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Ana Rita Fernandes Marques

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Dissertação de Mestrado
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The important thing is not to stop questioning.

Albert Einstein

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Abstract

Anxiety disorders affect a large portion of the world population and are frequently long-lasting and debilitating. Chronic stress is highly implicated in the etiology of these disorders, leading to a hypothalamic–pituitary–adrenal (HPA) axis dysfunction and structural changes in the brain. The bed nucleus of the stria terminalis (BNST) is directly involved in the regulation of the stress response, acting as a relay nucleus between the limbic structures and the paraventricular nucleus of the hypothalamus (PVN). The mechanisms by which chronic stress alters central circuits that mediate anxiety-like behavior are still largely unknown. Recent studies have implicated the medial prefrontal cortex (mPFC), together with BNST, in the genesis of anxiety-like behaviors. Taken this into account, the present work attempted to explore the influence of chronic stress upon a specific brain circuitry involved in the stress response: PFC-BNST-PVN. For this purpose, we recorded the neuronal activity of the BNST dorsomedial/fusiform (dm/fu) subnuclei with or without a chemical manipulation of the infralimbic region of the mPFC (ILCx) in both control and stressed rats. Subsequently, the HPA axis activity was assessed by measuring the c-Fos expression in the PVN and the levels of corticosteroids in the blood after an injection of glutamate in the ILCx. The inhibition of the ILCx with lidocaine did not affect the BNST neuronal activity. By contrast, the stimulation of the ILCx with glutamate decreased the noxious-evoked activity of BNST cells in control animals, but in CUS animals this effect was attenuated. Additionally, the pharmacological stimulation of ILCx enhanced the PVN activation pattern in CUS animals and induced the release of corticosterone only in control animals. Remarkably, the present results suggest that the ILCx has a phasic excitatory influence over the HPA axis and this effect seems to be mediated by the BNST. However, this pathway appears to be impaired in chronic stress conditions.

Alterações induzidas pelo stress crónico *in vivo* nas propriedades fisiológicas dos neurónios envolvidos em circuitos cerebrais mediadores do stress

Resumo

Os transtornos de ansiedade afectam uma grande parte da população mundial e são frequentemente duradouros e debilitantes. O *stress* crónico está muito implicado na etiologia desses transtornos, levando a uma disfunção do eixo hipotálamo-pituitária-adrenal (HPA) e mudanças estruturais no cérebro. O núcleo da estria terminal (NET) está directamente envolvido na regulação da resposta ao *stress*, agindo como um núcleo de passagem entre as estruturas límbicas e o núcleo paraventricular do hipotálamo (NPH). Os mecanismos pelos quais o stress crónico altera os circuitos que mediam o comportamento típico de ansiedade são ainda desconhecidos. Estudos recentes têm implicado o cortéx prefrontal medial (CPM), juntamente com o NET, na génese de comportamentos típicos de ansiedade. Tendo isto em consideração, o presente trabalho procurou explorar a influência do *stress* crónico sobre um circuito específico envolvido na resposta ao *stress*: CPM-NET-NPH. Para isso, gravamos a actividade neuronal dos subnucleos dorsomedial/fusiforme do NET com ou sem manipulação da região infralimbica do CPM em animais controlo e stressados. Posteriormente, avaliamos a actividade do eixo HPA através da medição da expressão de c-Fos no NPH e os níveis de corticosterona no sangue após uma injecção no CPM. A inibição do CPM com lidocaína não afectou a actividade neuronal do NET. Por outro lado, a estimulação do CPM com glutamato diminuiu a actividade evocada das células do NET em animais controlo, no entanto em animais stressados este efeito foi atenuado. Além disso, o estímulo farmacológico do CPM aumentou a activação do NPH em animais stressados e induziu a libertação de corticosterona apenas em animais controlo. Notavelmente, os resultados sugerem que o CPM tem uma influência fásica excitatória sobre o eixo HPA e este efeito parece ser mediado pelo NET. No entanto, esta via parece estar alterada em condições de stress crónico.

CONTENTS

ABBREVIATIONS

ACTH – Adrenocorticotrophic hormone

AS – Acoustic Startle

AVP – Arginine vasopressin

BNST – Bed Nucleus of the Stria Terminalis

dm/fu – dorsomedial/fusiform

CeA – Central amygdala

Cgl – anterior cingulated

CNS – Central Nervous System

CUS – Chronic Unpredictable Stress

CRF – Corticotrophin releasing factor

$CRFR_1$ – CRF receptor 1

$CRFR_2$ – CRF receptor 2

EPM – Elevated Plus Maze

Fos-IR – c-Fos-immunoreactive

GABA – Gamma aminobutyric acid

Glu – Glutamate

GR – Glucocorticoid receptor

HPA – hypothalamic-pituitary-adrenal

I – Injection

IL – Infralimbic

ILCx – Infralimbic prefrontal cortex

LI – Limbic-Insensitive

LS – Limbic-Sensitive

MeA – Medial amygdala

mPFC – Medial prefrontal cortex

MR – Mineraloid Receptor

OF – Open Field

PBS – Phosphate buffered saline

PI – Pre-injection

PoI – Pos-injection

PrL – Prelimbic

PFA – Paraformaldehyde

PFC – Prefrontal cortex

PVN – Paraventricular nucleus of the hypothalamus

RIA – Radioimmunoassay

SEM – Standard error of the mean

Ucn – Urocortin

vSUB – ventral subiculum of the hippocampus

Chapter 1 - Introduction

1. INTRODUCTION

1.1 Stress

1.1.1 Stress as a defense mechanism

Stress is a ubiquitous component of our life that challenges the physical and psychological integrity of mammalian animals. In normal conditions, stress is a positive experience since it induces hormonal and behavioral changes that aim at increasing the individual's chances for survival. Activation of stress response must be initiated rapidly, maintained for a proper amount of time and turned off after cessation of the stimuli. If this acute response is overused or inefficiently managed, it might lead to negative biological consequences to the health and well-being of the organism[1,2].

1.1.2 The stress response

Activation of the hypothalamic–pituitary–adrenal (HPA) axis is a vital component of the body's stress response. This behavioral and hormonal reaction is essential to restore and maintain the individual homeostasis in response to external or internal adverse stimuli. In basal conditions the HPA axis exhibits a circadian rhythm, with different frequency of secretory episodes during the day that is disrupted by stress [3,4]. The paraventricular nucleus of the hypothalamus (PVN) has an important regulatory role upon HPA axis, since it integrates the neurochemical pathways of the central nervous system and converts it to a hormonal signal, leading to the activation of several peripheral components involved in the stress response[5]. PVN neurons synthesize several adrenocorticotrophic

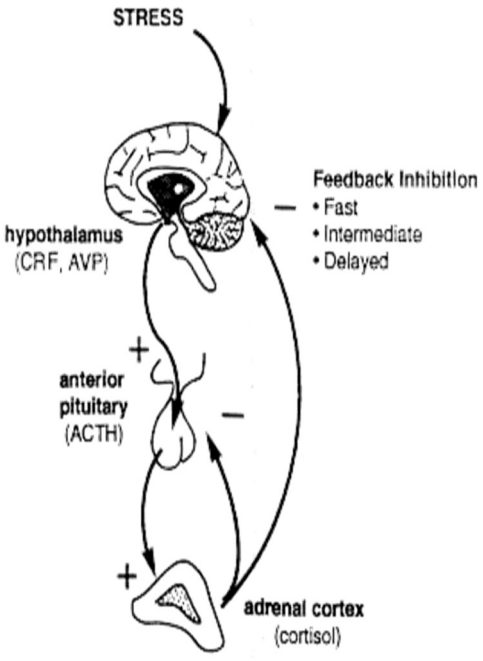

Figure 1. Schematic representation of the HPA axis. Neurons from the PVN nucleus secrete neuroregulatory peptides, especially CRF and AVP, to the pituitary. This leads to the release of ACTH into the circulatory system which promotes the release of corticosteroids by the adrenals.

hormone (ACTH) secretagogues, predominantly corticotrophin releasing factor (CRF) and arginine-vasopressin (AVP), and upon stimulation secrete them to the pituitary gland (Figure 1). These neurohormones stimulate the release of ACTH into the systemic circulation, which will lead to the release of corticosteroids (cortisol in humans, corticosterone in rodents) by the adrenal glands [6,7]. It is essential to control both initiation and cessation of HPA activity, otherwise if this secretion is prolonged in time or extent it will lead to a state of homeostatic imbalance marked by immune deficiency, neuroendocrine/autonomic disturbances and tissue atrophy [2].

1.1.3 Regulation of the stress response

The activity of the PVN (through the release of CRF/AVP) is essential to the stress response by mediating the release of corticosteroids that in turn leads to an increase in body metabolism in preparation for coping with stress, recovery and adaption. This loop in turn is fine-tuned by glucocorticoid-mediated negative feedback mechanisms that act upon the PVN and the pituitary itself. Additionally, CRF and related peptides are paramount mediators of the behavioral response associated to stress. Therefore the levels of both corticosteroids and CRF and their affinity to bind receptors influence the initiation and cessation of the HPA axis[8].

Together, glucocorticoids, the CRF family and their receptors are part of the neuroendocrine system essential for the adjustment of basal and stress-activated HPA axis [9] that we will address in the following sections.

1.1.3.1 Hormonal mediators of stress

Corticosteroids are the final effectors of the HPA axis and participate in the preservation of body homeostasis and the organism's response to stress, by controlling energy metabolism, disposition and storage. However if the levels of corticosteroids are maintained elevated for a long period of time, they may lead to pathophysiological changes in the brain, endocrine dysregulation and immune dysfunction [10]. Corticosteroids exert their actions by controlling gene expression at several cellular targets, an action that is mediated by two types of corticosteroid receptors. These receptors bind to the same ligand but have different pharmacological properties and

are differently distributed in the brain and other tissues. Type I receptors, also called mineralocorticoid receptors (MR), are expressed mainly in the hippocampus, septal nucleus and some cortical areas while the type II receptors or glucocorticoid receptors (GR) are ubiquitously distributed in the brain [11]. Remarkably both receptors are highly distributed in limbic areas involved in the modulation of stress response [12]. MRs become activated with normal blood levels of corticosteroids and are implicated in the onset of stress response, promoting pro-survival actions. By contrast, GRs only become activated when the MRs are saturated, as it occurs during the circadian peak or under stress conditions [8,10,13]. Upon activation GRs exert a negative feedback by inhibiting the synthesis of CRF in the PVN and ACTH in the pituitary [14] . An imbalance between MR/GR activation may lead to brain damage increasing the risk of psychopathology [15].

1.1.3.2 CRF: a major player of the HPA axis

CRF, a 41 amino acid peptide, was firstly isolated from mammalian brain by Vale and coworkers establishing its role in the regulation of the HPA axis [16]. Since then, CRF has been not only recognized for mediating endocrine stress-responsivity within the PVN, but also for being the major coordinator of autonomic and behavioral adaptive response to stress via extrahypothalamic circuits in the brainstem and limbic system [17-19]. CRF-expressing neurons are heterogeneously distributed throughout the central nervous system (CNS) and play an essential role within the limbic circuitries involved in the mediation of HPA axis. An overactive or inappropriate activation of CRF neuronal activity can have severe consequences for mental and physical health [20].

The CRF signaling system is composed of CRF and its related peptides: Urocortin (Ucn) 1, Ucn 2 and Ucn 3 [21-23] and they exert their actions by the activation of two distinct G-protein-coupled receptors: $CRFR_1$ and $CRFR_2$ [24-26]. These receptors share approximately 71% amino acid sequence similarity [27] but have different binding affinities to CRF peptides. Although Ucn 1 binds to both receptors with similar affinities, CRF binds with more affinity to $CRFR_1$ than to $CRFR_2$, while Ucn 2 and Unc 3 are high-affinity ligands to $CRFR_2$ [28]. CRF receptors also differ in their physiological functions and in their localization throughout the brain, reflecting the different actions that CRF exerts at the CNS level. $CRFR_1$ is mainly expressed in the cerebral cortex,

cerebellum, limbic structures and anterior pituitary [29], and has been highly implicated in the regulation of anxiety-related behavior in both humans and animals [30,31]. $CRFR_1$ seem to be crucial in the initiation of stress response; within the anterior pituitary, $CRFR_1$ mediates the effects of CRF on ACTH releasing and consequent corticosteroids secretion, and there is evidence suggesting that limbic $CRFR_1$ is involved in feedback regulation of HPA axis [32]. There are studies showing that an overexpression of CRF in the CNS produces several signs of increased anxiety-related behavior and these effects seem to be mediated by $CRFR_1$, since it was shown a reduced anxiety-related behavior in mice deficient for $CRFR_1$ and in animals with central administration of $CRFR_1$ antagonists [20,33]. By contrast, the $CRFR_2$ expression is confined to subcortical areas and the periphery, and its role in anxiety-related behavior is still not clear. Some studies attribute an anxiolytic function to this receptor [34] and others suggest that $CRFR_2$ mediate anxiogenic effects [35], but the most important is the consensus that $CRFR_2$ has a modulatory function upon the initial effects of stress-induced $CRFR_1$ activation [36]. CRF receptors have also different roles at the periphery level; there is evidence that $CRFR_1$ have pro-inflammatory effects, promoting inflammatory angiogenesis, whereas $CRFR_2$ inhibits these activities, being involved in the anti-inflammatory response [37].

