cells in the main divisions/nuclei of the BNST of rats under basal conditions and after anxiogenic stimuli

	Condition					
	Basal		Anxiogenic stimuli			
Division/nucleus	Cont	CUS	Cont	CUS	*F*-value	Significance *P*-value
Anteromedial						
ad	38.0 ± 13.2	27.7 ± 20.2	41.7 ± 27.9	11.7 ± 4.0	0.854	0.499
av	11.0 ± 3.5	11.3 ± 5.4	19.0 ± 9.0	10.3 ± 2.5	0.589	0.637
dm	10.0 ± 1.0	23.0 ± 4.7	29.0 ± 0.6	15.5 ± 5.2	4.129	0.043
mg	5.5 ± 1.4	6.0 ± 4.5	9.0 ± 4.2	4.6 ± 0.8	0.410	0.750
dl	6.5 ± 0.9	5.3 ± 3.3	3.7 ± 0.9	1.0 ± 0.6	2.264	0.150
v	13.0 ± 0.0	11.0 ± 3.2	24.7 ± 8.8	18.3 ± 5.5	1.136	0.386
Anterolateral						
al	5.5 ± 2.6	14.7 ± 3.8	16.3 ± 8.4	4.8 ± 2.1	1.769	0.223
ju	3.0 ± 0.6	2.0 ± 0.0	3.0 ± 1.5	0.8 ± 0.8	1.605	0.256
ov	5.5 ± 2.0	8.3 ± 0.9	5.3 ± 3.4	1.0 ± 1.0	2.662	0.111
fu	0	5.0 ± 3.2	4.0 ± 3.1	9.0 ± 4.1	1.312	0.330
rh	1.0 ± 0.0	1.3 ± 1.3	2.7 ± 1.5	0.5 ± 0.5	0.961	0.452
Posterior						
tr	0	1.3 ± 0.9	5.7 ± 2.7	1.0 ± 0.4	3.502	0.063
if	1.0 ± 0.6	8.0 ± 1.5	20.7 ± 6.9	10.8 ± 2.5	4.781	0.029
pr	9.3 ± 4.9	7.7 ± 1.9	21.0 ± 4.5	7.3 ± 0.9	4.032	0.045

ad, anterodorsal; al, anterolateral; av, anteroventral; dl, dorsolateral; dm, dorsomedial; fu, fusiform; if, interfascicular; ju, juxtacapsular; mg, magnocellular; ov, oval; pr, principal; rh, rhomboid; tr, transverse; v, ventral.

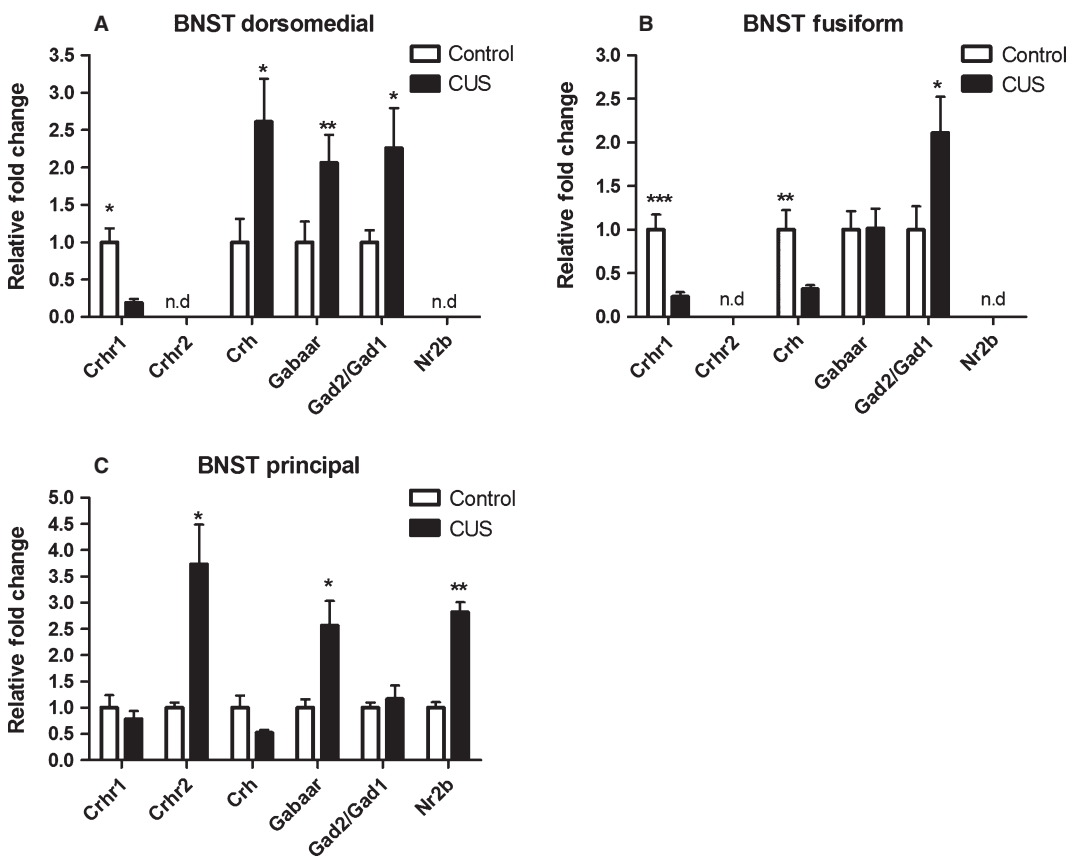

FIG. 6. Gene expression analysis of the BNST in response to stress. Expression levels for (A) dorsomedial nucleus of the BNST; (B) fusiform nucleus of the BNST; (C) principal nucleus of the BNST. CUS leads to decreased *Crh1* expression in the dorsomedial and fusiform nucleus and also to an increase in the expression of *Crfh2* in the principal nucleus. After stress exposure there is a significant shift of the ratio between *Gad2* and *Gad1* (which has been described to be a marker of stress in the BNST) in the dorsomedial and fusiform subnuclei accompanied by an increased expression of $GABA_A$ receptor in the dorsomedial and principal nuclei. Gad, Glutamic acid decarboxylase; n.d, non detectable. Data presented as mean + SEM. $*P < 0.05$; $**P < 0.01$: $***P \leq 0.001$.

activation of the $BNST_{dm}$ ($P = 0.061$) in control animals after stimuli (Cont + AxStim). Additionally, following the stressful stimulus, specific subnuclei (principal and interfascicularis) of the $BNST_p$ displayed a significant treatment effect in the number of cFOS-positive cells (principal: $F = 4.032$, $P = 0.045$; interfascicularis: $F = 4.781$, $P = 0.029$) with *post-hoc* comparison showing a significant increase in Cont + AxStim animals for the interfascicularis nucleus ($P = 0.028$).

Chronic stress recapitulated the effects of an acute anxiogenic stimulus in Cont animals in terms of the pattern of activation in the various BNST subnuclei. Under basal conditions, CUS animals already showed greater activation (increased cFOS) of the $BNST_{am}$ division as a whole, in particular of nuclei located ventral to the anterior commissure ($BNST_{dm}$: $P = 0.043$) when compared with Cont; this closely resembled the changes observed in Cont animals subjected to the acute acoustic stimulus (Fig. 5, Table 3). Whereas exposure to an acute anxiogenic stimulus resulted in a marked increase in cFOS staining in the $BNST_{dm}$ of control animals, this nucleus showed only moderate activation in stressed stimulated (CUS+AxStim) rats (Fig. 5, Table 3) and no significant activation of subnuclei of the posterior division (Fig. 5, Table 3). Together, this set of results suggests that CUS induces a tonic activation of specific ($BNST_{dm}$) nuclei in the BNST; in fact, these same subnuclei showed increased activation when Cont rats were subjected to an acute anxiogenic stimulus. In addition, they showed a reduced activation of the $BNST_{pr}$, which may account for the impairments in terminating the stress response that is normally observed in chronically stressed subjects (Steimer *et al.*, 2007; Mizoguchi *et al.*, 2008). In accordance with this, we found a significant increase in cFOS-positive neurons in the PVN of controls after exposure to anxiogenic stimuli (Cont+AxStim) ($P < 0.006$); curiously, such an increase was not detected in stressed animals.

Molecular correlates of stress-induced anxiety

We next analysed the expression patterns of key receptors in BNST subnuclei (Fig. 6). To establish a relationship between mRNA expression and the activation pattern, we conducted an analysis of individual subnuclei mRNA expression. To this end, qRT-PCR analyses were undertaken on laser microdissection samples from the dorsomedial, fusiform and principal nuclei of CUS and Cont animals. These nuclei were chosen in light of the function-related changes in cFOS as well as the specific role of each of these subnuclei (Choi *et al.*, 2007).

We observed significantly decreased levels of *Crhr1* in the dorsomedial and fusiform nuclei ($BNST_{dm}$: $t = 4.57$, $P = 0.013$; $BNST_{fu}$: $t = 4.67$, $P < 0.001$), but not in the principal nucleus ($t = 0.77$, $P = 0.456$) of the BNST. In contrast, *Crhr2* mRNA was significantly increased in the principal nucleus ($t = 1.96$, $P = 0.086$) although it was under detection levels in the dorsomedial and fusiform nuclei. Suggestive of disturbed glutamatergic innervation of the BNST, we observed that CUS increases *Nr2b receptor* mRNA in the principal nucleus ($BNST_{pr}$: $t = 2.78$, $P = 0.007$). Considering that glutamatergic projections to the posterior division (in particular the principal nucleus) arise mainly from the PFC, this disturbance suggests changes in the PFC–BNST pathway.

Confirming previous reports in the BNST (Herman, 2001), we showed that the ratio of *Gad2* : *Gad1* was increased in CUS-treated animals in both the dorsomedial and fusiform nuclei ($BNST_{dm}$: $t = 1.68$, $P = 0.045$; $BNST_{fu}$: $t = 2.12$, $P = 0.048$) but not in the principal nuclei ($t = 0.59$, $P = 0.565$). The expression of mRNA encoding the $GABA_A$ receptor was unaltered in the fusiform nucleus but was significantly up-regulated in the dorsomedial and principal nuclei ($BNST_{dm}$: $t = 4.23$, $P = 0.002$; $BNST_{pr}$: $t = 3.12$, $P = 0.011$) of CUS-treated animals.

Overall, these data revealed a clear molecular fingerprint induced by chronic stress in the BNST, suggesting enhanced activity of excitatory pathways (CRH) to pro-stress subnuclei, whereas antistress pathways are impaired.

Discussion

The BNST has been implicated in anxiety (Davis *et al.*, 2010), although the exact role of its specific subnuclei in this process remains unknown. The first aim of this study was to characterise the specific pattern of BNST activation in response to anxiogenic stimuli. The analysis of the expression of cFOS in response to anxiogenic stimuli within the BNST revealed a heterogeneous activation pattern: whereas several nuclei showed increased cFOS expression in response to the anxiogenic stimuli, others revealed decreased activation. This up- and down-activation of distinct cell groups inside the BNST attests to the complex role of this brain area. The observation that, in non-stressed animals, there is a preferential activation of the $BNST_{am}$ (particularly the $BNST_{dm}$) subnuclei in response to an anxiogenic stimulus suggests that these nuclei are involved in the immediate response to anxiogenic situations as proposed by Dong & Swanson (2004, 2006a,b,c). Moreover, due to their putative excitatory role over the PVN, they are also likely implicated in the simultaneous activation of the HPA axis (Herman *et al.*, 1994). In addition, Choi *et al.* (2008) showed that an excitotoxic lesion in these two subnuclei will lead to decreased cFOS in the PVN after an acute stress, although having a different regulation after chronic stress exposure. In fact, it is well established that: (i) $BNST_{am}$ neurons lying closest to the anterior commissure appear to densely innervate the hypothalamic periventricular region (Dong & Swanson, 2006a); (ii) CRH-immunoreactive cells are found in neuronal groups composing the anteromedial area (Ju *et al.*, 1989)

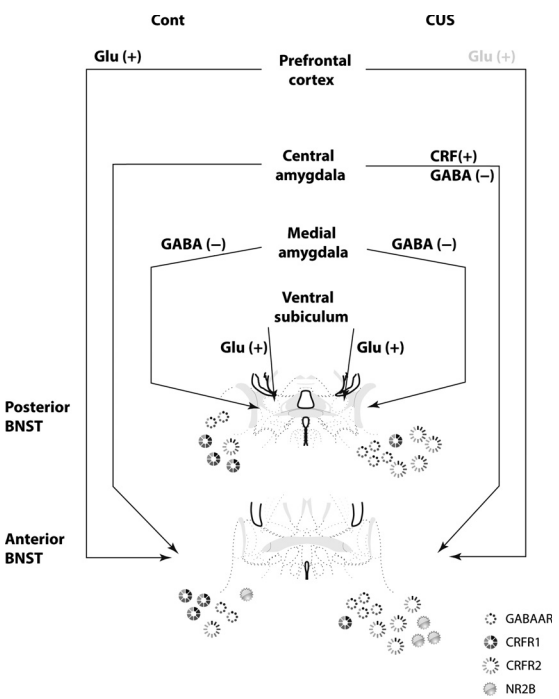

FIG. 7. Schematic representation of the changes occurring after stress exposure (right side) when compared with baseline (left side), which define the molecular signature of stress in the BNST. CRF, corticotrophin-releasing factor; Glu, glutamate.

and regulate hypothalamic functions; and (iii) electrical stimulation of the anteromedial aspects of the BNST is associated with increased corticosteroid secretion (Dunn & File, 1987). In agreement with this, we found that exposure to an anxiogenic stimulus triggered increased cFOS expression in the parvocellular component of the PVN.

Remarkably, a similar pattern of overactivation of the $BNST_{dm}$ and $BNST_{fu}$ nuclei was observed in stressed animals even before exposure to an anxiogenic stimulus. This finding clearly reveals that prolonged stressful exposure triggers an 'anxious-like' pattern of activation in the BNST that most likely underlies the reported increase in anxiety behavior displayed by these subjects. Such an increase in basal activation of the $BNST_{am}$ is likely a consequence of the observed increase in the expression of CRH in neurons in the central amygdaloid nucleus. Additionally, as the $BNST_{dm}$ and $BNST_{fu}$ send CRH projections (Phelix & Paull, 1990) to several areas of the limbic system, namely the PFC, nucleus accumbens, lateral septal nucleus and central amygdaloid nucleus (Davis *et al.*, 1997; Carvalho *et al.*, 2005; Jaferi & Bhatnagar, 2007; Rempel-Clower, 2007), the increase that we found in the expression of CRH in the dorsomedial subnuclei after stress is probably playing a critical role in the stress-induced anxious phenotype.

