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Influence of Stress in the Structure and Function of the Amygdala

Tese de Doutoramento
Ciências da Saúde - Ciências Biológicas e Biomédicas

Trabalho efectuado sob a orientação do
Professor Doutor Nuno Sousa

Novembro de 2007

Mãe!
Passa a tua mão pela minha cabeça!
Quando passas a tua mão na minha cabeça,
é tudo tão verdade!

Almada Negreiros

À memória do Carlos

... obrigado por todos os diálogos silenciosos

Aknowledgements

I would like to thank Professor Nuno Sousa, most of all, for being a friend, for being there, for his constant support and positive attitude. I am so glad that I could share part of my life with you and your family. A warm hug to Rita, Hugo and Isabel. Obrigado!

To my family, the cornerstones of my character and will. To my mother, for all her love, understanding and wisdom. Thank you for pointing me the direction but let me make my own choices and mistakes. To my father, for his support and care. To João Pedro and Ana Paula, loving brother and sister, for sharing your lives and experiences, and pushing me forward. To Pedro, Fernanda and Alberto, who have always supported me.

To Professor Cecília for believing in me and for all her support...

To Professor Osborne Almeida for his wise and insightful advices, delightful comments, friendship and hospitality (also to Leila and Isabel)...

To Professor Joana Palha for all her suggestions and accurate remarks and for all her happiness...

To Professor João Cerqueira (Joauche) for dragging me into this adventure, for all the time we spent together that never fades, for his work and for his contagious laugh...

To Professor João Sousa for being who he is, for his character, for all the work, for the challenging ideas and for always being there when I could not...

To Professors Armando Almeida, Margarida Neves, Jorge Pedrosa, António Salgado and Patrícia Maciel for their support...

To Drs. Mário Carvalho and Maria José for believing in me, for understanding and always supporting me...

To Dr. Pedro Morgado (and family) for his friendship, for his work and character and for teaching me the importance of little things...

To Drs. Ana Raquel and Rui Silva for their precious help and support ...

To Dr. Filipa Ribeiro for the time we spent together, laughs and support...

To Eng. Goreti Pinto and Luís Martins for all the help and work that made my research possible...

To Drs. Pedro Leão, Mário Oliveira, João Bessa, Hugo Tavares, Ricardo Taipa, André Carvalho, Jie Lu, Iannis Sotiropoulos, Caterina Catania for their help and support...

To all the researchers at ICVS and Neuroscience Research Domain with whom I have learned so much...

To the technical staff at ICVS, the scaffolds upon which we stand to make dreams come true...

To all my friends for the support, for enduring my absences, for bringing me back to reality and always having a warm welcome...

My last words go to Sara, my beloved companion and unconditional friend. I could never find the words to express my gratitude for all the patience, care, tenderness and support. You are so much more than I could ever wish for. Obrigado por seres quem és...

The present dissertation is the result of my scientific research at ICVS, although, no such task could ever be conducted without the help of these people, to whom I am forever indebted.

Braga, November 2007

José Miguel Pêgo

Abstract

Stress is a powerful modulator of emotional behavior and is implicated in the etiology of mood disorders like anxiety, depression and phobias. Mood disorders are particularly common and affect a large percentage of the world population with relevant implications to individual's health and to the society. Fear and anxiety represent specific aspects of emotional behavior that show behavioral resemblances but are triggered by events that activate distinct neuronal pathways: while the amygdala plays a key role in fear-conditioned to cue stimuli, the bed nucleus of the stria terminalis (BNST) is implicated in anxiety behavior and responses to contextual stimuli. In addition, the BNST is directly involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis being an important relay station of limbic inputs. In the present work, we have thoroughly characterized how emotional behavior, namely fear and anxiety, is affected by chronic stress. In order to uncover structural correlates of behavioral findings stereological estimates of the BNST and amygdaloid complex were performed, followed by 3-dimensional morphometric dendritic analysis. To clarify the relative importance of hormonal (corticosteroids) and central mediators (CRF_1R or CRF_2R agonists) of the stress response, and their contribution to neuroanatomical changes induced by stress, two additional sets of experiments were designed. Finally, the functional relevance of the observed changes was determined by characterizing the expression of immediate-early genes in control and stressed animals in basal conditions and after anxiogenic stimulus. Results show that chronic stress induces anxiety behavior without influencing fear-conditioning and locomotory/exploratory activity. Stress-induced anxiety correlated with increased volumes of the BNST, but not of the amygdala. Dendritic remodeling was found to make a significant contribution to the stress-induced increase in BNST volume, primarily due to changes in the anteromedial area of the BNST, an area strongly implicated in emotional behavior and in the neuroendocrine control of the stress response. Most of the effects of stress were recapitulated by exogenous corticosterone, while CRF_1R reproduced particular aspects of spinogenesis. At last, chronic stress was associated to altered expression of *c-fos* in the BNST in both basal conditions and after anxiogenic stimuli. In conclusion, this study shows that chronic stress impacts on BNST structure and function establishing a possible correlation between anxiety behavior and structural remodeling. Finally, the functional impairment of the BNST may reflect the loss of inhibitory tone over the HPA and contribute to the perpetuation of corticosteroid neuronal restructuring.

Resumo

O *Stress* é um modulador potente do comportamento emocional e está implicado na etiologia das perturbações do humor como a ansiedade, a depressão e as fobias. As perturbações do humor são particularmente comuns e afectam uma percentagem grande da população mundial com implicações relevantes para a saúde do indivíduo e da sociedade. O medo e a ansiedade representam aspectos específicos do comportamento emocional que apresentam semelhanças comportamentais mas são despoletados por eventos que activam circuitos neuronais distinctos: enquanto que a amígdala tem um papel central no condicionamento ao medo a estímulos específicos, o núcleo da estria terminal (NET) está implicado na ansiedade e em repostas a estímulos contextualizados. Adicionalmente, o NET está directamente envolvido na regulação do eixo hipotálamo-pituitária-surprarenal (HPA) sendo uma importante estação de passagem para a informação proveniente do sistema límbico. No presente trabalho, caracterizamos como é que o comportamento emocional, nomeadamente medo e ansiedade, é afectado pelo *stress* crónico. Por forma a estabelecer correlações estruturais com os achados comportamentais realizámos estimativas esterológicas do NET e da amígdala, seguido da análise dendrítica morfométrica de reconstruções 3-D de neurónios. Para clarificar qual a importânica relativa dos mediadores hormonais (corticosteróides) e centrais (agonistas CRF_1R ou CRF_2R) da resposta ao *stress*, e qual o seu contributo para as alterações neuroanatómicas observadas, realizámos duas experiências adicionais. Finalmente, a importância functional dos achados foi determinada caracterizando a expressão de genes de activação imediata em condições basais e após exposição a estímulos ansiogénicos. Os resultados mostraram que o *stress* crónico induz comportamento ansioso sem fectar o comportamento de medo ou actividade exploratória/locomotora. A ansiedade induzida pelo *stress* correlaciona-se com aumentos no volume do NET, mas não da amígdala. A remodelação dendrítica provou contribuir para o aumento significativo do NET, primariamente associado a alterações na área anteromedial do NET, uma área fortemente implicada no comportamento emocional e no controlo neuroendócrino da resposta ao *stress*. A maior parte dos efeitos observados pode ser replicado pela administração exógena de corticosteróides, enquanto que os agonistas dos CRF_1R reproduziram aspectos particulares da formação de espinhas. Por fim, o *stress* crónico está associado a alterações na expressão de *c-fos* no NET quer em situações basais como após estímulos ansiogénicos. Concluindo, este estudo mostra que o *stress* crónico tem um impacto

significativo na estrutura e função do NET estabelecendo uma possível ligação entre o comportamento ansioso e a remodelação estrutural. Finalmente, a disfunção do NET pode reflectir uma perda do tonus inibitório sobre o HPA e contribuir para a perpetuação da reestruturação neuronal pelos corticosteróides.

CONTENTS

ABBREVIATIONS LIST

ACTH – adrenocorticotrophic hormone

AHP – after-hyperpolarization

AL – allostatic load

AVP – arginine-vasopressin

BLa – basolateral anterior amygdaloid nucleus

BNST – bed nucleus of the stria terminalis

BNSTam – anteromedial division of the bed nucleus of the stria terminalis

CeA – central amygdaloid nucleus

CNS – central nervous system

CRF – corticotrophin-releasing factor

GC – glucocorticoids

CUS – chronic unpredictable stress

EPM – elevated-plus maze

GABA – gama-amino-butiric-acid

GAD – glutamic acid decarboxylase

GC – glucocorticoids

GR – glucocorticoid receptor

HPA – hypothalamus-pituitary-adrenal

icv – intracerebroventricular

LTD – long-term depression

LTP – long-term potentiation

MeA – medial amygdaloid nucleus

MR – mineralocorticoid receptor

NMDA – N - methyl - D – aspartic acid

PFC – prefrontal cortex

PTSD – post-traumatic stress disorder

PVN – paraventricular nucleus of the hypothalamus

tPA – tissue plasminogen activator

Ucn – urocortin

Chapter 1

Introduction

1. INTRODUCTION

1.1. Stress and emotional behavior

Stress has been implicated in the etiology of emotional disorders, such as depression (McEwen, 2004; Hammen, 2005), phobia (Risbrough & Stein, 2006) and anxiety (Charney *et al.*, 1993). Stress-related disorders affect an important portion of the active working population and therefore exert an important burden on the individual's health and on the society. The World Health Organization estimates that emotional/mood disorders are among the leading causes of disability worldwide and that by 2020 will be the second leading global burden of illness, only surpassed by cardiovascular disorders.

1.1.1. Definition

Stress is defined as a challenge to homeostatic equilibrium, by physical or psychological events (McEwen, 2003). Stress generates a cascade of hormonal and behavioral changes aimed to exert control over the threatening events. Adaptation in the face of potentially stressful challenges involves activation of neural, neuroendocrine and neuroimmune mechanisms. Effective coping implies that a stress response is effectively triggered when needed and terminated afterwards (de Kloet *et al.*, 2005). However, if the stress response is inadequate or prolonged in time, a series of pathophysiological changes occur in the brain, the immune system and viscera (Musselman & Nemeroff, 2000; Sapolsky, 2000; Mayer & Fanselow, 2003; Sorrells & Sapolsky, 2007) that may ultimately lead to several disorders.

1.1.2. The stress response

The central component of the stress response is activation of the hypothalamus-pituitary-adrenal axis (HPA) (Sapolsky *et al.*, 1986). Upon stimulation, neurosecretory neurons of the paraventricular nucleus of the hypothalamus (PVN) secrete several adrenocorticotrophic hormone (ACTH) secretagogues, the most important of which are corticotrophin-releasing factor (CRF) and arginine-vasopressin (AVP) (Keller-Wood & Dallman, 1984; Herman & Cullinan, 1997). Release of

ACTH will, in turn, lead to the secretion of corticosteroids by the adrenal glands (Figure 1). Therefore, by integrating the output of different stress-sensitive brain circuitries (Herman & Cullinan, 1997), the PVN plays a key role in regulating the HPA stress response (Whitnall, 1993).

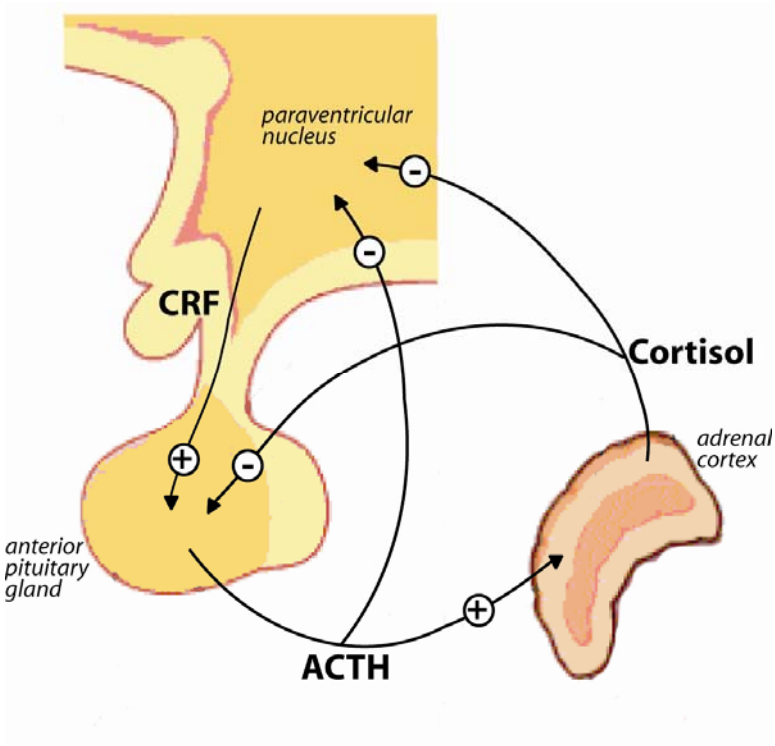

Figure 1. Schematic representation of the hypothalamus-pituitary-adrenal axis and its regulation.

Indeed, stressful events can activate the PVN by distinct neuronal pathways (Herman & Cullinan, 1997; Herman *et al.*, 2003): stressors like hemorrhage, respiratory distress or systemic inflammation, represent an immediate threat to the homeostatic balance of the individual, are perceived by somatic, visceral or circunventricular sensory pathways (Chan *et al.*, 1993; Cole & Sawchenko, 2002) and directly activate the PVN bypassing cortical and limbic areas. More precisely, the excitatory ascending pathways originating in brainstem nuclei that convey noradrenergic inputs from the nucleus of tractus solitarius (Gann *et al.*, 1977; Abercrombie & Jacobs, 1987; Smith *et al.*, 1991; Cullinan *et al.*, 1995), the serotonergic inputs from the raphe nuclei (Sawchenko *et al.*, 1983; Feldman *et al.*, 1987) or from adjacent hypothalamic nuclei are in a privileged situation to receive visceral and autonomic inputs and to mount a rapid "reactive" neuroendocrine response. Other stressors activate the HPA in the absence of primary sensory stimuli signaling homeostatic disruption. These responses are centrally generated and represent an effort of the organism to mount a response that mobilizes energetic, metabolic and immune

resources, in anticipation to homeostatic disruption. The activation of these complex pathways seems to occur by contextual, conditioned (memory) or species-specific cues that predict adversity. Ultimately, there will be an activation of the HPA and autonomic systems to set up an adequate stress response (de Kloet *et al.*, 2005). The mnemonic aspects of this response are important determinants of the magnitude of the HPA response as these stimuli become stressful only by comparison with previous experience. The limbic system plays a central role in the coordination of this "anticipatory" stress response (Herman *et al.*, 2005). Stimuli that require assembly and integration of multiple sensorial modalities like restraint, stress or fear conditioning are processed by limbic centers related to emotional behavior (amygdala related to fear and anxiety) (LeDoux, 2000; Ledoux, 2007), memory and learning (hippocampus) and executive and cognitive functions (prefrontal cortex, PFC) (Maier *et al.*, 2006). Acting in a coordinated way, the intricate neuronal circuitries that characterize these networks establish a temporal and contextual framework of stimuli interpretation that determines the aversive/rewarding value of a stimulus. Characteristically, the modulatory action that limbic structures exert on the HPA is not conveyed by direct excitatory pathways; it is rather through the modulatory action of other neurons located in the hypothalamus or in the bed nucleus of the stria terminalis (BNST) (Herman *et al.*, 1994; Herman *et al.*, 2003), that the net result of limbic inputs influences the activity of neurosecretory neurons of the PVN. More specifically, the GABAergic projection neurons from the BNST exert an inhibitory tone over the PVN, under the control of an excitatory glutamatergic input from the PFC and hippocampus (Cullinan *et al.*, 1993), and an inhibitory GABAergic input from the central and medial amygdala nuclei (Prewitt & Herman, 1998; Herman *et al.*, 2005).

1.1.3. Hormonal mediators of stress

Circulating corticosteroids yield a central role in the adaptative response to stress, changing energy metabolism and dampening the immune and inflammatory responses. An adequate stress response, implicates that they act in the short term to prevent overshooting of the innate response (Sapolsky *et al.*, 2000; de Kloet *et al.*, 2005). However, prolonged exposure to elevated levels of corticosteroids leads to immune dysfunction (Sapolsky *et al.*, 2000; Sorrells & Sapolsky, 2007), endocrine dysregulation (Sapolsky *et al.*, 2000) and, ultimately, to neuropathology (Sapolsky, 1999).

Corticosteroids actions are mediated by two different subtypes of receptors which bind the same hormone (cortisol in humans, corticosterone in rodents) with distinct affinities. Mineralocorticoid (MR) and glucocorticoid (GR) receptors are ubiquitously distributed in the body, including the brain, especially in the limbic areas that are responsible for the modulation of the stress response (Reul & de Kloet, 1986). The MR has an higher affinity than the GR and it is therefore highly occupied even in basal conditions (Reul & de Kloet, 1985). While GR is activated mainly during elevation of circulating corticosteroids, MR has been implicated in the appraisal process and onset of the stress response, is responsible for the mobilization of energy substrates and most of the behavioral changes observed afterwards. The later include anxious-like behavior but also facilitated learning and memory (in particular consolidation of memories). However, the long-term activation of GR has been associated with deleterious effects on several mnesic and cognitive functions (Sapolsky *et al.*, 1986; Sousa *et al.*, 2000; Cerqueira *et al.*, 2005; McEwen, 2005; Cerqueira *et al.*, 2007a; Cerqueira *et al.*, 2007b; Sousa *et al.*, 2007). Interestingly, these effects have been correlated with neuroarchitectural changes in several regions of the central nervous system (CNS), including the hippocampal formation, prefrontal cortex and amygdala (McEwen, 2007). Of notice, the hippocampal formation and prefrontal cortex are themselves back-regulating the HPA axis, preventing the prolongation of stress response beyond adaptation (De Kloet & Reul, 1987).

1.1.4. Stress beyond corticosteroids: the CRF family and its receptors

The elevation of circulating corticosteroids is a central component of the stress response. This fact has even misled the field of neurobiology of stress, in as much as, some have erroneously assumed that high levels of corticosteroids can mimic stress. This is certainly not the case as many other processes are altered by stressors, including several transmitters such as CRF. CRF is a main hypothalamic neuropeptide involved in the control of synthesis and release of ACTH by the pituitary and plays a pivotal role in the regulation of the HPA axis and stress response (Lau *et al.*, 1983). CRF and the related peptides Urocortin (Ucn), UcnII and UcnIII form a family of peptides that act through G-protein-coupled receptors, CRF_1R and CRF_2R. While CRF binds CRF_1R selectively, Ucn binds both CRF_1R and CRF_2R. The more recently discovered UcnII and UcnIII bind

the CRF_2R with high selectivity. Each receptor displays a different expression pattern and has distinct physiological functions (Chen *et al.*, 1993; Chalmers *et al.*, 1995; Lovenberg *et al.*, 1995; Dautzenberg & Hauger, 2002; Reul & Holsboer, 2002). In the brain, CRF is expressed in neurons of the PVN, cerebral cortex, cerebellum, amygdala and hippocampus; Ucn is limited to the Edinger-Westphal, lateral olivary and supraoptic nuclei; UcnII has a distinct subcortical expression in regions related to stress-response such as PVN, the locus coeruleus, the supraoptic and arcuate nuclei of the hypothalamus and several motor nuclei of the brainstem and spinal cord; and, UcnIII is expressed in the rostral perifornical area, posterior part of the BNST, medial nucleus of the amygdala and lateral septum.