All of these findings point to the use of CRF antagonists in the treatment of human pathologic states associated with chronic hyperactivity of the stress system [3,31].

1.1.4 Neurocircuitry of stress

Inappropriate regulation of stress has severe consequences to the health and well-being of the organism, thus it is crucial to limit the stress response. The importance of regulation HPA axis, through modulation of PVN activity, requires efficient mechanisms working in parallel with steroid feedback. This process seems to be mediated by two different types of stressors: Limbic-Sensitive (LS) and Limbic-Insensitive (LI) stressors. LI stressors are physiological threats, such as hemorrhage or systemic inflammation, which interact directly with the PVN by way of brainstem circuitry, bypassing cognitive processing. They act through somatic nociceptors, visceral afferents or humoral sensory pathways evoking a rapid and reflexive activation of the HPA axis. By contrast, although the LS stimulus does not represent a direct threat to the body homeostasis the experience that it represents is assembled at

cortical level and interacts with structures from the limbic system, which in turn mediate PVN activity [6,38]. Therefore the limbic structures are essential to the anticipatory stress responses based on prior experiences and the interaction between hippocampus, amygdala (including the bed nucleus of the stria terminalis [BNST] a part of extended amygdala) and prefrontal cortex (PFC) determine the magnitude of HPA response [3940]. See figure 2.

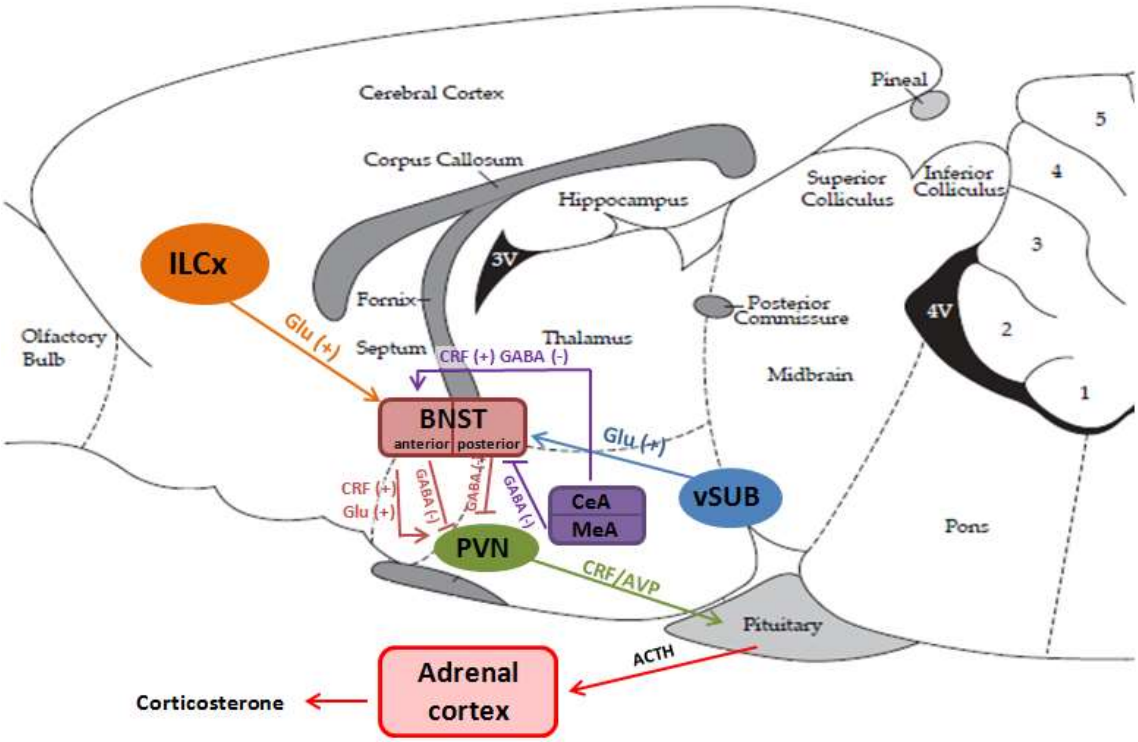

Figure 2. Schematic representation of the limbic inputs to the BNST and its regulatory influence over the HPA axis. ILCx, infralimbic cortex; BNST, bed nucleus of the stria terminalis; PVN, hypothalamic paraventricular nucleus; CeA, central amygdala; MeA, medial amygdala; vSUB, ventral subiculum of the hippocampus; Glu, glutamate; CRF, corticotrophin releasing factor; GABA, gama aminobutyric acid; AVP, arginine-vasopressin; ACTH, adrenocorticotrophic hormone

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The influence of limbic structures upon HPA axis appears to depend on the type of the stressor and on the region of the nuclei, however the mechanisms by which limbic system mediates the stress response are far from clear. Several studies point to a multi-faceted inhibitory action of the hippocampus on HPA axis activity [41]. The hippocampus expresses high levels of both MRs and GRs [42] being a good target to glucocorticoids actions. Indeed several studies have shown an association between

hippocampal damage and attenuated inhibition of HPA axis suggesting that the hippocampus inhibit the stress-induced HPA activation via GRs negative feedback inhibition [43,44]. A growing number of studies now suggest that these behavioral effects may be associated with different hippocampal subregions [45]. Through the years, the importance of a specific hippocampal region – the subiculum – has been highlighted. Restricted lesions in the ventral subiculum appear to enhance the response to stress [7,46], suggesting that this region inhibits HPA axis. Subiculum is thought to modulate HPA activity through the interaction with other brain regions as amygdala, lateral septum, paraventricular thalamus, PFC and BNST, which in turn have direct and indirect pathways that regulate PVN-secreting neurons [41,47-50].

There is substantial evidence that, besides its role in working memory and behavioral flexibility, the medial PFC (mPFC) is also implicated in stress regulation [51]. The mPFC expresses high numbers of GRs that become activated after a LS stimulus [52] suggesting that this region is a target site for the glucocorticoid negative-feedback, and this hypothesis is supported by studies showing an attenuated response of HPA axis after an injection of corticosterone in the mPFC [53]. In addition, specific lesions in the mPFC have been correlated with an increase activation of PVN and consequent increase of secretion of ACTH and corticosterone upon stress exposure [54], which also point to the inhibitory role of mPFC upon HPA axis. Of interest, lesions of the right infralimbic cortex (ILCx) decrease the activity of HPA axis following restrain stress [55] suggesting that there is a lateralized susceptibility to the effects of stress or specific roles according to the hemisphere. Other reports show that ILCx is implicated in stress excitation while the dorsal subareas seem to have an inhibitory role upon HPA axis [56], suggesting that ventral and dorsal mPFC areas have opposite functions in the regulation of the stress response. Of notice, the influence of mPFC over the PVN is not directly linked as there are no direct projections between mPFC and the PVN [57]. However, this relationship may be appreciated when its anatomical connections with relay nuclei such as the BNST, amygdala, dorsomedial hypothalamus, ventrolateral preoptic area and peri-PVN regions are considered.

The amygdala is also implicated in the stress response and appears to have an excitatory influence upon the HPA axis. Direct evidence for the involvement of this brain region in the stress regulation comes from studies demonstrating that the

amygdala becomes highly activated in response to LS stressors [54] and that damage to the central or medial amygdaloid nuclei results in impaired HPA axis activity [58-60] . The excitatory effects of the amygdala seem to be mediated by the central (CeA) and medial (MeA) amygdaloid nuclei, which represent the major amygdalar projections to basal forebrain, hypothalamic and brainstem structures [61]. These amygdaloid nuclei are also potential targets to glucocorticoids since they express both GRs and MRs [62,63]. However the glucocorticoid signaling in these nuclei appears to be different from the typical negative feedback, since it was described that there is an overexpression of CRF in the CeA associated with a decrease of CRF in PVN after a treatment with corticosteroids [64]. This finding along with other studies led to postulate that CRF-expressing neurons in the CeA have an important role in the generation and maintenance of physiological stress responses [65]. Similarly to the hippocampus and the PFC, the influence of the amygdala on HPA axis is mediated mainly through relay synaptic circuits and not through direct projections.

A potential relay nucleus between the aforementioned limbic structures and the PVN in the stress response is the BNST [66]. This tiny collection of nuclei receives excitatory glutamatergic/CRFergic and inhibitory GABAergic limbic inputs from these regulatory areas and has direct projections to the PVN [48,67-70]. The influence of BNST on HPA axis seems to be region-specific. Some lesions studies have shown that damage in the anterior BNST attenuated PVN excitation in response to [70]stress while damage in the posterior BNST augmented HPA axis activation [71,72]. These results suggest that the anterior BNST has an excitatory role over the PVN whereas the posterior BNST inhibits the PVN activity in response to stress.

1.2 Chronic stress and emotional disorders

Stress triggers a cascade of hormonal and behavioral changes whenever an organism is exposed to a threatening event and, in normal conditions, this is essential for its survival. However when there is an increase in stressful life events and these acute responses happen to be inadequate or prolonged in time, stress becomes chronic and may lead to affective disorders, such as depression and anxiety [73-75]. Chronic stress has been correlated with abnormalities in the HPA axis, inducing

structural changes in the brain, particularly in the limbic system, along with dysfunctions in the body metabolic, immune and cardiovascular systems [76]. Clinical and animal studies have shown a wide variety of HPA axis alterations related with a very heterogeneous group of emotional disorders [77].

The importance of emotional disorders must be highlighted since they affect a large portion of the world population, being frequently long-lasting and debilitating, and the World Health Organization estimates that by 2020 these disorders will be the second leading global burden of illness worldwide [78].

1.2.1 Anxiety disorders

Anxiety is characterized by a feeling of nervousness, apprehension, fear or worry in response to unconditioned diffuse cues [79] and represents a biological advance since it prepares the individual to potentially life-threatening situations. Healthy individuals commonly experience anxiety in response to everyday life events with no obvious reasons for worry, such as loud noises and fast approaching objects. However, when this "flight or fight" response is excessive as it occurs in chronic stress, it may have a serious impact on daily life [80].

Anxiety disorders represent a heterogeneous group of disorders that range from fobias to generalized anxiety disorders [81] and are the most common psychiatric illnesses [82] with over 10% of individuals experiencing an anxiety disorder at some point in their life time [83]. These disorders lead to a state of ongoing physiological arousal which may have profoundly disabling consequences, leading to significant suffering and a reduced quality of life [84]. People suffering from such disorders tend to expect disaster and their worry is often unrealistic or out of proportion for the situation. Besides the neurological symptoms these individuals also suffer physical consequences such as dysfunctions in gastrointestinal, cardiovascular and respiratory systems, being unable to live a normal life. Such disorders commonly occur in the general population, though they seem to be more incident in women and elderly people, and result from the interaction between the individual genetic background and the exposure to daily life stressors[77]. Animal models of anxiety along with clinical studies have been used to

unveil the genetic, environmental or neurobiology causes of anxiety disorders and to test potential anxiolytic substances [85-89].

1.2.2 Limbic stress circuits and HPA dysfunction

Animal and clinical studies have shown that stress-related disorders such as anxiety are correlated with abnormalities in HPA axis activity and consequent alterations in glucocorticoids secretion. Animals exposed to chronic stress exhibit an overactivation of HPA axis, characterized by a baseline hypersecretion of corticosterone and ACTH, associated with a baseline decrease in GR expression and increase of CRF and AVP in the parvocellular PVN nucleus [90]. The participation of the limbic system in the stress response suggests that these changes in HPA axis may be due to structural and functional alterations in the brain regions involved in the control of the stress response (Table 1). Indeed, it was shown that chronic stress exposure alters the structure and function of hippocampus, mPFC, amygdala and BNST.