The role of CRH in anxiety, namely in the BNST network, is well established (Arborelius *et al.*, 1999; Sahuque *et al.*, 2006). Although the BNST is endowed with both CRHR1 and CRHR2 (Phelix & Paull, 1990; Chalmers *et al.*, 1995), the relative ratio clearly favors CRHR1. Importantly, CRHR1 also display a higher affinity for CRH (Vaughan *et al.*, 1995). In the light of this, the down-regulation of the CRHR1 in $BNST_{dm}$ and $BNST_{fu}$ herein reported is likely to be ascribed to down-regulation of the receptors due to increased availability of the ligand. In contrast, we found an up-regulation of CRHR2 levels, namely in the $BNST_{pr}$. Interestingly, an opposing role of these receptor subtypes in anxiety behavior has been proposed (Heinrichs *et al.*, 1997; Liebsch *et al.*, 1999; Radulovic *et al.*, 1999; Risbrough *et al.*, 2004). In fact, although the role of CRHR1 in anxiety is well established, there are conflicting results on whether CRHR2 activation triggers anxiogenic or anxiolytic behavior (Bale *et al.*, 2000; Coste *et al.*, 2000; Pelleymounter *et al.*, 2002, 2004). Part of this discrepancy might reside in regional specificities in the role of CRHR2. Indeed, there is now evidence that CRHR2 in the BNST is mediating conditioned behavior responses, but not unconditioned anxiety-like behaviors (Sahuque *et al.*, 2006). Importantly, the latter study also highlighted that the anxiolytic actions of both CRHR1 and CRHR2 antagonists in the BNST were not observable in basal conditions and required previous exposure to stress (Sahuque *et al.*, 2006), further reinforcing the relevance of our present findings after chronic stress exposure.

In addition to the alterations found in the $BNST_{am}$, this study also highlights a diminished cFOS activation in the $BNST_{pr}$. The $BNST_{pr}$ contains GABAergic neurons (Dong & Swanson, 2004) that exert an inhibitory control over the PVN (Choi *et al.*, 2007). Activation of these subnuclei occurs, amongst others, through a glutamatergic hippocampal input (Zhu *et al.*, 2001; Herman *et al.*, 2005) that is also targeted by stress (Sousa *et al.*, 2000; Cerqueira *et al.*, 2007). Importantly, the finding of a blunted activation of the $BNST_{pr}$ in stressed subjects is of relevance to the observed disinhibition of the HPA axis.

In support of an altered GABAergic innervation in the BNST, the present analysis also reveals a shift in Glutamic Acid Decarboxylase expression towards Glutamic Acid Decarboxylase 2 in the BNST as a consequence of chronic stress exposure, which fits with previous reports (Herman, 2001), but also an increased expression of $GABA_A$ receptor in the $BNST_{dm}$ and $BNST_{pr}$. Although it is well known that a global reduction in GABAergic transmission, namely through chronic inhibition of GABA synthesis, leads to an anxious-like phenotype (Sajdyk *et al.*, 2008), recent reports have shown an increase in GABA transmission (Li *et al.*, 2012). In dorsal raphe neurons, CRH has been shown to be a modulator of the GABAergic transmission (Kirby *et al.*, 2008).

Glutamatergic *N*-methyl-D-aspartate receptors, in particular those containing the NR2B subunit, have also been implicated in anxiety behavior (Kash *et al.*, 2009). It has also been shown that CRHR1 is also essential for the modulation of glutamatergic transmission within the BNST (Kash *et al.*, 2009; Nobis *et al.*, 2011). It is of relevance that, after chronic ethanol exposure, there are alterations in the glutamatergic plasticity associated with an increase in the number of NR2B receptors (Kash *et al.*, 2009), with these receptors also being associated with alterations in anxiety induced by alcohol withdrawal (Kiefer *et al.*, 2003). Again, our present finding of increased NR2B expression in the $BNST_{pr}$ supports a glutamatergic dysregulation in stress-induced anxiety and opens new perspectives for intervention in anxiety-related conditions.

In summary, the present data show that the BNST activation pattern in stressed subjects is remarkably similar to that found in controls in response to acute anxiogenic stimuli (Fig. 7). This stress-induced alteration in the activation of the BNST is paralleled by subnuclei-specific molecular changes that contribute to our understanding of the neural mechanisms involved in stress-induced hyperanxiety and HPA axis overactivation.

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Abbreviations

AxStim, anxiogenic stimulus; BNST, bed nucleus of the stria terminalis; $BNST_{al,}$ anterolateral division of the bed nucleus of the stria terminalis; $BNST_{am,}$ anteromedial division of the bed nucleus of the stria terminalis; $BNST_{dm,}$ dorsomedial nucleus of the bed nucleus of the stria terminalis; $BNST_{fu,}$ fusiform nucleus of the bed nucleus of the stria terminalis; $BNST_{p,}$ posterior division of the bed nucleus of the stria terminalis; $BNST_{pr,}$ principal nucleus of the bed nucleus of the stria terminalis; Cont, control; CRH, corticotropin-releasing hormone; CRHR, corticotropin-releasing hormone receptor; CUS, chronic unpredictable stress; HPA, hypothalamus–pituitary–adrenal; PBS, phosphate-buffered solution; PFC, prefrontal cortex; PVN, paraventricular nucleus of the hypothalamus; qRT-PCR, quantitative real-time polymerase chain reaction.

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Chapter 2.3

Ventura-Silva AP, Glangetas C, Sousa N, Georges F, Pêgo JM

Stress triggers a hyperactivation of neurons in the anterior Bed Nucleus of the Stria Terminalis

(manuscript under preparation)

Stress triggers a hyperactivation of neurons in the anterior Bed Nucleus of the Stria Terminalis

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Abstract

Prolonged stress exposure induces structural changes in several brain regions with behavioral and neuroendocrine implications. The anterior division of the bed nucleus of the stria terminalis (BNST), an area involved in the regulation of anxiety and the hypothalamic-pituitary-adrenal (HPA) axis activity, is also targeted by stress. This division of the BNST receives major inputs from the infralimbic cortex (ILCx) and central nucleus of the amygdala (CeA), but their modulatory actions on the neuronal activity of anterior BNST after chronic stress is not known. Using an in vivo electrophysiology approach in rats, we studied the effect of ILCx and CeA stimulation in the activity of anterior BNST neurons in basal conditions and after exposure to a chronic unpredictable stress protocol. Our results show that chronic stress triggers a hyperactivation of anterior BNST neurons both by increasing the excitatory responses after ILCx stimulation and by decreasing the inhibitory responses after CeA stimulation. These results contribute to understand the neurophysiological mechanisms underlying the stress-induced anxiety and overactivation of the HPA axis.

Introduction

Chronic stress induces behavioral and morphological changes in both humans and rodents (File et al., 1996; Drevets, 2000; D'Andrea et al., 2011). The limbic system is particularly vulnerable to stress exposure as demonstrated by studies from different laboratories (Vyas et al., 2002; Cerqueira et al., 2005; Cerqueira et al., 2007; Herman et al., 2005; Bessa et al., 2009); importantly, we have shown that the bed nucleus of the stria terminalis (BNST) is also targeted by stress (Pêgo et al., 2008). The BNST is anatomically and functionally quite heterogeneous and the effects of chronic stress were predominantly observed in the anterior division of the BNST, in particular, chronic stress induces hypertrophy of dendrites and spine sprouting in this division (Pêgo et al., 2008). Taking into account that the projection from the anterior division of the BNST to the hypothalamic paraventricular nucleus (PVN) exerts an excitatory action on the corticotrophin-releasing factor (CRF) producing neurons in the PVN, it is not surprising its role in controlling the activation of the HPA axis (Zhu et al., 2001; Dong & Swanson, 2004; Dong & Swanson, 2006; Choi et al, 2007). Thus, it becomes of relevance to better understand how the different inputs to this component of the BNST are modulated by stress.

There are two major sources of input to the anterior BNST. One originates in medial prefrontal cortex (mPFC), in particularly from its infralimbic (ILCx) subdivision (Sesack et al., 1989; Hurley et al., 1991). Specifically, the ILCx exerts an excitatory influence over the anterior BNST (Massi et al., 2008) mediated through its glutamatergic projections. The other is the amygdalar input that originates largely in the central nucleus of the amygdala (CeA) and incorporates the stria terminalis (Dong et al., 2001). The CeA is rich in GABAergic neurons that show co-expression of GABA and several neuropeptides such as corticotrophin-releasing factor (CRF), encephalin or dynorphin (Champagne et al., 1998; Marchant et al., 2007; Poulin et al., 2008). Lesions of CeA have been shown to lead to a reduction in adrenocorticotrophic hormone (ACTH) levels in the PVN after stress, revealing that the output of this region may also result in the activation of the HPA axis (Beaulieu et al., 1987).

Despite a growing number of studies focusing on the BNST surprisingly there are few studies addressing the impact of ILCx stimulation to the anterior BNST and almost no studies showing the in vivo effect of CeA stimulation on the electrical activity of the BNST. The present work aims to assess how the electrophysiological proprieties of BNST neurons are affected by stimulation of the CeA and ILCx and also how chronic stress alters these networks.

Materials and Methods

Animals

Animal experiments were conducted in accordance with the European Union Directive (2010/63/EU) and the NIH guidelines on animal care and experimentation.

Adult male Sprague-Dawley (300-350g; Elevage Janvier) were housed in groups of 2 or 3 per cage under standard laboratory conditions (artificial light/dark cycle of 12/12h; lights on at 8 a.m) and with ad libitum access to commercial chow and water.

Treatment

For the experiments regarding the alterations induced by chronic stress in the synaptic transmission between ILCx-BNST and CeA-BNST rats were randomly assigned to a CUS or Cont group (n=8 per group). For the cFos expression analysis, 8 rats were divided into the same groups: CUS (n=4) and Cont (n=4). Chronic Unpredictable Stress (CUS) protocol was performed as described before (Cerqueira et al, 2007). Treatment started when the animals were 8 weeks of age and lasted for 4 weeks. Briefly, CUS animals were exposed daily to a stressor (30min/d) scheduled in a random order for the duration of the experiment. Stressors were one of the following: exposure to a hot stream of air, cold water (18°C), immobilization, overcrowding and shaking. Cont animals were gently handled for 5min on a daily basis.

Surgery

For the electrophysiological experiments, animals were submitted to stereotaxic surgery for the placement of stimulating electrodes in ILCx and CeA and a recording pipette in the BNST. The surgeries were performed under isoflurane anesthesia (Centravet). An incision was made through the skin and connective tissues over the skull and three holes were drilled in the skull and the electrodes and pipette were placed at the following coordinates: ILCx: 3.0mm from bregma, 0.5mm from midline, 4.5mm from brain surface; CeA: -2.2mm from bregma, 4.2mm from midline, 7.0mm from brain surface; BNST: -0.2mm from bregma, 1.5mm from midline, 6.0 to 7.4mm from brain surface.

For the tract tracing experiment, the surgeries were performed under ketamine/medeteomidine anesthesia. The injections were performed in ILCx, CeA (coordinates as above) and PVN (1.6mm from bregma, 0.3mm from midline, 7.8mm from brain surface).

Electrical Stimulation

Concentric electrodes (250 mm diameter overall, 100 mm diameter inner electrode which extended 100 mm beyond the outer electrode; Phymep) were inserted in the ILCx or CeA, the stimulation was administered using a square pulse stimulator (CED 1401,SPIKE 2; Cambridge Electronic Design) and stimulus isolator (DS3; Digitimer). The stimulation consisted of 100 pulses of 0.5Hz with 0.5ms duration with intensity range from 0.2-1.0mA for the ILCx and 0.1-0.5mA for the CeA stimulation.

BNST recordings

A glass micropipette (tip diameter, 1–2 mm; 10–15mohm) filled with a 2% pontamine sky blue solution in 0.5 M sodium acetate was lowered into the BNST. The extracellular potential was recorded with an Axoclamp2B amplifier in the bridge mode versus a reference electrode maintained in contact with the skull by a sponge moistened with a 0.9% NaCl solution. The extracellular potential amplified by the Axoclamp2B amplifier was further amplified and filtered (low-pass filter at 300 Hz and high-pass filter at 10 kHz) via a differential AC amplifier (model 1700; A-M Systems). Spikes of single neurons were discriminated, and digital pulses were led to a computer for on-line data collection using a laboratory interface and software (CED 1401, SPIKE 2; Cambridge Electronic Design). After isolating a single BNST neuron, prestimulation spontaneous activity was recorded to establish baseline activity for at least 5 min. Subsequently, single pulses were delivered to the ILCx or CeA every 2s. At least 100 trials were administered per cell.

Pharmacological Manipulation

Picrotoxin (Sigma) and antalarmin (Sigma) were used to test the involvement of GABA and CRF in the synaptic transmission between CeA and BNST. Double-barrel pipettes were used to perform microinjection of the drug in the vicinity of the neuron recorded. Double-barrel pipette consist of an assembly of an injection pipette together with a recording one (Akaoka and Aston-Jones, 1991). The recording pipette consists of a glass micropipette with a tip diameter of 1-2µm. The injection pipette is pulled and broken to a tip diameter of 30µm and then heated and bent at an angle of 30 degrees. After preparing the 2 pipette separately they are brought together using precision micromanipulators. The pipettes are assembled so that the tips are parallel and the recording microelectrode tip extends 140µm beyond the injection. The assembly is then secured using photopolymerizing resin. Electrical stimulation of CeA and BNST recordings were performed as described above. Activity of BNST neurons was recorded before and after the microinjection of 60nl of one of the drugs (picrotoxin : 1mM;

antalarmin ; 100μM). The drugs were injected using brief pulses of pneumatic pressure (Picospritzer; General Valve).

Tract Tracers methods

A subset of animals (n=4) was injected with anterograde and retrograde tracers. The 4 animals were injected with a retrograde tracer in the PVN and with an anterograde tracer either in the ILCx (n=2) or the CeA (n=2). For the retrograde tracing, 100nL of 0.5% cholera toxin subunit B conjugated with FITC (CTb; Sigma) were injected by pressure in the PVN (20nl/min). For the anterograde tracing, 100nl of biotinilated-dextran (Sigma) was injected in either the ILCx or the CeA (20nl/min). The needles were left in place for 10 min after the injection.

Histological Procedures

At the end of each electrophysiological experiment, pontamine sky blue (Sigma) was iontoforetical injected in the recording place (negative continuous current at 20mA for 30 min). To mark the placement of the stimulation electrodes, 10uA of positive current was passed through each electrode for 1min. Animals were sacrificed under isoflurane anhestesia and brains were collected and snap frozen in isopentane at -80°C. Coronal sections (30μm) were cut in a cryostat (Leica), mounted and stained with neutral red for histological determination of the localization of recording and stimulation sites.