CRF_1R is mainly expressed in the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampus and olfactory bulbs (Dautzenberg & Hauger, 2002; Reul & Holsboer, 2002) and has been consistently implicated in the stress-related behavior (Liebsch *et al.*, 1995; Heinrichs *et al.*, 1997; Skutella *et al.*, 1998; Liebsch *et al.*, 1999; Heinrichs & Koob, 2004), revealing anxiety-like responses in different animal paradigms like the elevated-plus maze (File *et al.*, 1988), social interaction test (Dunn & File, 1987) and acoustic startle (Swerdlow *et al.*, 1986); additionally it plays a role in hormonal (Dunn & File, 1987) and autonomic activation (Nakamori *et al.*, 1993) following stress. CRF_1R seems to be crucial to the initiation of the stress response but not to the baseline drive of the HPA axis (Reul & Holsboer, 2002). CRF_2R is mainly expressed in subcortical areas like the PVN, lateral septum, amygdala, hippocampus and BNST. The specific role of CRF_2R is not yet clearly understood, as results are controversial: while some studies suggest that central activation of CRF_2R triggers anxiogenis (Radulovic *et al.*, 1999) others have observed an anxiolytic effect (Valdez *et al.*, 2003; Zhao *et al.*, 2007). Several confounds might explain such discrepancy, including specific regional activation or duration of administration. More consistent, appears to be the role of CRF_2R in the extinction of HPA activation (Bale *et al.*, 2000; Coste *et al.*, 2000).

1.2. Chronic stress, anxiety, fear and the aging brain

1.2.1. Anxiety

Anxiety disorders are very common and affect a large percentage of the population (Kessler *et al.*, 2007; Merikangas & Kalaydjian, 2007; Moussavi *et al.*, 2007). These range from phobias to

generalized anxiety disorders (American Psychiatric Association, 1994). Anxiety is characterized by a sensation of discomfort and apprehension in response to unconditioned diffuse cues (Koch, 1999). It is commonly experienced by healthy individuals in response to unspecific frightening cues, although without clear threatening outcomes (e.g. loud noises, fast approaching objects, etc.). The biological advantage of anxiety status subsides on preparing the individual to eventual harmful encounters in a normal process that is called the 'flight or fight' phenomenon. The symptomatic response to anxiogenic stimuli is characterized by increased heart rate, tensed muscles, and eventually an acute sense of focus as the individual tries to determine the source of threat. This response outlasts the exposure of the anxiogenic stimulus, suggesting long-term activation of the neural substrates and processes that regulate anxiety (Lee *et al.*, 1994; Lee & Davis, 1997a; b). Anxiety disorders develop when symptoms occur without any recognizable stimulus or when the stimulus does not warrant such a reaction.

1.2.2. Fear

Fear, in contrast, involves the conditional learning that a specific cue, or a more complex context, predicts an imminent adversity (Brown *et al.*, 1951; Davis, 1986; 1992). It is present even in more primitive animal species (Davies *et al.*, 2002; Eisenberg & Dudai, 2004; Portavella *et al.*, 2004) as it represents a clear advantage to the survival of the individual and continuity of species. By learning that a particular situation poses threat to the individual, future encounters are avoided by keeping away from specific objects or contexts. Fear response is characterized by a set of symptoms relayed by neuroendocrine and autonomic centers and that reflect the activation of common neuronal pathways to the anxiety response (Walker *et al.*, 2003). However, these common pathways are activated in a particular set-up of conditions that reflect the activation of distinctive initial centers. Fear responses are elicited by explicit, adversity-predicting, short-lasting cues, in which the danger period has a clear offset. These responses are, in general, self-limited in time and magnitude, the symptoms resulting from its activation being abolished by the termination of stimuli. In summary, fear and anxiety behaviors are mediated by common pathways that are activated in distinctive conditions; that is: duration and conditioning (of stimuli), although the two variables are often intertwined.

1.2.3. The aging brain

Anxiety disorders are particularly common in otherwise healthy old individuals (McEwen, 2003), both in humans and in animals (McEwen, 2003; Bessa *et al.*, 2005; Mohlman & Price, 2006; Riedel-Heller *et al.*, 2006; Weissman & Levine, 2007). Normal aging is associated with the gradual deterioration of biological systems including the brain. The burden exerted on the body through cumulative attempts to adapt to life's demands, in the context of a chronically challenged system, led to the conceptualization of cumulative load during aging, a concept termed allostatic load (AL) (McEwen & Stellar, 1993; McEwen, 1998; McEwen & Seeman, 1999). When the adaptive responses to challenge lie chronically outside the normal operating ranges, wear and tear on regulatory systems occurs and AL accumulates, ultimately leading to disease. The observation that the behavioral changes in normal aging resemble those of chronically stressed individuals has prompted researchers to propose bridging hypothesis that clarify the mechanisms underlying AL in the aged individual. Evidence has stemmed from different approaches: i) aging is associated with a progressive increase in basal cortisolemia (Sapolsky, 1999); ii) corticosteroid levels normalization takes longer in the aged HPA which reveals impaired shut-off mechanisms (Issa *et al.*, 1990); iii) neuropathological changes, namely in the hippocampal formation, show structural degeneration resembling overexposure to glucocorticoids (GC) (Nichols *et al.*, 2001); iv) impairment of hippocampal-dependent functions is correlated to the burden of GC exposure in humans (Lupien *et al.*, 1994; Seeman *et al.*, 2001); v) normalization of GC levels correlates with a decrease in the cognitive and electrophysiological abnormalities observed in aged animals (Landfield *et al.*, 1981; Meaney *et al.*, 1988; Talmi *et al.*, 1996).

This simplistic view that cognitive and behavioral aspects of the aging process result from the deterioration of stress-sensitive brain areas as the result of repetitive glucocorticoid insults is attractive, but, probably, it represents only part of the aging process. In spite of this, the "glucocorticoid cascade" theory of aging has found many similarities in the clinical settings. There is considerable evidence that normal aging impacts upon neuroendocrine stress responses, and studies of the molecular and cellular mechanisms underlying the pathophysiology of age-related disorders such as Alzheimer's disease and anxiety, are revealing novel insight into the involvement of perturbed neuroendocrine stress responses in these disorders (Pedersen *et al.*, 2001). Memory disorders and hippocampal shrinkage in the elderly are associated with polymorphisms in serotonin transporters system that interact with cortisol levels (O'Hara *et al.*,

2007) and are involved in the generation of anxiety behavior. In addition, the progressive elevation of GC and dysfunctional shut-down of HPA, seen in the normal senescent individual, increases the chances that sensitive brain systems are damaged by hormonal stress mediators. This hypothesis is corroborated by studies demonstrating that the genetic background and early life events, which lead to overactivation of the HPA, render the individual vulnerable to particular psychopathological entities as a consequence of the stress (Agid *et al.*, 1999; Gispen-de Wied, 2000).

In summary, aging is a complex process that involves, but is not restricted to, the progressive dysregulation of the HPA axis and subsequently damage to stress-sensitive brain areas. As a consequence, cognitive and behavioral disorders subside, a processed that is influenced by early life events and biological burden of the individual.

1.2.4. The role of stress

Animal (File, 1996; Arborelius *et al.*, 1999; Vyas *et al.*, 2002; Anisman & Matheson, 2005) and clinical (Dranovsky & Hen, 2006; Chrousos & Kino, 2007; Greaves-Lord *et al.*, 2007) studies have consistently correlated chronic stress with altered emotional behavior. Stress is considered a risk factor for disorders like depression and anxiety. Most importantly, a significant percentage of depressed patients displays signs of impaired function of the HPA axis (Shelton, 2007), as assessed by the levels of cortisol and by the response to dexamethasone suppression test (Gillespie & Nemeroff, 2005). Coherently, cognitive and antidepressant therapy, that result in improved clinical symptoms, correlate with normalization of the HPA function (Mason & Pariante, 2006; Schule, 2007). Additionally, inappropriate adaptation to stress can result in a state of anxiety and facilitation of phobic behavior acquisition (Garakani *et al.*, 2006). Most of these effects can be attributed to sustained increases in corticosteroid levels (Arborelius *et al.*, 1999) which subsequently activate receptors in brain areas implicated in the regulation of emotional behavior. GC have been shown to enhance the excitability of amygdalar neurons and to influence the acquisition of fear behavior (Skorzewska *et al.*, 2006; Duvarci & Pare, 2007; Yang *et al.*, 2007). Other studies, recurrently, demonstrated that stress induces anxiety-like behavior (Vyas *et al.*, 2002; McEwen, 2003; 2004; Vyas & Chattarji, 2004) and that GR modulate anxiety-associated behavior (Boyle *et al.*, 2006). The amygdala and extended amygdala (including the

BNST), both of which display a rich population of corticosteroid receptors that are activated during stress (Cullinan *et al.*, 1995; Figueiredo *et al.*, 2003), have a putative role in the integration of polysensorial information that culminates in the expression of emotional behavior. As a result, the correlates (structural or neurochemical) of the altered emotional behavior associated with chronic stress should be searched in these brain structures (Please see Chapter 2.1).

1.3. The amygdala and the extended amygdala – implications in anxiety behavior

The amygdala, a deep temporal structure with rich interconnections with the hippocampus and PFC, has long been known to be involved in emotional behavior (Weiskrantz, 1956; Robinson, 1963; Goddard, 1964). Lesions in the amygdala (which included the BNST) induce disturbances in emotional and social behavior, attention disorders and deficits in memory consolidation/extinction (Bucy & Kluver, 1955; Weiskrantz, 1956). However, recently, more selective lesion studies, combined with pharmacological approaches have helped to clarify the differential role of the amygdala and BNST in emotional behavior (Davis, 1992; Lee & Davis, 1997a; Davis, 1998; 2006).

1.3.1. Amygdala: Structural and functional organization

The early view that the amygdala is a relatively homogeneous structure has been challenged by embryological, anatomical and functional studies. To the initial description by Burdach (1819-1822) of the amygdalar nucleus (Mandelkern or almond-shaped nucleus), which is now known to represent the basolateral complex, subsequent studies added the adjacent olfactory cortex and the medial and central nuclei, forming the currently designated amygdalar complex. The later claim by Meynert (Swanson & Petrovich, 1998), that Burdarch's amygdala was a ventral temporal lobe extension of the claustrum (the deepest layer of cortex), set up a dispute that took several decades to solve until the extensive comparative work of the forebrain by Johnston (1923). The later study proposed that the amygdalar complex would form a collection of grey masses that develops around a primitive amygdalar fissure in the lateral olfactory area and subdivided it into a primitive group of nuclei associated to the olfactory system (central, medial

and cortical nuclei, and nucleus of the lateral olfactory tract), and a phylogenetically new group of nuclei (lateral and basal). However, more recent studies (Swanson & Petrovich, 1998; De Olmos *et al.*, 2004), proposed a new subdivision (Figure 2). This takes into consideration more extensive information based on connectional, neurochemical and detailed anatomical works: *i)* the caudal olfactory system (nucleus of the lateral olfactory tract, cortical nucleus and postpiriform and piriform-amygdalar areas), *ii)* a ventral extension of the claustrum (lateral, basal and posterior nuclei) that forms a frontotemporal system, and *iii)* a specialized ventromedial expansion of the striatum (central [CeA) and medial [MeA] amygdaloid nuclei, and anterior amygdaloid area) (Swanson & Petrovich, 1998).

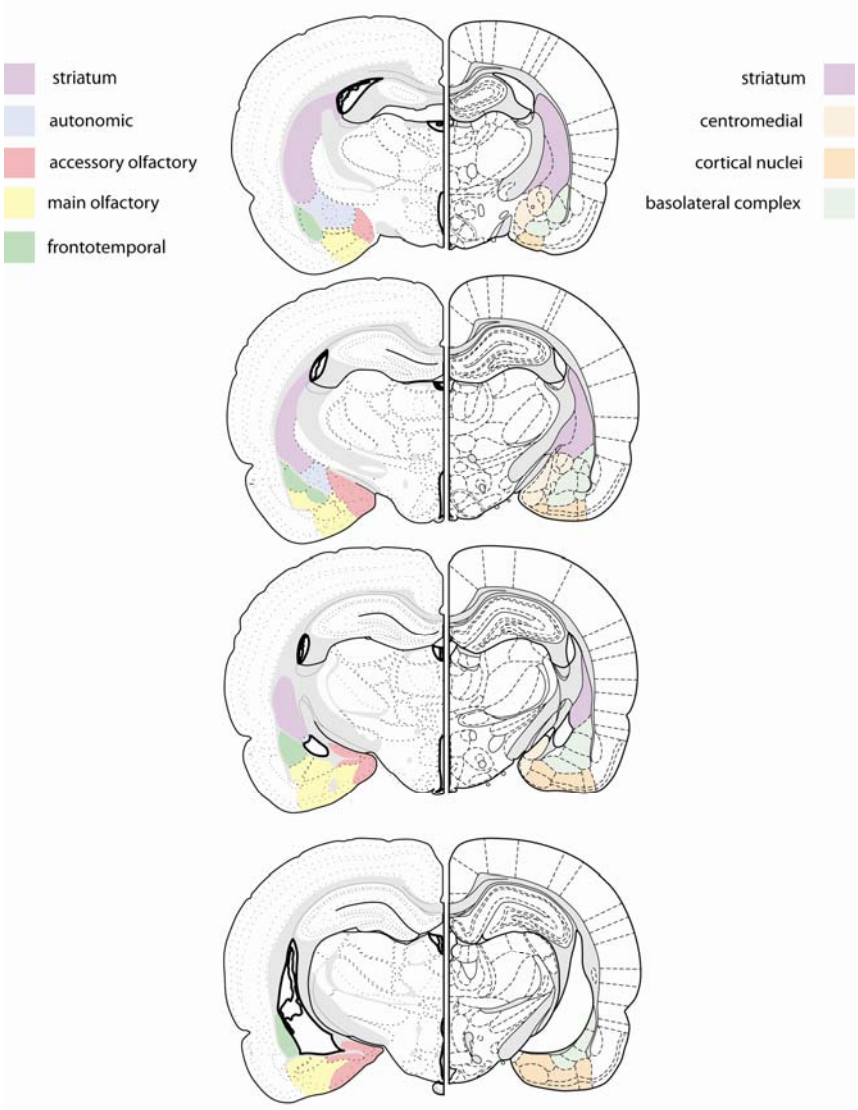

Figure 2. Diagrammatic representation of the main divisions of the amygdala according to Swanson (1998; left) and Paxinos & Watson (2005; right). From top to bottom, drawings represent coronal sections of the rat brain at approximately 1.73 mm, 2.45 mm, 3.25 mm and 3.90 mm (left) and 2.04 mm, 2.92 mm, 3.48 mm and 3.96 mm (right) posterior to bregma.

The olfactory systems resemble less the nuclear arrangement of the remainder divisions of the amygdala but rather display the typical laminar arrangement of cortical areas. They form the caudal part of the piriform lobe, receiving projections originating in the main and accessory olfactory lobes (Alheid & Heimer, 1988). This sensorial modality represents one major modulator of animal behavior, in particular reproductive and social behavior (Newman, 1999; Moffatt, 2003). The frontotemporal is the most extensively studied division of the amygdala. It forms part of the deepest cortical layers of temporal, endopiriform and frontal lobes (Swanson & Petrovich, 1998), receives privileged information from several sensorial modalities (somato-sensorial, auditory, visual) (Ledoux, 2007) and is intricately connected to the hippocampus and to the prefrontal cortex (Canteras & Swanson, 1992; Petrovich *et al.*, 2001; Akirav & Maroun, 2007). It is activated by specific cues that are associated to aversive situations, mounting a rapid response to eminent threats, through the activation of several autonomic (e.g. ventral tegmental area, periaqueductal grey) and neuroendocrine centers (hypothalamic areas), mainly through CeA. Typically this immediate response system is only transiently activated, playing a significant role in the individual's survival. Importantly, its neurons show plastic proprieties (Samson *et al.*, 2005; Fuchs *et al.*, 2006; Kim *et al.*, 2006; Sigurdsson *et al.*, 2007) that have a putative role in the formation of fearful memories and conditioned behavior; therefore creating a system of value categorization in the interpretation of situations through comparison with past experiences. Additionally, the activity of the frontotemporal division is influenced by the outputs of executive (PFC) (Akirav & Maroun, 2007) and mnesic systems (hippocampus); these in turn are modulated by the emotional value of experiences through the reciprocal pathways that interconnect the amygdala and with those brain regions (Bishop, 2007; McEwen, 2007). The striatal amygdala system forms a caudoventral extension of the striatum (Swanson & Petrovich, 1998) as judged by its topographic, histochemical and structural resemblances with the supradjacent striatum (Cassell *et al.*, 1986; Simerly *et al.*, 1989; Esclapez *et al.*, 1993; McDonald & Augustine, 1993; Sun & Cassell, 1993; Risold & Swanson, 1997). Its GABAergic projecting neurons form an inhibitory output that modulates basal activity of several brainstem and basal forebrain nuclei. The CeA nucleus is the main output of the amygdala relaying processed information from most amygdaloid nuclei. It projects massively to several brainstem autonomic nuclei and is responsible for several vegetative responses (tachycardia, hypertension, midriasis, startle, etc) that are triggered by emotionally charged stimuli. The MeA nucleus receives direct inputs from the accessory olfactory bulb, and is viewed as part of an accessory olfactory system (Swanson &

Petrovich, 1998) that modulates pherormonal information and projects to the BNST and hypothalamic areas. It displays some degree of sexual dimorphism and is involved in the neuroendocrine control of sexual and reproductive behavior. In summary, the amygdala cannot be seen as a structural or functional unit, but rather a complex network of systems (olfactory, autonomic and frontotemporal) that convey information relevant for survival.