Table 1. Neuroplastic responses to chronic stress (adapted from Ulrich-Lay 2009)

Site	Chronic-stress-induced plasticity	Effect
Hippocampus	Dendritic atrophy and decreased GR expression	Decreased HPA activity and decreased spatial memory
Medial prefrontal cortex	Dendritic atrophy and decreased GR expression	Decreased HPA activity, decreased spatial working memory and behavioral flexibility
Central amygdala	Increased CRF expression and release	Increased HPA and autonomic excitability, and anxiety
BNST anteromedial	Dendritic hypertrophy, spine sprouting and increased CRF expression	Increased HPA and autonomic excitability, and anxiety
Paraventricular nucleus of the hypothalamus	Increased secretagogue synthesis, increased stress responsiveness and decreased GR expression	Increased excitability to novel stress

Several studies have shown that chronic restrain stress as well as corticosterone treatment induce a decrease in the volume of the hippocampus due to dendritic atrophy and debranching of CA3 pyramidal neurons [91-93]. These structural alterations were correlated with deficits in spatial memory [94]. Additionally, it was shown that rats exposed to glucocorticoids in the neonatal period exhibited a decrease in the number of neurons and in the volumes of the CA3 hippocampal field[95]. Chronic stress also

triggers structural changes in the PFC. It was shown that chronic stress or glucocorticoids induce a significant atrophy of the apical dendritic branches and loss of dendrite spines in the pyramidal neurons in lamina II-II of mPFC [96,97]. These dendrites rearrangements were correlated with impairments in spatial working memory and behavior flexibility [98]. The amygdala and the BNST are also targets of chronic stress. It was reported that high levels of glucocorticoids increase CRF mRNA expression in the CeA [64], an effect that is mimicked by some chronic stress regimens. Data unpublished from our group reveal that chronic stress exposure leads to an increase of CRF levels in both CeA and BNST correlated with a decrease of $CRFR_1$ expression in the BNST subnuclei that receives CRF-projections from the CeA, suggesting that chronic stress induces an overactivation of CRF neurotransmission between the CeA and the BNST. Additionally, two independent studies reported a significant level of synaptic plasticity in the BNST after exposure to chronic stress, primarily due to an overall increase in dendritic arborization [99] and a particular dendritic hypertrophy and spine sprouting in the anteromedial area of the BNST [100]. Both studies described a good preservation of the morphology of the amygdala after chronic stress.

1.3 PFC and BNST - implications in anxiety behavior

This study looks at the particular influence of the PFC-BNST-PVN pathway upon the stress response. Substantial evidence has pointed to an excitatory role of ILCx upon HPA axis [56] which may be mediated by a bisynaptic glutamate-CRF/glutamate signal via the dorsomedial/fusiform BNST [57,67,69]. This pathway may be altered in animals exposed to chronic stress, since it was shown that chronic stress elicits structural and functional impairments in both BNST and PFC[96,97,99,100]. Together these findings suggest that the anxiety-like behavior induced by chronic stress may be due to impairments of the brain structures involved in the genesis of the emotional behavior, such as BNST and PFC. Indeed, several studies have shown that the BNST and the mPFC are highly implicated in the genesis of anxiety-like behaviors [101-103]. For example, studies in primates demonstrated that lesions in the PFC decreased the expression of anxiety [104] and these behavioral effects are mediated by descending modulatory inputs to the BNST region [105].

1.3.1 PFC: Structural and functional organization

Prefrontal cortex is the commonly designation for the cortex of the anterior pole of the mammalian brain. In humans the PFC represents about 30% of the cerebral cortex and encompasses a heterogeneous group of structurally and functionally related areas that integrate the processed information from various sensory modalities to form the physiologic constructs of memory, perception, and diverse cognitive processes [106,107].

For many years the PFC was considered to be a uniquely primate structure, due to its size and complexity, but nowadays this theory is no longer tenable. Anatomical and functional data indicate that rats also have a region at the frontal pole of the brain, though less differentiated than in primates, that is considered to be the rodent equivalent of the primate PFC [108]. The rat PFC is composed by several distinct regions that can be grouped in two main subdivisions: a medial frontal division (mPFC), which share similar functions with the primate dorsolateral and medial PFC, and a ventrolateral region that is involved in the control of socioaffective behavior, resembling the primate orbitofrontal cortex [108,109]. The mPFC has been highly implicated in the control of executive functions which requires attention, such as working memory and behavioral flexibility, and appears to be consistently activated during the stress response [51]. Of particular importance are the opposing effects of two distinct subareas within the mPFC (Figure 3) in the regulation of neuroendocrine and autonomic responses to emotional stress. The ventral division of the mPFC (mainly the infralimbic) seems to be more important to the initial response to stress since it stimulates the emotional behavior, the activation of sympathetic system and the HPA activity while the dorsal regions (prelimbic (PrL) and anterior cingulated (CgL)) tend to dampen the activity of the ILCx, being involved in the inhibition of the stress response [56]. This suggests that mPFC controls the emotional-cognitive aspects of behavior by the interaction between these two regions, which display different connectivity patterns. The efferent projections from ILCx are mainly distributed to autonomic/visceral-related sites, supporting its role in visceromotor behaviors, whereas PL primarily projects to limbic sites that reportedly affect cognition, which is consistent with its role in limbic-cognitive functions [57,110].

Other important feature is the functional hemispheric asymmetries exhibited by mPFC, particularly in the context of stressful or highly emotional situations. Sullivan and Gratton report that the suppression of the stress-induced corticosterone response as a result of mPFC bilateral lesions can be faithfully reproduced by unilateral lesions of the right mPFC, but not of the left mPFC [55]. Additionally they showed that selective ILCx lesions of the right, but not the left PFC, were found to have an anxiolytic effect, as assessed by increased time spent on the open arms of an elevated plus maze [111]. These findings suggest that intact right PFC is necessary mount a maximal HPA response, although an appropriate balance of activity between the hemispheres appears essential in achieving such optimal levels of function [112].

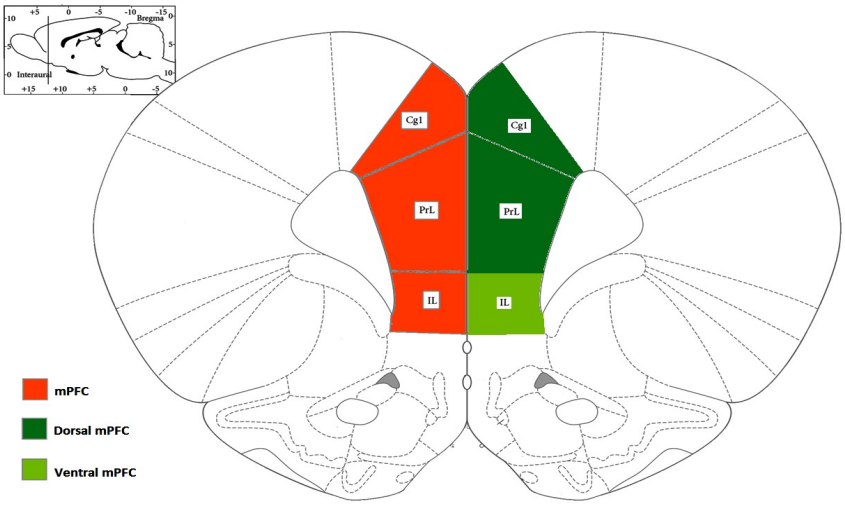

Figure 3. Diagrammatic representation of the mPFC divisions according to Paxinos & Watson (2007). This drawing represents a coronal section of the rat brain at approximately 3,24 mm posterior to bregma. Cgl. anterior cingulated; PrL, prelimbic; IL, infralimbic; mPFC, medial prefrontal cortex.

1.3.2 BNST: Structural and functional organization

The BNST was firstly described by Jonhson [113] as the gray matter surrounding the stria terminalis that forms a continuum extent since the base of the olfactory peduncle and nucleus accumbens anteriorly until the amygdala posteriorly. Since then there were some attempts to subdivide the BNST however its anatomical organization is still under debate. Swanson and coworkers suggested a main separation between anterior and posterior divisions [114,115], with the anterior division being later arranged into

medial and lateral groups [116]. Based on their cytoarchitectonic studies, they also concluded that BNST is a very complex structure which can be subdivided into 15 distinct cell groups or nuclei [114] as it shown in figure 4.

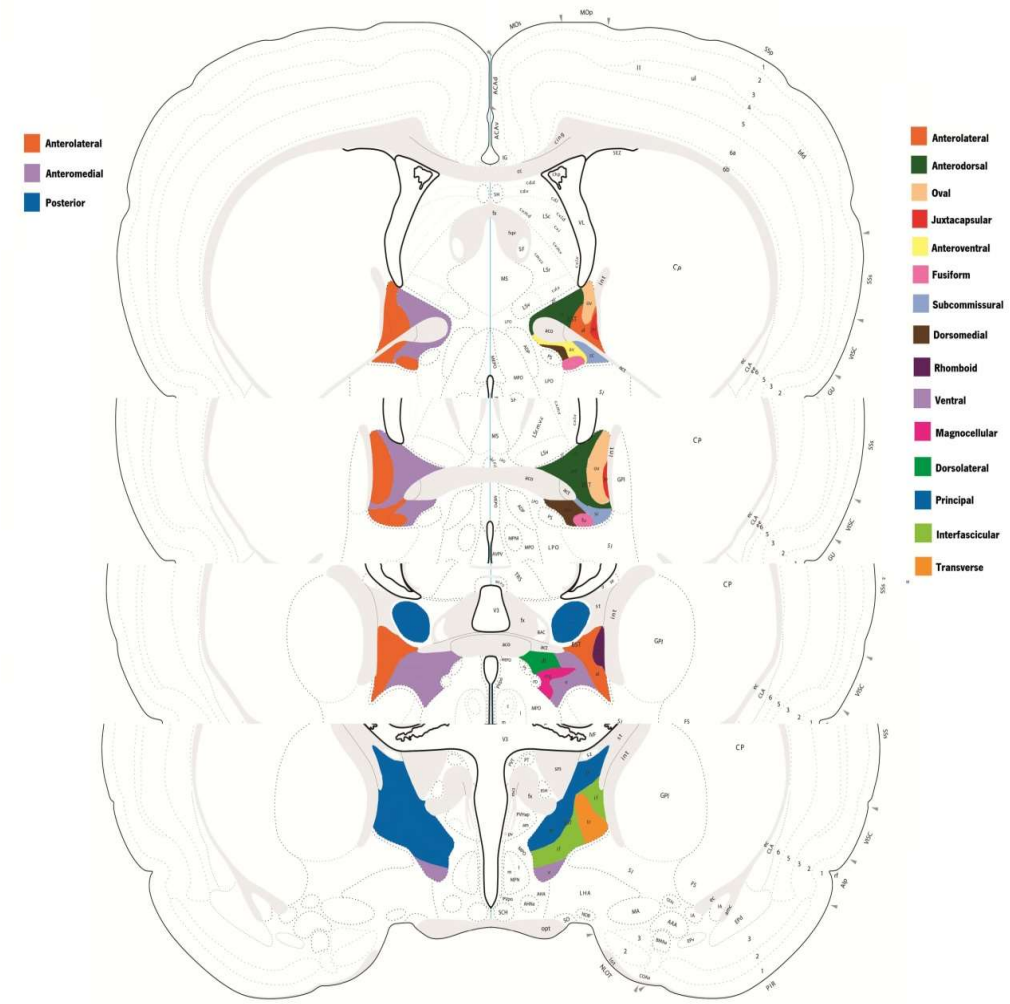

Figure 4. Diagrammatic representation of the main divisions of the BNST (left) and specific BNST subnuclei (right) according to Swanson (1998). From top to bottom, drawings represent coronal sections of the rat brain at approximately 0.00, 0.26, 0.51 and 1.08 mm posterior to bregma.

These anatomical and cytoarchitectonic differences are also correlated with different biological roles. The anterolateral division receives strong inputs from central amygdala and projects densely to hypothalamic areas concerned with autonomic and energy homeostasis and feeding behavior[116,117]. The anteromedial division is highly innervated by the amygdala, specially the central and medial nuclei, and has dense projections to hypothalamic regions closely associated with neuroendocrine regulation

[116,118,119]. The posterior division receives projections from the medial and posterior amygdala but also from the ventral subiculum of the hippocampus and is involved in the control of defensive and reproductive behavior [116,120].