For the tract-tracing experiments, one week after the surgery rats were deeply anesthetized with pentobarbital and the brains collected in 4% PFA. Coronal sections (50 μm) were cut in a vibratome and collected in PBS. Alternate sections were incubated for 1h with fluorescent streptadivin (1:500 in PBS; 547nm, Invitrogen) and for 5 min with DAPI before mounting. The sections were later observed and photographed in an Olympus –IX81 microscope.

cFos expression

Another set of animals (Cont: n=4; CUS: n=3) was submitted to stereotaxic surgery as described above. After the end of the surgery they were left in the stereotaxic apparatus under isoflurane anesthesia for 90 min. Then, a series of electrical stimulation in CeA were performed (5 times 100 pulses at 0,5Hz and intensity of 0,5mA). 90 min after the last pulse, animals were perfused with PFA 4%. Brains were collected and later processed for immunohistochemistry.

Immunohistochemistry

Coronal sections (60 µm thick) were serially collected in phosphate-buffered solution (PBS). Alternate sections were immunostained for cFOS by overnight incubation with rabbit anti-cFOS polyclonal antibody (1:8000; Tebu-Bio, Sc-52) after blocking (90min) in a PBS solution, containing 1.5% Triton X-100 and 2% normal donkey serum. Following washes in PBST, sections were incubated overnight in biotinylated donkey anti-rabbit antibody (AP182B, Millipore) followed by an ABC solution (Vectorstain Elite), and finally visualized with diaminobenzidine (DAB). Sections were counterstained with neutral red to help delimit regional boundaries before mounting and coverslipping. cFOS-immunoreactive (Fos-IR) neurons were marked by a dark brown DAB precipitate. The number of Fos-IR neurons in the PVN was counted and the number per area was calculated.

Data analysis

For the electrophysiology experiments, cumulative peristimulus time histograms (PSTHs; 5 ms bin width) of BNST activity were generated during electrical stimulation of the ILCx or CeA, for each neuron recorded. PSTHs were analyzed to determine excitatory and inhibitory epochs (Georges and Aston-Jones, 2002). In brief, the mean and standard deviation (SD) of counts per bin were determined for a baseline period, defined as the 500ms epoch preceding stimulation. The onset of excitation was defined as the first of five bins whose mean value exceeded mean baseline activity by 2 SD, and response offset was determined as the time at which activity had returned to be consistently within 2 SD of baseline. Response magnitudes for excitation were calculated with the following equation: (counts in excitatory epoch) - (mean counts per baseline bin 3 number of bins in excitatory epoch). The onset of inhibition was defined as the first of 5 bins whose mean value were below 30% of the baseline activity and the response offset when the activity of the neurons was consistently above 30% of the baseline activity. The total duration of the inhibition was determined for each neuron.

Statistical analysis of data was performed using t-test to compare means and Tukey test as post hoc analysis. Results are presented as mean ± standard error of the mean (SEM). Statistical significance was accepted for a probability level (p) below 0.05.

Results

BNST as a relay station between cortical and amygdalar inputs to the PVN

It has been well described a potential role of the BNST as a relay point from inputs from limbic structures that will be transmitted to the PVN. We have tested this by injecting anterograde tracers in limbic structures (ILCx or CeA) and a retrograde tracer in the PVN and we observed if there was a convergent of innervation from these structures in the BNST. Our results show that for the CeA-BNST-PVN pathway, 59% of the dextran-marked cells (amygdalar innervation) were in close contact with cells with retrograde labeling. At the same time, 54% of cells with ILCx innervation were in contact with cells labeled retrogradely. These results show us that a large percentage of the inputs received from the ILCx or CeA will reach the PVN, confirming the role of the BNST as a relay of information between these areas (Figure 1).

The effects of CeA stimulation upon the neuronal activity in the anterior division of the BNST

Electrical stimulation of CeA triggered an alteration of the activity of neurons in the anterior division of the BNST. In control animals, the stimulation of the CeA led to a decreased neuronal activity in the BNST neurons. Importantly, the onset latency of inhibition was 17.9 ms after the stimuli and last, in average, 68.3 ms (Figure 2B, Table 1). In stressed animals, CeA stimulation also triggered an inhibition of BNST, but there was a significant decrease of the duration of the inhibition ($p<0.0001$), which lasted in average 27.1ms (Figure 2B, Table 1).

Taking into account that the CeA is a nucleus richly endowed with GABAergic neurons, we assessed the contribution of the GABAergic projections to the response observed in the neurons of the anterior division of the BNST. By using double-barrel pipettes, we next recorded the neurons of the anterior division of the BNST after a microinjection in the same location of picrotoxin, a GABAA receptor antagonist; in this way, we blocked the GABAergic input to these neurons. Importantly, we confirmed that an injection of 60nl of picrotoxin (1mM) was able to block the inhibitory response in both experimental groups (Fig. 2D), thus showing that the input from CeA to the anterior division of the BNST is GABAergic. More interestingly, was the finding that after the stimulation of CeA, a significant percentage of neurons in control animals also presented a long latency increase in activity when compared to baseline; importantly, this percentage was even higher in stressed animals (Cont: 46%; CUS: 63%, Table 1). In addition, to the difference in number of neurons expressing such excitatory

response, we found that its magnitude (Figure 3B, Table 1; p=0.018) was bigger in stressed animals than in controls. Interestingly, the onset of this rebound excitatory response was faster in stressed animals (started at 90ms in stressed vs 113ms in controls; p=0.05).

To evaluate the effect of stress in the activation of the HPA axis, we performed a cFOS analysis of neurons in PVN in control and stressed animals that were submitted to CeA Stimulation. CUS animals show a tendency for increased number of cFOS marked cells when comparing with controls albeit this difference not being significative (Figure 4).

CUS induces hyperexcitability of BNST neurons following ILCx stimulation

Previously, we showed that ILCx stimulation, through a glutamatergic pathway, is able to drive a short latency increase in the activity of the BNST neurons (Massi et al., 2008). Herein, we aimed to understand if chronic stress alters the synaptic transmission between these two regions. First, we performed electric stimulation of ILCx while recording BNST neurons in control animals and were able to confirm our previous results; in 24 of the recorded neurons, ILCx was able to induce an excitation of neurons 9.3ms after the stimuli was applied and that lasted 11.3ms (Table 1). This response was augmented as we increased the intensity of stimulation: for a low intensity (0.2-0.5 mA) of stimulation there was a 27% increase in the response magnitude; for an intensity of 1.0mA (high intensity) the increase in the excitatory activity raised to 58% (p=0.018) (Figure 4C, Table 1). Of notice, in stressed animals, the increase in magnitude of the excitatory response of neurons in the anterior division of the BNST was significantly higher, particularly for higher intensities of stimulation of the ILCx (Figure 5C, Table 1; p=0.044).

In approximately 30% of the neurons recorded in the anterior division of the BNST, we have identified an inhibition of the neuronal activity after the initial excitatory response. Again a significative difference in this response was found between experimental groups: while in stressed animals the duration of this rebound inhibition was 31.2 ms, in controls it lasted for 88ms (Figure 5D, Table 1; p=0.0013).

Discussion

The BNST is part of the extended amygdala, sharing many neurochemical and morphological properties with medial (MeA) and central amygdala (Alheid and Heimer, 1988; de Olmos and Heimer, 1999). These two amygdaloid regions send dense projections to the BNST (Dong et al., 2001); while the projections from MeA go mainly to the posterior BNST, the CeA projection targets largely the anterior BNST. Both MeA and CeA are rich in GABAergic neurons which suggests that the role of these nuclei is mainly inhibitory; in fact stimulation of MeA was shown to induce inhibitory response in neurons of the BNST (Sanchez et al., 1995). On the other hand, and even though the CeA constitutes the principal output of the amygdala, little was known about the effects of CeA stimulation in the activity of neurons in downstream pathways. Similarly to MeA, the central nucleus is rich in GABAergic neurons but it has also a high expression of neuropeptides like CRF, often colocalized in GABAergic neurons (Veinante et al., 1997; Champagne et al., 1998). By showing in control animals, that the stimulation of CeA induced a long inhibition of the activity of BNST neurons, we demonstrate that the role of this nucleus is to inhibit the anterior BNST.

The confirmation that picrotoxin could block this effect confirms the inhibitory influence of the CeA over the anterior BNST and its dependence on GABAA receptors. GABAA receptors are known to play a fundamental role in the inhibitory transmission conveyed by the CeA (Esmaeili et al., 2009), which is relevant for anxiety behavior and its pharmacological modulation. Benzodiazepines, a class of drugs with a potent anxiolytic effect, enhance GABA effects through actions on GABAA receptors. Thus, it is likely that the effect of benzodiazepines will be mediated, in part, through the effects at the CeA-anterior BNST synapse. In fact, the anterior BNST has been implicated in anxiety behavior (Shepard et al., 2009; Walker et al., 2009) and lesions in subnuclei of this region (Choi et al., 2007) have shown to induce a decreased activation of the HPA axis. The inhibitory tone of CeA in this area in control animals is probably contributing to maintain the corticosteroid homeostasis of the organism.

Interestingly, however, exposure to CUS might compromise this physiological balance. Indeed, CUS induces several morphological and molecular alterations in the CeA and BNST (Vyas et al., 2003; Pêgo et al., 2008, Ventura-Silva et al., 2012). We have previously showed that CUS triggers an increased volume and increase in spine density in the anterolateral BNST without inducing major morphological alterations in amygdala (Pêgo et al., 2008). With the present experiments we demonstrated that after CUS there is a decrease in the inhibitory response of BNST neurons to stimulation of the CeA. This

observation is a likely consequence of a decreased in GABAergic transmission from the CeA to the BNST; importantly, it might constitute an important target for therapeutic intervention.

Apart from a strong inhibitory response after CeA stimulation we also identified a long latency excitation of anterior BNST neurons after CeA stimulation. In fact, this excitation, starting at an average of 100ms, was increased in CUS (both in terms of duration and intensity). The long latency of excitation is characteristic of either a polysynaptic response or a response mediated by G-protein coupled receptors. Taking into account that the CeA is the major source of extra-hypothalamic CRF, it is plausible that this effect is mediated by release of this peptide, especially after the demonstration that stress increases the expression of CRF in the CeA and that CRFR1 receptors are decreased in the BNST (Sutherland et al., 2010; Ventura-Silva et al., 2012). However, other factors may also contribute to this response, in particular, peptides as dynorphin. Indeed, Li and colleagues (2012) have shown that both CRF and dynorphin play an important role in the modulation of glutamatergic transmission in the BNST. Overall, stress seems to shift the pathway between CeA to BNST from an inhibitory to a more excitatory tone by downgrading the GABAergic tone and increasing the excitatory peptidergic response produced in CeA.

Stimulation of the ILCx, the other major input to the anterior BNST, produced a strong excitatory response in BNST neurons. This fits our previous observations that that the stimulation of the ILCx triggers a glutamatergic release in the anterior BNST (Massi et al., 2008). As it is known that stress leads to an increase of glutamate release in several brain regions, the observation that stress animals display an increased excitatory response after ILCx stimulation fits with the concept that prolonged stress triggers an overexcitation of the anterior BNST, which may contribute to explain the stress-induced hyperanxiety as well as the overactivation of the HPA observed after prolonged stress exposure.

With this work we were able to characterize the impact of stimulation of the ILCx and CeA in the electrophysiological proprieties of BNST neurons in both baseline conditions and after chronic stress exposure. Importantly, we found that in basal conditions, the anterior BNST neurons are under an inhibitory influence of CeA and excitatory inputs from ILCx and CeA; chronic exposure to stress promotes an hyperactivation of the anterior BNST neurons as a result of a stronger excitatory tone of the projections both from CeA and ILCx and a decrease in the inhibitory tone originating in the CeA. When considering the function of the anterior BNST, these results seem to be relevant to understand, and eventually modulate, the stress-induced hyperanxiety and deregulation of the HPA axis.

Abbreviations

ACTH – Adrenocorticotrophic Hormone

BNST – Bed Nucleus of the Stria Terminalis

CeA – Central Nucleus of the Amygdala

Cont - Control

CRF – Corticotrophin-Releasing Factor

CUS – Chronic Unpredictable Stress

HPA – Hypothalamic-Pituitary-Adrenal

ILCx – Infralimbic Cortex

MeA – Medial Amygdala

mPFC – media Prefrontal Cortex

PBS - phosphate-buffered solution

PSTH – Peristimulus Time Histogram

PVN – Paraventricular Nucleus of the Hypothalamus

SD – Standard Deviation

SEM – standard error of the mean

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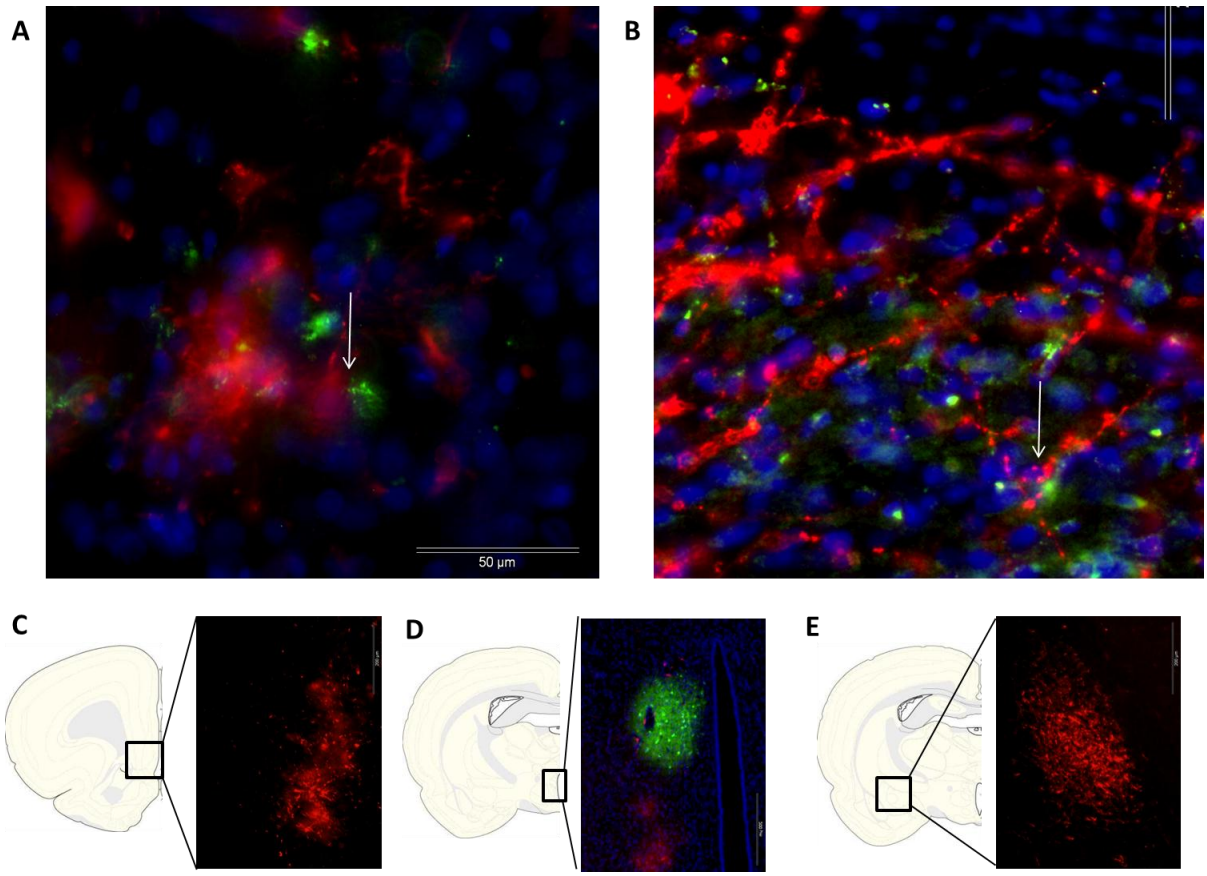

Figure 1. Tract-tracing studies. A and B represent images of the anterior BNST after injection of the retrograde tracer CTb (green) in the PVN and the anterograde tracer dextran (red) in the ILCx (A) or the CeA (B). Arrows indicate cells showing both tracers in close proximity. C- Injection of dextran in the ILCx; D- Injetion of CTb in the PVN; E- Injection in CeA of dextran. BNST- Bed nucleus of the stria terminalis, CTb- cholera toxin subunit B, PVN- paraventricular nucleus of the hypothalamus, ILCx- infralimbic cortex, CeA – central amygdala.