1.3.2. BNST: Structural and functional organization

The anatomical organization of the BNST has been subject of constant revision and its definite parcellation is not, yet, consensual. Johnston (1923) initially described it as a ventral extension of pallidum, an area formerly considered part of the substantia inominata, and forms a continuum that extends from the olfactory tubercule and nucleus accumbens anteriorly until the amygdala posteriorly. It receives massive projections from adjacent areas including the amygdala through the bundle of fibres that forms the stria terminalis. In fact, its lateral and medial parts form two corridors of sublenticular neurons that are in continuity with the central and medial nuclei of the amygdala. They share common neurochemical and neuroarchitectural characteristics and have, therefore, lead to the designation of central and medial extended amygdala (Alheid, 2003). Nevertheless, extensive studies by Swanson and collaborators (Ju & Swanson, 1989; Ju *et al.*, 1989; Swanson, 1998), have proposed a more recent parcelation into major anterior and posterior divisions (relative to stria terminalis main fiber bundles); the former can be further parcelled into dorsal, lateral and ventral areas, relative to the anterior comissure fibers (Figure 3). However, in a subsequent revision of the anatomical, cytoarchitectural and projection studies, the cell groups of the anterior division were arranged into medial and lateral groups (Dong *et al.*, 2001). The medial division, which includes the anterodorsal and anteroventral areas (Dong & Swanson, 2006a; b; c), is characterized by dense projections to hypothalamic regions closely associated with neuroendocrine control; the lateral group of the anterior division (which includes the anterolateral area) is characterized by projections to hypothalamic areas concerned with autonomic and energy homeostasis and feeding behavior (Dong & Swanson, 2004). It is important to note that the BNST is continuous with the CeA and MeA and that these structures, together, function as a parallel pathway to that in the adjacent striatal area. Such anatomical and functional interrelationships support the inclusion of the BNST, as well as the substantia innominata, into the so-called “extended amygdala” (Alheid, 2003). In a global view, the

extended amygdala seems to be critical for mediating behavioral and autonomic (CeA), reproductive (MeA) and neuroendocrine (BNST) responses (Swanson & Petrovich, 1998).

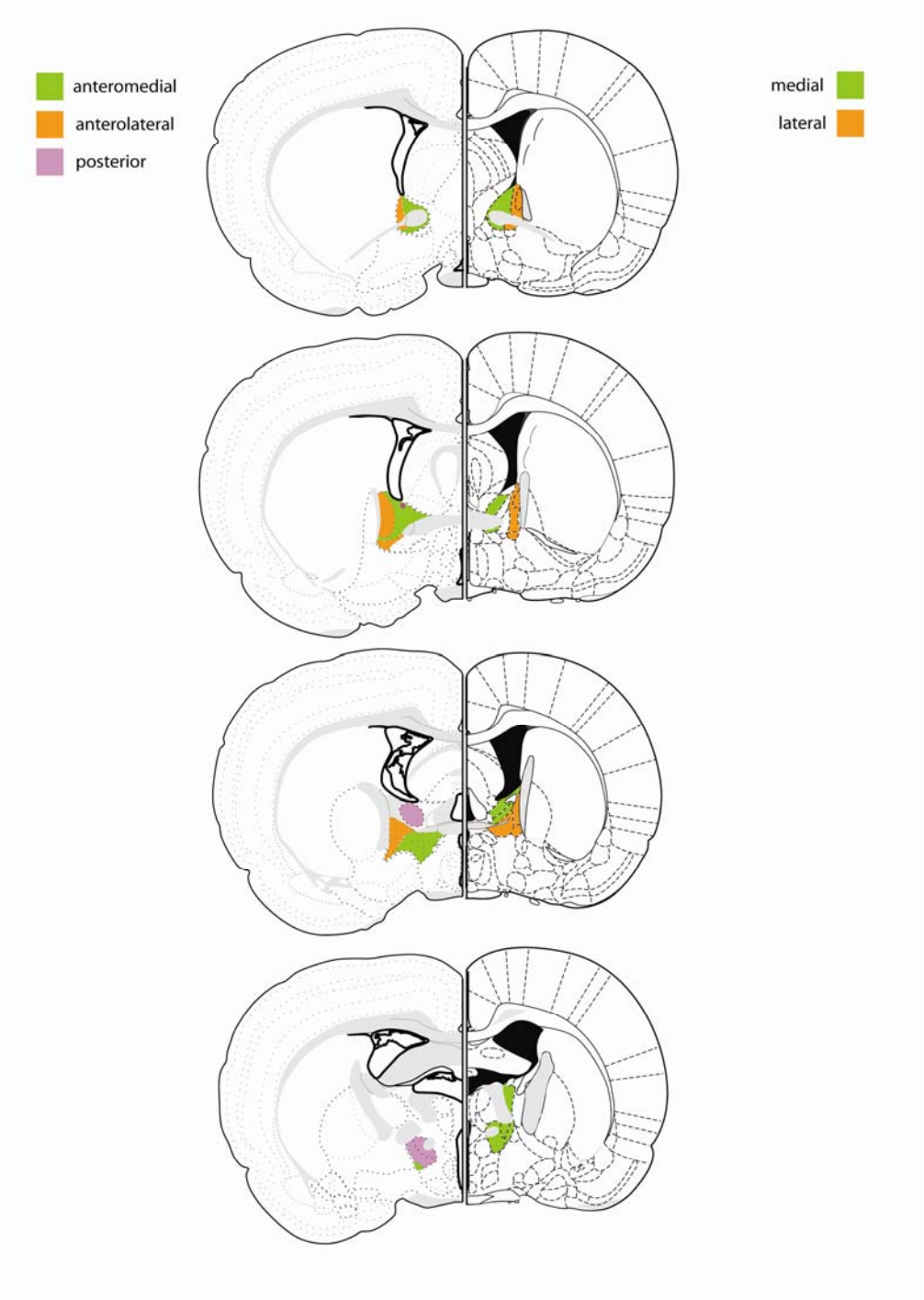

Figure 3. Diagrammatic representation of the main divisions of the BNST according to Swanson (1998; left) and Paxinos & Watson (2005; right). From top to bottom, drawings represent coronal sections of the rat brain at approximately 0.00 mm, 0.26 mm, 0.51 mm and 1.08 mm (left) and 0.00 mm, 0.24 mm, 0.48 mm and 1.08 mm (right) posterior to bregma.

The role of the BNST in the activation of the HPA axis (Herman *et al.*, 2003; Choi *et al.*, 2007) and its anatomical connections with the neuroendocrine hypothalamus (Dunn, 1987; Dong & Swanson, 2006a) as well as with other brain regions such as the brainstem and ventral striatopallidal, areas that regulate defensive, sexual, ingestive, and exploratory behaviors, have been previously established. Additionally, the BNST (Herman *et al.*, 2003; Herman *et al.*, 2005), along with the nucleus of tractus solitarius, preoptic area and dorsal hypothalamus, is one of the relay stations where inputs from stress-sensitive areas of the cortex and limbic systems are conveyed and integrated to mount an adequate activation of the HPA (Herman *et al.*, 1994; Herman & Cullinan, 1997; Herman *et al.*, 2003). See Figure 4.

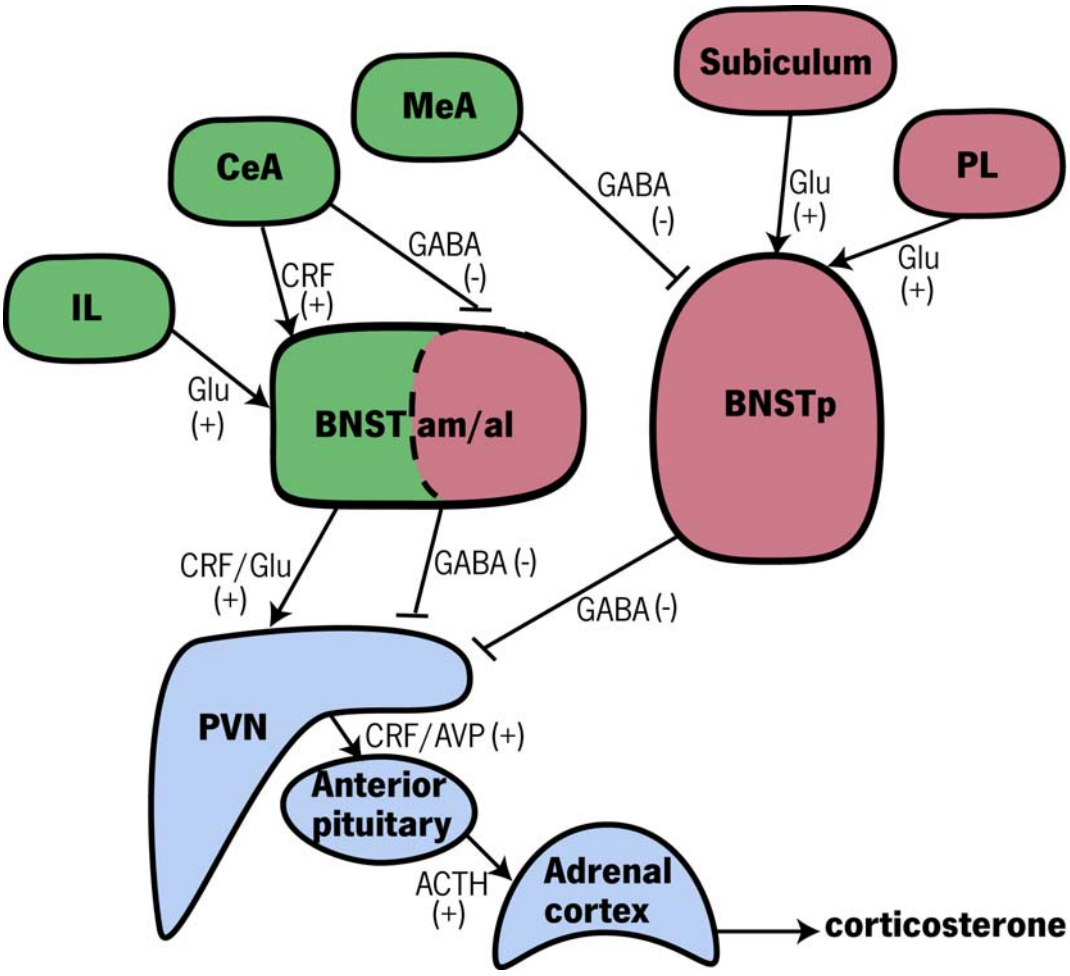

Figure 4. Schematic representation of the limbic inputs to the BNST and its regulatory influence over the HPA.

The role of the BNST in emotional behavior has been extensively explored by Davis and collaborators (Davis, 1986; 1992; Davis *et al.*, 1997; Lee & Davis, 1997a; Davis, 1998). Although there are resemblances in the phenotypic expression of BNST and amygdala activities,

there are also differences responsible for the distinct role of these structures on emotional behavior. An involvement of the BNST is evident in paradigms in which behavior is influenced by long-duration stimuli (e.g., CRF- or light-enhanced startle) and in paradigms that assess the persistent behavioral effects of even a brief unconditioned stressor (e.g., long-term shock-dependent increases in baseline startle, conditioned defeat, the effects of inescapable shock in the learned helplessness model or on subsequent eyeblink conditioning). Despite behavioral outcomes that are often similar in form (i.e., increased startle), the BNST has not been shown to mediate behaviors elicited by specific short-lasting threats in which the danger period has a clear offset (i.e., fear-potentiated startle or freezing to a discrete conditioned fear stimulus) (Walker *et al.*, 2003). These characteristics of BNST-dependent behavior suggest a special role in anxiety, as opposed to fear, insofar as anxiety, unlike fear, is typically viewed as a sustained state of apprehension unrelated to immediate environmental threats (Walker *et al.*, 2003).

In summary, whereas the amygdala is transiently activated in fear conditioning (an emotional state elicited by explicit neutral clues), anxiety (a similar emotional state thought to be elicited by diffuse contextual clues) seems to result from persistent activation of the BNST (Davis *et al.*, 1997).

1.4. Aims of the study

Stress is an important trigger of emotional behavior disarray both in young individuals and throughout life. Hormonal and central mediators play an important role in the initiation and termination of the stress response. These mediators are powerful modulators of the neuronal circuitries in stress-sensitive areas, including those underlying emotional behavior. However, a clear correlation between this behavioral change and the anatomical substrates of such behavior in the stressed individual is not, yet, established.

Current knowledge has not, yet, established an explicit relationship between allostatic overload induced anxiety and remodeling of brain structures that underlie emotional behavior. Indeed, both the amygdala and BNST, which are putative targets of stress endorsed alterations, can mediate emotional aspects of behavior (anxiety and fear) that resemble each other. However, it is

unclear if the emotional changes observed in these models are functionally dependent on the isolated impairment of the amygdala, BNST or both. Furthermore, a clear correlation between functional impairment and structural changes in these brain areas is still to be ascertained in the context of prolonged biological burden. Lastly, although both central and peripheral components of the stress response have been implicated in the altered emotional behavior observed, the relative importance of such contributions to the reshaping of the brain areas implicated has not been determined or is still unknown.

The present work aims at:

- Thoroughly characterize how emotional behavior, namely fear and anxiety, is affected by chronic stress and with advanced age (Chapters 2.1 & 2.4).
- Determine the neuroanatomical substrates upon which stress acts to induce anxiety and fear-conditioned behavior, namely the BNST and the amygdala, respectively (Chapter 2.1).
- Clarify the relative importance of hormonal (corticosteroids) and central mediators (CRF_1R or CRF_2R agonists) of the stress response, in their contribution to neuroanatomical changes induced by stress (Chapters 2.1 & 2.3).
- Investigate whether there is functional impairment of these neuroanatomical areas in the chronically stressed individual (Chapter 2.2).

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Chapter 2

Experimental work

Chapter 2.1

Pêgo JM, Morgado P, Pinto LG, Cerqueira JJ, Almeida OFX & Sousa N

Dissociation of the morphological correlates of stress-induced anxiety and fear

(Manuscript under review)

(2007)

Dissociation of the morphological correlates of stress-induced anxiety and fear

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Number of pages: 46

Number of figures: 8

Number of tables: 4

Supplementary material: 3 tables

Keywords: amygdala; BNST; chronic-stress; rat; stereology

Abstract

Chronic stress is a powerful modulator of emotional behavior. Previous studies have shown that distinct neuronal pathways modulate different emotional behaviors: while the amygdala plays a key role in fear-conditioned to cue stimuli, the bed nucleus of stria terminalis (BNST) is implicated in anxiety behavior and responses to contextual stimuli. In addition, the BNST is directly involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis. In the present study, we assessed anxiety (measured in the elevated plus maze and acoustic startle apparatus) and fear-conditioned responses to light stimuli in rats that had been exposed to either chronic unpredictable stress or corticosterone for 28 days after which stereological estimates of the BNST and amygdaloid complex were performed, followed by 3-dimensional morphometric dendritic analysis. Results show that chronic stress induces hyperanxiety without influencing fear-conditioning and locomotory/exploratory activity. Stress-induced hyperanxiety correlated with increased volumes of the BNST, but not of the amygdala. Dendritic remodeling was found to make a significant contribution to the stress-induced increase in BNST volume, primarily due to changes in the anteromedial area of the BNST, an area strongly implicated in emotional behavior and in the neuroendocrine control of the stress response. Importantly, all of the effects of stress were recapitulated by exogenous corticosterone. In conclusion, this study shows that chronic stress impacts on BNST structure and function; its findings pertain to the modulation of emotional behavior and the maladaptive response to stress.

Introduction

Chronic stress induces a hyperanxious state (File, 1996; Arborelius *et al.*, 1999; Vyas *et al.*, 2002; Anisman & Matheson, 2005). Inappropriate adaptation to stress severely compromises the organism's coping mechanisms, resulting in a state of hyperanxiety and the facilitation of phobic behavior acquisition (Garakani *et al.*, 2006). Most of these effects can be attributed to sustained increases in corticosteroid levels (Arborelius *et al.*, 1999) which subsequently activate receptors in brain areas implicated in the regulation of emotional behavior. The hippocampus and prefrontal cortex are important for coordinating the adaptative response to stress (Figueiredo *et al.*, 2003b; Cerqueira *et al.*, 2007); their functions are complemented by the amygdala and extended amygdala (including the bed nucleus of stria terminalis [BNST]), both of which display a rich population of corticosteroid receptors that are activated during stress (Cullinan *et al.*, 1995; Figueiredo *et al.*, 2003a). In addition, the BNST plays a direct role in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis by modulating corticortropin-releasing-hormone (CRH) expressing neurons in the hypothalamic paraventricular nucleus (Dunn, 1987; Herman *et al.*, 1994).

The amygdala has long been known to be involved in emotional behavior (Weiskrantz, 1956; Robinson, 1963; Goddard, 1964). Lesion studies in the amygdala (which included the BNST) described disturbances in emotional and social behavior, attention disorders and deficits in memory consolidation/extinction (Bucy & Kluver, 1955; Weiskrantz, 1956; Kluver & Bucy, 1997; Baron-Cohen *et al.*, 2000). More recent lesion and pharmacological studies have helped differentiate between the relative importance of the amygdala and BNST in the control of fear and anxiety, respectively (Davis, 1992a; b; Lee & Davis, 1997a; Davis, 1998; 2006). Briefly, whereas the amygdala is transiently activated in fear conditioning (an emotional state elicited by explicit neutral clues), anxiety (a similar emotional state thought to be elicited by diffuse contextual clues) results from persistent activation of the BNST (Davis *et al.*, 1997).

Many previous studies in rats have attempted to correlate stress-induced hyperemotionality with alterations in the structure of the amygdala and BNST. One notable finding was that chronic restraint stress leads to dendritic remodelling of amygdala and BNST neurons (Vyas *et al.*, 2002;

Vyas *et al.*, 2003). However, that study did not examine how the volume and neuronal number of the amygdala and BNST are affected by stress; also, it did not discriminate between the effects of stress on the two components of emotional behavior, fear-conditioning and anxiety. The present study used the acoustic startle chamber and elevated-plus maze to discriminate between the two types of emotional behavior; additionally, it sought the morphological correlates of these behaviors by including a stereological analysis of the amygdala and BNST of rats that had been submitted to either a chronic unpredictable stress paradigm or chronic corticosteroid administration.

Materials and Methods

Animals and Treatments

All experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) were housed in groups of 2/3 under standard laboratory conditions with an artificial light/dark cycle of 12/12 hours (lights on at 7 a.m), 22°C room temperature, *ad libitum* access to food and water. Chronic stress protocols were initiated when animals were 8 weeks old and continued over a period of 4 weeks, after which behavioral tests were conducted (at 12 weeks of age). All drugs were purchased from Sigma (St. Louis, MO).

Body weights were recorded weekly and post-mortem thymus weights were used as an index of treatment efficacy. Corticosterone levels were measured in blood serum, obtained between 9:00 and 10:00 a.m. (12 h after the last exposure to stress), by radioimmunoassay (R & D Systems, Minneapolis, MN).