Substantial evidence has implicated BNST as critical structure mediating anxiety-like behavior in both humans and animals [102,121], and some animal studies suggest that CRF levels within the BNST play a central role in the genesis of this behavior [122,123]. This small and complex nucleus is involved in the regulation of HPA axis, acting as a relay station between the limbic system and the PVN [48,68-70,124]. Depending on the region BNST can either activate or inhibit HPA axis. Lesion and stimulation studies have been showing that the anterior division of the BNST is associated with the activation of HPA axis whereas the posterior BNST has an inhibitory role[71,125-128]. Within these main divisions, BNST subnuclei also have specific inputs upon HPA axis, for example, posterior subnuclei like principal have inhibitory GABAergic projections to the PVN [48,115] while dorsomedial/fusiform (dm/fu) subnuclei (anteromedial division) express CRF and send the heaviest BNST projections to the PVN [116,118,129]. These BNST dm/fu subnuclei have been implicated in the activation of HPA axis, since it was shown that lesions in this area decreased corticosterone and PVN c-Fos mRNA responses to acute stress. However these lesions did not alter the basal tone of CRF and AVP mRNA expression in the PVN, suggesting that BNST dm/fu primarily regulates HPA responses to stress rather that basal HPA tone [130]. These findings point to an essential role of BNST dm/fu in the modulation of the stress response.

Chapter 2 - Objectives

2. OBJECTIVES

Substantial evidence has implicated the mPFC in the regulation of the PVN response to stress through the modulation of a relay nucleus – the BNST [69,105]. Taken this in account, we attempted to further explore the role of a specific region of the mPFC, the ILCx, upon the activity of BNST dm/fu subnuclei, and its consequences to the HPA axis activity.

The present work aimed at:

- Characterize neuronal basal activity of the BNST dm/fu subnuclei;
- Evaluate the effects of chronic stress exposure upon the basal activity of the BNST dm/fu neurons;
- Investigate the role of ILCx upon the activity of BNST dm/fu neurons and if this is altered in anxious animals;
- Determine the activation pattern of the PVN neurons in response to a pharmacological stimulation of ILCx in both control and chronic stress conditions;
- Characterize the effects of a pharmacological activation of ILCx upon the corticosterone secretion.

Chapter 3 – Materials and Methods

3. MATERIALS AND METHODS

3.1 Animals and treatments

This experimental protocol was conducted in accordance with European regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation. All efforts were made to minimize the number of animals and their suffering. All experiments were conducted with adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) which were housed two per cage with food and water delivered *ad libitum* in a temperature and humidity controlled vivarium, maintained on a 12/12-h light/dark cycle (lights on at 8 a.m.).

To assess the influence of chronic stress upon BNST neuronal activity, rats with 8 weeks of age were randomly divided into two groups: (i) **Control,** which were handled on a daily basis for 4 weeks and (ii) **CUS**, which were exposed to Chronic Unpredictable Stress (CUS) protocol for 4 weeks. This protocol (CUS) consisted in exposing the animals to a daily randomly stressor (1h/day), which could be one of the following stimuli: cold water (18ºC), shaking, restraining, overcrowding and exposure to a hot air stream. This stress paradigm was selected based on previous studies [100,131,132]. Behavior analysis was performed to confirm the anxious phenotype and included the open field (OF) test for locomotion and exploratory activity and the acoustic startle (AS) along with the elevated plus maze (EPM) for anxiety-like behavior.

Following these behavioral tests rats were assigned into 2 sets of animals (Figure 5). Animals from the first set (**SET1**, n=25 per group) were used in electrophysiological recording sessions of neuronal activity in the BNST, as described in section 3.4. The other set (**SET2**, n=4 per group) underwent a similar electrophysiological protocol as SET1, but it was also used to analyze c-Fos expression in the PVN and to measure corticosterone levels in the blood serum. To determine the activation pattern of the PVN after ILCx stimulation, an injection of glutamate was made in the ILCx and the rats were sacrificed 90 min later as described in section 3.6. To analyze whether the pharmacological manipulation of the ILCx has influence upon the corticosterone secretion, left femoral vein of each animal from SET2 was catheterized (section 3.3) to allow repeated blood collections at different time points during the electrophysiological procedure.

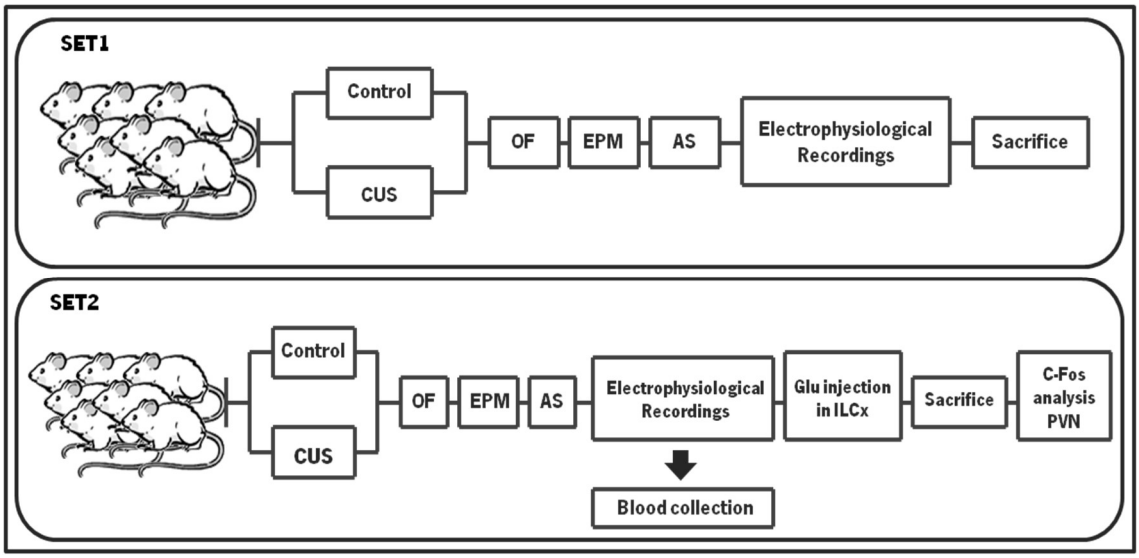

Figure 5. Schematic representation of the experimental design. **SET1**. Rats were divided in two groups: Control and CUS. The behavior of each animal from both groups was evaluated on the OF, EPM and AS tests prior to an electrophysiological recording session of BNST activity. **SET2.** Rats were also divided in two groups: Control and CUS, their behavior was evaluated in the same way as group SET2 prior to an electrophysiological recording session of BNST activity. Blood samples were collected at different time points during the electrophysiological protocol. Afterwards an injection of glutamate was made in ILCx and 90 min later the rats were sacrificed. Their brains were used for histological analysis in the PVN. OF, Open Field; EPM, Elevated Plus Maze; AS, Acoustic Startle; Glu, glutamate; ILCx, Infra-Limbic Cortex; PVN, hypothalamic paraventricular nucleus.

3.2 Behavioral assessment

3.2.1 Open Field

Open Field arena was used to assess locomotor and exploratory behavior. Each animal was initially placed in the center of an empty and bright square arena (43.2 x 43.2 cm) surrounded by transparent acrylic walls (model ENV-515, MedAssociares Inc, St. Albans, VT 05478) and was free to explore it for 5 minutes. Horizontal activity and instant position were registered, using a system of 16-beam infrared arrays connected to a computer. Total distances were used as indicators of locomotor activity.

3.2.2 Elevated Plus Maze

Animals were placed in the center of a black polypropylene “plus”-shaped maze (ENV-560, MedAssociates Inc, St. Albans, VT 05478) elevated 72.4 cm above the floor (EPM).The maze was arranged in a cross with two opposite open arms (50.8cm x 10.2cm) and two closed arms (50.8 x 10.2 x 40.6cm). Each animal was allowed to freely

explore the maze for 5 minutes. The time spent in open arms, junction area and closed arms, as well as the numbers of entries on both arms were measured using an infrared photobeam system connected to a specific software (MedPCIV, MedAssociates). Although normal exploratory behavior is in favor of the closed arms, this preference is more evident in anxious animals [133,134]. Thus, time spent in the open arms versus the time spent in the closed arms was considered to evaluate anxious-like behavior.

3.2.3 Acoustic Startle

SR-LAB startle response system (San Diego Instruments, San Diego, CA, USA) was used to measure the motor response to a sudden intense stimulus – startle reflex – which is higher in anxious animals [135]. Each rat was individually placed in a non-restrictive Plexiglas cylinder (inner diameter 8.8cm, length 22.2cm), mounted on a Plexiglas platform inside a ventilated sound-attenuated chamber. Startle stimuli (each lasting 50ms, but varying in intensity from 70 to 120dB, in 10-dB increments) were presented through a high frequency speaker located 33 cm above the startle chambers and the test session lasted approximately 20 minutes, after a 5 minutes acclimation period. Cylinder movements were detected and measured by a piezoelectric element mounted under each cylinder. Startle magnitudes were sampled every millisecond (ms) over a period of 200 ms, beginning with the onset of the startle stimulus. A Startle response is defined as the peak response during 200 ms recording period. One day before the test animals were habituated to the apparatus for 5 minutes.

3.3 Cannulation of the femoral vein

3.3.1 Catheter system

Polyethylene catheters (PE, Plastics One, Roanoke, USA) 10 (0.28 mm inner diameter, 0.61 mm outer diameter) and 50 (0.58 mm inner diameter, 1.27 mm outer diameter) were cut to the following length: PE-10: 8-9 mm and PE-50: 3-4 mm. The proximal end of PE-10 was obliquely cut using a sharp blade and the other end was connected to PE-50 which was attached to a 25G needle, as it is shown in figure 6A. Sterilized saline containing heparin (50 UI/1ml of

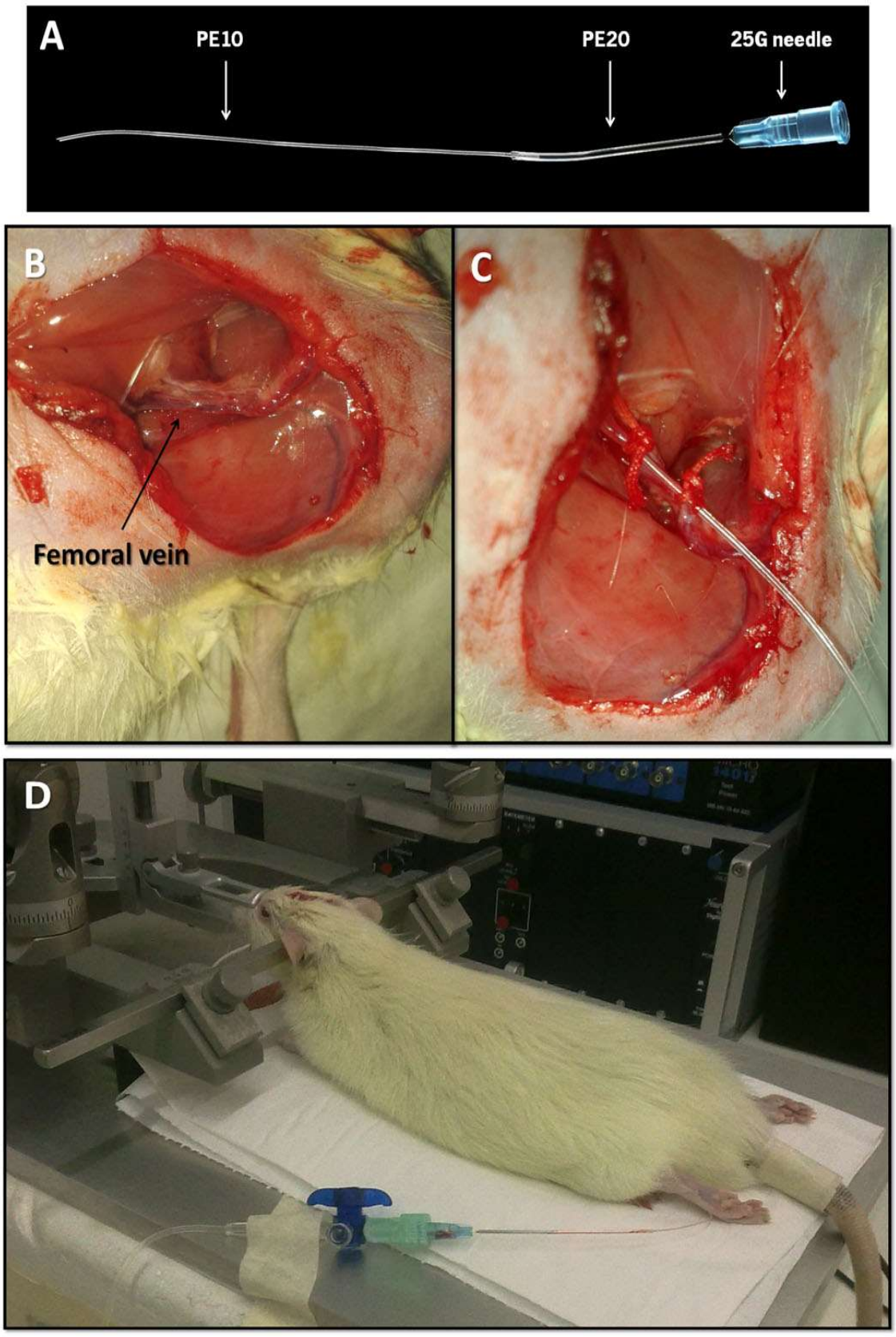

Figure 6. Cannulation of femoral vein. **A**. The catheter system was prepared with a PE-10, a P-50 and a 25G needle **B.** An incision was made in the inguinal region, the connective tissue was teared away and the femoral vein was isolated. **C.** The catheter was inserted inside the vein and two sutures were tied up surrounding the vein-catheter system. **D** In the end, the animal was placed in the stereotaxic apparatus and the 25G needle was attached to a three-way stopcock, which was then linked to a saline (0.9%) perfusion system.

saline, Winthrop, Porto Salvo, Portugal) was pumped into this system, and the three-way stopcock (Braun, Melsugen, Germany) was closed.