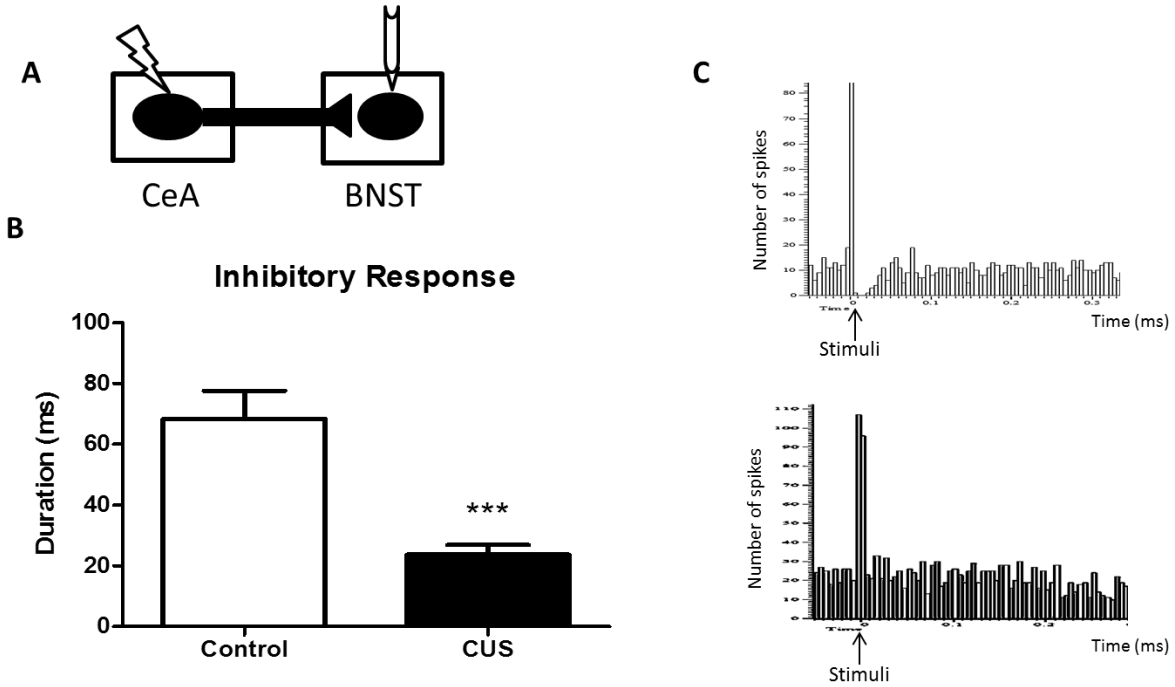

Figure 2. Inhibitory Response after CeA Simulation. Electrical Stimulation of CeA induces a large inhibitory response in control animals that is decreased when rats are submitted to a chronic stress protocol. This response is mediated by GABA receptors. A– representative scheme of the places of stimulation and recording. B- Duration of the inhibitory response in control and CUS. C and D- representative PSTH of the recording of a neuron in an animal after saline injection (C) or picrotoxin (D). Data presented as mean ± SEM.. *** $p<0.001$. CeA – Central Amygdala; BNST – Bed Nucleus of the Stia Terminalis; CUS – Chronic Unpredictable Stress.

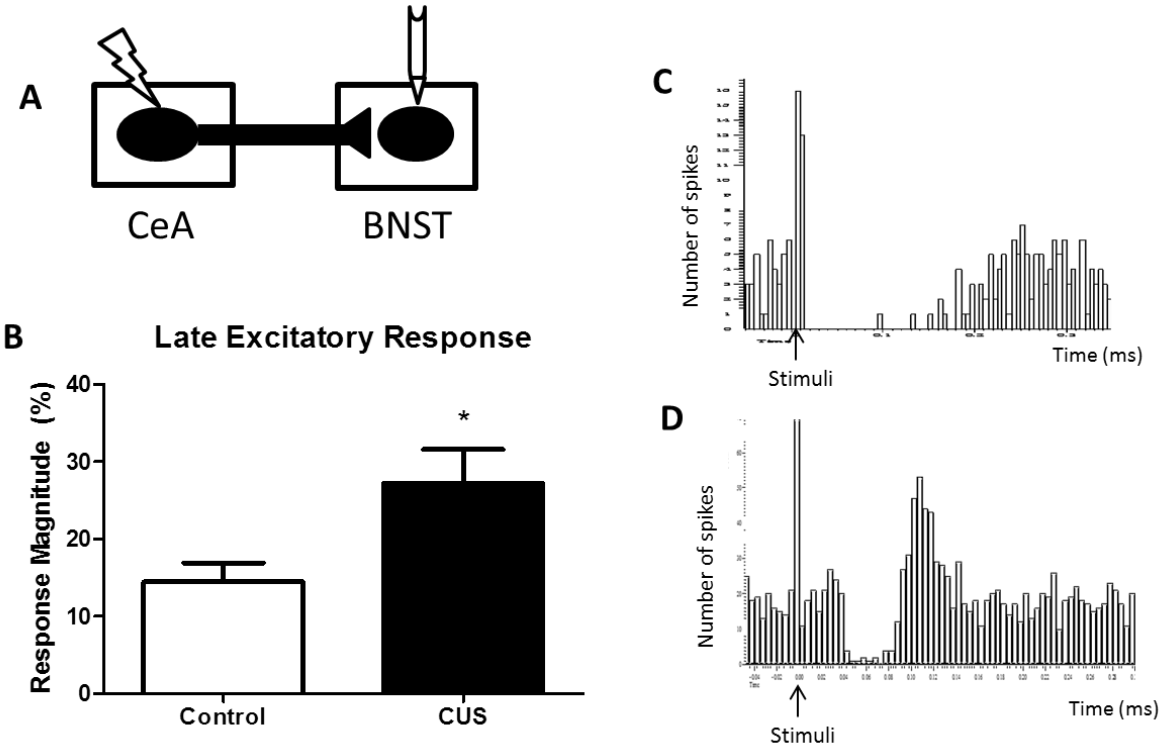

Figure 3. Excitatory Response after CeA Stimulation. An electrical stimulation in CeA drives a late excitation of neurons in the anterior BNST that is increased by chronic stress exposure. A- Schematic representation of the locale of stimulation and recording. B- Excitatory response after CeA stimulation in Controls and CUS. C and D- Representative PSTH of the recording of a neuron in a Control Animal (C) or CUS (D). Data presented as mean ± SEM. * $p<0.05$. CeA – Central Amygdala; BNST – Bed Nucleus of the Stria Terminalis; CUS – Chronic Unpredictable Stress.

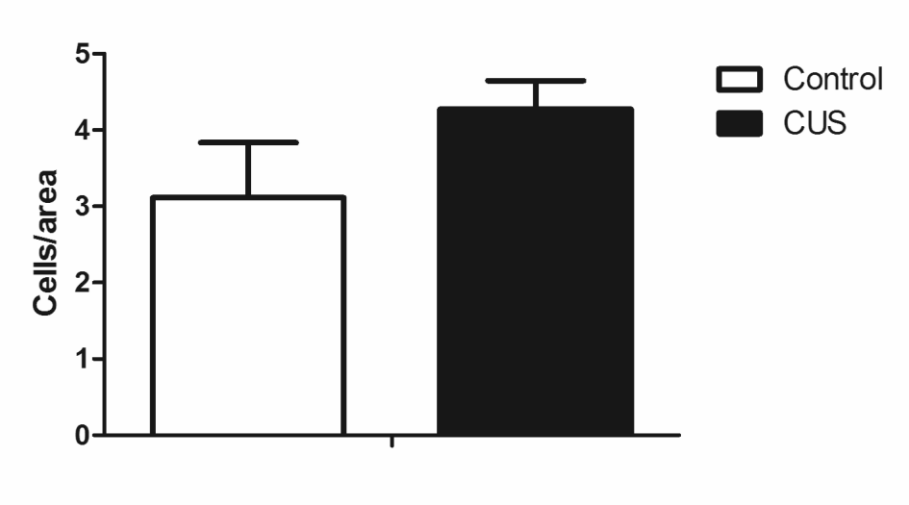

Figure 4. Number of cFOS positive cells in the PVN per area. After CeA Stimulation, stressed animals show a tendency for an increase in the activation of PVN neurons. Data presented as mean ± SEM. CUS – Chronic Unpredictable Stress. CeA – Central Amygdala; PVN – Paraventricular Nucleus of the Hypothalamus.

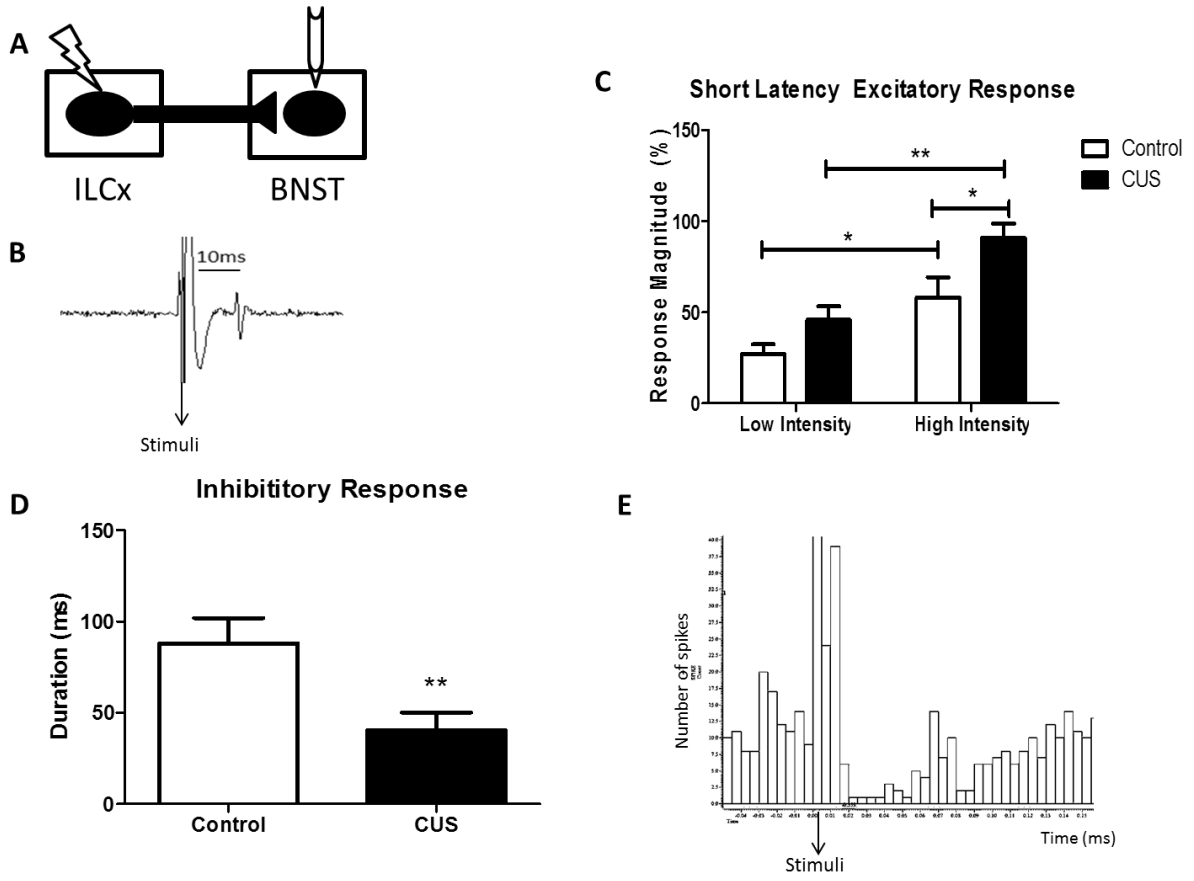

Figure 5. Stimulation of ILCx in CUS animals induces an increased hyperactivity of BNST neurons when comparing with controls by increasing the excitatory inputs while decreasing the inhibition. A- Schematic representation of the stimulation and recording areas. B- ILCx stimulation induces a short latency excitation of BNST neurons. C- Excitatory response in Control and CUS animals after ILCx stimulation. D- Duration of the inhibitory response in control and CUS. E- Representative PSTH of the recording of one neuron in a control animal. Data presented as mean ± SEM. * $p<0.05$; ** $p<0.01$. ILCx – Infralimbic Cortex. BNST – Bed Nucleus of the Stria Terminalis; CUS – Chronic Unpredictable Stress.

Table 1. Summary of the basic electrophysiological characteristics, onset latencies and durations of excitatory and inhibitory responses of neurons recorded in the BNST after CeA or ILCx stimulation. All values are mean ± SEM. Response magnitude calculation as described in materials and methods. # corresponds to 1.0mA intensity of stimulation. BNST- Bed Nucleus of the Stria Terminalis; CeA- Central Amygdala; CUS- Chronic Unpredictable Stress; ILCx- Infralimbic Cortex.