To assess the influence of chronic stress on behavior and amygdala and BNST morphology, rats were exposed for 4 weeks to a chronic unpredictable stress (CUS) protocol, as described previously (Cerqueira *et al.*, 2007). Rats were randomly assigned to control (Cont) or CUS groups (n=10 per group). Stressful stimuli were scheduled in a random order, with exposure to 30 min of a different stressor everyday. Control animals were handled on a daily basis over the 4 week treatment period. This particular CUS model was selected because previous studies (Cullinan *et al.*, 1995; Herman *et al.*, 1995; Sousa *et al.*, 1998; Cullinan & Wolfe, 2000; Sousa *et al.*, 2000) have shown that CUS results in a state of chronic hypercorticalism, characterized by increased adrenal weight and serum corticosterone levels, reduced thymus weight, and reduced body weight gain. Furthermore, the mixture of psychological and physical elements in the CUS paradigm not only reduces the chances of adaptation but also better mimics the variability of stressors encountered in daily life (Sousa *et al.*, 1998; Joels *et al.*, 2004).

To assess the influence of the corticosteroid milieu on behavior and brain structure, rats were randomly assigned to two experimental groups (n=10 per group). Controls (Veh) received daily subcutaneous injections of vehicle (sesame oil, Sigma) while corticosterone-treated (CORT) animals received daily subcutaneous injections of corticosterone (25 mg/kg in sesame oil) over a period of 4 weeks.

Behavioral analysis included the open field test for locomotion and exploratory activity, the elevated plus maze for anxiety-like behavior, and the acoustic startle paradigm to assess anxiety-like (Pego *et al.*, 2006) and fear behavior; testing was carried out during the daily light phase, between 9 a.m. and 4 p.m.

Following the behavioral tests, animals were deeply anesthetized with pentobarbital and perfused transcardially with either 4% paraformaldehyde solution for glycolmethacrylate inclusion (n= 6 per group), or saline for Golgi-Cox staining (n=4 per group). Brains were dissected and post-fixed (4% paraformaldheyde) for at least 30 days, or kept in Golgi-Cox solution until processed for histology.

Elevated plus maze

Animals were tested over 5 min in a black polypropylene "plus"-shaped maze (ENV-560, MedAssociates Inc, St. Albans, VT 05478) at a height of 72 cm above the floor (EPM). The maze consisted of two facing open arms (50.8 x 10.2 cm) and two closed arms (50.8 x 10.2 x 40.6 cm). Testing was performed under bright white light.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 EPM are presented as percentage of the total duration of the trial.

Acoustic startle

Startle reflex (ASR) was measured in startle response apparatuses (SR-LAB, San Diego Instruments, San Diego, CA, USA), each consisting of a non-restrictive Plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated,

sound-attenuated chamber. Animals were habituated to the apparatus (5 min daily) for 2 days before actual testing. Cylinder movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system (San Diego Instruments, San Diego, CA, USA) was used to ensure comparable startle magnitudes. Startle stimuli were presented through a high frequency speaker located 33 cm above the startle chambers. Startle magnitudes were sampled every millisecond (m*s*) over a period of 200 m*s*, beginning with the onset of the startle stimulus. A startle response is defined as the peak response during 200 m*s* recording period.

Startle response as a function of stimulus intensity

Background white noise, with an intensity of 63 dB, was used to minimize the impact of external acoustic stimuli. Rats were placed in the startle chamber and allowed to acclimatize to chamber for 5 min. They were then presented with 5 baseline startle stimuli (50 m*s* pulse of white noise at 120 dB) at an inter-stimulus interval of 30 *s* so as to familiarize them with the startle stimulus, thus facilitating the accuracy of measurement of overall startle amplitudes. Following this baseline trial, animals were randomly presented with 60 startle stimuli (each lasting 50 m*s*, but varying in intensity from 70 to 120 dB, in 10-dB increments). Animals were then returned to their home cage. Tests on individual animals were conducted sequentially; between tests, chambers and the acrylic holders were thoroughly cleaned (70% ethanol and water) to eliminate residual olfactory cues.

Fear-potentiated startle

Rats were placed in the first test chamber, the floor of which consisted of a stainless steel grid through which a software-controlled electric current could be passed. Animals were rehabituated to the startle chamber for 5 min before being presented with 20 light-shock pairings, at 30 *s* intervals. The shock (0.6 mA) was presented during the last 500 m*s* of the 5 *s* light pulse. The light stimulus was delivered via a 3-watt incandescent light bulb fastened to the inside wall of the startle chamber. After completion of the conditioning trials, animals were returned to their home cages. The same testing procedure was applied on the following day, except that 20, rather than

5, baseline trials were administered before testing. Additionally, startle measurements were made in the same grid holder that was used to condition the animals. After delivery of the final baseline trial, animals were randomly presented 10 startle stimuli, each with an intensity of 120 dB and duration of 50 m*s*. In half of the trials, the startle stimulus was presented concomitantly with the conditioned stimulus (CS; light). Startle stimuli paired with the CS were delivered during the last 50 m*s* of the 5 *s* light presentation.

Prepulse inhibition

Rats were transferred to the experimental room approximately 1 h before the test. The full test session lasted approximately 20 min, preceded by a 5 min acclimatization period (rat in testing tube). The chambers were supplied with white background noise (70 dB). Each session started and ended with 5 startle trials consisting of 120 dB bursts of white noise, lasting 40 m*s* each. These trials were used for assessing habituation over the test session and were not included in calculations of prepulse inhibition (PPI). Following the 5 introductory startle trials, a total of 35 test trials were delivered according to the following schedule: (i) 10 startle trials of 120 dB; (ii) 5 of each of four prepulse+startle trials (PP72, PP74, PP78, and PP86; prepulse intensities were respectively, 2, 4, 8, and 16 dB above background noise level, with a duration of 20 m*s* each; prepulses were presented 100 m*s* before the onset of the 120 dB startle pulse); and (iii) 5 trials with background noise but no stimulus.

The trials were delivered in a pseudo-randomized order, avoiding 2 identical trials in succession. The inter-trial intervals were randomized between 10 and 20 *s*, with a mean interval of 15 *s*. The movement of the tube was registered for 100 m*s* after the onset of the startle stimulus (sampling frequency 1 kHz) and was amplified, and the peak startle amplitude and the average response over 100 m*s* were computed. The maximum startle amplitude (V_{max}) and the average response (AVG) over 100 m*s* were calculated as the mean of the 10 startle trials. The AVG for each prepulse intensity was averaged and used for the analysis of PPI. PPI was expressed as the percent reduction in AVG compared with the startle trials, according to the formula:

$$\% \text{ PPI} = 100 - [(\text{AVG at prepulse+startle trial})/(\text{AVG at startle trial})]\text{x}100$$

Open field

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

Histological procedures

Brains (n=6 per group) from the 4% paraformaldehyde-perfused animals (see above) were embedded in glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Werheim, Germany) before sectioning on a microtome, as described previously (Cerqueira *et al.*, 2005). Every other 30 µm-thick coronal section was placed on a gelatinized slide, stained with Giemsa, mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped. The shrinkage factor (SFv) was calculated according to Madeira *et al* (1990).

Brains (n=4 per group) from animals that had been transcardially perfused with 0.9% saline (see above) were processed for Golgi-Cox staining according to a published protocol (Gibb and Kolb,(1998). Briefly, brains were removed and immersed in Golgi-Cox solution (a 1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate (Glaser & Van der Loos, 1981) for 14 days; brains were then transferred to a 30% sucrose solution (3 days), before being cut on a vibratome. Coronal sections (200 µm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Linda-a-Velha, Portugal), fixed in Kodak Rapid Fix (prepared as manufacturer instructions), dehydrated through a graded series of ethanols, and cleared in xylene before being mounted and coverslipped.
Slides were coded before morphometric analysis in both sets.

Regional boundaries

Stereological parameters were analyzed on 6 regions of the amygdaloid complex, including the basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA) and lateral (La) nuclei/regions/areas, as outlined in Paxinos and Watson (2005) (Fig. 1). Both La and CeA nuclei were discernible, according to established definitions (De Olmos *et al.*, 2004). Parcellation of the anterior and posterior divisions of the basolateral amygdaloid nucleus (BL) can be problematic in Giemsa-stain specimens examined at low magnifications; we therefore used higher magnifications and differentiated this nucleus form other areas on the basis of cell size and staining intensity (Krettek & Price, 1978; De Olmos *et al.*, 2004). Definition of the anterior and posterior components of the basolateral amygdaloid nucleus (BM) was based on the slightly larger and more darkly staining neurons in its posterior part, as compared to the anterior BM. To delineate the BMp from the surrounding neuronal groups, namely the cortical amygdaloid nuclei, the the following criteria were applied: loss of layered organization typical of cortical nuclei, presence of a mesh of scattered neurons and differences in cell size (De Olmos *et al.*, 2004). Regional boundaries were drawn by the same observer for all animals so as to avoid observer bias and to allow comparison between groups. However, because of the above-mentioned potential difficulties in defining anterior and posterior divisions of BM and BL, we also calculated statistics for these nuclei as a whole, in order to exclude the introduction of differences due to possibly ill-defined anterior-posterior parcellation.

The anatomical organization of the BNST has been thoroughly characterized by Swanson and collaborators (Ju & Swanson, 1989; Ju *et al.*, 1989; Swanson, 1998), being subdivided into major anterior and posterior divisions (relative to stria terminalis main fiber bundles); the former can be further parcelled into dorsal, lateral and ventral areas, relative to the anterior comissure fibers. However, in a subsequent revision of the anatomical, cytoarchitectural and projection studies, the cell groups of the anterior division were arranged into medial and lateral groups (Dong *et al.*, 2001). The medial division is characterized by dense projections to hypothalamic regions that are closely associated with neuroendocrine control, and includes the anterodorsal and anteroventral areas (Dong & Swanson, 2006); in contrast, the lateral group of the anterior division (which includes the anterolateral area) is characterized by projections to hypothalamic areas concerned with autonomic and energy homeostasis and feeding behavior (Dong & Swanson, 2004). The above-mentioned regions were outlined according to anatomical references

and recognized on the basis of clear cytoarchitectural differences, namely the density of cells, size of the perikarya and relative position (Swanson, 1998) (Fig. 2).

Stereological procedures

Volume estimations were performed using StereoInvestigator® software (Microbrightfield, VT, USA) and a camera attached to a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany).

Cavalieri's principle was used to assess the volume of each level. Briefly, every 4th section was used and the cross-sectional area of each region of interest was estimated by point counting (final magnification x112). We used a test-point system in which the inter-point distance, at tissue level, was 100 μm for the BMp and BLp, 250 μm for the BNST and 150 μm for the remaining nuclei. The volume of the region of interest was determined from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Average cell numbers were estimated using the optical fractionator method (West *et al.*, 1991). Briefly, a grid of virtual, equally-spaced 3D-boxes (30 μm x 30 μm x 20 μm) (same grid spacing as for volume estimations) was superimposed on every 4th section of the lamina of interest and the number of neurons falling inside the boxes was counted, according to standardized stereological procedures (Gundersen *et al.*, 1999; Coulin *et al.*, 2001). The estimated total number of neurons in each region was then calculated on the basis of the number of counted neurons, grid spacing, volume of the box, and number of boxes counted. Neurons were differentiated from other cells on the basis of perikaryon size and shape (Peinado *et al.*, 1997).

Coefficients of error were computed according to the formulas of Gundersen *et al.* (1999) for cell numbers and Gundersen and Jensen (1987) for volume estimates.

Dendritic trees analysis

Three-D reconstructions of representative Golgi-impregnated neurons from the BLa and BNST were made. The criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without truncated branches, except on the most superficial layer; (iii) relative isolation from neighboring

impregnated neurons to avoid interference with the analysis (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain. Golgi studies of specific divisions of the BNST are sparse (McDonald, 1983; Larriva-Sahd, 2004; 2006). The first study describing the distribution of different neuronal types in the BNST (McDonald, 1983) was based on the lateral/medial division of the nucleus. This parcellation has, however, been subsequently revised (Ju & Swanson, 1989). Accordingly, we chose neurons for dendritic analysis in the anteromedial area, using the following criteria: (i) presence of transverse anterior comissure; (ii) rostral location to the stria terminalis main bundle; (iii) selection of neurons adjacent to the anterior comissure. These landmarks correspond to the rostral portion of the medial division described by McDonald (1983) as being populated by cells with characteristic ovoid soma and polarized dendritic trees that branch sparingly. These cells are characteristically sparsely to moderate spiny, contrasting with the spine- and dendrite-rich cells of the lateral division.

Golgi-impregnated pyramidal-like neurons (type I) of the basolateral amygdala were readily identified by their characteristic pyramidal or piriform soma, spine-sparse primary dendrites and spine-dense secondary dendrites (McDonald, 1982) (Fig. 3 for representative photomicrographs). For each selected neuron, all branches of the dendritic tree and the location of all dendritic spines were reconstructed at 600X magnification, using a motorized microscope (Carl Zeiss Axioplan 2, Hamburg, Germany, with oil-objectives), attached to a camera (DXC-390, Sony Co., Japan) and Neurolucida software (Microbrightfield, VT, USA). Three-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). In each brain, 10 BLa neurons (160 in total) and 10 BNSTam neurons (160 in total) were studied. Several aspects of dendritic morphology were examined. To assess overall changes, total dendritic length and number of dendrites were compared between groups. The total number of spines on dendrites and spine density (total spines divided by dendritic length) were also compared between groups. To assess differences in the arrangement of dendritic material, a 3-D version of a Sholl analysis (Sholl, 1956; Uylings & van Pelt, 2002) was performed; for this, the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm were recorded.

Data analysis

Except for coefficients of error, all results are expressed as group means ± standard error. A repeated measures test was used to evaluate data from the Sholl analysis, acoustic startle, prepulse inhibition and fear-potentiated data. Student's *t* test was used to compare means between groups for the remaining variables.

The coefficients of error (C.E.) of the individual stereological estimates were calculated according to Gundersen and Jensen (1987). Coefficients of variation (C.V.=standard deviation/mean) were determined for the stereological estimates. Statistical significance was accepted when the probability level was < 0.05.

Results

Verification of efficacy of CUS and CORT treatment procedures

The CUS protocol decreased body-weight gain (Cont, 96.1 ± 3.1 g; CUS, 79.3 ± 2.4 g; $t = 4.26$, $P < 0.001$) and reduced the thymus/body-weight ratio (Cont, 1.03 ± 0.09 x 10^{-3}; CUS, 0.77 ± 0.08 x 10^{-3}; $t = 2.10$, $P < 0.05$). Exposure to chronic stress resulted in persistently raised plasma corticosterone levels, which were still significantly higher than those found in controls more than 12 h after the last stress exposure (Cont, 31 ± 6.0 ng/ml; CUS, 117 ± 11 ng/ml; $t = 6.80$, $P < 0.001$).

CORT-treatment slowed body-weight gain (Veh, 88.4 ± 12.9 g; CORT, 24.0 ± 9.9 g; $t = 3.32$, $P < 0.01$) and reduced the thymus/body-weight ratio (Veh, 1.03 ± 0.27 x 10^{-3}; CORT, 0.68 ± 0.05 x 10^{-3}; $t = 6.64$, $P < 0.001$). Corticosterone levels were persistently raised in CORT-treated animals and were significantly higher than those found in Veh-treated (Veh, 41 ± 4 ng/ml; CORT, 203 ± 11 ng/ml; $t = 13.6$, $P < 0.001$)

Chronic stress triggers hyperanxiety but does not affect fear conditioning or locomotory /exploratory activity

Chronic stress induced an anxiety-like phenotype insofar that, as compare to Cont animals, CUS rats spent significantly less time ($t = 2.77$, $P < 0.009$) and made fewer entries ($t = 2.03$, $P < 0.04$) into the open arms of the EPM. Furthermore, the ratio of open/closed arm times ($t = 2.40$, $P < 0.02$) was significantly smaller in CUS animals. Nevertheless, the number of closed-arm entries did not significantly differ between the groups ($t = -0.07$, $P = 0.94$), indicating that exploratory/locomotory activity was preserved (Fig. 4A,B and Supplementary material Table 1).

Responsiveness to acoustic stimuli was increased after CUS ($F = 5.13$, $P < 0.05$). There was a significant trend for the startle amplitudes of the CUS rats to increase faster compared to Cont animals. However, between-group comparisons failed to reveal significant differences on individual noise intensities (Table 1).

Response to inhibitory prepulse was not affected by chronic unpredictable stress, as shown by analysis of prepulse inhibition of acoustic startle response which did not vary as a function of treatment x prepulse intensity interaction ($F = 0.150$, $P = 0.92$). Comparison between groups revealed no differences on any prepulse intensity (Fig. 5). In addition, CUS did not affect responsiveness to fear in the acoustic startle test. Analysis of the fear-potentiated startle data among rats tested in the shock chamber, revealed that startle amplitude did not vary as a function of the treatment x stimulus interaction ($F = 0.107$, $P = 0.74$). As shown in Table 2, the magnitude of the fear-potentiated startle was similar among the CUS rats to that observed in Cnt rats.

Finally, locomotory activity was not different between control as assessed by using the OF (Outer distance, 503 ± 32; Center distance, 107 ± 18; Outer time, 273 ± 5; Center time, 27 ± 5) and CUS (Outer distance, 495 ± 31; Center distance, 97 ± 12; Outer time, 273 ± 4; Center time, 27 ± 4) animals. However, a significant decrease was found in the time (Cont, 68 ± 6; CUS, 49 ± 5; $t = 2.40$, $P < 0.02$) that animals exposed to the CUS spent in rearing activity, although there was no significant difference in the number of rearings (Cont, 37 ± 3; CUS, 33 ± 3; $t = 1.15$, $P = 0.22$) (Supplementary material Table 1).

Stress-induced hyperanxiety correlates with BNST but not amygdala hypertrophy

The volumes of the amygdaloid nuclei (Fig. 6A) did not differ between Cont and CUS animals (BLa, $t = -0.78$, $P = 0.45$; BLp, $t = -0.28$, $P = 0.78$; BMa, $t = -0.08$, $P = 0.93$; BMp, $t = 0.57$, $P = 0.58$; CeA, $t = -0.09$, $P = 0.92$; La, $t = -1.68$, $P = 0.12$). The same was true when the basolateral and basomedial nuclei volumes were computed without regard to their anterior-posterior divisions (BL, $t = -0.53$, $P = 0.60$; BM, $t = 0.23$, $P = 0.82$). However, CUS animals presented a significant increase (23%) in volume of anteromedial division of BNST ($t = -3.03$, $P < 0.02$) compared to Cont animals (Fig. 6B). There were no significant differences in the volumes of the BNSTal ($t = -0.59$, $P < 0.58$) and BNSTp divisions ($t = -0.54$, $P < 0.60$). To clarify the impact of cell numbers in the volumetric changes in the BNST of CUS-treated animals, the total number of neurons was

estimated. Stereological estimates of total number of neurons failed to reveal significant differences between experimental groups in any of the BNST divisions or amygdaloid nuclei (Fig. 7A,B). It is important to note that the low coefficients of error, which did not exceed the biological variance (Supplementary material Tables 2 & 3), attest to the validity of the stereological determinations (Coulin *et al.*, 2001).