3.3.2 Catheter placement

Rats from SET2 were anaesthetized with intraperitoneal injection of pentobarbital solution (100mg/kg, Ceva Sante Animale, Libourne, France). A long skin incision was made in the left inguinal region and the connective tissue was teared away. Under a microscope, the femoral vein was gently separated from the femoral artery and femoral nerve (Figure 6B). Two 2-0 ligatures (J&J Ethicon, New Jersey, USA) were passed under the vein. The caudal ligature was tied around the vein to stop the blood flow and the proximal ligature was tied with one loose knot. A 23G needle was used, with very caution, to make a stab wound in the vein. The proximal end of the catheter was gently slid into the vein and was threaded along it until a gentle pressure was detected. The catheter was then retracted to a position where blood could be withdrawn and 500 µl of heparinized saline (50 IU/mL) was slowly injected through the catheter to prevent blood clotting to occur within the system. The proximal ligature that was surrounding the vein-catheter system was tied up (Figure 6C). In the end, the catheter was tunneled subcutaneously and the incision wound was closed with surgical sutures. The blood loss during the surgery procedures was negligible.

3.4 Electrophysiology

Animals from the SET2 were firstly submitted to the cannulation surgery and then continued with the electrophysiology protocol as the other rats (Figure 6D).

During the electrophysiological procedure several blood collections were made, as it described in figure 7B, to measure the levels of corticosterone in the serum.

3.4.1 Cannula and Electrode Implantation

The animals were anesthetized with sodium pentobarbital solution (100 mg/kg i.p.) for the electrophysiological procedure. During recording experiments the level of anesthesia was frequently monitored by the animal response to tail pinch and an additional dose of pentobarbitone (30-35 mg/kg) was administered whenever needed.

Each animal was placed in a stereotaxic frame, and its rectal temperature was maintained at 36–37°C by a thermistor-controlled electric heating blanket. The skull of the animal was exposed and the bregma reference point was identified. Two holes were drilled to place a recording electrode in the right BNST dm/fu subnuclei (RC: -0,26 mm, LM: +1,5 mm, DV: -7 mm) and a guide cannula in the right ILCx (RC: +2,8 mm, LM: -0,5 mm, DV: -4 mm) according to the Swanson atlas [136] (Figure 8A). The coordinates were calculated relative to bregma with the incisor bar set at -3.3 mm.

3.4.2 Electrophysiological recordings

Recordings in the BNST dm/fu subnuclei were made with lacquer-coated tungsten electrodes (tip impedance 5-7 MΩ, FHC, ME, USA) and the electrical activity of the neurons was amplified, filtered and displayed on an oscilloscope. Spikes of single neurons were discriminated, and digital pulses were digitalized to a computer interface (CED Micro 1401, Cambridge Electronic Design, Cambridge, U.K.) and analyzed using Spike 2 software (CED). Signals were amplified and filtered (high-pass filter at 500 Hz and low-pass filter at 5 kHz) via a differential AC pre-amplifier (model NL104A; Digitimer, Hertfordshire, England). In the electrophysiological recordings the following parameters were evaluated: (i) spontaneous activity of BNST cells, (ii) spontaneous activity of BNST cells after drug injection in the ILCx, and (iii) evoked response of BNST neurons to mechanical stimulation (tail pinch, 5s). These parameters were considered for both control and CUS animals.

3.4.3 Pharmacology and drugs administration

The drugs chosen for intracerebral administration were glutamate (Merck, Darmstadt, Germany), an excitatory neurotransmitter of the nervous system, and lidocaine 2% (B. Braun Medical, Portugal), a blocker of NA^+ channels and consequent inhibitor of signal propagation in neurons. Glutamate was prepared with sterilized saline 0.9% (Unither, Amiens, France), pH = 7.2, and lidocaine 2% was acquired as a solution. Each injection volume (0,5 µL) contained: 50 nM of glutamate or 2% of lidocaine. These doses were chosen according to previous studies (Pinto-Ribeiro 2008). Control injections were performed with saline 0,9% in order to avoid any confounding

effect that might result from the injection itself. The chosen volume for drug injection was 0,5 µL based on previous studies showing that this volume spread at least 1 mm within the injection site [101,137].

Drugs administration was performed through an injection cannula (33 gauge; Plastics One, Roanoke, USA) inserted into and protruding 1mm beyond the tip of the guide cannula (26 gauge; Plastics One, Roanoke, USA), which was previously placed in the ILCx coordinates, as referred above. The microinjections were made using a 5 µL Hamilton syringe (Hamilton Inc., Reno, USA) connected to an injection cannula by a polyethylene catheter (PE-10) and lasted for a period of 20 seconds, to avoid the possible neuronal activation by pressure. The efficacy of the injection was monitored by observation of the movement of a small air bubble through the polyethylene catheter. The injection needle was retained within the guide cannula until the end of each electrophysiological recording to prevent backflow of the drug into the cannula and to avoid collateral effects induced by the movement of the needle.

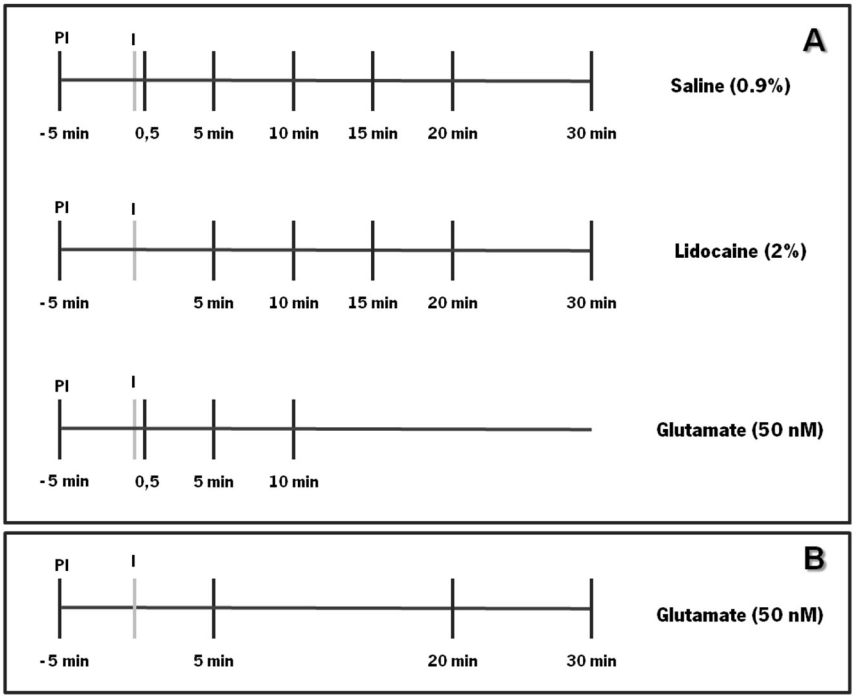

Figure 7. Schematic representation of the time points that tail pinch (**A**) and the blood samples (**B**) were performed during the electrophysiological recording sessions. **A.** Tail pinch was performed before the drugs administration to set the basal evoked activity. Then it was performed at different time points after the injection, depending on the drug tested. **B.** Blood collections were performed at different time points during the stimulation of the ILCx with glutamate: 5 min before the drug administration and 5, 20 and 30 min after the microinjection. PI, pre-injection; I, injection.

Pharmacological tests were performed as described in figure 7A. An electrophysiological recording of BNST dm/fu neurons (see section 3.4.2) was done for each drug administration. Throughout the complete protocol we applied a mechanical stimulation (tail pinch, 5s) at different time points, depending on the time of action of the drug tested. The first time point (pre-injection, PI) was used to measure the basal response, before the intra-cerebral drug administration. The drugs were randomly administrated on each animal, with at least 1h-interval between injections to allow the drug clearance from the injection site [138,139].

3.5 Histology

At the end of each electrophysiological protocol, the ILCx injection site (figure 8B) was marked with an 0.5 µL injection of 2% pontamine sky blue dye (Sigma, St Louis, MO, USA) in 0.5 M sodium acetate, according with established protocols [140]. A lesion was performed to mark the BNST recording site by passing 1mA current through the recording electrode for 5s (Figure 8C). After the experimental procedures, animals from the SET2 were treated for immunohistochemistry as described in section 3.6 and the others were euthanized with a lethal dose of pentobarbital solution (200 mg/kg i.p.) and their brains were extracted and conserved in a fixative solution containing 4% Paraformaldehyde (PFA) in Phosphate buffered saline (PBS) for 3 days. Brains were then dissected in coronal sections (70µm thick) and slices containing the ILCx area were stained with Cresyl violet while the BNST slices were stained with Giemsa to enable histological determination of injection and recording sites. Representative locations of the effective BNST recording sites are presented in figure 8D. Only the results from animals whose ILCx and BNST dm/fu nuclei location was confirmed were used for the data analysis.

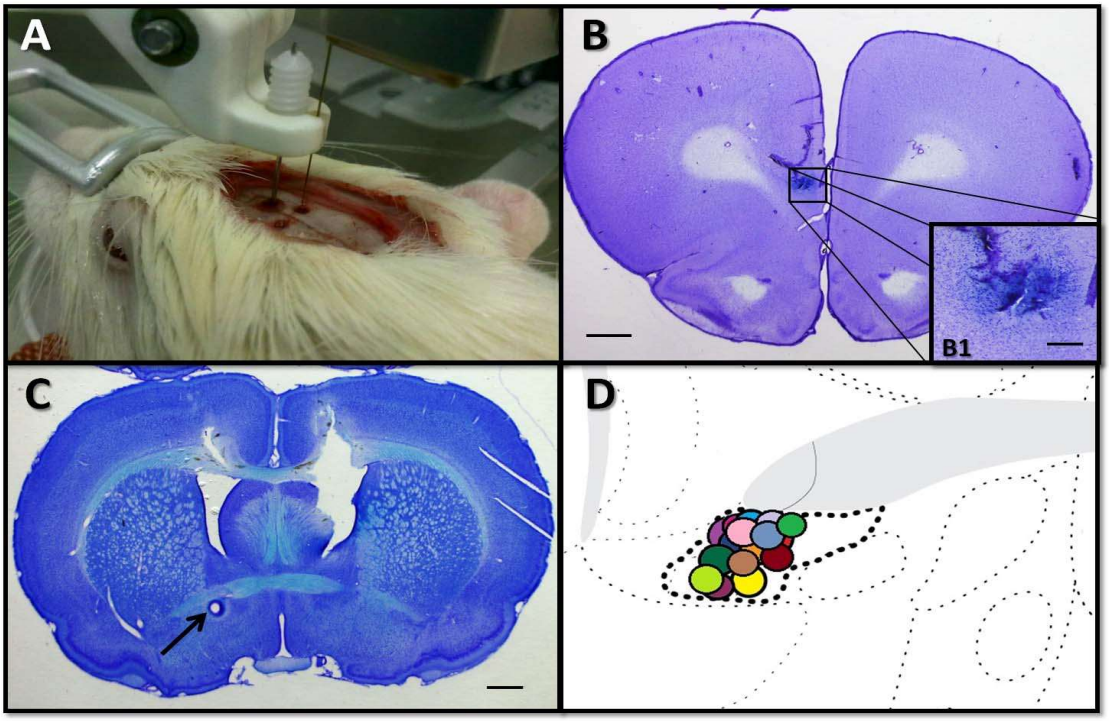

Figure 8. Stimulation and recording sites. **A**. Representation of the placement of an electrode in the BNST and a guide cannula in the ILCx. **B.** Photomicrograph of a coronal section of the ILCx, representing an effective injection site which was verified by a microinjection of pontamine sky blue. **C.** Photomicrograph of a coronal section of the BNST, representing an effective recording site marked by passing current through the electrode. **D.** Schematic representation of the effective recording sites in the BNST dm/fu subnuclei. Scale bars: B, 1 mm; B1, 0.22 µm; C, 1 mm.