BNST neurons recorded	**CeA Stimulation**		**ILCx Stimulation#**	
	Cont	**CUS**	**Cont**	**CUS**
Spontaneous Firing Rate (Hz)	8,41 ± 1,2	10,0 ± 2,3	7,6 ± 1,6	9,9 ± 1,8
Neurons responsive to stimulation (n)	24	27	24	28
Excitatory Response to Stimulation (% of neurons)	46	63	83	84
Response magnitude of excitation (% increase)	15 ± 2,4	27 ± 4,1	58,2 ± 6,7	90,8 ± 7,9
Onset of excitation (ms)	113 ± 9.7	90 ± 3,4	9,3 ± 1,1	11,7 ± 1,4
Duration of excitation (ms)	32 ± 4,3	40± 5,2	11,3 ± 1,4	9,3 ± 1,2
Neurons with inhibitory activity (%)	83	85	29	35,7
Onset of inhibition (ms)	17,9 ± 3,4	27,1 ± 5,6	22,1 ± 3,7	32,2 ± 4,8
Duration of inhibition	68,3± 9,3	23,7 ± 3,1	88 ± 13,9	31,2 ± 3,6

Chapter 2.4

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Excitotoxic lesions in the central nucleus of the amygdala attenuate stress-induced anxiety behavior

(manuscript under preparation)

Excitotoxic lesions in the central nucleus of the amygdala attenuate stress-induced anxiety behaviour

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Abstract

The extended amygdala, composed by the amygdaloid nuclei and the bed nucleus of the stria terminalis (BNST), plays a critical role in anxiety behavior. In particular, the link between the central nucleus of the amygdala (CeA) and the BNST seems to be critical to the formation of anxiety-like behavior. Chronic unpredictable stress exposure is recognized as a validated animal model of anxiety and is known to trigger significant morphofunctional changes in the extended amygdala. Quite surprisingly, no study has ever analyzed the role of the CeA in the onset of stress-induced anxiety and fear conditioning behaviors; thus, in the present study we induced a bilateral excitotoxic lesion in rats that were subsequently exposed to a chronic stress protocol. Data shows that lesioned animals display attenuation of the stress response and of stress-induced anxiety-like behavior when compared with stressed animals with sham lesions; in contrast, the lesion of the CeA precluded the appearance of fear conditioning in non-stressed animals. These results unravel the relevance of the CeA in the regulating of the HPA axis activity and in onset of anxiety behavior triggered by stress and fear conditioning behaviors and suggests that modulation of its activity might be of relevance to the control of emotional disturbances.

Introduction

Anxiety disorders are very prevalent. Anxiety is characterized by a sensation of discomfort and apprehension in response to unconditioned diffuse cues (Koch, 1999). It is well established that exposure to chronic stress is a triggering factor for development of anxiety. Stress induces several alterations in the central nervous system, with particular relevance to areas in the limbic system that regulate the stress response and emotional behavior. It has been shown that different models of stress can alter dendritic and synaptic plasticity in contrasting patterns, namely atrophy in the prefrontal cortex (Cerqueira et al., 2005; Cerqueira et al., 2007; Dias-Ferreira et al., 2009) and hippocampus (Bessa et al., 2009) and hypertrophy in the bed nucleus of the stria terminalis (BNST; Pêgo et al., 2008) and the amygdala (Vyas et al., 2002; Vyas et al., 2003).

Among the limbic structures, the extended amygdala, which comprises, among other areas, the BNST and the central nucleus of the amygdala (CeA) (Alheid et al., 1998) plays a major role in the modulation of anxiety behavior. In particular, CeA is the major output area of the amygdala and is involved in both fear conditioning and anxiety behavior (Walker et al., 2009; Davis et al., 2010). Although the CeA does not have strong direct projections to the hypothalamus (Moga & Saper, 1994; Prewitt and Herman, 1998), its activation will lead to the stimulation of hypothalamic nuclei and areas that are responsible for fear and stress responses (Beaulieu et al., 1987; Shepard et al., 2006). This stimulation occurs largely through a massive projection from CeA to the BNST, a region that in turn projects densely to the paraventricular nucleus of the hypothalamus (PVN) (Dong et al., 2001). Indeed, it is presently recognized that the BNST acts as a relay station between upper limbic areas and the PVN, playing a fundamental role in the modulation of the stress response and anxiety behaviors (Herman et al., 2005; Choi et al., 2007).

The connection between the CeA and the BNST occurs through the stria terminalis, a bundle of projection fibres that include GABAergic neurons co-expressing peptides such as corticotrophin releasing factor (CRF) or encephalin (Veinante et al., 1997; Day et al., 1999). Of relevance, CRF is highly expressed in the CeA, being this region the major extra-hypothalamic source of this peptide. Due to the role of CRF in stress response, it has been proposed that neurons expressing CRF in the CeA are involved in stress related anxiety and fear behavior (Davis et al., 1992;

Makino et al., 1995). It was shown that exposure to stress induces increased expression of CRF in several brain regions, including the extended amygdala (Kalin et al., 1994; Makino et al., 1994; Cook et al., 2004; Shepard et al., 2006). In addition, lentiviral overexpression of CRF in the CeA results in the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and alterations in the baseline response to acoustic stimuli (Keen-Rineheart et al., 2009). On the other hand, it was shown that lesions of CeA induce reduction of ACTH levels after immobilization stress (Beaulieu et al., 1987). Quite surprisingly, to the best of our knowledge, there is a lack of reports about the consequences of lesions in the CeA in a rodent model of chronic stress. To further understand the role that this area plays in anxiety and fear conditioning behavior we assessed how excitotoxic lesions of CeA affect the development of anxiety induced by a chronic unpredictable stress (CUS) paradigm.

Materials and Methods

Animal experiments were conducted in accordance with the European Union Directive (2010/63/EU) and the NIH guidelines on animal care and experimentation.
Adult male Wistar rats were housed in groups of 2 per cage under standard laboratory conditions (temperature 22°C; artificial light/dark cycle of 12/12h; lights on at 8 a.m) and with ad libitum access to commercial chow and water.

Twenty six male rats (8 weeks old) were submitted to stereotaxic surgery under ketamine/medetomidine anaesthesia. The animals were randomly distributed to one of two groups. A group of animals was injected with phosphate buffer solution (PBS) (n=13) and other (n=13) with ibotenic acid (Sigma) in the central amygdala (-2.2mm from bregma, 4.2mm from midline, 7.0mm from brain surface). Ibotenic acid (10mg/ml) was injected at a rate of 0.05µl/min for a total volume of 0.2µl.

After the surgery animals were given one week to rest and then were subdivided into 4 groups: Control-Sham (Cont-Sham; n=10 randomly chosen from animals injected with vehicle); control-lesion (Cont-Lesion; n=10 randomly chosen from animals that received ibotenic acid); a CUS-Sham (n=10 corresponding to animals injected with vehicle) and CUS-Lesion (n=10 from the animals injected with ibotenic acid).

Animals that were treated with CUS protocol were exposed during 4 weeks once daily to a stressor (30min/d) of one of the following aversive stimuli: cold water (18°C), vibration, restraint, overcrowding an 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). Corticosterone levels were measured in blood serum, obtained between 9 and 10 a.m and between 6 and 7 p.m., by radioimmunoassay (R & D Systems, Minneapolis, MN).

After the end of the stress exposure a behavioural evaluation was performed to assess anxiety-like behavior (elevated-plus maze (EPM) and acoustic startle), fear conditioning (fear potentiated-

startle) and locomotory activity (open field) as described previously (Pêgo et al., 2008). Following behavioural tests the animals were deeply anaesthetized with pentobarbital and perfused transcardiacally with saline. Brains were collected, involved in OCT and frozen. The brains were kept at -20°C until histological processed; 20µm coronal sections were obtained in a cryostat and stained with cresyl violet to assess the location of lesions (Figure 1).

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

Results

The treatment induced an overall alteration in body weight gain ($F=16.31$; $p<0.001$; Table 1). Exposure to CUS protocol lead to a significant decrease in body-weight gain when compared with control groups ($p<0.05$; Table 1) although animals with lesion in central amygdala (CUS-Lesion) had a smaller reduction in body-weight gain when compared with CUS-Sham (Table 1). Both CUS groups showed a non-significant decrease in thymus weight when compared with control animals (Table 1). CUS-Sham animals showed a non-significant increase in adrenal weight when compared with both control groups and CUS-Lesion animals (Table 1). The efficacy of the stress was also measured by assessing the corticosterone levels in the blood. The treatment induced an overall alteration of the plasma corticosterone levels one hour after "lights on" (from 9 to 10 a.m.) ($F=13.31$; $p<0.001$) with stressed animals showing an increase in corticosterone when comparing with controls (Cont-Sham vs CUS-Sham: $p<0.001$; Cont-Sham vs CUS-Lesion: $p=0.01$; Cont-Lesion vs CUS-Sham: $p<0.001$). The presence of a lesion in the CeA was able to attenuate the increase of the levels of corticosterone in the plasma induced by stress (CUS-Sham vs CUS-Lesion: $p=0.02$) (Figure 2). There were no differences between the plasma corticosterone levels measured from 6 to 7p.m. (data not shown).

In the EPM, the experimental procedures induced an alteration in the time spent in open arms ($F=8.31$; $p=0.001$). CUS induced anxiety-like behaviour in non-lesioned animals when comparing with controls as revealed by the reduction of time spent in open arms ($p<0.01$) and by the reduced number of entries in the open arm ($F=7.89$; $p<0.001$). In contrast, in CeA lesioned animals, the same CUS protocol did not induce a significant decrease in the time spent in the open arms (Cont-Lesion vs CUS-Lesion: $p=0.164$); importantly, the comparison amongst CUS groups show a decreased anxiety-like behaviour with stressed-lesioned animals spending more time in open arms than stressed non-lesioned animals (CUS-Sham vs CUS-Lesion $p<0.048$). The comparison amongst control groups did not reveal an effect of the CeA lesion in this parameter ($p=0.44$), which demonstrates that the lesion on its own is not able to induce behaviour alterations in the EPM. Furthermore, there was no significant difference between groups in the number of explorations or the number of entrances in closed arms, showing that the animals presented similar exploratory/locomotor activity (Entrances: $F=0,513$, $p=0.68$; Explorations: $F=0.427$, $p=0.74$) (Figure 3).

In the acoustic startle test there is an overall increase in responsiveness to the stimuli with the experimental procedures ($F=3.99$, $p=0.02$). Despite a trend for an attenuation in the responsiveness to the startle in stressed animals injected with ibotenic acid (CUS-Lesion) when compared with CUS-Sham group, these difference did not reach statistical significance (at 120db: $p=0.16$). There was also no significant difference between both control groups (Figure 4). These results suggest that the lesion in the CeA may attenuate the stress effects in this reflex response.

We have also evaluated the fear-potentiated startle of these animals with the treatment inducing an alteration in fear-conditioning ($F=5.60$; $p=0.006$). The lesion, in both Control-Lesion and CUS-Lesion induced a disruption of the conditioning in relation with Control-Sham (Cont-Sham vs Cont-Lesion: $p<0.05$; Cont-Sham vs CUS-Lesion: $p<0.05$). CUS-Sham also did not present any significant alteration when compared with controls (Figure 5).

Locomotory activity was assessed with the open field test and no differences were found between control groups and animals submitted to CUS protocol. The animals with excitotoxic lesions in CeA also did not show any difference when compared with animals injected with vehicle ($F=1.07$, $p=0.37$). Furthermore there were no significant differences between groups in the number of rearings and in the time spent in rearing activity, indicating no alterations in exploratory activity ($F=1.87$, $p=0.46$). (Figure 6).

Discussion

Herein, we show for the first time that excitotoxic lesions in the CeA attenuate stress-induced anxiety behaviour and also attenuated the activation of the HPA axis. Previous studies have demonstrated that lesions in CeA affect the manifestation of fear-behaviour but not light-enhanced startle, a behaviour more associated with a display of anxiety (Davis et al., 1997; Walker & Davis, 2008). In fact, it is widely accepted that while amygdala is essential for the manifestation of fear-behaviour, the BNST is more determinant for anxiety-like behaviour (Walker et al., 2003; Hammack et al., 2004; Lee et al., 2008). It is important to note that the attenuation of the stress-induced anxiety in the CeA lesioned animals was observed under stress conditions but not in basal conditions. In order words, it demonstrates that the integrity of the CeA is required for the installation of the stress-induced hyperanxiety, but does not trigger anxiety behaviour per se. It also deserves to highlight that the attenuation of stress-induced anxiety is not complete, which suggests that other pathways are still conveying the changes in the neuronal networks that rule stress-induced anxiety behavior. This is not surprising if one considers that there are several factors contributing to the activity of anxiety circuits, namely at the level of the BNST. Of relevance, the anterior BNST receives a strong glutamatergic projection from the infralimbic cortex that will likely contribute to anxiety-like behavior (Sesack et al., 1989; Hurley et al., 1991, Massi et al., 2008).

In contrast, in the fear conditioned test we confirm that the integrity of the CeA is critical, as its lesion in non-stressed animals triggered itself an altered behavior. Moreover, and also in accordance with our previous reports (Pêgo et al., 2008), the CUS protocol did not induce an alteration in the fear-potentiated behavior. These facts demonstrate that the contribution of the main output of the amygdala (CeA) is quite distinct in these behaviors: while it is determinant to fear conditioning in non-stressed conditions, it only is required for the setting up of anxiety behavior when this is triggered by a complementary insult (in the present case, stress). This is not surprising when considering that the neuronal circuits involved in fear-potentiated startle critically depend on the projection of the CeA to the caudal pontine reticular nucleus (Davis et al., 2006), whereas for anxiety behavior, and for the control of HPA activity, the output of the CeA to the BNST represent only one of the possible modulators of its activity.

Given the topographical organization of the projections of the CeA, it is likely that an excitotoxic lesion in CeA will lead to a reduction of GABA and peptidergic inputs, particularly CRF, into the anterior BNST (Veinante et al., 1997; Day et al., 1999). Taking into account that the increase in CRF following chronic stress has a fundamental role in the activation of anterior BNST and consequent activation of the HPA axis (Silva et al., 2012), this might be one plausible explanation for the attenuation of the stress-induced anxiety behaviour in CeA lesioned animals. In further support of this hypothesis is the fact that a lesion in CeA did not affect anxiety-behaviour in baseline conditions, which is in line with previous observations by other labs (Moller et al., 1997; McHugh et al., 2004; Cai et al., 2012).

In summary, these findings contribute to better understand the role of the CeA in the pathogenesis of anxiety and fear behaviour and in the sense to know how its modulation might be of relevance for the control of emotional disturbances involving such behaviors but also for the control of HPA activity.

Abbreviations:

BNST – Bed Nucleus of the Stria Terminalis

CeA – Central Nucleus of the Amygdala

Cont - Control

CRF – Corticotrophin Releasing Factor

CUS – Chronic Unpredictable Stress

EPM – Elevated-Plus Maze

HPA – Hypothalamus-Pituitary-Adrenals

PBS – Phosphate buffer solution

PVN – Paraventricular Nucleus of the Hypothalamus

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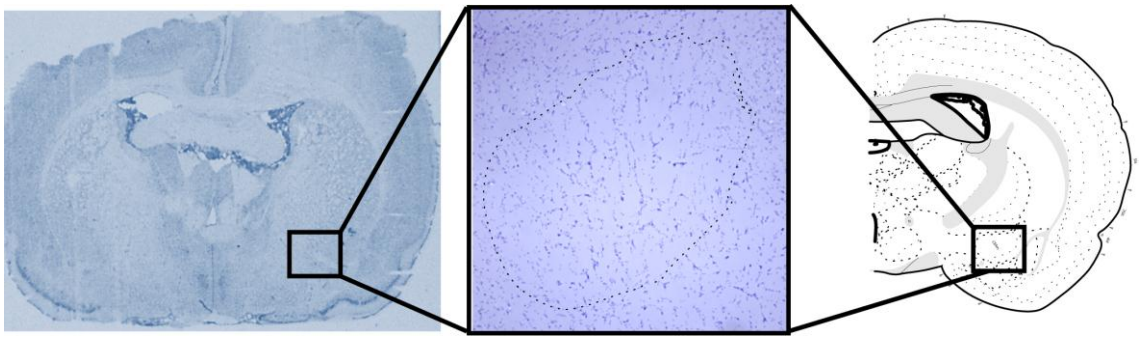

Figure 1. Representative picture of the placement of lesion in the CeA. Atlas section according to Swanson (1998).