To ascertain the role of plastic changes in the neuronal morphology in the volumetric changes, we undertook a 3D morphological analysis of dendritic arborizations in each region of interest. Data revealed that CUS produced a significant increase in the total length of dendrites of bipolar neurons in the BNST ($t = 2.20$, $P < 0.04$), and in the total number of spines ($t = 3.15$, $P < 0.006$), although the spine density was not significantly changed ($t = 0.97$, $P = 0.34$) (Table 3). Sholl analysis of the number of intersections as a function of their distance from the soma (Fig. 8A) did not reveal a significant effect of CUS on bipolar neurons dendrites ($F = 2.03$, $P = 0.17$) although increased proximal ramification and longer dendritic lengths were observed.

3-D analysis of dendrites of pyramidal-like neurons in the amygdala showed that CUS did not influence total dendritic length ($t = 0.29$, $P = 0.77$), total number of spines ($t = -1.07$, $P = 0.29$) or spine density ($t = -1.78$, $P = 0.08$). Sholl analysis of the number of intersections as a function of their distance from the soma did not reveal ($F = 2.50$, $P = 0.12$) any effect of CUS (Fig. 8C).

Corticosteroids mediate the behavioral and structural effects of stress in the BNST

Corticosterone treatment replicated the behavioral phenotype of CUS group in the OF, EPM and acoustic startle (AS) (Fig. 4C,D, Table 4 and Supplementary material Table 1). However, in the fear-potentiated startle (FPS) paradigm, the magnitude of the fear-potentiated startle was not significantly greater ($F = 3.49$, $P < 0.79$) in CORT-treated rats compared to Veh-treated rats (Table 2).

Stereological estimates on CORT-treated animals revealed a strong resemblance to the results obtained for CUS-treated rats. While the volumes of the amygdaloid nuclei did not differ between the Veh and CORT groups (BLa, $t = -1.03$, $P = 0.32$; BLp, $t = -0.58$, $P = 0.57$; BL, $t = -2.66$, $P = 0.024$; BMa, $t = -1.42$, $P = 0.18$; BMp, $t = 1.39$, $P = 0.19$; BM, $t = 0.16$, $P = 0.65$; CeA, $t = -0.21$, $P = 0.84$; La, $t = -0.61$, $P = 0.95$), there was a significant increase (20%) in the volume of the BNSTam in the CORT- *vs.* Veh-treated animals ($t = 2.88$, $P < 0.02$). There were no significant differences in the volumes of the BNSTal ($t = -2.20$, $P = 0.06$) and BNSTp divisions ($t = 0.52$, $P = 0.61$) (Fig. 6C,D and Supplementary material Table 2). Stereological estimates of the total number of neurons (Fig. 7C,D) did not reveal any significant between-group differences in any of amygdaloid or BNST divisions (Supplementary material Table 3).

Data derived from the 3D dendritic morphological analysis demonstrated that, CORT treatment induced similar changes to CUS in bipolar neurons of the BNST. Total dendritic lengths were significantly increased ($t = 2.22$, $P < 0.03$), but only non-significant increases were found in the total number of spines ($t = 1.43$, $P = 0.16$) and spine density ($t = 0.52$, $P = 0.60$). Sholl analysis did not reveal an effect of CORT on bipolar neurons dendrites ($F = 0.006$, $P = 0.93$) (Fig. 8B,D and Table 3).

Similarly to what was found for CUS, CORT treatment did not affect the dendritic morphology of pyramidal-like neurons of the BL ($t = -1.46$, $P = 0.14$). However the total number of spines ($t = -3.81$, $P < 0.0004$) and spine density ($t = -4.13$, $P < 0.0001$) were decreased when compared to Veh-injected animals (Fig. 8B,D and Table 3). Sholl analysis failed to reveal significant differences between groups ($F = 2.01$, $P = 0.16$).

Discussion

The present study reveals that chronic stress triggers hyperanxiety, without affecting fear-potentiated startle. These behavioral changes are associated with volumetric increases in the BNSTam division but not in the amygdala. The enlarged BNSTam in stressed animals resulted from a hypertrophy of dendrites in this region. Additionally, the present results demonstrate that stress induces anxiety, along with structural re-arrangements in the BNST, through the mediation of corticosteroids.

A primary aim of this study was to determine the neuroanatomical substrates upon which stress acts to induce anxiety and fear-conditioned behavior. Anxiety is characterized by a generalized sensation of discomfort and apprehension in response to unconditioned diffuse cues (Koch, 1999). This response outlasts the exposure of the anxiogenic stimulus, suggesting long-term activation of the neural substrates and processes that regulate anxiety (Lee *et al.*, 1994; Lee & Davis, 1997b; a). In contrast, fear-conditioning involves learning that a specific cue, or a more complex context, predicts imminent adversity (Brown *et al.*, 1951; Davis, 1986; 1992a). The neuronal pathways that mediate the acquisition and expression of anxiety versus fear-conditioning to cues are known: while the latter involves intra-amygdaloid connections, anxiety behavior predominantly activates the BNST (Davis *et al.*, 1997).

The EPM paradigm is based on the conflict between a rodent's natural aversion to open spaces with its drive to explore a new environment (Dawson & Tricklebank, 1995; Fernandes & File, 1996; Hogg, 1996). Despite being the most popular and reliable test for evaluating anxiety levels in animals (Hogg, 1996; Mechiel Korte & De Boer, 2003; Carobrez & Bertoglio, 2005; Sousa *et al.*, 2006), the EPM is influenced by a number of environmental factors (Pereira *et al.*, 2005; Lewejohann *et al.*, 2006) and should thus be complemented by other behavioral tests. In the present study, the acoustic startle reflex (ASR) in response to loud acoustic stimuli was the complementary measure of anxiety-like behavior. This response is also enhanced in humans that suffer from anxiety disorders (Grillon *et al.*, 1994; Grillon *et al.*, 1996) and is considered a

diagnostic criterion for post-traumatic stress disorder (American Psychiatry Association, 1994). The ASR pathway includes several brainstem nuclei (auditory nerve, ventral cochlear nucleus, caudal pontine reticular nucleus) that receive projections from forebrain areas, including the central amygdaloid nucleus (Koch, 1999), which together modulate the startle response. In rodents, the ASR can be conditioned by anxiogenic (contextual) as well as fearful stimuli (cues). Interestingly, the neuronal pathways involved tend to differ according to the stimulus (Davis *et al.*, 1997). Previous studies have demonstrated that anxiogenic stimuli that enhance the ASR response depend on the activation of the BNST (Davis *et al.*, 1997; Koch, 1999). In contrast, the fear-potentiated ASR response depends mainly on activation of amygdaloid nuclei, with the BNST playing a lesser role (Lee *et al.*, 1996; Davis *et al.*, 1997).

The present results confirm that chronic stress exposure induces anxiety-like behavior (Shekhar *et al.*, 2005; Bondi *et al.*, 2007). They also show that, consistently with the findings in the EPM test, chronic stress exposure enhances responsiveness to acoustic stimuli, thus lowering the threshold of response to anxiogenic stimuli. Further, this work shows that corticosterone can mimick the effects of CUS in both test paradigms. In accord with previously-published work (Dagnino-Subiabre *et al.*, 2005), the effects of stress on anxiety behavior were paralleled by neither changes in locomotory and exploratory behavior nor alterations in the fear-potentiated startle response. The study by Dagnino-Subiabre *et al* (2005) showed that, chronic stress differentially affects the acquisition of fear-like behavior in a manner that depends on the nature of the conditioned stimulus. The strikingly different impacts of CUS on anxiety *vs.* fear-conditioned behavior reported in the present work prompted our subsequent analysis of the underlying neuroanatomical substrates of these behaviors, namely, the amygdaloid complex and BNST.

Consistent with the lack of CUS- and CORT-induced changes in the fear-potentiated startle response, we failed to observe significant differences in any of the amygdaloid nuclei of animals subjected to CUS and CORT treatment. In contrast, BNSTam volumes were significantly increased after both treatments, suggesting involvement of the BNST in the generation of a hyperanxious state. The role of the BNST in the activation of the HPA axis, its anatomical connections with the neuroendocrine hypothalamus (Dunn, 1987; Dong & Swanson, 2006) as

well as with other brain regions such as the brainstem and ventral striatopallidal, areas that regulate defensive, sexual, ingestive, and exploratory behaviors, have been previously established (Dong & Swanson, 2006). The BNST can be subdivided into several areas, each of which is likely to subserve specific functions on the basis of the following: *i)* BNSTam neurons lying closest to the anterior comissure appear to densely innervate the hypothalamic periventricular region (Dong & Swanson, 2006); *ii)* CRH-immunoreactive cells are found in the neuronal groups composing the anteromedial area (Ju *et al.*, 1989) and have a putative role in regulating the hypothalamic function; and *iii)* electrical stimulation of the anteromedial aspects of the BNST is associated with increased corticosteroid secretion (Dunn, 1987). Thus, it is highly probable that chronic stress acts on the BNSTam to generate a hyperanxious phenotype and, at the same time, a hypercorticalismic state.

The lack of differences in BNSTam neuronal numbers of CUS-treated rats led us to examine for morphological signs of neuroplasticity in this area. A 3D morphological analysis revealed that the increase in BNSTam volume in stressed animals was largely due to increased arborization of bipolar neurons dendrites in animals exposed to either stress or exogenous corticosterone. Although the total lengths and dendritic branching patterns of spiny pyramidal-like neurons in the amygdaloid BLa did not change after either treatment we observed a significant reduction of spine number (as well as spine density) in the dendrites of BLa neurons; these results suggest that synaptic transmission in the amygdala is altered by both CUS and corticosterone.

Our finding that neither stress nor corticosterone treatment lead to major structural changes in the amygdala (nuclear volumes, total number of neurons, dendritic lengths) seems be at odds with previous studies that implicate a role of specific amygdaloid nuclei in triggering the stress response and the expression of emotional behavior. For example, Vyas *et al* (2002) reported hypertrophy of BLa pyramidal-like neurons after chronic immobilization stress (CIS). On the other hand, that same study showed that exposure to a CUS protocol for a period similar to that used by us, did not result in any structural changes. Together, these findings highlight the stimulus-dependent specificity of the neuronal circuitry responsible for generating anxiety (Gewirtz *et al.*, 1998; Rosen *et al.*, 1998; Dagnino-Subiabre *et al.*, 2005; Miracle *et al.*, 2006). We suggest that

the inescapability associated with the CIS paradigm triggers an emotional phenotype that results in the expression of fear responses rather than anxiety; this interpretation would explain the CIS-induced structural changes in the amygdala.

The early view that the amygdala is a homogeneous structure (Johnston, 1923) has been challenged by embryological, anatomical and functional studies (Swanson & Petrovich, 1998). The newer work proposed its subdivision into: *i)* the caudal olfactory system (nucleus of the lateral olfactory tract, cortical nucleus and postpiriform and pirirform-amygdalar areas), *ii)* a ventral extension of the claustrum (lateral, basal and posterior nuclei) that forms a frontotemporal system, and *iii)* a specialized ventromedial expansion of the striatum (CeA and medial amygdaloid [MeA] nuclei, and anterior amygdaloid area) (Swanson & Petrovich, 1998). It is important to note that the BNST is continuous with the CeA and MeA and that these structures together function as a parallel pathway to that in the adjacent striatal area. Such anatomical and functional interrelationships support the inclusion of the BNST, as well as the substantia innominata, into the so-called "extended amygdala" (Alheid, 2003). While the frontotemporal and olfactory systems are important for the establishment of associations between events and emotional value, the extended amygdala seems to be critical for mediating behavioral and autonomic (CeA), survival (MeA) and neuroendocrine (BNST) responses (Swanson & Petrovich, 1998). Against this structure-function background, the fact that stress-induced morphological changes are largely confined to a specific area of the extended amygdala, strongly implicated in neuroendocrine regulation, is striking.

In summary, our results add new information, from a neuromorphological perspective, to the understanding of how chronic stress elicits anxiety behavior. ***1)*** Our findings clearly demonstrate that stress-induced hyperanxiety is associated with structural and functional changes in the BNST, but not in the amygdala. ***2)*** Most of the effects induced by CUS were reproducible by exogenous corticosterone administration, indicating corticosteroid mediation of the effects of CUS. ***3)*** Given the role of the BNST in the regulation of the HPA axis, the herein reported CUS-induced hypertrophy of the BNST bears on our understanding of stress-induced hyperactivity of the HPA axis, providing an anatomical basis for dysregulated closure of this neuroendocrine loop

under conditions of chronic stress. ***4)*** Our description of a structure-based framework that contributes to the dichotomous activation of the BNST and amygdala during chronic stress states is expected to increase our understanding of the neurobiological basis of resilience to stress. Another interesting finding that emerges from this work is that the amygdala, which is implicated in the acquisition and expression of fear behavior, appears to be relatively insensitive to stressful insults; nevertheless, our results show that CUS can impose more subtle damage to this region by impairing its synaptic plasticity. In contrast, the BNST reaction to CUS is more pronounced, expressed as overt reorganization of the BNSTam and resulting in hyperactivity of the HPA axis. By identifying the fine structural alterations (i.e. dendritic morphology and synaptic numbers) that result from exposure to chronic stress, our study suggests new targets through which the pathophysiological cycles that lead to behavioural dysfunction may be interrupted.

Abbreviations

AS – acoustic startle

ASR –acoustic startle reflex

AVG – average startle response

BL – basolateral amygdaloid nucleus

BLa - basolateral anterior amygdaloid nucleus

BLp - basolateral posterior amygdaloid nucleus

BM – basomedial amygdaloid nucleus

BMa - basomedial anterior amygdaloid nucleus

BMp - basomedial posterior amygdaloid nucleus

BNST - bed nucleus of stria terminalis

BNSTam - anteromedial division of BNST

BNSTal - anterolateral division of BNST

BNSTp – posterior division of BNST

CE – coefficient of error

CeA - central amygdaloid nucleus

CIS – chronic immobilization stress

Cont - control rats

CORT - corticosterone-treated animals

CRH - corticotrophin-releasing-hormone

CS – conditioned stimulus

CUS - chronic unpredictable stress animals

CV – coefficient of variation

EPM – elevated plus maze

FPS – fear potentiated startle

HPA – hypothalamus-pituitary-adrenal

La – lateral amygdaloid nucleus

OF – open field

PPI – prepulse inhibition

SD – standard Deviation

Veh - vehicle-injected rats

V_{max} – maximum startle response

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Table 1. Acoustic Startle data.

	Startle amplitude (arbitary units)	
Noise (dB)	Cont	CUS
70 dB	36.943 ± 7.76	26.150 ± 4.08
80 dB	27.571 ± 2.24	22.700 ± 2.28
90 dB	63.650 ± 15.15	41.586 ± 5.77
100 dB	429.400 ± 111.32	806.733 ± 179.78
110 dB	5029.543 ± 755.72	6573.575 ± 226.23
120 dB	6446.753 ± 705.47	6752.409 ± 335.78

Startle amplitude in response to acoustic stimulus. Cont, control rats; CUS, chronic unpredictable stress rats. Results are presented as mean ± SEM.

Table 2. Fear Potentiated Acoustic Startle data.

	Startle amplitude (arbitary units)	
	Cont	CUS
Startle	2827.637 ± 314.51	3435.300 ± 238.77
CS + Startle	4252.900 ± 645.48	4642.900 ± 531.211
	Veh	CORT
Startle	3732.575 ± 406.90	3074.925 ± 303.77
CS + Startle	3653.850 ± 412.14	4010.050 ± 510.73

Startle amplitude in response to an acoustic stimulus. CUS did not affect responsiveness to fear in the acoustic startle test. Startle amplitude in response to acoustic stimuli in the presence of conditioned stimulus startle was enhanced in CORT-treated rats compared to the Veh-treated animals; however, the two groups did not differ significantly. Cont, control rats; CORT, corticosterone-treated rats; CS – Conditioned stimulus; CUS, chronic unpredictable stress rats; Veh, vehicle-injected rats. Results are presented as mean ± SEM.

Table 3. Morphometric analysis of dendrites of neurons of the amygdala and BNST.

		Cont	CUS	t ; p
BLA (pyramidal)	Total dendritic length (μm)	948 ± 59	981 ± 110	0.29 ; 0.77
	Total number of spines (n)	539 ± 39	472 ± 46	-1.07 ; 0.29
	Spine density (n/μm)	0.57 ± 0.03	0.49 ± 0.02	-1.78 ; 0.08
BNST (bipolar)	Total dendritic length (μm)	450 ± 65	644 ± 58	2.20 ; 0.04
	Total number of spines (n)	190 ± 24	307 ± 28	3.15 ; 0.006
	Spine density (n/μm)	0.44 ± 0.03	0.48 ± 0.02	0.97 ; 0.34
		Veh	CORT	t ; p
BLA (pyramidal)	Total dendritic length (μm)	947 ± 61	824 ± 59	-1.46 ; 0.14
	Total number of spines (n)	535 ± 40	335 ± 35	-3.81 ; 0.0004
	Spine density (n/μm)	0.57 ± 0.03	0.40 ± 0.03	-4.13 ; 0.0001
BNST (bipolar)	Total dendritic length (μm)	351 ± 20	431 ± 29	2.22 ; 0.03
	Total number of spines (n)	153 ± 10	183 ± 18	1.43 ; 0.16
	Spine density (n/μm)	0.42 ± 0.13	0.44 ± 0.12	0.52 ; 0.60

BLA, Basolateral amygdaloid nucleus; BNST, Bed Nucleus of Stria Terminalis; Cont, control rats; CUS, chronic unpredictable stress rats. Data are presented as mean ±SEM.

Table 4. Acoustic Startle data.