3.6 Immunohistochemistry and quantification

Animals were perfused transcardially, under deep pentobarbital anesthesia (200mg/kg i.p.), with fixative solution containing 4% PFA in PBS and brains were collected and post-fixed (4% PFA in PBS) for 1 day, followed by sucrose 8% in 0.1M PBS for 2 days, at 4ºC. Brains were dissected in coronal sections (50 µm thick) and slices containing the PVN area were serially collected in PBS.

The sections from ILCx and BSNT areas were used to verify the brain injection and recording sites as described in section 3.5. For c-Fos staining, sections were incubated in 1% H_2O_2 for 30 minutes and then blocked in incubation solution (10% Fetal Bovine Serum and 0.3% Triton X-100 in PBS) for 1 hour to decrease background. Sections were incubated overnight with a rabbit polyclonal antibody against c-Fos (1:10000, Calbiochem, Darmstadt, Germany), followed by 1 hour incubation in biotinylated goat anti-rabbit IgG (1:200, Dako, Denmark), and 1 hour incubation in

avidin-horseradish peroxidase complex (1:200; ABC Elite Kit, Vector Laboratories, USA). Finally, sections were incubated with 0.05% diaminobenzidine (DAB, Sigma-Aldrich, St. Louis, MO) and counterstained with hematoxylin to delimit regional boundaries. Slides were mounted with Entellan (Merk, Darmstadt, Germany) and coverslipped.

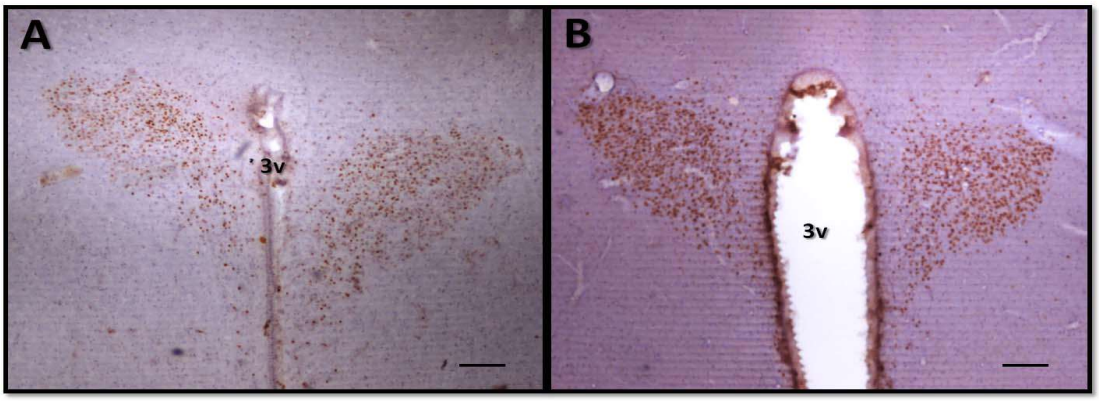

Figure 9. Photomicrographs showing Fos-IR neurons in the PVN after a microinjection of glutamate in the ILCX**.** **A**. Sections from control (**A**) and CUS (**B**) rats. 3v, third ventricle. Scale bar: 400 µm.

Bright-field microscopy was used to confirm the presence of c-Fos-immunoreactive (Fos-IR) neurons by the presence of a dark brown DAB precipitate (figure 9). PVN right and left areas were drawn (Swanson, 1998) and the number of Fos-IR neurons was counted within these areas using the Optical Fractionator method (Stereo Investigator, MBF Bioscience, USA) [141]. To establish comparisons between groups, the number of Fos-IR neurons per area was calculated.

3.7 Corticosterone measurements in the blood serum

To assess whether a pharmacological stimulation of the ILCx has an effect upon the release of corticosterone, blood samples were collected at different time points during the pharmacological modulation of the ILCx. In the end of the electrophysiological protocol, the samples were stored at 4ºC and were centrifuged 24 hours later at 13000 rpm for 10 minutes. Serum was then removed and stored at -80ºC to further analysis. Plasma corticosterone levels were measured using 125I radioimmunoassay (RIA) kits (MP Biomedicals, Inc., Orangeburg, NY). Briefly, the serum samples were diluted in steroid diluent (1:200), and then were incubated (triplicates)

with corticosterone 125-I and anti-CORT for 2 hours at RT, followed by an addition of a precipitant solution. In the end the precipitated was measured in an automatic gamma counter (Perkin Elmer 1470, Machester, United Kingdom).

3.8 Statistical/data analysis

Behavioral and FOS-IR data was analyzed using Student's t test to compare means between groups, except the AS, that was analyzed by repeated measures with the stimulus intensity as within-group variables. For *in vivo* electrophysiological experiments, cumulative peristimulus time histograms (PSTHs) (0.5 sec bin width) of BNST dm/fu activity were generated for each neuron recorded, in both control and CUS animals. These PSTHs were also generated for BNST dm/fu activity during pharmacological manipulation of the ILCx. Analysis of the changes in the spontaneous and noxious-evoked activity of BNST dm/fu neurons after drug administration in the ILCx was performed only by comparing the changes in the basal discharge frequency throughout the time for each drug ($ANOVA_{rm}$ followed by Bonferroni post-hoc test) and by comparing the changes throughout the time between saline and glutamate/lidocaine ($ANOVA_{2w}$ followed by Bonferroni post-hoc test). Results are expressed as mean ± SEM. Differences were considered significant to $*P < 0.05$; $**P < 0.01$ and $***P < 0.001$.

Chapter 4 – Results

4. RESULTS

4.1 Verification of the biological efficacy of CUS treatment

The CUS protocol decreased body-weight gain (t = 3.21, P = 0.002), as depicted by the ratio of the animal's weight four weeks after the treatment over the animal's weight before the stress (Control: 1.44 ± 0.04; CUS: 1.31 ± 0.01). Additionally, there was an almost significant increase in the baseline levels of corticosterone of CUS (assessed in SET2) treated animals (Control: 231 ± 54 ng/mL; CUS: 426 ± 39 ng/mL; t = 2.54, P = 0.06).

4.2 CUS induces an anxious phenotype

The animals submitted to CUS protocol exhibited increased signs of anxiety when compared to controls. In the EPM, CUS animals spent significantly less time (t = 3.79, P = 0.002) and made fewer entries (t = 2.43, P = 0.03) in the open arms than control animals (Figure 10A,B). In addition, the ratio of open/closed arm entries (t = 2.90, P = 0.012) was significantly smaller in CUS animals (Figure 10C).

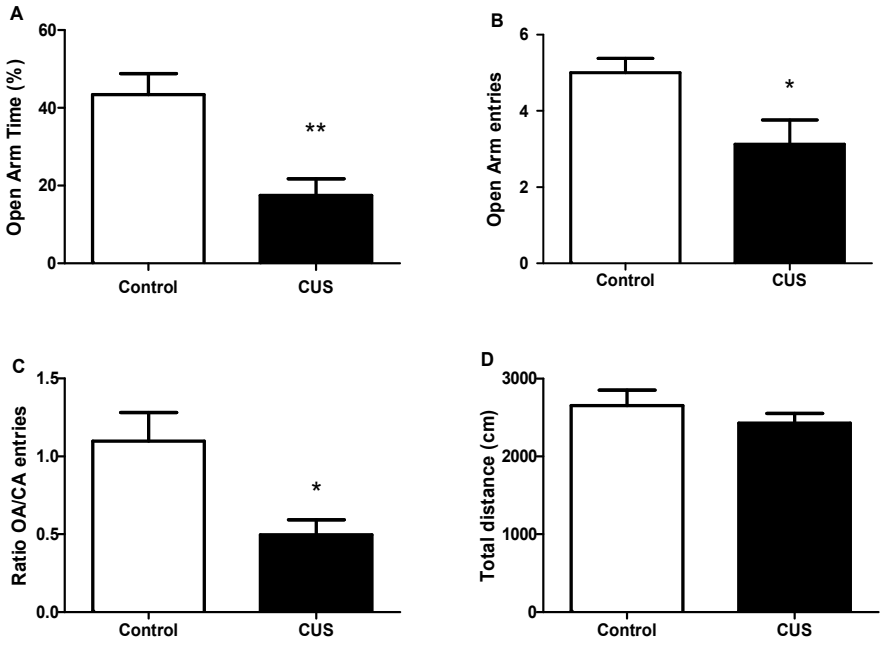

Figure 10. Results from Elevated Plus Maze (A, B and C) and Open Field (D) tests. **A.** Time spent in the Open Arms given as percentage of total time. **B.** Number of entries in the Open Arm. **C.** Ratio of open/closed arm entries. **D.** Total distances in the Open Field arena. CUS animals spent significantly less time and made fewer entries in the open arms of the EPM. No differences were found for the total distances travelled by animals in the OF arena. Control, control rats; CUS, chronic unpredictable stress rats; OA/CA, open arm/closed arm. Results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$.

Nevertheless, there was no differences in total distance travelled by animals in the OF arena (t = 0.95, P = 0.35), indicating that exploratory/locomotory activity was preserved (figure 10D).

The startle response in the AS paradigm varied as a function of treatment x startle intensity (F = 7.17, P < 0.001). There was a significant increase of the startle amplitudes from both groups in response to progressive stimuli intensity (F = 94.7, P < 0.001), however the startle amplitude of CUS rats trend was significantly steeper when compared to controls (F = 5.95, P = 0.022). Comparison between groups revealed that the startle amplitude of CUS rats was significantly higher as compared to controls at the 110 and 120 dB (figure 11).

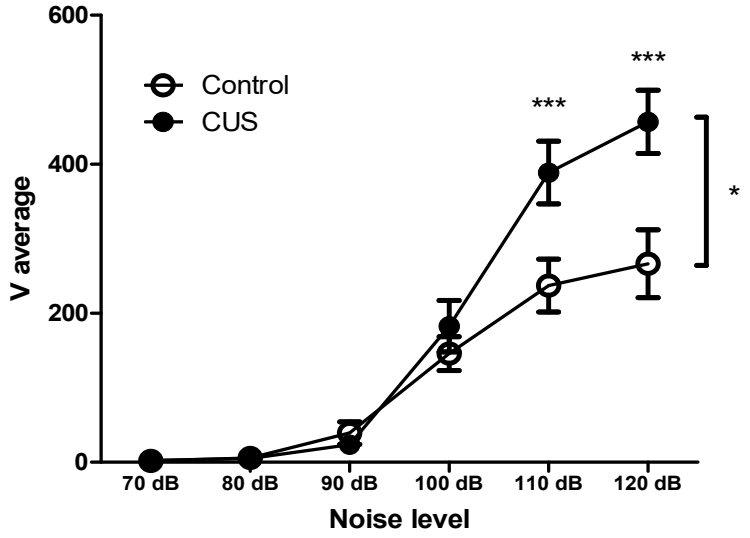

Figure 11. Acoustic startle results presented as startle amplitude in response to acoustic stimulus. CUS treated animals showed a significant increase in startle response when compared to controls at the 110 dB and 120 dB. Control, control rats; CUS, chronic unpredictable stress rats. Results are presented as mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

4.3 ILCx modulation of BNST neurons – comparison between control and stressed animals

To evaluate whether CUS induces alterations in BNST dm/fu neuronal activity and to determine the role of ILCx upon these alterations, we recorded the spontaneous and noxious-evoked activity of BNST dm/fu neurons before and after ILCx pharmacological manipulation. 62 cells (37 from controls and 25 from CUS) were analyzed.

4.3.1 Spontaneous activity

There was a trend for spontaneous activity of BNST dm/fu to be higher in CUS animals (Figure 12) however no significant differences were found (Control x CUS: F = 2.14, P = 0.15). The spontaneous activity of BNST dm/fu cells, before any intracerebral drug administration in the ILCx, was 1.17 ± 0.14 spikes/s for control animals (n = 20) and 1.51 ± 0.20 spikes/s for CUS (n = 15). The microinjection of saline in the ILCx did not alter spontaneous discharge rate of the BNST dm/fu cells in both groups (Control, F = 1.03, P = 0.36; CUS, F = 0.65, P = 0.52) (Figure 12A). Glutamate administration in the ILCx increased significantly the activity of BNST dm/fu cells 30 seconds after the injection in CUS rats (F = 11.59, P < 0.001; GluPI x GluPoI: P < 0.001; GluPoI x GluPpoI: P < 0.001), however it had no effect in control animals (F = 0.32, P = 0.81) (Figure 12B). No changes were observed in both groups 5 minutes after glutamate in the ILCx. Lidocaine microinjection in the ILCx did not alter the spontaneous activity of the BNST dm/fu cells from CUS (F = 1.33, P = 0.28) and control animals (F = 0.34, P = 0.72) (data not shown).