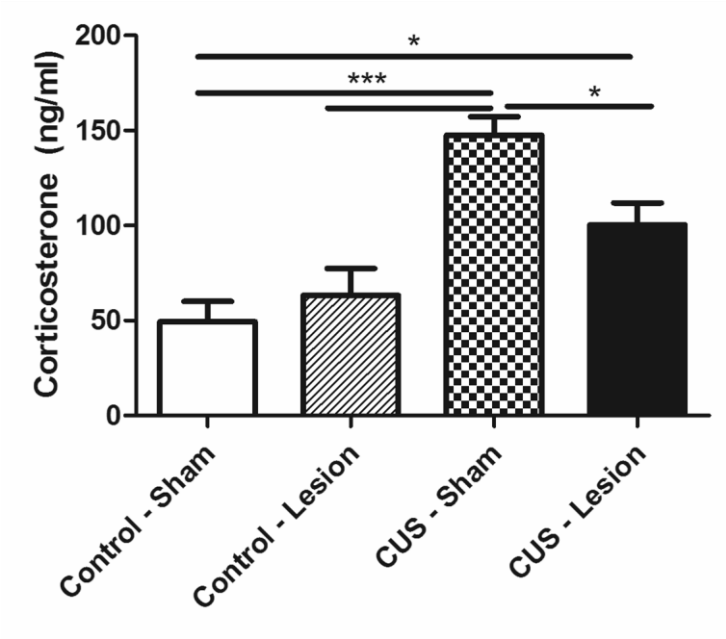

Figure 2. Corticosterone levels measured in the plasma of rats collected from 9 to 10 a.m. CUS induces an increase in corticosterone levels when comparing with controls, the lesion attenuates this increase in stressed animals. CUS- Chronic Unpredictable Stress; * $p<0.05$; *** $p<0.001$. Results are presented as Mean+SEM.

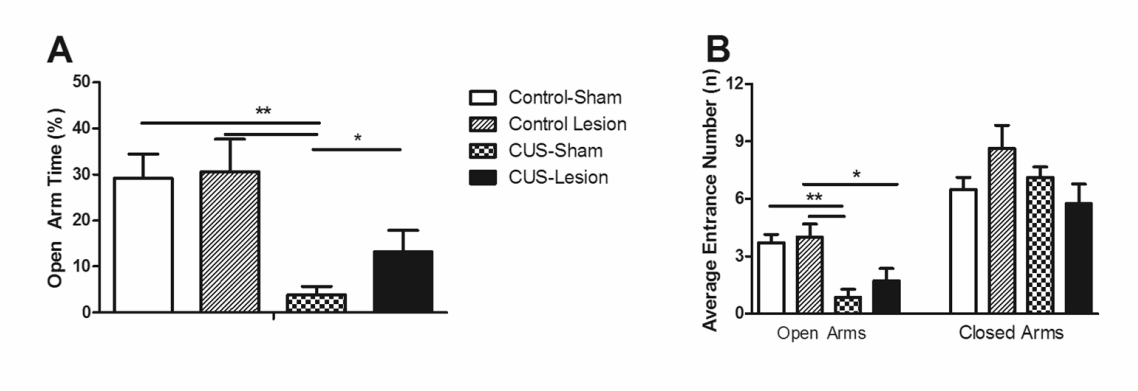

Figure 3. Anxiety-like behavior measured in the Elevated Plus-Maze. A. Percentage of time spent in open arms. B. Total number of entrances in the open and closed arms. CUS- Chronic Unpredictable Stress; * $p<0.05$; ** $p<0.01$. Results are presented as Mean+SEM.

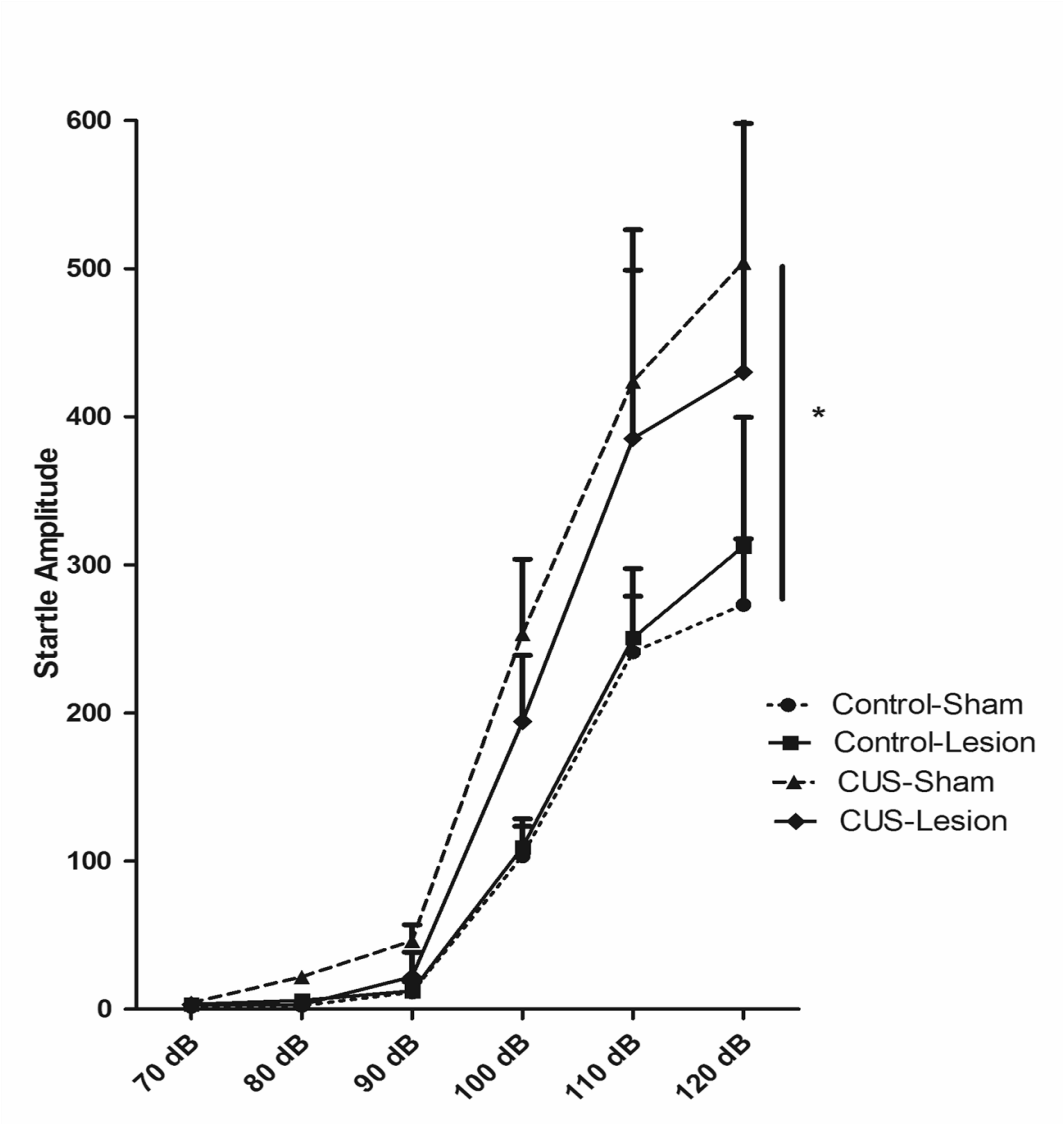

Figure 4. Acoustic Startle Data. Startle amplitude in response to an acoustic stimulus. CUS induces an increase in the startle when comparing with controls. CUS- Chronic Unpredictable Stress; *, $p<0.05$. Results are presented as mean ± SEM.

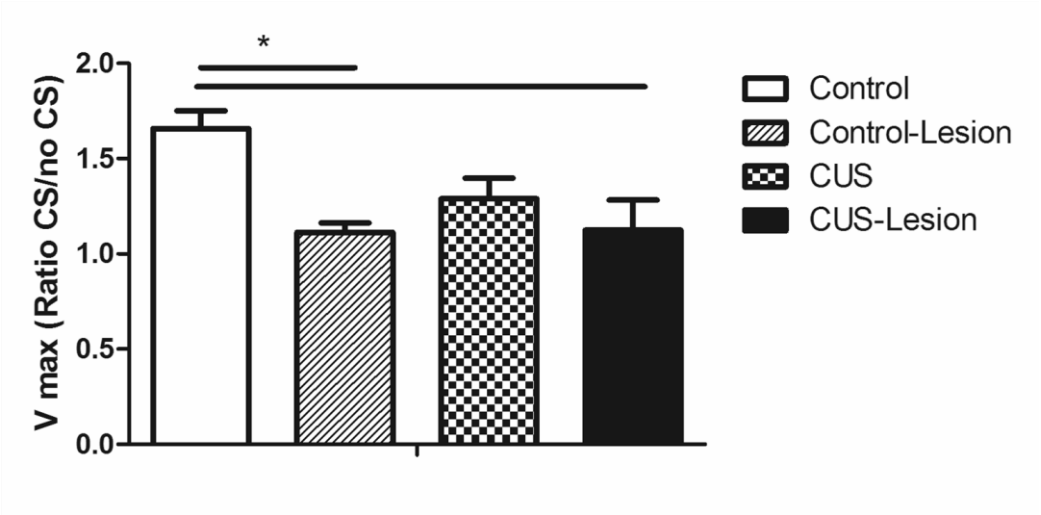

Figure 5. Fear-Potentiated Data. Startle amplitude in response to an acoustic stimulus. No statistical differences were found between groups. CS – Conditioned stimulus; CUS- chronic unpredictable stress rats. Results are presented as mean ± SEM.* $p<0.05$.

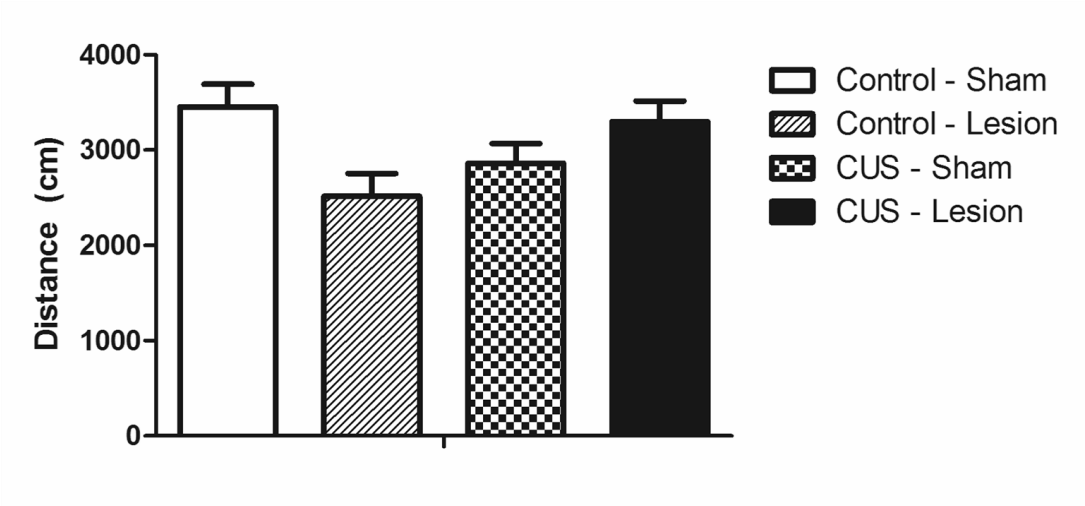

Figure 6. Open Field Test. Total distance ran in the open field test. No statistical differences were found between groups. CUS- Chronic Unpredictable Stress. Results are presented as Mean + SEM.

Table 1. Biometric markers revealed that the CUS protocol decreased body-weight gain and thymus weight. CUS caused slight increase in adrenal weight. Data presented as Mean ± SEM.

	Control-Sham	Control-Lesion	CUS-Sham	CUS-Lesion		Significance
Body weight gain (g)	98.2±2.5	106.2±2.4	83.8 ± 3.6	90.3 ± 2.9	t= 3.48	P< 0.05
Thymus weight (gr/BW)	0.46±0.02	0.47±0.01	0.37 ± 0.01	0.38 ± 0.01	t= 4.45	P< 0.110
Adrenal weight (gr/BW)	0.044±0.01	0.046±0.01	0.52 ± 0.01	0.48 ± 0.01	t= 1.35	P< 0.32

Chapter 3

Discussion

Discussion

Throughout this Thesis we have unraveled new insights in the role of the BNST in the modulation of stress-related anxiety using a series of behavioral, molecular and electrophysiological techniques. In order to reach our goals, we used chronic unpredictable stress as an animal model of anxiety which allowed us to mimic the alterations that this disorder induces at the central nervous system level. We confirmed that this animal model is able to induce behavioral alterations in rats, particularly by inducing anxiety-behavior as measured in the elevated-plus maze and the acoustic startle (Pêgo et al., 2008). The behavioral alterations induced by stress were correlated with a series of alterations at a structural, molecular and electrophysiological level in a brain region that has been shown to be relevant for anxiety-behavior, the BNST. Specifically, exposure to CUS in rodents induces increased spine density and hypertrophy of dendrites in the anterolateral BNST, a division of this region that is important for the activation of the HPA axis (Pêgo et al., 2008; Choi et al., 2007; Choi et al., 2008). Additionally, we observed that exposure to chronic stress or to an anxiogenic stimulus is able to induce a differential pattern of c-fos activation throughout the BNST. In summary, we saw that a stressful stimuli was able to induce increased activation in subnuclei localized in the anterior division of the BNST while in opposition, decreased the activation of nuclei in the posterior division. The complexity of the BNST and its subnuclei, both in biological roles and in the different way they can be affected by stress lead us to search for the molecular fingerprinting of stress in individual subnuclei of the BNST and for that, we performed a molecular analysis of the BNST conjugated with laser capture microdissection.. With the help of this technique, we were able to show that, in general, stress induces a decrease of CRFR1 expression in nuclei located in the anterior division (dorsomedial and fusiform nuclei) while increasing CRFR2 in a nucleus located in the posterior division (principal nucleus). At the same time we saw alterations in both glutamatergic and GABAergic systems with an increase of NR2B in principal nucleus and increase of GABAA receptor in the dorsomedial and principal nucleus, respectively. Due to the role of the anterior division of the BNST in the activation of the HPA axis, therefore contributing to the stress response, and together with the alterations we observed at a structural and molecular level, we focused on this division of the BNST to perform an electrophysiological analysis. For that, we placed stimulating electrodes in the Infralimbic Cortex (ILCx) and Central Amygdala (CeA), two brain

regions that are important in the modulation of the stress response and that have strong projections to the anterior BNST. By stimulating, either the ILCx or CeA while we performed recordings of the activity of neurons in the anterior BNST, we were able to see that in basal conditions ILCx is able to drive the excitation of BNST neurons by increasing their activity, a response that is increased by chronic stress exposure. On the other hand, in control animals, there is a strong inhibition of the activity of neurons of the BNST after CeA stimulation, inhibition that is decreased after stress exposure. Together, these findings show us that, stress is able to induce a hyperactivation of neurons of the anterior BNST and impair putative inhibitory mechanisms.