	Startle amplitude (arbitary units)		
Noise (dB)	Veh	CORT	
70 dB	21.57 ± 2.60	30.05 ± 3.47	
80 dB	45.22 ± 4.17	152.74 ± 44.56	*
90 dB	192.56 ± 33.56	689.92 ± 182.60	*
100 dB	954.67 ± 212.30	5020.34 ± 674.51	*
110 dB	5918.38 ± 628.91	8010.44 ± 501.00	*
120 dB	6477.20 ± 443.10	7588.14 ± 661.43	

Startle amplitude in response to an acoustic stimulus. Results are presented as mean ± SEM. The startle response varied as a function of treatment x startle intensity interaction ($F = 13.38$; $P < 0.003$). There was a significant trend for the startle amplitudes of the CORT rats to increase more rapidly as a function of stimulus intensity. Comparison between groups revealed that as compared to Veh rats, the startle amplitude of CORT rats was significantly elevated at the 80, 90 dB, 100 and 110 dB. * $P < 0.05$. Veh, vehicle-injected animals; CORT, corticosterone-treated animals.

Figures

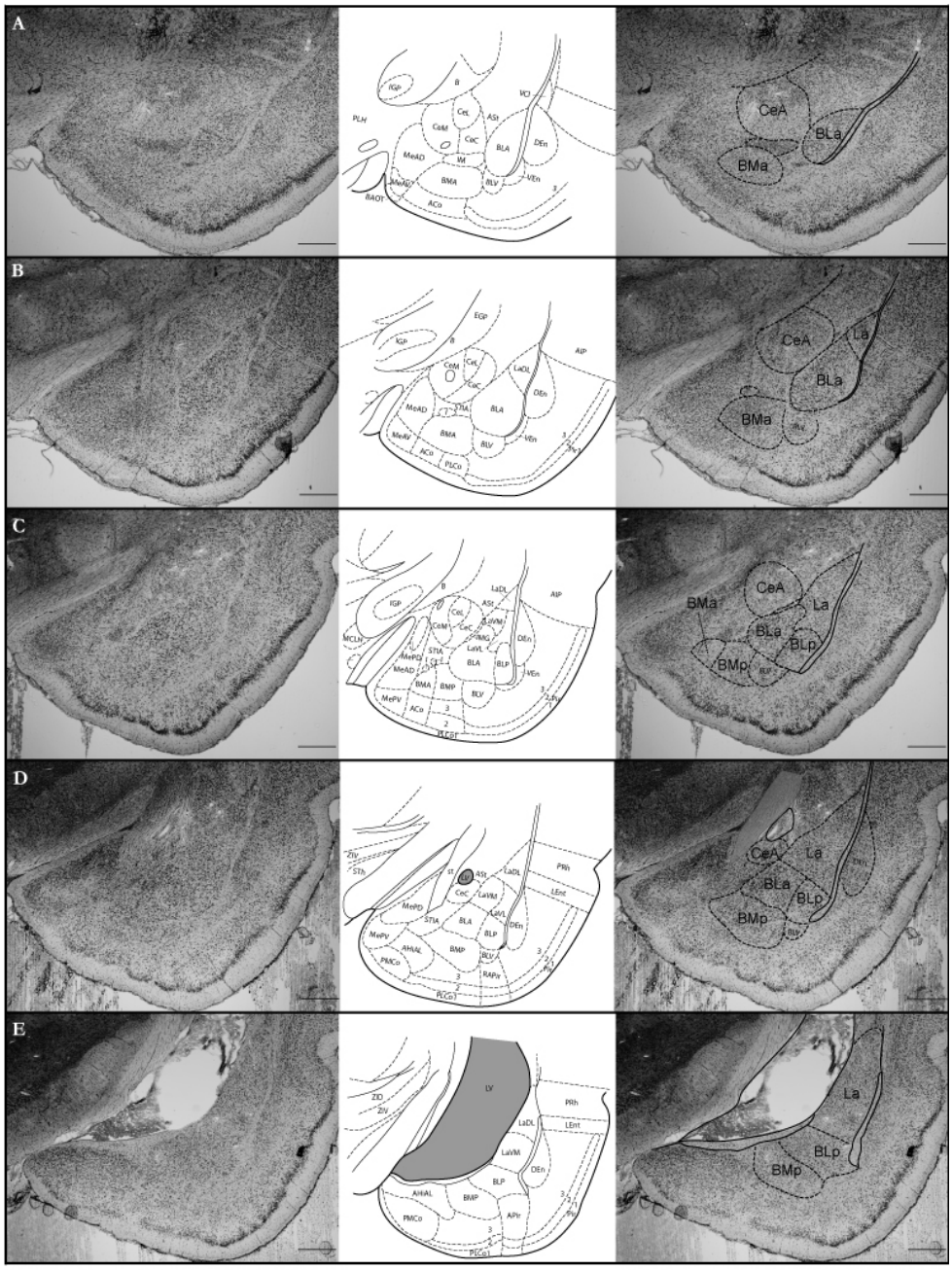

Figure 1. Low power micrographs of a glycolmethacrylate-embedded coronal section of the temporal lobe of the rat stained with Giemsa (left panels); also shown are the corresponding Paxinos atlas schemes (middle panels) used for delineation (right panels) of different amygdaloid nuclei. **A**, Figure 49 (Bregma -1.92). **B**, Figure 52 (Bregma -2.28). **C**, Figure 55 (Bregma -2.64). **D**, Figure 61 (Bregma -3.36). **E**, Figure 66 (Bregma -3.96). BLa, Basolateral anterior; BLp, Basolateral posterior; BMa, Basomedial anterior; BMp, Basomedial posterior; CeA, Central; La, Lateral. Scale bar represents 200 μm.

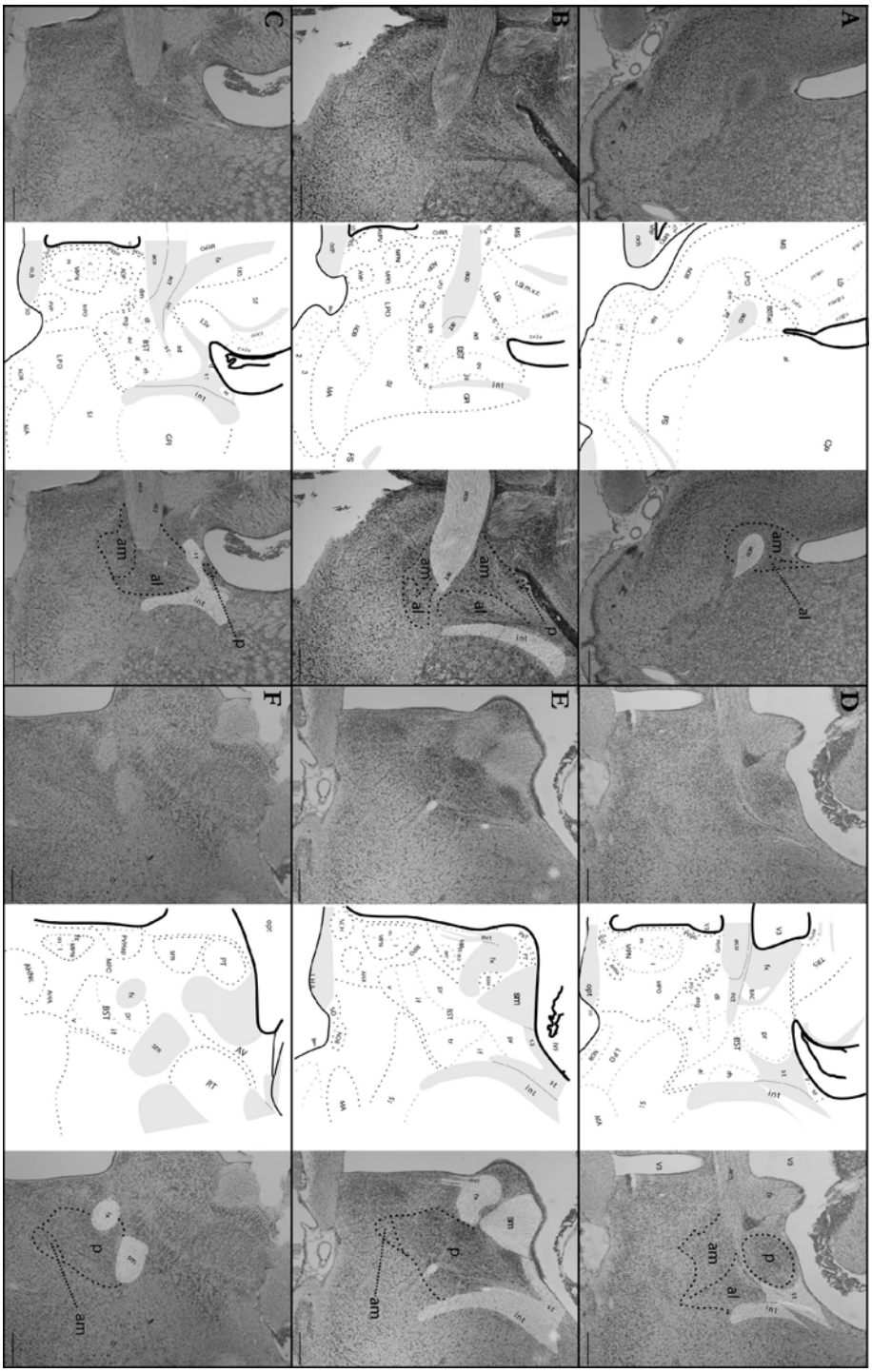

Figure 2. Low power micrographs of a glycolmethacrylate-embedded coronal section of the BNST of the rat stained with Giemsa (left panels); also shown are the corresponding Swanson atlas schemes (middle panels) used for delineation (right panels) of different BNST divisions. **A**, Plate 16 (Bregma +0.10). **B**, Plate 19 (Bregma –0.26). **C**, Plate 20 (Bregma -0.46). **D**, Plate 21 (Bregma -0.51). **E**, Plate 22 (Bregma -0.60). **E**, Plate 23 (Bregma -1.08). am, anteromedial division of BNST; al, anterolateral division of BNST; p, posterior division of BNST. Scale bar represents 200 μm.

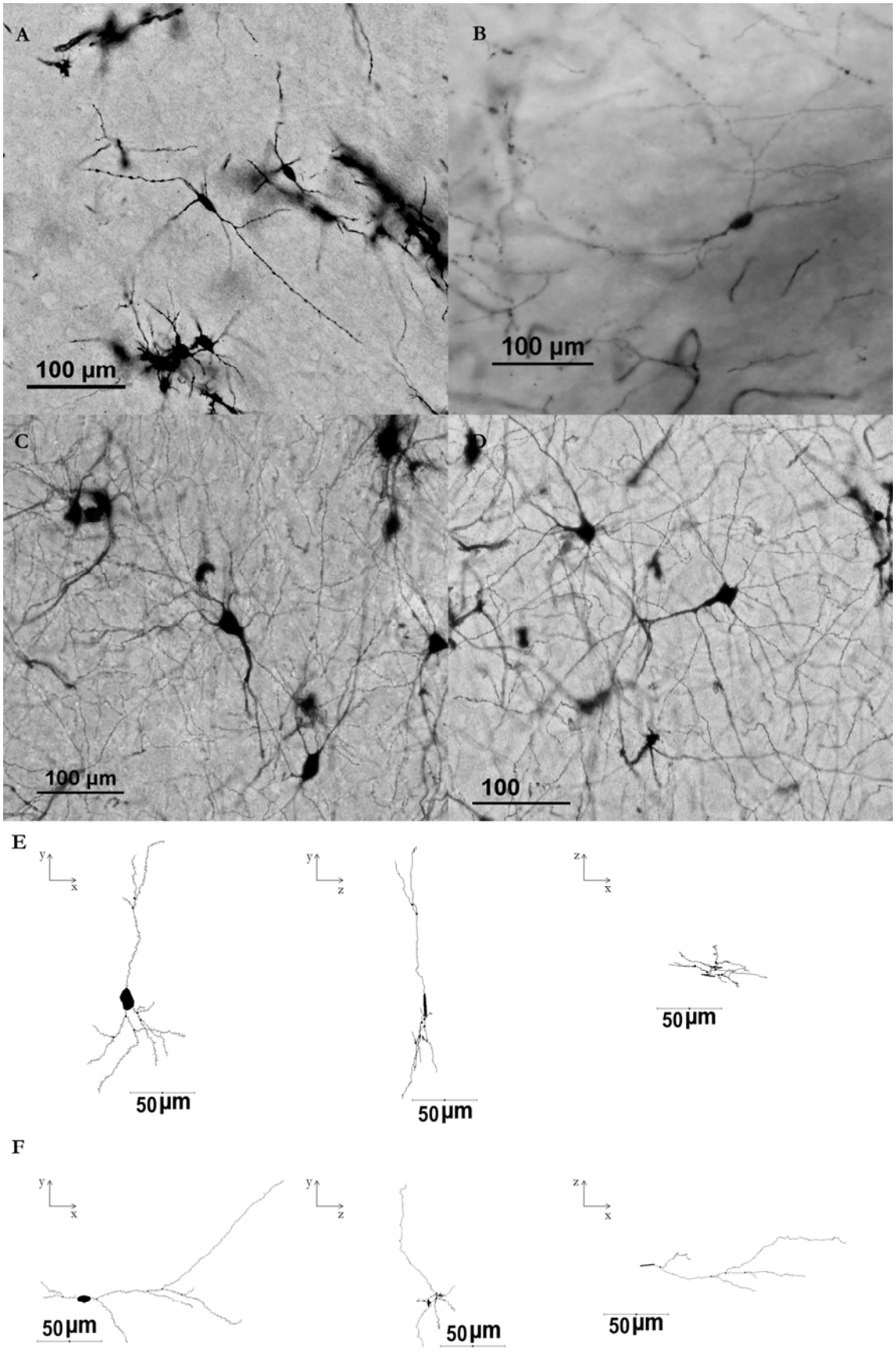
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Figure 3. High power micrographs of Golgi-Cox-stained neurons of the BNST and BLa sections **A**, **B**, Bipolar neurons of BNST (A-Cont; B-CUS). **C**, **D**, Pyramidal neurons of BLa (C-Cont; D-CUS). **E**, **F**, 3D reconstructions of BLa pyramidal and BNST bipolar neurons in the 3 spatial dimensions.

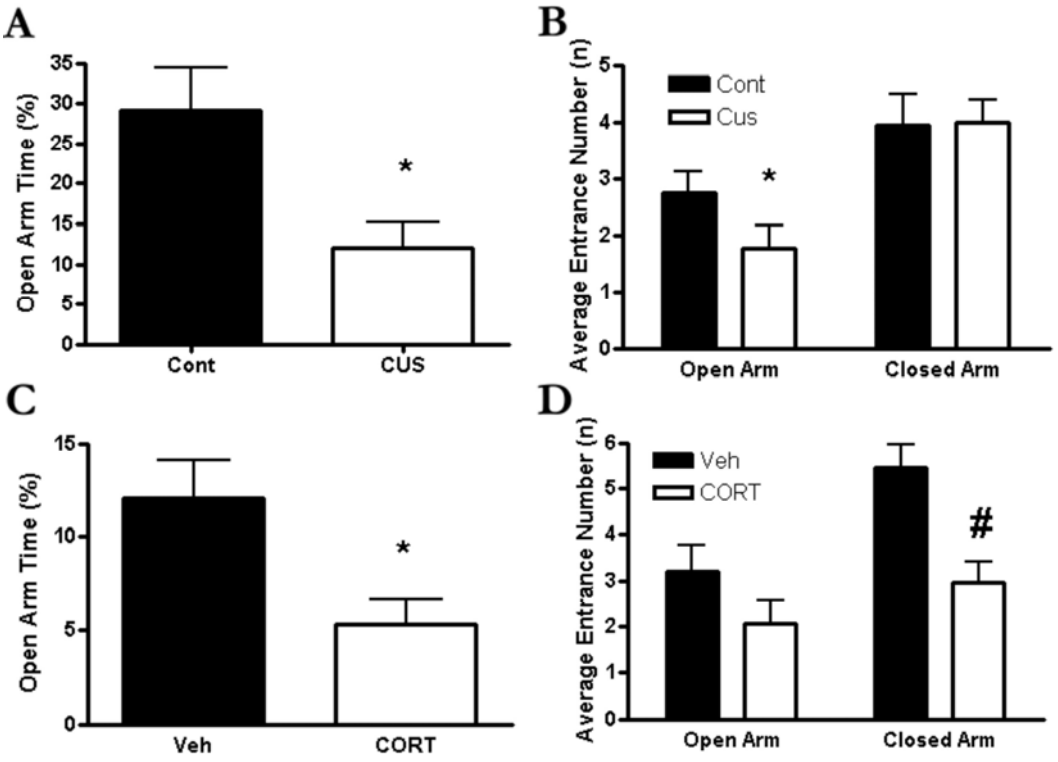

Figure 4. Results from the Elevated Plus Maze test results. **A**, **C**, Time spent in the Open Arms given as percentage of total time. **B**, **D**, Number of entries in the Open and Closed Arms. Cont, control rats; CUS, chronic unpredictable stress rats; Veh, vehicle-injected rats; CORT, corticosterone-treated rats. Results are presented as mean ± SEM. * $P < 0.05$, # $p < 0.001$.

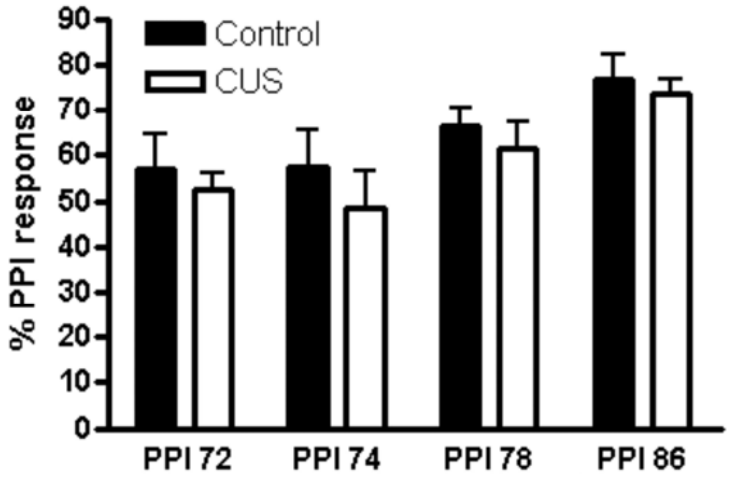

Figure 5. Results from the prepulse inhibition test results. Cont, control animals; CUS, chronic unpredictable stress animals. No significant differences were found between Cont and CUS-treated animals at any prepulse intensity.