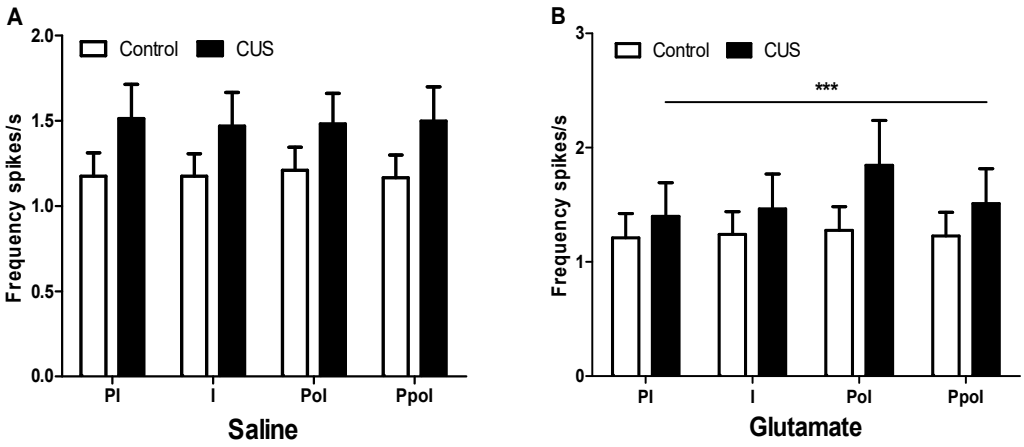

Figure 12. Spontaneous activity of BNST dm/fu neurons of control and CUS rats after the administration of saline (**A**) and glutamate (**B**) in the ILCx. The microinjection of saline (0.9%) did not alter the spontaneous activity of BNST dm/fu cells of both control (n = 20) and CUS (n = 15) animals. Glutamate administration in the ILCx increased the spontaneous activity of BNST dm/fu cells of CUS (n = 18) treated animals, and had no effect in control rats (n = 20). Control, control rats; CUS, chronic unpredictable stress rats; PI, pre-injection; I, injection; PoI, 30s post-injection; PpoI, 5 min post-injection. Results are presented as mean ± SEM. ***$p < 0.001$.

4.3.2 Noxious-evoked Response

All BNST dm/fu cells that were recorded increased their discharge rate in response to pinch, however there were no differences between the noxious-evoked response of BNST dm/fu neurons from control and CUS rats (t = 0.66, P = 0.51) before any drug administration (data not shown). In addition, the microinjection of saline in the ILCx did not alter the noxious-evoked response of BNST dm/fu cells from either groups (Control: F = 0.10, P = 0.99; CUS: F = 0.02, P = 0.99; Control x CUS: F = 0.01; P = 0.93). The inhibition of the ILCx with lidocaine had similar effects as saline (F = 0.21, P = 0.96). There were no changes in the noxious-evoked response of BNST dm/fu cells of both control (F = 0.09, P = 0.99) and CUS (F = 0.29, P = 0.92) rats after lidocaine administration (Figure 13).

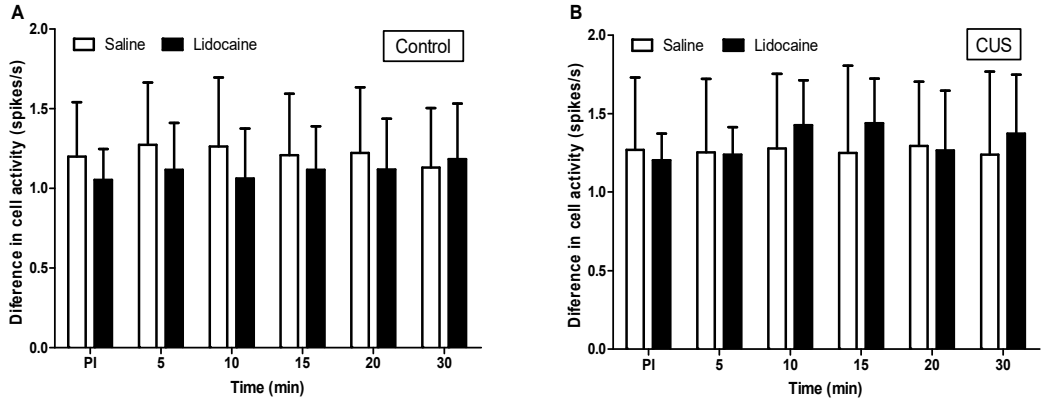

Figure 13. Noxious-evoked response of BNST dm/fu cells to mechanical stimulation (pinch) after the microinjection of lidocaine in the ILCx of control (**A**) and CUS (**B**) rats. Injection of saline (0.9%) was used as a control (Control, n = 20; CUS, n = 15). The microinjection of lidocaine did not alter the noxious-evoked response of BNST dm/fu cells of both control (n = 15) and CUS (n = 14) animals. Control, control rats; CUS, chronic unpredictable stress rats; PI, pre-injection. Results are presented as mean ± SEM.

The activation of ILCx neurons with glutamate resulted in a decrease of the noxious-evoked responses of BNST dm/fu cells from control (F = 9.42, P < 0.001; PI x 0.5: P < 0.01; 0.5 x 5: P < 0.001; 0.5 x 10: P <0.001) and CUS animals (F = 3.63, P < 0.02; 0.5 x 10: P < 0.05). This response occurred immediately 30 seconds after the injection and then there was a recover to basal activity (Figure 14).

Comparison between saline and glutamate injections revealed that the BNST dm/fu neuronal activity varied as a function of drug x time (F = 4.15, P = 0.008) only in control animals.

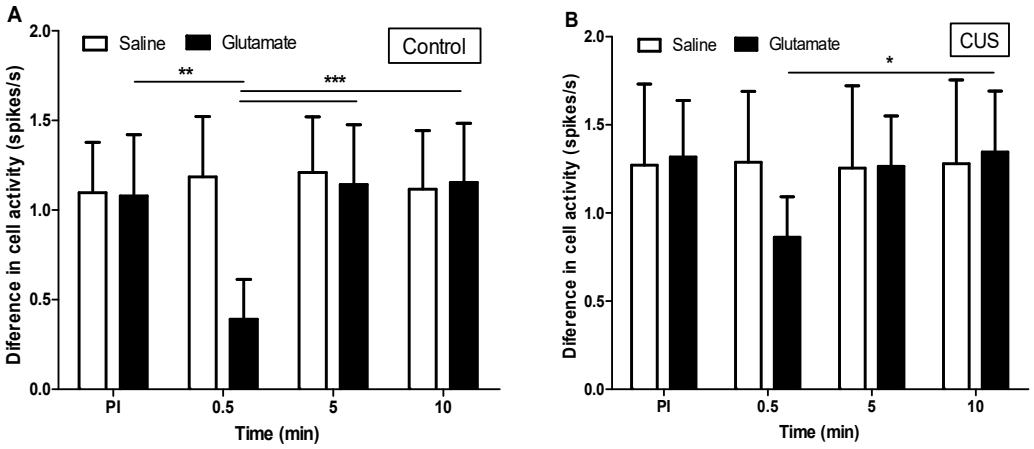

Figure 14. Noxious-evoked response of BNST dm/fu cells to mechanical stimulation (pinch) after the microinjection of glutamate in the ILCx of control (**A**) and CUS (**B**) rats. Injection of saline (0.9%) was used as a control (Control, n = 20; CUS, n = 15). Glutamate administration in the ILCx decreased significantly the noxious-evoked response of BNST dm/fu cells of both CUS (n = 17) and control (n = 15) rats. Control, control rats; CUS, chronic unpredictable stress rats; PI, pre-injection. Results are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

4.4 Activation of ILCx differentially affects c-Fos expression in the PVN of control and CUS animals

To evaluate if the alterations in the BNST dm/fu spontaneous neuronal activity and noxious-evoked response that occur after the pharmacological manipulation of ILCx have an effect over the HPA axis, we analyze the c-Fos expression in the PVN of both control and CUS rats after an administration of glutamate in the ILCx.

There were no differences in the total number of Fos-IR neurons in the PVN between control and CUS animals (t = 1, P = 0.09). Nevertheless, there was an increase in the number of Fos-IR PVN neurons in the right hemisphere of CUS animals when compared to the right hemisphere of the controls (t = 3.64, P = 0.011). Additionally, comparison between hemispheres revealed that in CUS animals the number of Fos-IR neurons was increased in the right side of the PVN (t = 5, P = 0.002), while no differences were found in control animals (t = 1, P = 0.31). See figure 15.

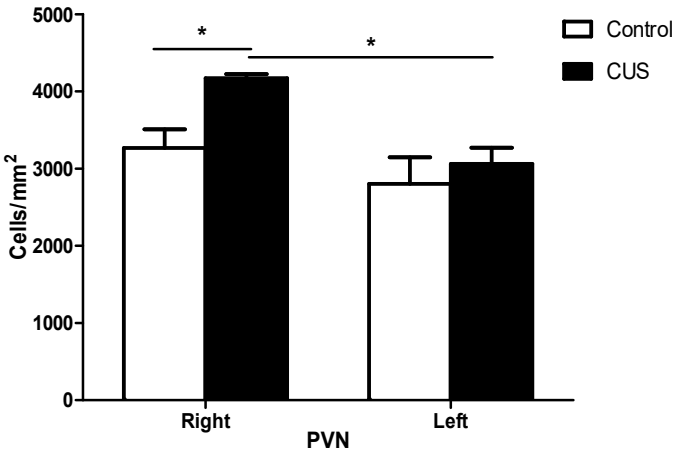

Figure 15. Distribution of Fos-IR neurons in the right and left hemispheres of the PVN, in control and CUS animals. There was an increase in the number of Fos-IR neurons in the right hemisphere of CUS animals. Control, control rats; CUS, chronic unpredictable stress rats. Results are presented as mean ± SEM. *$p < 0.05$

4.5 CUS impairs the normal corticosterone response to the stimulation ILCx

To further explore whether the alterations in the PVN c-Fos expression that occur after the pharmacological manipulation of ILCx have an effect over the corticosterone secretion, we analyze the corticosterone serum levels of both control and CUS rats during an administration of glutamate in the ILCx.

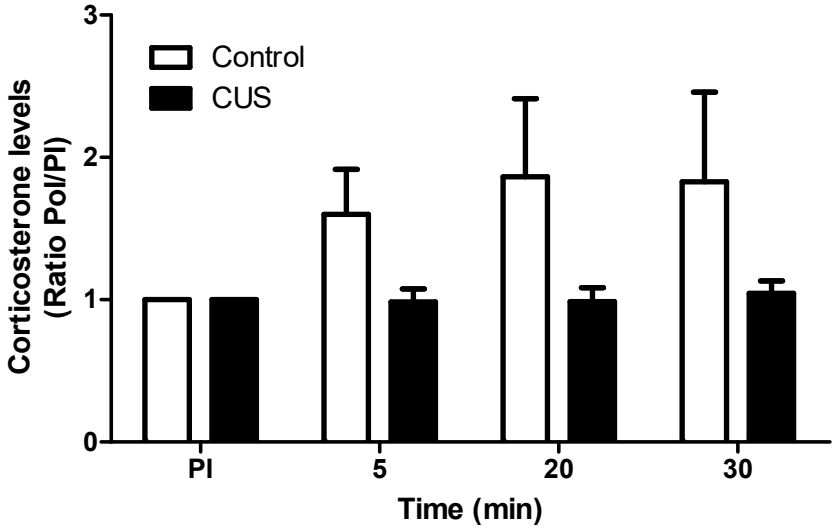

Figure 16. Effects of the ILCx chemical stimulation with glutamate on the release of corticosterone. Results were transformed in ratio PoI/PI to normalize the basal levels for all the animals. There was a trend for the levels of corticosterone to increase after the injection in the ILCx in control animals. Control, control rats; CUS, chronic unpredictable stress rats; PI, preinjection; PoI, post-injection. Results are presented as mean ± SEM.

There were no significant differences in the levels of corticosterone during the pharmacological manipulation of ILCx in both control ($F = 1.84$, $P = 0.24$) and CUS animals ($F = 0.25$, $P = 0.86$). In addition, comparison between groups revealed that the levels of corticosterone did not vary as a function of group x time ($F = 2.40$, $P = 0.11$). Nevertheless, there was a trend for the corticosterone levels from the control animals to increase when compared to CUS, after the injection of glutamate in the ILCx.