Finally, to better understand the role of the strong connection between CeA and the BNST in the development of stress-related anxiety, we performed excitotoxic lesions in CeA of animals that were later submitted to chronic stress. We were able to observe that lesions in CeA are able to partially reduce the effects of stress in the expression of anxiety-behavior.

Animal Model of Anxiety

Throughout this work we have aimed to further understand the expression of anxiety-behavior in the brain, and particularly in the BNST. For this, it was essential to use an animal model that allowed us to induce an anxiety phenotype in rodents.

The use of stress exposure as inducer of anxiety is well characterized but there is not an unique model; several animal models have been used by different authors with different characteristics. The differences in animal models can be found at several levels, particularly: duration of treatment (acute or chronic), type of stressor, variability of stressor (using only one stressor or using a series of several stressors). In this work, we have opted to use a chronic unpredictable model of stress where the animals were exposed to a different stressor every day. The use of a chronic model induces a sustained increase of corticosteroids due to hyperactivity of the HPA axis (Cerqueira et al., 2007; Pêgo et al., 2008) while an acute model induces a transient increase in circulating corticosteroids aimed to restore the homeostasis of the organism. The sustained increase in corticosteroids is responsible for several of the

deleterious effects of stress in the brain, and for that, the use of a chronic model presents advantages when trying to assess the full effects of stress exposure in the brain.

The induction of chronic stress in rodents is followed by changes in several biological markers such as decreased body weight gain, deterioration in fur condition, increased adrenal glands weight or presence of gastric ulcers. The use of a longer model of chronic stress (4 weeks) allowed us to observe the presence of these markers of stress since, from our observations, the phenotypically markers of stress do not fully develop until 2 or 3 weeks after of stress exposure. In fact, previous studies using a shorter model of chronic stress (2 weeks) failed to observe the development of a chronic stress phenotype (Vyas, et al., 2002; Vyas et al., 2003).

Together with the use of a chronic model of stress, the use of an unpredictable model also contributes to the persistent increase in circulating corticosteroids. The application of a single stressor or the application of several stressors in the same order has been shown to fail to induce a sustained stress phenotype (Gamaro et al., 1998, Rivat et al., 2010). This is a consequence of the adaptation of the animal to the stressor, resulting in a normalization of the HPA axis activity (Gadek-Michalska et al., 2003). The application of stressors in an unpredictable order and time of the day induces a state of permanent alertness that prevents the habituation of the animal and also prevents the development of coping mechanisms leading to a continuous activation of the HPA axis and a permanent increase in corticosteroids secretion.

The use of a chronic and unpredictable model of stress also presents clinical relevance. In fact, in humans, the development of anxiety disorders if often associated with a chronic exposure to anxiogenic stimuli (generalized anxiety disorder) or with a late onset of the condition after the exposure to the anxiogenic situation (PTSD). At the same time, patients with an anxiety disorder show a lower threshold of alertness reacting to daily events that will not trigger an anxiety response in healthy individuals.

In the present work, we have, therefore, chosen to use chronic unpredictable stress as a rodent model of anxiety. In chapter 2.2, CUS was used in order to assess the molecular correlates of stress-induced anxiety within the BNST, in chapter 2.3 to assess the influence of

ILCx and CeA in the electrophysiological activity of BNST neurons. Finally, in chapter 2.4, the use of this stress model was used to explore how a lesion in central amygdala affects the development of stress-induced anxiety.

Molecular signature of stress in the BNST

There are several molecules that play an important role in the mediation of the stress response. In particular, two different hormones are considered the major molecules involved in the stress response; corticosteroids and CRF. The BNST possesses receptors for both these families of molecules and the two are essential components of the stress modulation. Corticosteroids (cortisol in humans and corticosteroids in rodents) can act through two different receptors, mineralocorticoid (MR) and glucocorticoid receptors (GR), which present different affinity for the ligand. While MRs are only present in some brain regions, particularly the hippocampus, cortical areas and septal neurons, GRs are distributed ubiquitously throughout the brain (Reul & de Kloet, 1985; Kwak, 1993). As a result of the different affinity, MRs are occupied in basal conditions while GRs, that present a lower affinity for corticosteroids, are activated when there is an increase in the levels of circulating corticosteroids, as in the case of exposure to a stressful stimuli (de Kloet, 2000). Similarly to corticosteroids, CRF and related peptides (urocortin 1, 2 and 3) can act through two different receptors, CRFR1 and CRFR2. While CRFR1 is more widely distributed, CRFR2 expression is more restricted to limbic structures. These two receptors also present different affinities for their ligands; CRF binds almost exclusively to CRFR1 while urocortin 2 and 3 bind with higher affinity to CRFR2 and urocortin 1 binds with similar affinity to both receptors. Among the different outcomes of exposure to stressful stimuli, CRF strikes out as playing a very important role in the expression of anxiety by being able to contribute to its development through the activation of CRFR1. Therefore, in this Thesis we have tried to further understand the role of the CRFergic system in stress-related anxiety.

Nevertheless, it is important to not forget that the complexity of the stress response involves several other neurotransmitters, and that the BNST, besides receiving a strong CRF projection from CeA, also receives important GABA and glutamatergic inputs from limbic structures, namely CeA and medial amygdala for GABA and prefrontal cortex and hippocampus for glutamate. In order to unravel a molecular signature of stress and assessing the alterations

induced in these three (CRF, GABA and glutamate) neurotransmitter systems in the BNST we have set up the experimental design described in chapter 2.2.

As mentioned before, the BNST, albeit relatively small, presents an elevated number of subnuclei as divided by Swanson (1998). Importantly, the different nuclei have different biological roles and characteristics (Choi et al., 2007; 2008) and, therefore, it is important to consider the potential impact of stress on specific variations between nuclei when performing a study of the BNST. The differences between the BNST subnuclei in the stress response can also be seen at the activation level of each subnuclei after stress and after an anxiogenic stimuli (chapter 2.2). While nuclei that are in the anterior division as the dorsomedial nuclei show an increased activation (measured by the number of cells marked with c-Fos), nuclei that are in the posterior division (posterior and interfascicular nuclei) show a reduced activation after stress.

As the BNST subnuclei show differences between them, either in their biological role (Choi et al, 2007) or their different patterns of activation, it becomes critical to use laser capture microdissection to perform an individual analysis of individual subnuclei of the BNST instead of analyzing the BNST as a whole. For this analysis, we focused on three subnuclei that showed the most relevant pattern of activation/deactivation after stress exposure (dorsomedial and fusiform nuclei/principal nucleus) and that play a role in the regulation of the HPA axis.

We started by assessing how the CRFergic system within the BNST was affected by stress. The role of CRF in the modulation of stress is well known and particularly in the development of anxiety behavior. CRF is widely expression in several stress related areas, with the ones more relevant for this work being the CeA, BNST and PVN. Several authors have also reported that central CRF injections are able to induce a phenotype similar to stress (Sherman and Kalin, 1988; de Pedro et al., 1993; Liang et al., 1992). Therefore, it was not surprising to find changes in the expression of CRF and CRF receptors in our animal model. We have found alterations in mRNA levels of CRF in both dorsomedial and fusiform nuclei, although in opposite directions; while there is an increase in CRF in BNSTdm, there is a decrease in BNSTfu. The dorsomedial nucleus sends CRF projections to several limbic structures (Phelix and Paul, 1990) as the hypothalamus, CeA, PFC, therefore the increase in CRF we saw in this

structure is a likely contributor to the observed anxious phenotype we observed in chapter 2.2. In addition, the increase in CRF inputs from this nucleus to the PVN in the hypothalamus will likely contribute to the activation of CRFergic neurons in the PVN that will result in an activation of the HPA axis.

Despite the difference in the expression level of this neuropeptide, both the dorsomedial and fusiform nuclei presented a similar pattern of the expression of CRFR1 and CRFR2 (the latter being expressed under detectable levels in both nuclei). We observed a decreased expression of CRFR1 in both these nuclei while we did not observe any alteration in the levels of this receptor in the principal nucleus. It is known that CRFR1 presents an elevated affinity for CRF (while in contrast CRFR2 has a very low affinity for this ligand) and we believe that the decrease in CRFR1 can be a direct consequence of increased levels of CRF projections from CeA to the BNST. In fact, previous studies have shown that increased availability of the ligand leads to a decrease expression of the receptor showing that CRFR1 expression may also be mediated by a negative feedback mechanism (Pozzoli et al., 1996). Interestingly, the administration of a synthetic corticosteroid (dexamethasone) is also able to decrease the expression of CRFR1 in the hypothalamus corticosteroids (Zhou et al., 1996) suggesting that the decrease in CRFR1 expression could also be a result of increased levels of circulating corticosteroids. Neverthless, as we only observed a decrease in the expression of CRFR1 in two of the three subnuclei analyzed, it is more likely that the cause of altered expression of the receptor is an increase in the availability of the ligand and not only an effect mediated by corticosteroids.

As a consequence of the higher affinity of CRF for CRFR1, the increase availability of CRF after stress exposure will act mostly through these receptors contributing to the increased anxiogenesis. Interestingly, the decrease in the expression of CRFR1 was confined to the anterior level of the BNST and not at the posterior level. As the anterior division receives important projections from the CeA while amygdalar projections to the posterior division come mostly from the medial amygdala (Dong et al., 2001), we hypothesize that an increase in CRF expression in CeA is the responsible for the alterations in CRFR1 expression in the BNST and a key player in the manifestation of anxiety behavior in stressed animals. A direct effect of CRFR1 activation is likely to induce alterations in synaptic plasticity induced by stress. In fact, Pêgo and colleagues (2008) showed that stress induces an increased number of synapses in

the anterolateral BNST and that the injection of CRFR1 agonists was able to mimic this effect (Pêgo et al., unpublished data). We hypothesize that these increases in spine number will likely result in the increased neuronal activity that we assessed in chapter 2.3. Additionally, other authors report negative effects of CRFR1 occupancy in dendritic arborization in other brain areas as the hippocampus (Ivy et al., 2010) or locus coeruleus (Cibelli et al., 2001).

In contrast, the principal nucleus is subject to an increase in the expression of CRFR2 following stress. It is important to note that, CRFR1 is more predominant in the anterior BNST, whereas CRFR2 is mostly presented in the posterior BNST (Chalmers et al., 1995; Van Pett et al., 2000). While CRFR1 has been implicated in anxiety behavior (Smith, 1998), data from several authors (Bale et al., 2000, Coste et al., 2000; Pelleymounter et al., 2002; Pelleymounter et al., 2004) report that CRFR2 role seems to be associated with a decrease in . Our results seem to contribute to this dual action of each CRF receptor, with the type 2 receptor being overexpressed in a nucleus (principal) that is more associated with the termination of the stress response (Choi et al., 2007), suggesting that this overexpression can be a consequence of a compensatory mechanism resulting from the long stress exposure.

As mentioned before, CRFR2 presents a low affinity for CRF and in turn, presents higher affinity for urocortin 3. Interestingly, previous data from our lab showed that while intraventricular injections of CRF and urocortin 1 (a mixed ligand of both CRF receptors) were able to induce anxiety-like behavior, the injection of urocortin 3 failed to induce behavioral alterations. Other authors also report increased CRFR2 in models of anxiety, particularly, in a mice model of PTSD there is a strong increase in CRFR2 expression in the BNST (Lebow et al., 2012).

As we mentioned before, apart from looking at the CRFergic system, we have also looked into the expression of receptors for two of the main neurotransmitters in the brain, glutamate and GABA, and that are also important components of the stress response.

The BNST receives strong projections from highly glutamatergic areas. In particular, the anterior BNST receives projections from the ILCx and the posterior BNST from the ventral hippocampus (Sesack et al., 1989). Of all glutamate receptors, NR2B in particular, seem to be essential in the central nervous system response to stress and for the expression of anxiety behavior (Kash et al., 2009). We were not able to detect the expression of NR2B in the

BNSTdm and BNSTfu due to the expression being bellow detection levels but we observed an increase in the expression of this receptor in the principal nucleus. We hypothesize that the increase in NR2B may be a consequence of decreased glutamatergic inputs from the ventral hippocampus and an attempt to balance the decreased activation seen with cfos immunoreactivity. As this nucleus seems to have an impaired activity in stressed animals this will contribute to a deregulation of the HPA activity, in particular by the lack of inhibitory inputs from the BNSTpr to the PVN, helping to facilitate the increased HPA activity.

The role of GABAA receptors in the expression of anxiety is well known since some of the major anxiolytics drugs are agonists for GABA receptors (benzodiazepines). We observed two changes at the GABAergic level. First an increase in GABAA receptors in both the dorsomedial and principal nucleus, which may be a consequence of a potential reduction in GABA levels as a result of the stress exposure, and result in an anxious phenotype (Sajdyk et al., 2008). Secondly, we observed an alteration in the ratio between the expression of GAD2 and GAD1 with an increased in the expression of GAD2 when comparing with GAD1 in the dorsomedial nuclei. These results are in accordance with previous reports by Herman and colleagues (2001) that showed that, at a protein level, acute stress shifts the expression towards GAD2.

It is also important to consider that the alterations in one neurotransmitter have implications on the activity of the others; recently several reports have been pointing to the role of CRF in the modulation of glutamatergic inputs in (Kash et al., 2009; Nobis et al., 2011) and also of GABAergic inputs (Kirby et al., 2008). Therefore, the altered expression of CRF and its receptors is likely to also affect the transmission of the other neurotransmitters analyzed.

Functional Correlates of Stress-Induced Anxiety

After dissecting the morphological (Pêgo et al., 2008) and molecular correlates (chapter 2.2) of stress-induced anxiety in the BNST, we have searched for a functional alteration in this region after stress (chapter 2.3). With the help of single-cell recording electrophysiology we were able to record the activity of neurons located in the anterior BNST while stimulating the ILCx or the CeA in control and stressed animals. This technique, allowed us to have a more

integrative perspective on the neuronal circuits underlying anxiety, being an important tool to further understand how the BNST activity can be modulated by upstream inputs.