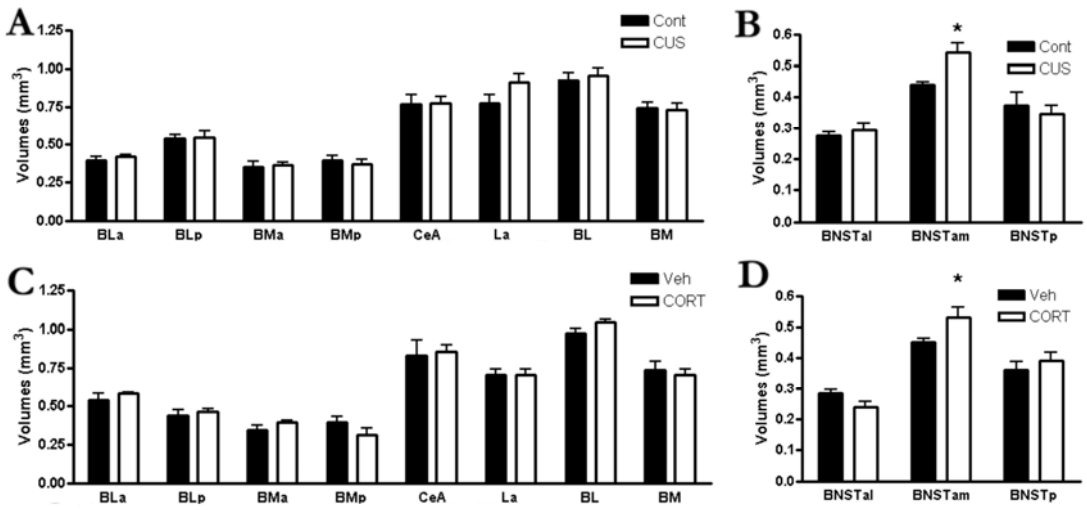

Figure 6. Stereological data on volumetry measuraments. **A**, **C**, Volumes of amygdaloid nuclei. **B**, **D**, Volumes of the bed nucleus of stria terminalis (BNST). BLa, Basolateral anterior; BLp, Basolateral posterior; BMa, Basomedial anterior; BMp, Basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, Central; Cont, control animals; CORT, corticosterone-treated animals; CUS, chronic unpredictable stress animals; La, Lateral; Veh, vehicle-injected animals.* $P < 0.05$. Results are presented as mean ± SEM.

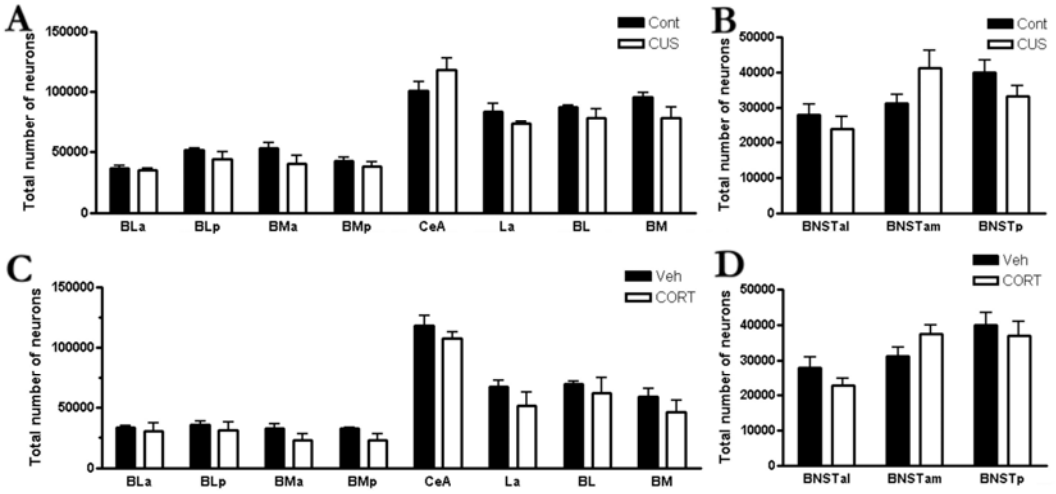

Figure 7. Stereological estimation of total number of neurons. **A**, **C**, Total number of neurons of amygdaloid nuclei. **B**, **D**, Total number of neurons of the bed nucleus of stria terminalis (BNST). BLa, Basolateral anterior; BLp, Basolateral posterior; BMa, Basomedial anterior; BMp, Basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, Central; Cont, control animals; CORT, corticosterone-treated animals; CUS, chronic unpredictable stress animals; La, Lateral; Veh, vehicle-injected animals.* $P < 0.05$. Results are presented as mean ± SEM.

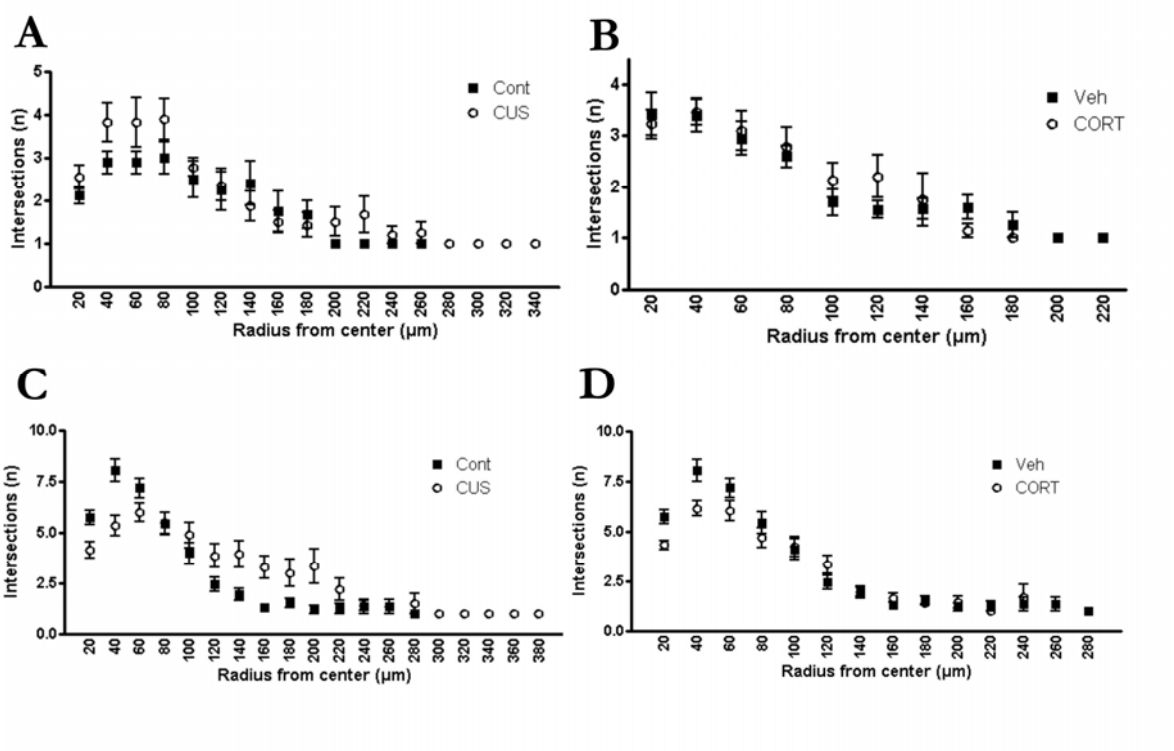

Figure 8. Sholl analysis data. **A**, **B**, BNST bipolar neurons dendritic tree. **C**, **D**, Amygdala BLa pyramidal neurons dendritic tree. Cont, control rats; CUS, chronic unpredictable stress rats; Veh, vehicle-injected rats; CORT, corticosterone-treated rats. Results are presented as mean ± SEM.

Supplementary material

Table 1. Statistical data on results of Elevated Plus Maze and Open-Field tests.

		Cont vs CUS	Veh vs CORT
		t ; p	t ; p
Elevated Plus Maze	Open Arm Time	2.77 ; 0.009	2.66 ; 0.01
	Open Arm Entries	2.03; 0.05	1.79 ; 0.08
	Closed Arm Time	-2.34 ; 0.02	-3.72 ; 0.001
	Closed Arm Entries	-0.07 ; 0.94	-3.62 ; 0.001
	Open/Closed Arm Time Ratio	2.40 ; 0.02	3.03 ; 0.006
Open-Field	Center Time	0.00 ; 0.99	0.04 ; 0.96
	Outer Time	-0.09 ; 0.99	-0.04 ; 0.96
	Center Distances	0.45 ; 0.65	1.01 ; 0.33
	Outer Distances	0.19 ; 0.84	0.55 ; 0.58
	Time Ratio (center/outer)	0.15 ; 0.88	0.04 ; 0.96
	Distances Ratio (center/outer)	0.25 ; 0.79	0.34 ; 0.74
	Rearing Number	1.15 ; 0.22	0.62 ; 0.54
	Rearing Time	2.405 ; 0.02	0.80 ; 0.43

Cont, control animals; CORT, corticosterone-treated animals; CUS, chronic unpredictable stress animals; Veh, vehicle-injected animals.

Table 2. Statistical data on volumetric measurements of individual nucleus of the amygdala and BNST.

	Cont		CUS		Veh		CORT	
	CE	**BV**	**CE**	**BV**	**CE**	**BV**	**CE**	**BV**
BLa	0.08	0.16	0.09	0.08	0.08	0.21	0.07	0.08
BLp	0.03	0.13	0.04	0.19	0.09	0.23	0.10	0.09
BL	-	0.05	-	0.23	-	0.08	-	0.03
BMa	0.06	0.25	0.07	0.09	0.07	0.21	0.05	0.15
BMp	0.07	0.21	0.05	0.19	0.10	0.23	0.14	0.35
BM	-	0.09	-	0.29	-	0.20	-	0.13
CeA	0.07	0.22	0.07	0.13	0.04	0.21	0.07	0.11
La	0.04	0.18	0.04	0.14	0.05	0.12	0.03	0.11
BNSTam	0.07	0.14	0.04	0.17	0.06	0.14	0.05	0.13
BNSTal	0.09	0.17	0.10	0.14	0.09	0.10	0.06	0.14
BNSTp	0.08	0.21	0.06	0.15	0.08	0.19	0.09	0.13

Cont, control animals; CUS, chronic unpredictable stress animals; Veh, vehicle-injected animals; CORT, corticosterone-treated animals. Coefficient of error (CE) and biological variance (BV) are presented for volumes of individual nuclei. BL, basolateral; BLa, basolateral anterior; BLp, basolateral posterior; BMa, basomedial anterior; BMp, basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, central; La, lateral. The shrinkage factor was 1.00 for control and Veh groups, 1.05 for CUS and 1.04 for CORT animals.

Table 3. Statistical data on neuronal number estimates of individual nuclei of the amygdala and BNST.

	Cont		CUS			Veh		CORT		
	CE	**BV**	**CE**	**BV**	***t ; p***	**CE**	**BV**	**CE**	**BV**	***t ; p***
BLa	0.10	0.13	0.11	0.17	-0.40 ; 0.70	0.09	0.12	0.09	0.24	-0.71 ; 0.50
BLp	0.05	0.35	0.06	0.09	-1.01 ; 0.34	0.08	0.16	0.10	0.07	-0.67 ; 0.52
BL	-	0.23	-	0.05	-1.00 ; 0.34	-	0.09	-	0.12	-1.00 ; 0.35
BMa	0.08	0.42	0.09	0.17	-1.55 ; 0.15	0.07	0.30	0.05	0.16	0.98 ; 0.35
BMp	0.07	0.22	0.07	0.16	-0.92 ; 0.38	0.07	0.15	0.09	0.25	1.46 ; 0.19
BM	-	0.29	-	0.09	-1.56 ; 0.15	-	0.24	-	0.10	0.43 ; 0.68
CeA	0.06	0.20	0.07	0.16	1.37 ; 0.20	0.08	0.17	0.07	0.09	1.01 ; 0.34
La	0.07	0.15	0.07	0.18	-1.70 ; 0.12	0.06	0.17	0.06	0.15	0.82 ; 0.44
BNSTam	0.15	0.32	0.13	0.25	0.77 ; 0.47	0.15	0.25	0.12	0.13	2.00 ; 0.08
BNSTal	0.14	0.25	0.17	0.32	-1.56 ; 0.17	0.13	0.19	0.16	0.16	-1.76 ; 0.15
BNSTp	0.12	0.24	0.14	0.17	1.17 ; 0.29	0.12	0.21	0.13	0.21	1.59 ; 0.12

Cont, control animals; CUS, chronic unpredictable stress animals; Veh, vehicle-injected animals; CORT, corticosterone-treated animals. Coefficient of error (CE) and biological variance (BV) are presented for total number of neurons of individual nuclei. BL, basolateral; BLa, basolateral anterior; BLp, basolateral posterior; BMa, basomedial anterior; BMp, basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, central; La, lateral.

Chapter 2.2

Pêgo JM, Sousa JC, Martins L, Cerqueira JJ, Almeida OFX & Sousa N

Stress alters the pattern of c-fos activation in the bed nucleus of stria terminalis: implications for anxiety behavior and HPA dysfunction

(Manuscript to be submitted)

(2007)

Rationale of this chapter and on-going work

The work described in the previous chapter has established a correlation between stress-induced anxiety and structural remodeling of the BNST anteromedial division. The main question addressed in this study is to understand if the structural remodeling observed is associated with an altered activation of the BNST that would contribute to better understand the stress-induced changes in behavior and HPA axis regulation. To address this question we compared the expression of c-fos in control and chronically stressed rats on basal conditions. To determine if anxiogenic stimuli would induce a different pattern of activation we created a second set of animals that were exposed to anxiogenic acoustic stimuli. We observed specific patterns of activation in specific areas/nuclei of the BNST, on basal conditions and following anxiogenic stimuli. Importantly, these patterns were changed by chronic stress.

This on-going work includes immunohistochemical characterization of activated neurons and determination of gene expression in specific BNST nuclei using laser microdissection techniques.

Stress alters the pattern of c-fos activation in the bed nucleus of stria terminalis: implications for anxiety behavior and HPA dysfunction

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Number of pages: 24

Number of figures: 3

Number of tables: 1

Keywords: stress, anxiety, BNST, c-fos

Abstract

Chronic stress is an underlying cause of prevailing emotional disorders like anxiety. Stress induced anxiety is paralleled by structural remodeling of several brain areas, namely in the bed nucleus of the stria terminalis (BNST). The BNST is a complex structure composed by different divisions/nuclei that display distinct roles in anxiety behavior and in the control of the stress response. However, it is not known if the stress-induced structural and behavioral changes are associated with an altered pattern of activation in the BNST. To address this question we have compared the expression of c-fos, an immediate-early gene, in different divisions/nuclei of the BNST in rats submitted to chronic unpredictable stress (CUS) and in controls. A set of controls and CUS rats was evaluated on basal conditions and another set of animals was compared after acoustic anxiogenic stimuli. Data showed an altered activation of the BNST in stressed-animals under basal conditions (increased c-fos expression in the dorsomedial, ventral and fusiform nuclei, but decreased activation in the principal nucleus). Interestingly, the activation of the anteromedial (dorsomedial and ventral nuclei) and posterior divisions of the BNST triggered by anxiogenic stimuli in control animals was attenuated in stressed animals. These results confirm that the altered structure of the BNST following CUS is associated with functional impairments, which are likely to be of relevance for the stress-induced anxiety but also for the dysregulation of the HPA after prolonged stress.

Introduction

Stress has been implicated in the etiology of emotional disorders (Hammen, 2005; Risbrough & Stein, 2006), including anxiety (Charney *et al.*, 1993). Importantly, a significant percentage of these patients display signs of impaired function of the hypothalamus-pituitary-adrenal axis (HPA) function (Shelton, 2007), as assessed by the levels of cortisol and by the response to dexamethasone suppression test (Gillespie & Nemeroff, 2005). The hyperactivation of the HPA axis will ultimately exacerbate the burden on neuronal centers that regulate its control, namely the hippocampus, prefrontal cortex (PFC), the amygdala and the bed nucleus of the stria terminalis (BNST) as a result of the sustained activation of corticosteroids receptors in these brain areas (Sousa, *et al.* 2000; Cerqueira *et al.*, 2005; Pêgo *et al.* unpublished data, Chapter 2.1). Interestingly, these same regions are implicated in emotional behavior (Davis *et al.*, 1997; McEwen, 2005) and, thus, the interplay between stress, HPA dysfunction and emotional disorders perpetuates.

The structure and function of the BNST is complex and heterogeneous (Ju & Swanson, 1989; Ju *et al.*, 1989; Choi *et al.*, 2007). Some authors recognize 3 main divisions (anteromedial, anterolateral, posterior) in the BNST, based on structural landmarks (Ju & Swanson, 1989; Dong *et al.*, 2001a). However, when using such parcellation there is some degree of functional overlap. As an example, it was known the important GABAergic projection from the anteromedial division of the BNST (BNSTam) which exerts an inhibitory influence over the paraventricular nucleus of the hypothalamus (PVN) but, more recently, it was described an excitatory projection originating in the same division of the BNST that activates 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 (CeA) and medial (MeA) nuclei of the amygdala (Prewitt & Herman, 1998; Herman *et al.*, 2005). These apparent discrepancies can only be solved by using parcellations that subdivide these divisions in its subnuclei because this combines structural with stochastic and functional data.

Previous studies have shown that the BNST is involved in the processing of anxiety, an emotional behavior thought to be elicited by diffuse contextual clues (Davis *et al.*, 1997). Recently, we have shown that chronic unpredictable stress (CUS) induces a state of increased anxiety which is paralleled by increases in volumes of the BNSTam as a result of dendritic hypertrophy. Thus, we thought of interest to map the expression of the immediate early gene c-fos in different

divisions/nuclei of the BNST in controls and stressed rats to compare their activation both in basal conditions and after exposure to anxiogenic stimuli.

Materials and Methods

Animals and treatments

All experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) were housed in groups of 2 under standard laboratory conditions with an artificial light/dark cycle of 12/12 hours (lights on at 7 a.m.), 22°C room temperature, *ad libitum* access to food and water. Body weights were recorded weekly and post-mortem thymus weights were used as an index of treatment efficacy.

To assess the influence of chronic stress on BNST activation, 8 week-old rats were exposed for 4 weeks to a CUS protocol, as described previously (Cerqueira *et al.*, 2007). Briefly, rats were randomly assigned to control (Cont) or CUS groups (n= 8 per group). Stressful stimuli were scheduled in a random order, with exposure to 30 min. of a different stressor every day. Control animals were gently handled for 5 min on a daily basis over the 4 week treatment period. Treatments were performed during the daily light phase, between 9 a.m. and 4 p.m. following a random schedule.

This particular CUS model was selected because previous studies (Cullinan *et al.*, 1995; Herman *et al.*, 1995; Sousa *et al.*, 1998; Cullinan & Wolfe, 2000; Sousa *et al.*, 2000) have shown that CUS results in a state of chronic hypercorticalism, characterized by increased adrenal weight and serum corticosterone levels, reduced thymus weight, and reduced body weight gain. Furthermore, the mixture of psychological and physical elements in the CUS paradigm not only reduces the chances of adaptation but also better mimics the variability of stressors encountered in daily life (Sousa *et al.*, 1998; Joels *et al.*, 2004).