Chapter 5 –Discussion

5. DISCUSSION

The present work has characterized the influence of ILCx projections to the BNST dm/fu subnuclei, and consequent influence upon HPA axis in both control and chronic stress conditions. The BNST dm/fu spontaneous and noxious-evoked neuronal activity was recorded in both control and chronic stressed animals to evaluate the influence of CUS exposure in BNST dm/fu neuronal activity. Subsequently, the specific contribution of ILCx upon the BNST dm/fu neurons was assessed by recording the spontaneous and noxious-evoked activity of BNST dm/fu neurons before and after ILCx pharmacological manipulation. Finally, the influence of ILCx modulation of BNST dm/fu neurons upon the HPA axis activity was assessed by measuring the c-Fos expression in the PVN and the levels of corticosteroids in the blood after a pharmacological activation of ILCx.

5.1 Technical Considerations

The choice of the animal model of anxiety for this experiment was based in previous studies [100,131,132] that validated the CUS paradigm as a good animal model to mimic the conditions observed in the clinical settings. Indeed, clinical [80,84,142] and animal [7,93,100,132] studies have shown that the presentation of random and unpredictable stimuli induces a state of permanent alertness, which is supported by the continuous activation of the HPA axis. The overactivation of the stress response will in turn lead to structural and functional alterations of several brain regions, including the BNST [99,100]. In order to assess treatment efficacy a series of behavioral tests and biological variables were used. Anxiety-like behavior was assessed in the AS and in the EPM, which is considered to be the "gold standard test" for measuring anxiety behavior in rats [133,134,143]. Additionally, the OF test was used to evaluate the exploratory and locomotory activity, an important component that must be preserved when the animals are tested in the EPM.

The technique that was chosen to evaluate BNST neuronal activity was the *in vivo* extracellular single-cell recording. There are several benefits of this technique when compared to others, for example, with single-cell recording we are able to distinguish neurons within the same nuclei that respond differently to the same stimulus, while with local field potentials we can only assess the cooperating synaptic

inputs into a recorded area. Furthermore, with in vivo approaches we could analyze the body response to a specific brain manipulation, for example, the alterations in the levels of corticosteroids, an effect that we cannot assess with the patch-clamp technique. Following the choice of the electrophysiological technique, particular care was given to the several experimental factors, in order to better understand the role of the ILCx upon the BNST neurons.

ILCx was pharmacologically manipulated with glutamate and lidocaine to evaluate a possible phasic and tonic influence of the ILCx upon BNST dm/fu neuronal activity. Glutamate is the most predominant excitatory neurotransmitter of the CNS and it is commonly used in pharmacological studies due to the fact that constitutive mechanisms quickly remove it from the synaptic cleft [139]. By contrast, lidocaine is a synthetic compound commonly used as a local anesthetic [144], which leads to a temporary inhibition of the signal propagation by blocking fast voltage gated sodium (Na+) channels [138,145]. Transient inactivation of the ILCx, induced by local intracerebral administration of lidocaine, may unveil whether there is a tonic influence of this area upon others, in this case the BNST.

Other important feature that we have taken in account to this study was the calculation of the volume to be administrated in the ILCx. The chosen volume for the drug microinjection was 0,5 µL, based on previous studies which reported that this microinjection volume restricted the drug spreading diameter to the size of the ILCx [137]. Additionally, we found that the drug should be delivered for a period of 20 seconds, to avoid the possible neuronal activation by pressure. A short microinjection period usually results in an abrupt increase in focal pressure and may cause depolarization by itself, confounding the true effect of the drug. Nevertheless, we used the saline (0.9%) injection as a baseline control of the drug administration in order to exclude the hypothesis that the microinjection itself was having an effect during electrophysiological recordings.

To analyze the evoked activity of BNST/dm neurons we used a noxious stimulus based on previous studies which reported that BNST neurons were responsive to tail pinch [146,147].

3.2. Behavioral measurements of anxiety

In the present study we intended to explore whether a particular brain pathway (ILCx-BNST-PVN) is altered in stress-related anxiety disorders. For that we used two different groups: a control and a model of anxiety, which were validated using behavior tests. Indeed, the behavioral data clearly revealed a state of increased anxiousness in CUS animals both when assessing anxiety in the EPM and in the AS. The EPM paradigm is based on the conflict between a rodent natural drive to explore a novel environment and its tendency to avoid the open spaces [134,143,148]. Data gathered in the EPM confirms that CUS animals spent less time and made fewer entries in the open arms of the maze, despite preserving a normal locomotion activity measured in the OF. In the AS we also found an increased startle response in the CUS group, demonstrating increased reactivity to anxiogenic acoustic stimuli [149]. Taken together, our behavioral data showed that we indeed had two different groups before the electrophysiological procedure.

5.3. The role of ILCx in the modulation of BNST dm/fu neurons in Health and Disease

The behavioral alterations induced by chronic stress, namely anxiety, are often correlated with specific structural and functional changes in the limbic structures involved in the stress response [92,93,98]. The structure and function of BNST, a putative relay nucleus between limbic structures and the PVN, is also affected in chronic stress conditions [99,100]. Nevertheless, little is known about BNST neuronal basal activity and how it is affected by chronic stress exposure. Indeed, compared with other brain components, few studies have examined the electrophysical properties of BNST neurons [150-154]. In this study we attempted to explore the *in vivo* properties of BNST dm/fu neurons in both control and anxious animals, and to analyze whether they are affected by ILCx pharmacological manipulation.

Our electrophysiological data revealed a non-significant trend for the spontaneous activity of BNST dm/fu neurons to be increased in CUS animals. This data is in agreement with previous studies that reported a significant level of synaptic plasticity in the BNST after the exposure to chronic stress[99,100]. Moreover, we found that there is a significant trend for the spontaneous activity of BNST dm/fu neurons of

CUS animals to be enhanced by the stimulation of the ILCx. In accord with previously published work [96,99], this suggests that chronic stress induces structural and functional changes in the ILCx, which in turn will lead to an overactivation of its efferent brain regions, namely the BNST. Our results also revealed that when it is stimulated, the ILCx modulates the noxious-evoked response of BNST dm/fu neurons. In control animals there was a significant decrease of the noxious-evoked activity of BNST dm/fu neurons, an effect that seems to be abolished in chronic stress conditions.

Nevertheless, the inhibition of ILCx with lidocaine did not have an effect over neither the spontaneous nor the noxious-evoked activity of BNST dm/fu neurons, which suggests that the ILCx exerts a phasic rather than a tonic excitatory influence over the firing characteristics of BNST dm/fu neurons. This data is consistent with other electrophysiological studies which demonstrated a phasic excitatory input arising from the ILCx to the BNST neurons [154]. Therefore, it is hypothesized that the BNST relays the excitatory inputs of the ILCx upon the HPA axis. Several anatomical studies reported a substantial direct projection from the deeper layers of the mPFC to the ventral region of the BNST [57,69,116,154], suggesting a direct pathway between the ILCx and the BNST. The ILCx may exert its actions upon the HPA axis through a direct excitation of the CRF/glutamate BNSTdm/fu projections to the PVN or by a desinhibition of the GABA BNST dm/fu population that inhibits the PVN activity. The results from control animals suggest that the inhibition of the noxious-evoked response of the BNST dm/fu neurons in response to a chemical stimulation of the ILCx might be explained by an inhibitory pathway. This pathway is likely to target the GABAergic neurons in the BNST that are inhibiting the PVN activity, a mechanism that seems to be abolished in CUS animals. On the other hand, we observed an enhanced spontaneous activity of BNST neurons and a decreased noxious-evoked response following a pharmacological stimulation of the ILCx in CUS animals. The exposure to chronic stress, along with the structural changes, may also induce a rearrangement of the brain pathways involved in the stress response. Our results suggest that chronic stress may impair the regulatory pathway from the ILCx to the BNST causing a shift from facilitatory to inhibitory influence of the ILCx upon the HPA axis. Additionally, the excitation or desinhibition of the BNST dm/fu subnuclei may be mediated by the

interactions between the ILCx and other limbic structures, such as the amygdala, which is involved in emotional behavior and strongly innervates the BNST [124,155,156].

5.4 The impact of chronic stress upon the ILCx modulation of HPA axis

Taken in account the influence of ILCx stimulation upon BNST dm/fu neurons, we attempted to further explore this effect upon the HPA axis activity. For this, we analyze the c-Fos expression in the PVN and the levels of corticosterone in the blood after an injection of glutamate in the ILCx. The analysis of the c-Fos expression revealed no differences in the overall activation of the PVN neurons in response to the pharmacological stimulation of the ILCx when comparing control with CUS animals. Comparison between groups of the interhemispheric c-Fos expression, we found no differences between groups in the left hemisphere, however we observed an enhanced activation of PVN neurons from the right hemisphere in CUS animals when compared to controls. This suggests that chronic stress induces changes in the brain circuits that modulate the stress response, and that ILCx participates in this mechanisms. It was reported by Radley and coworkers that specific lesions in the ILCx lead to a decrease in c-Fos expression within the neurosecretory region of PVN in response to stress [56], and this excitatory role of ILCx upon PVN activity might be enhanced by chronic stress. Indeed, it was shown that there is a rearrangement of dendrites in the afferent areas of mPFC in animals exposed to chronic stress [157]. Importantly, the differences in the PVN activity of CUS animals were only detected in the right hemisphere. To better understand these effects, it is important to highlight that only the right brain hemisphere was pharmacologically manipulated. Therefore, if there were no differences between the projections from the right ILCx to both PVN hemispheres, we can explain this asymmetry by interhemispheric differences in the activity of PVN induced by chronic stress. On the other hand, we may assume that by manipulating the right ILCx we only induced an effect in the right PVN, suggesting a lateralized influence of the ILCx upon the HPA axis. However it is known that there are interhemisferic differences within the mPFC and that they are altered in chronic stress conditions [51], for example, the proximal apical dendrites of control animals are longer in right ILCx but this hemispheric difference is abolished by chronic stress [157].

However, little is known about the consequences of the ILCx lateralized susceptibility to the effects of chronic stress upon the PVN activity.

The injection of glutamate in the right ILCx did not significantly altered plasma levels of corticosterone at any time post-injection. Although it was not significant, there was a trend for the pharmacological stimulation of the ILCx to increase the levels of corticosterone in control animals. This data is in accordance with previous studies which reported an increase in the plasmatic corticosterone levels after an electrical stimulation of the mPFC [158159]. Importantly, the control of the mPFC upon the HPA axis is impaired when animals are subjected to chronic stress, and this might explain the results for the CUS animals. Indeed, Sullivan and Gratton reported a blunting in the peak of adrenocortical secretory response in animals submitted to chronic restrain stress, an effect that was accentuated when the mPFC was lesioned [55]. The lack of significantly differences in our results can be due to the small number of animals tested.

In summary, our results add new information about the impact of chronic stress in a specific brain circuitry (ILCx-BNST-PVN) which is implicated in the normal response to stress. The present results point to an exacerbation of the ILCx excitatory inputs to the BNST dm/fu subnuclei in chronic stress conditions, an effect that is correlated with an overactivation of the HPA axis.

Chapter 6 – Conclusions and Future Perspectives

6. CONCLUSIONS AND FUTURE PERSPECTIVES

In this work we analyzed for the first time the spontaneous and the noxious-evoked neuronal activity of a specific region within the BNST, the dorsomedial and fusiform subnuclei, in both control and CUS-treated animals. Additionally, we also analyzed the influence of the right ILCx projections to the BNST and its effect upon the HPA axis activity.

In summary, the present work shows that in basal conditions the ILCx modulates the activity of BNST dm/fu neurons by inhibiting their response to a noxious stimulus. This, in turn, is correlated with the activation of the HPA axis. Importantly, chronic stress seems to impair this regulatory pathway causing a shift from inhibitory to excitatory influence of the ILCx upon the BNST dm/fu subnuclei, leading to an overactivation of the HPA axis.

Taken together, the conclusions withdrawn from this work give new insight about the impact of chronic stress in a specific brain circuitry (ILCx-BNST-PVN) which is implicated in the normal response to stress. However, several questions arise from these observations. Future work should involve:

- Further analysis on the impact of chronic stress upon the left ILCx modulation of BNST dm/fu neurons and consequent activation of HPA axis, using the same electrophysiological protocol;
- The confirmation of an anatomical connection between the PVN and the BNST dm/fu neurons that are modulated by the ILCx, through the use of electron microscopy and tract tracing approaches;
- The discrimination of a possible role of other limbic structures, namely the amygdala and the hippocampus, upon the ILCx modulation of BNST neurons;
- The evaluation of the electrophysiological properties of BNST dm/fu neurons and its modulation by the ILCx in freely moving rodents;

Chapter 7 – References

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