As mentioned above, the anterior BNST is a key region for the activation of the HPA axis that receives strong projections from CeA and ILCx and for this we wanted to assess the contribution of each of these regions to the neuronal activity of BNST neurons. While little was known about the influence of the stimulation of CeA in BNST neuronal activity, previous work showed that ILCx stimulation is able to drive an hyperactivation of BNST neurons (Massi et al., 2008). In chapter 2.3, we were able to confirm these results, showing that the electrical stimulation of ILCx induces a short latency excitatory response in a high percentage of the neurons recorded (83%) that is mediated by glutamate receptors (Massi et al., 2008). Interestingly, after the initial excitatory response, around 29% of neurons recorded in control animals presented a decrease in their activity. The exposure of animals to a chronic stress paradigm induces an even bigger increase in the activity of BNST neurons by increasing the response magnitude of the excitatory response while decreasing the duration of the inhibition. The increase in excitatory activity can be a consequence of a potential increase in glutamatergic transmission into the anterior BNST. Though this may seem contradictory with our LMD data it is also important to notice that glutamatergic inputs to the principal nucleus (posterior BNST) arise from hippocampal inputs while inputs to the anterior BNST derive from PFC. It is also known that the deleterious effects of stress progress from hippocampus to the PFC and lead to decreased hippocampal efferent activity that unleashes the glutamatergic outputs of PFC (Cerqueira et al., 2007a). Interestingly, NMDAR in the BNST play a role in the development of long term plasticity (LTP) seen in this structure (Weitlauf et al., 2004) which lead us to hypothesize that an increased glutamatergic transmission after stress is important for the alterations in synaptic plasticity in the BNST. Of relevance, synaptic plasticity in the amygdala is important for the development of anxiety (Bauer et LeDoux., 2004) making more likely that LTP or LTD phenomenon in the BNST also contribute to the manifestation of this disorder.

Previous studies (Massi et al., 2008) were not able to identify the nature of the inhibitory response; injection of picrotoxin (a GABA receptor agonist) was unable to alter the inhibitory response. Further studies should be made in order to identify the cause of the inhibition, potentially mediated by another inhibitory neurotransmitter as glycine. This response may also

be the result of an indirect projection from the ILCx to another area that is also able to modulate the activity of the BNST. One such potential pathway is mediated via the amygdala since it is known that the PFC projects to the amygdala (Likhtik et al., 2005). It is reasonable to believe that this indirect pathway may be a parallel regulatory circuitry that modulates the influence of PFC over the BNST through inhibitory neurons namely GABA or other neurotransmitters. Additionally, it should not be discarded a potential role of CRF-related peptides in the modulation of this response as several reports show that CRF receptors are able to modulate glutamatergic transmission. CRFR1 localized post-synaptically, when activated, is able to facilitate glutamate release and increase in excitatory transmission while the presence of CRFR2 may have the opposite effect (Liu et al., 2004). This fits our current model since we observed decreased CRFR1 expression and increased CRFR2 expression in the BNST.

In parallel, the alterations observed after CeA stimulation also contribute to an increased activation of the BNST. In control animals, CeA stimulation induces a strong inhibition of the neuronal activity in the BNST; 83% of neurons recorded presented a long inhibition (68,3ms) after the stimuli. The inhibitory influence of CeA is largely decreased after stress, albeit a similar percentage of neurons present an inhibition of their activity, the duration of this response was reduced to 23,7ms. Using double-barrel micropipettes to perform pharmacological manipulation of BNST neurons while recording them, we were able to identify this inhibitory response as being mediated by GABAA receptors since the injection of picrotoxin was able to block the inhibition induced by CeA stimulation. Of relevance, we can associate this decreased inhibition in stressed animals with an increase in GABAA receptors in the BNST subnuclei following stress exposure. Even if the results are apparently discordant, the increase in GABAA receptor expression may be a direct consequence of decreased GABAergic innervation into the BNST. As mentioned before, exposure to stress is able to induce an increase in CRF expression in several brain regions (Sutherland et al., 2010) and it is also known that CeA neurons, despite being mostly GABAergic present co-expression of GABA and CRF (Day et al., 1999, Veinante et al., 1997). Of relevance, it was shown before that an immobilization stressor is able to induce c-Fos activation of amygdalar neurons that co-express CRF (Gray et al., 2010).We propose that chronic stress exposure shifts the expression of these neurotransmitters in CeA neurons by favoring the expression of CRF and decreasing the levels

of GABA. This would have the direct result of reducing the GABAergic inhibitory inputs from the CeA to the BNST that we observed with our electrophysiological experiments. As we are observing a picture of the BNST after 28 days of chronic stress exposure, we propose that the increased in GABAA receptor expression is the result of a long lasting up-regulation of receptors in an attempt to balance the system by providing more receptors due to the lack of ligand available. Coherently, it has been described that stress is able to reduce GABAergic transmission in different brain areas (Verkuyl et al., 2004; Biggio et al., 1990; Martisova etal., 2012).

Similarly to ILCx, it was often possible to observe a 2-stage response following CeA stimulation: after the initial inhibitory response, 46 (control) to 63% (CUS) of neurons presented an increased activity. Stress was able to further increase the activity of the neurons from a 15% increase comparing to the baseline in controls to 23% increase in activity for stressed animals. The kinetics of the increased in excitation after CeA expression are relevant when attempting to understand the cause for this response. A direct effect of a neurotransmitter happens in a much shorter time frame, while we see a late excitatory response; this can be the result of a polysynaptic mechanism or the consequence of a slower modulatory mechanism mediated by G-protein coupled receptors. CeA is the major output region of the amygdala and its projections include areas that also project to the BNST as the PFC (Sah et al., 2003) and for that the excitatory response we see may be an indirect result of CeA stimulation of other areas. Of relevance, CRF interaction with the dopaminergic system is able to modulate the excitatory synaptic transmission from the basolateral amygdala to PFC (Orozco-Cabal et al., 2008; Gallagher et al., 2009). On the other hand, consistent with the observations of interactions of the CRF system with other neurotransmitters, we hypothesize that CRF may not have a direct effect in the electrophysiological transmission but instead is acting as a neuromodulator of synaptic transmission. To test this hypothesis we injected antalarmin, a CRFR1 antagonist, but we were unable to show any difference in the activation response in stressed animals. However disappointing we feel that further studies should be made to determine the nature of this response and to confirm, or not, whether CRF is one of the components involved in this modulation. For this, a new dose of antalarmin should be tested as well as performing injections of different antagonists and agonists of CRFR1 and CRFR2.

As the modulation of the activity of the BNST is a complex process, we should not discard the idea that other neuropeptides expressed in the CeA may also present a role in the modulation of the influence this region has in BNST neurons. In fact, the amygdala is a rich source of several peptides that play a role in the modulation of several aspects of the stress response.In particular, recent studies have shown that NPY, a neuropeptide that seems to have a protector effect on the stress response (Heilig et al., 1995; Sadjyk et al., 1999) and is expressed in the amygdala (Tasan et al., 2010) is able to mediate GABAergic transmission in the BNST (Kash and Winder, 2006; Pleil et al., 2012). Other amygdalar neuropeptide, dynorphin, is also able to mediate the activity of BNST neurons with the activation of a receptor for this peptide being able to reduce GABAergic transmission in the BNST and specifically, in the CeA to BNST pathway (Li et al., 2012). Interestingly, recent evidences point to an interaction between CRF and dynorphin in the modulation of anxiety behavior (Bruchas et al., 2009).

Other neuromodulators that may be involved in this response are endocannabinoids since it is known that endocannabinoids are able to mediate the glutamatergic transmission in the BNST upon ILCx stimulation (Massi et al., 2008) and they are also important for the modulation of the HPA axis (Gorzalka et al., 2009). Additionally, the endocannabinoid system has strong links with the CRFergic system with antagonists for CB1 receptors being able to reverse the anxiolytic effect of central CRF lesions (Brown et al., 2012) while the activation of CB1 is able to increase the CRF-induced release of ACTH at the pituitary (Pagotto et al., 2001). This evidences point us to a likely role of CB1 receptors in the modulation of BNST activity following chronic stress, with the activation of these receptors being one additional factor contributing to the increased activity of the HPA axis.

Further studies should be made in order to fully clarify the role these molecules may have in the modulation of BNST activity. Interestingly, we should note that CRF seems to be a common link in the modulation of the effects of several different molecules, once more pointing to a very important role of CRF in the development of stress-related anxiety.

Interestingly, the anterior BNST, where the recordings were made, suffers morphological alterations induced by stress (Pêgo et al., 2008). In particular, this region shows an increase in dendritic length and increase in the number of spines, which can lead to an increased number of synaptic contacts, contributing to the increased excitability of neurons in this area. As mentioned before, the global increase in CRF induced by stress and activation of CRFR1 is able to mimic the effects of stress in spine sprouting in the anterolateral BNST. Therefore, we

suggest that one of the indirect contributors to the increased synaptic transmission in the BNST is CRF, by increasing the number of synaptic contacts. Our electrophysiological data indicating an increased activity of the anterior BNST neurons also correlates with the increase in the activation of subnuclei in the anterior BNST that we observed by the presence of c-Fos.

One key aspect that stems from the present dissertation is that, rather than the study of excitatory or inhibitory influences over the BNST it is of relevance to understand the balance between excitation and inhibition occurring in this brain region during the maladaptive response to stress. For both ILCx and CeA inputs, we see that stress is inducing an increase in excitation while decreasing the inhibition. This can provide an important insight for therapeutical strategies applied to anxiety disorders, as the treatment should not focus exclusively in controlling the levels of excitatory or inhibitory neurotransmitters but instead in attempting to restore the balance between excitation and inhibition.

The role of the Central Amygdala in the development of stress-induced anxiety

One aspect that became more clear with the work done so far in this Thesis, is that the connection between CeA and the BNST is one of the most important in the development of anxiety since CeA is the major source of CRF inputs to the BNST and its stimulation is also able to significantly alter the activity of BNST neurons. Due to this, we next wanted to have a better insight on the strong interconnectivity between CeA and the BNST and the role of amygdalar CRF inputs in the development of stress-induced anxiety. For that, in chapter 2.4, we performed an excitotoxic lesion of CeA and after submitting the animals to a chronic stress protocol we assessed their anxiety-like behavior. The goal in this chapter was then, to understand how the absence of projections from CeA to the BNST affects the development of anxiety, providing an insight into the role of GABA, CRF and potentially other neuropeptides in the induction of this disorder by stress. CeA lesions should prevent important projections for the stress response to arrive in the BNST. Importantly, there is an increase in CRF expression in CeA after stress, that is, likely, essential for the activation of the anterior BNST during the stress response With a lesion in CeA, this projection should be abolished, resulting in a decreased activation of the BNST and in turn, decreased expression of anxiety-behavior.

We were able to observe that a lesion in CeA is able to promote a reduction of the weight loss, one of the biomarkers of a chronic stress phenotype and at the same time reduces the increase in adrenal weight of lesioned stress animals when comparing with a sham-stress group. The lesion in CeA was also able to attenuate the increased levels of plasma corticosteroids in stressed animals. These findings suggest that CeA lesions seem to have an effect in ameliorate the phenotype-induced by stress. We were also able to see a similar tendency in the anxiety-behavior measured at the end of the stress protocol, with animals that were lesioned in the CeA showing a rescue of anxiety-behavior measured in the EPM and with a similar tendency in the acoustic startle test.

Nevertheless, we do not see a full recovery of the anxiety phenotype since there are several other factors to consider in this complex behavior. Even if CeA is one of the main sources of inputs into the BNST, one cannot disregard other inputs, namely from the ILCx, that, as seen in chapter 2.3 has an important contribution to a hyperactivation of the anterior BNST after chronic stress exposure.

Together, our findings showed that the BNST is significantly affected by exposure to chronic stress at several different levels, structural as it was shown by previous work from our lab (Pêgo et al., 2008) but also molecular (chapter 2.2) and functional (chapter 2.3). We were also able to identify alterations in several systems important for the stress response, namely, CRF, GABA and glutamatergic systems, leading us to conclude that there are strong alterations in these types of inputs received by the BNST during stress. This showed us that the stress network consisting of the PFC, amygdala, hippocampus and the BNST is severely impacted by chronic stress exposure. We have seen that, in particular, ILCx and CeA stimulation is able to differentially modulate neuronal activity in the anterior BNST in basal and stressful conditions. Additionally, CeA inputs seem to be essential for the expression of stress-related anxiety as a lesion in this area is able to attenuate the impact stress has in the development of anxiety-behavior. We believe that our findings provide an important additional knowledge to the important role of the BNST in the modulation of stress-related anxiety.

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Chapter 4

Conclusion and Future Perspectives

Conclusion and Future Perspectives

During the course of this work we have explored the development of stress-induced anxiety, focusing on the molecular and functional markers of the BNST after chronic stress exposure. Additionally, we assessed the importance of the projections from CeA to the BNST, both in electrophysiological terms and also in the development of an anxious phenotype induced by stress. In summary, we were able to conclude that:

1) Chronic Unpredictable Stress exposure is able to alter the molecular signature of specific subnuclei of the BNST that can be correlated with altered pattern of C-Fos expression. (chapter 2.2)

2) Stress induces decrease in the expression of a receptor associated with angiogenesis (CRFR1) in nuclei (dorsomedial and fusiform) involved in the activation of the stress response while increasing receptors associated with anxiolytic role in a nucleus (principal) involved in the termination of the stress response. (chapter 2.2)

3) In control animals, CeA drives a large inhibition of anterior BNST neurons that is mediated by GABA receptors. (chapter 2.3)

4) Stress induces an hyperactivation of anterior BNST neurons by increasing the excitatory inputs from ILCx while decreasing the inhibition induced by CeA. (chapter 2.3)

5) A lesion in CeA is able to partially reduce the anxious-like behavior induced by chronic stress exposure. (chapter 2.4)

We were able to provide further insights on the network between limbic structures (particularly ILCx and CeA) and the BNST and its role in the modulation of the stress response. However, new questions arise from our observations that should be explored in order to better characterize the development of anxiety-behavior by chronic stress exposure.

1) It remains unclear what is the exact contribution of amygdalar neuropeptides to the electrophysiological alterations in the BNST induced by stress. A more complete pharmacological manipulation of the BNST should be done in an attempt to completely dissect the mechanism responsible for the response of BNST neurons.

2) Together with point 1), a specific lesion of CRF neurons in CeA or a knockdown of the expression of this neuropeptide before submitting animals to chronic stress will be able to provide a better insight of the influence of this peptide in the modulation of BNST neurons and on the development of stress-induced anxiety.

3) We have always looked at a endpoint of 4 weeks of stress. It would be important to try to observe the presence of the stress signature we have unraveled in earlier timepoints to understand what can be a cause or a consequence of the anxious phenotype.

4) Finally, it is fundamental to understand if it is possible to alter the observed changes by the administration of drugs with potential anxiolytic effect (and specifically benzodiazepines or CRFR antagonists).