At the end of treatment protocols animals were randomly assigned to 2 sets of animals. In one set (n = 4 per group) animals were sacrificed 24 h after the last exposure to a stressful stimuli; in the other set (n = 4 per group) of animals, 24h after the last stressor exposure, rats were submitted to one session of anxiogenic stimuli in the acoustic startle apparatus and sacrificed 30 min. later.

Acoustic startle as an anxiogenic stimulus

The acoustic startle reflex was induced in a startle response apparatus (SR-LAB, San Diego Instruments, San Diego, CA, USA), consisting of a non-restrictive plexiglas cylinder (inner diameter 8.8 cm, length 22.2 cm), mounted on a plexiglas platform and placed in a ventilated, sound-attenuated chamber. To avoid the confounding effect of exposure to a novel environment, animals were habituated to the apparatus (5 min daily) for 2 days before actual trial.

Background white noise, with an intensity of 63 dB, was used to minimize the impact of external acoustic stimuli. Rats were placed in the startle chamber and allowed to acclimatize 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 inter-stimulus interval ranging from 10 s to 20 s. Total duration of procedure lasted 15 min. Animals were then returned to a resting cage for 30 min before being anesthetized and transferred to a different room where perfusion was carried when animals lost reaction to corneal reflex. Tests on individual animals were conducted sequentially; between tests, chambers and the acrylic holders were thoroughly cleaned (70% ethanol and water) to eliminate residual olfactory cues.

Immunohistochemistry

Animals were deeply anesthetized with pentobarbital and perfused transcardially with 250ml of saline followed by 750 ml of fixative solution containing 4% paraformaldehyde in 0.1M phosphate-buffered solution (PBS). Brains were dissected and post-fixed (4% paraformaldehyde in PBS) for 4h, followed by sucrose 8% in 0.1M PBS for 2 days, at 4°C. Coronal sections (50 μm thick) containing the septal area, were serially collected in PBS. Every other section (from the 50 μm sections) was removed for processing. The first section to be collected was chosen randomly. Sections were immunostained for c-fos by incubation with a rabbit anti-c-fos polyclonal antibody (Calbiochem, Darmstadt, Germany) at 1:10.000 dilution, overnight, preceded by 2 h blocking in a PBS solution, containing 0.3% Triton X-100, 0.1M glycine and 10% normal swine serum to decrease background. Following washes in PBST, sections were incubated in biotinylated goat anti-rabbit antibody (Dako, Denmark) followed by an ABC solution (Vectorstain Elite, Vector, USA), both at 1:20 and ultimately reacted for DAB. Sections immunoreacted for c-fos were placed on a

gelatinized slide, coded and counterstained with haematoxylin to delimit regional boundaries. Slides were then mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped.

Morphological estimations

The anatomical organization of the BNST has been thoroughly characterized by Swanson and collaborators (Ju & Swanson, 1989; Ju *et al.*, 1989; Swanson, 1998), and recently revised (Dong *et al.*, 2001a; Dong & Swanson, 2006a). It can be subdivided into major anterior and posterior divisions (relative to stria terminalis main fiber bundles); the former can be further parcelled into medial and lateral groups. The medial division is characterized by dense projections to hypothalamic regions that are closely associated with neuroendocrine control, and includes the anterodorsal and anteroventral areas; in contrast, the lateral group of the anterior division (which includes the anterolateral area) is characterized by projections to hypothalamic areas concerned with autonomic and energy homeostasis and feeding behavior (Dong & Swanson, 2004a). We have used the latter parcellation to establish a parallel to a previous work by Pêgo *et al.* (Chapter 2.1). The above-mentioned regions were outlined according to anatomical references (Swanson, 1998) and recognized on the basis of clear cytoarchitectural differences, namely the density of cells, size of the perikarya and relative position (Ju & Swanson, 1989; Dong *et al.*, 2001a; Dong & Swanson, 2004a; b; 2006a).

Morphometric estimations were performed using StereoInvestigator® software (Microbrightfield, VT, USA) and a camera attached to a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany). Cavalieri's principle was used to estimate volumes of each region of interest. Briefly, every other section was used and the cross-sectional area of each region of interest was estimated by point counting (final magnification x112). We used a test-point system in which the inter-point distance, at tissue level, was 250 μm. The area of the region of interest was determined from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

c-fos ***determinations***

c-fos-immunoreactive (Fos-IR) neurons were recognized by the dark brown DAB precipitate (Fig. 1). The number of Fos-IR neurons was counted and the number per area was calculated, to

establish comparisons. Fos-IR neurons were mapped out into drawings (Swanson, 1998) in order to depict their relative distribution in the nuclei constituting the specific divisions. Relative density of Fos-IR was graded in qualitative terms (number of Fos-IR/nucleus) and averaged between animals in the same treatment group, according to the following scale: -, absent/rare (<2); + light (2-5); ++, moderate (5-10); +++, dense (10-20); ++++, very dense (>20).

Data analysis

All results are expressed as group means ± standard error. Student's *t*- test was used to compare means between groups. Statistical significance was accepted when the probability level was <0.05.

Results

Verification of the biological efficacy of CUS treatment

The CUS protocol decreased body-weight gain (Cont, 96.1 ± 3.1 g; CUS, 79.3 ± 2.4 g; $t_{1,15}$ = 4.26, P < 0.001), reduced the thymus weight (Cont, 0.54 ± 0.02; CUS, 0.38 ± 0.02 x 10^{-3}; $t_{1,15}$ = 5.03, P < 0.001) and induced a non-significant increase in adrenal weight (Cont, 0.88 ± 0.01; CUS, 0.94 ± 0.01; $t_{1,15}$ = -0.86, P < 0.41).

Stereological estimates of BNST areas data showed that the total area of the anteromedial division was increased in CUS animals by 28% when compared to Cont animals (Cont, 0.55 ± 0.02 mm^3; CUS, 0.70 ± 0.03 mm^3; $t_{1,15}$ = -3.74, P < 0.01). Both the anterolateral (BNSTal)(Cont, 0.34 ± 0.02 mm^3; CUS, 0.41 ± 0.02 mm^3; $t_{1,15}$ = -2.02, P = 0.13) and the posterior (BNSTp) (Cont, 0.41 ± 0.03 mm^3; CUS, 0.49 ± 0.01 mm^3; $t_{1,15}$ = -2.06, P = 0.09) divisions showed a trend for volumetric increase in stressed animals but without reaching statistical significance. The observed changes are consistent with previous studies (Pêgo *et al.* unpublished data, Chapter 2.1) that showed that CUS and exogenous corticosteroids were correlated with enlargement of the anteromedial division of the BNST.

c-fos expression in the BNST in basal conditions and in response to anxiogenic stimuli

Data revealed that, both in basal conditions as well as after anxiogenic stimuli, the BNSTam was predominantly activated in relation to the remainder divisions, as depicted by the ratio of Fos-IR in the BNSTam over the average of Fos-IR in the BNST (Cont basal, 1.87 ± 0.19; CUS basal 1.80 ± 0.15; Cont + startle, 1.93 ± 0.17; CUS + startle, 2.07 ± 0.13). Overall, these results demonstrate that the BNSTam is activated on both basal conditions and in reaction to stimuli (Fig.2 A).

Comparative analysis of treatment groups on basal conditions and in response to anxiogenic stimuli was done in order to assess the influence of anxiogenic stimuli on c-fos expression patterns. Data revealed that anxiogenic stimuli triggered increased activation on the BNSTam ($t_{1,7}$ = 3.65, P < 0.05) and BNSTp ($t_{1,7}$ = 2.96 P < 0.05), but not in the BNSTal ($t_{1,7}$ = 1.4, P = 0.22) in control rats. CUS-treated animals, on the other hand, failed to significantly increase the activation

of c-fos after anxiogenic stimuli in the BNSTam ($t_{1,7}$ = -0.55, P = 0.60), BNSTal ($t_{1,7}$ = 2.13, P = 0.09) and BNSTp ($t_{1,7}$ = 0.15, P = 0.88) (Fig. 2 B).

Chronic stress alters activation of specific BNST nuclei

Comparison of the number of c-fos-positive cells between controls and stressed animals under basal conditions failed to reveal significant alterations in any of the main divisions of the BNST (BNSTam, P = 0.15; BNSTal, P = 0.33; BNSTp, P = 0.44) divisions. A similar comparison in animals exposed to anxiogenic stimulus showed a significant difference in the number of c-fos-positive cells in the BNSTam ($t_{1,7}$ = 2.66, P < 0.05) and BNSTp ($t_{1,7}$ = 2.94, P < 0.05), with control rats displaying higher activation than stressed animals in both divisions (Fig. 2).

Taken into account the complexity and heterogeneity of the BNST in stochastic and functional terms (Ju & Swanson, 1989; Ju *et al.*, 1989), we extended our analysis to the individual nuclei of the BNST. Interestingly, this detailed assessment revealed a greater activation of the dorsomedial and ventral nuclei of the anteromedial division ($BNST_{dm}$ and $BNST_{v}$, respectively) in stressed animals under basal conditions (Table 1). Additionally, two other nuclei of different divisions showed an altered activation pattern: while fusiform nucleus ($BNST_{fu}$) of the anterolateral division was highly activated in stressed rats, the principal nucleus ($BNST_{pr}$) of the posterior division displayed reduced c-fos staining in stressed animals (Table 1). Exposure to acoustic anxiogenic stimuli triggered remarkable c-fos activation in $BNST_{dm}$ and $BNST_{v}$ of control animals, but in stressed rats there was only a slight increase ("ceiling" effect). In response to anxiogenic stimuli, some nuclei (transverse and interfascicularis) of the posterior division of the BNST of control animals also revealed a significant increase in the number of c-fos positive cells; again, such anxiogenic-induced activation was blunted in stressed animals (Table 1, Fig. 3).

These results suggest that CUS induces a tonic activation of specific nuclei in the BNST. Of relevance, these nuclei seem to be important to anxiety-like behavior as they were activated/inhibited by anxiogenic acoustic stimuli in control animals. The differential regulation of these nuclei is likely to be dependent of the specific inputs of each nucleus and also determines their distinct influence on the stress response (including HPA axis activity) and in emotional behavior.

Discussion

The analysis of the expression of c-fos within the major divisions of the BNST revealed a heterogeneous activation pattern. In fact, while some groups of cells showed reduced activation in response to stress or anxiogenic stimuli, others revealed enhanced activation by the same factors. These variations disclose the complex relationships between the BNST and upstream regulatory inputs. Additionally, up- and down-activation of distinct cell groups inside the BNST attest to the particular specificities of each nucleus in their regulatory influence over behavior and HPA axis activity. Globally, these heterogeneities support a prior suggestion (Ju & Swanson, 1989; Ju *et al.*, 1989) that the BNST perhaps should more properly be referred to as the bed *nuclei* of the stria terminalis.

The first goal of our study was to determine the basal pattern of activation of the BNST. The observation of a preferential activation of the BNSTam, independently of the treatment condition, is of relevance because several studies have proposed an excitatory role for the BNSTam in the activation of the PVN and, thus, of the stress response (Herman *et al.*, 1994). In fact, *i)* BNSTam neurons lying closest to the anterior commissure appear to densely innervate the hypothalamic periventricular region (Dong & Swanson, 2006a); *ii)* corticotrophin-releasing facto (CRF) immunoreactive cells are found in the neuronal groups composing the anteromedial area (Ju *et al.*, 1989) and have a putative role in regulating the hypothalamic function; and *iii)* electrical stimulation of the anteromedial aspects of the BNST is associated with increased corticosteroid secretion (Dunn, 1987).

The analysis of the expression of c-fos on basal conditions has also revealed a distinctive pattern of activation in stressed animals. While there was not a significant difference in the overall activation of the BNSTam between controls and stressed rats, the latter showed greater activation of $BNST_{dm}$, $BNST_{v}$ and $BNST_{fu}$ nuclei of the anterior division in basal conditions. These data are in agreement with a previous report by Choi *et al.* (2007) that showed compartmentalized roles of specific BNST nuclei in HPA axis regulation; indeed, these authors report attenuated HPA axis activation following specific lesions of the $BNST_{dm}$ and $BNST_{fu}$ nuclei. These nuclei express CRF (Phelix & Paull, 1990) and send dense projections to the PVN (Dong *et al.*, 2001b; Dong & Swanson, 2006b). In addition, they also express glutamate (Glu) that stimulates the HPA axis (Forray & Gysling, 2004). Therefore, it is hypothesized that the stimulatory influence of the $BNST_{dm}$/$BNST_{fu}$ nuclei of the BNST may be mediated by an excitatory CRF/Glu signal to the PVN

(Paull & Gibbs, 1983; Silverman *et al.*, 1989; Champagne *et al.*, 1998). Interestingly, these nuclei are activated by CRF-expressing neurons in the CeA (Prewitt & Herman, 1998) or through a glutamatergic signal originated in the infralimbic PFC (Radley *et al.*, 2006). In addition, because the $BNST_{dm}$ and $BNST_{fu}$ also project (Dong *et al.*, 2001b; Dong & Swanson, 2006b) to several areas of the limbic system, namely the PFC, nucleus accumbens, lateral septal nucleus and CeA (Davis *et al.*, 1997; Carvalho *et al.*, 2005; Jaferi & Bhatnagar, 2007; Rempel-Clower, 2007) it is likely their implication in anxiety behavior.

Our second aim was to analyze how anxiogenic stimuli activate the BNST. c-fos expression characteristically occurs in a short period of time (approximately 30 min). Hence, it is particularly helpful in revealing areas that are activated shortly after exposure to stimuli. When analyzing treatment groups separately we found that, when control animals were presented with anxiogenic stimuli, the number of Fos-IR neurons was increased in the anteromedial (particularly in the dorsomedial and ventral nuclei) and posterior (namely in the transverse and interfascicularis nuclei) divisions of the BNST. This activation pattern suggests that these division/nuclei are involved in the immediate response to anxiogenic situations, which fits with previous reports showing the rapid involvement of these nuclei in response to the anxiogenic stimuli (e.g. acoustic startle) (Dong & Swanson, 2004b; 2006a; b; c). Importantly, comparison of the activation in the BNST in controls and stressed animals also revealed that the recruitment of other neurons of the anterior division in response to anxiogenic stimuli was smaller in the $BNST_{dm}$, $BNST_{v}$ and $BNST_{fu}$ of stressed rats, as they were already more active in basal conditions.

Besides the alterations found in the anterior division of the BNST, this study also highlights an altered c-fos activation in the posterior division of the BNST, particularly in the $BNST_{pr}$. The $BNST_{pr}$ contains GABAergic neurons (Dong & Swanson, 2004b) that putatively exert an inhibitory control over the PVN. In fact, lesions of the $BNST_{pr}$ induced elevations in the expression of c-fos as well as of CRF and AVP (arginine-vasopressine) mRNA in the PVN (Choi *et al.*, 2007). The $BNST_{pr}$ receives projections from the MeA (Dong *et al.*, 2001a) and from the hippocampus (Dong *et al.*, 2001a). The input from MeA is also GABAergic, which results in a trans-synaptic (GABA–GABA) disinhibition of the PVN (Bowers *et al.*, 1998; Prewitt & Herman, 1998); in contrast, the glutamatergic hippocampal input inhibits the HPA axis by stimulating the $BNST_{pr}$ (Zhu *et al.*, 2001; Herman *et al.*, 2004). Thus, our finding that, under basal conditions, stressed animals display a decreased activation of the $BNST_{pr}$ might reflect an attenuated hippocampal input that

results from stress-induced hippocampal damage (Sousa *et al.*, 2000; Cerqueira *et al.*, 2007). In addition, as the $BNST_{pr}$ projects to lateral septal nucleus, CeA and subiculum, this altered pattern of activation might be of relevance in the stress-induced anxiety behavior.

In conclusion, the present data clearly show that in basal and anxiogenic conditions the BNST activation pattern is altered by chronic stress exposure. Most importantly, the observed changes are coherent with the unconditioned and long-term modulation of neuronal pathways that underlie anxiety behavior. Furthermore, because the BNST is a relay nucleus that integrates multiple limbic inputs that regulate the activity of the PVN, the present observations also contribute to our understanding of the neural mechanisms/networks involved in the stress-induced HPA dysfunction.

Abbreviations

AVP – arginine-vasopressine

BNST – bed nucleus of the stria terminalis

BNSTam – anteromedial division of BNST

BNSTal – anterolateral division of BNST

$BNST_{dm}$ – dorsomedial nucleus of the BNST

$BNST_{fu}$ – fusiform nucleus of the BNST

BNSTp – posterior division of BNST

$BNST_{pr}$ – principal nucleus of the BNST

$BNST_{v}$ – ventral nucleus of the BNST

CeA – central nucleus of the amygdala

Cont – control rats

CRF – corticotrophin-releasing-factor

CUS – chronic unpredictable stress

Fos-IR – c-fos immunoreactive

GABA – gama-amino-butiric-acid

Glu – glutamate

HPA – hypothalamus-pituitary-adrenal

MeA – medial nucleus of the amygdala

PBS – phosphate-buffered solution

PFC – prefrontal cortex

PVN – paraventricular nucleus of the hypothalamus

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Table 1. Distribution of c-fos-positive cells in the main divisions/nuclei of the bed nucleus of stria terminalis of rats under basal conditions and after anxiogenic stimuli. Crosses indicate relative densities of Fos-IR neurons in qualitative terms: -, absent/rare (<2); + light (2-5); ++, moderate (5-10); +++, dense (10-20); ++++, very dense (>20).

ad, anterodorsal; al, anterolateral; av, anteroventral; cont, control animals; CUS, chronic unpredictable stress animals; d, dorsal; dl; dorsolateral; dm, dorsomedial; fu, fusiform; if, interfascicular; ju, juxtacapsular; mg, magnocellular; ov, oval; pr, principal; rh, rhomboid; tr, transverse; v, ventral.

Condition / Division / Nucleus		**Basal**		**Anxiogenic stimuli**	
		Cont	**CUS**	**Cont**	**CUS**
Anteromedial	**ad**	+++	++	+++	++
	av	++/+++	++	+++	+++
	dm	+	+++	+++	+++
	mg	+++	++	+++	+++
	dl	++	++	++	++
	v	+	++	++++	+++
Anterolateral	**al**	-/+	+	++	+
	ju	+	+	+	+
	ov	+	+	+/++	+
	fu	-	++	+	+++
	rh	+	+	+	+
Posterior	**tr**	-	+	++	+
	if	+	++	+++	++
	pr	+++	+	+++	+
	d	-	-	-	-

Figures

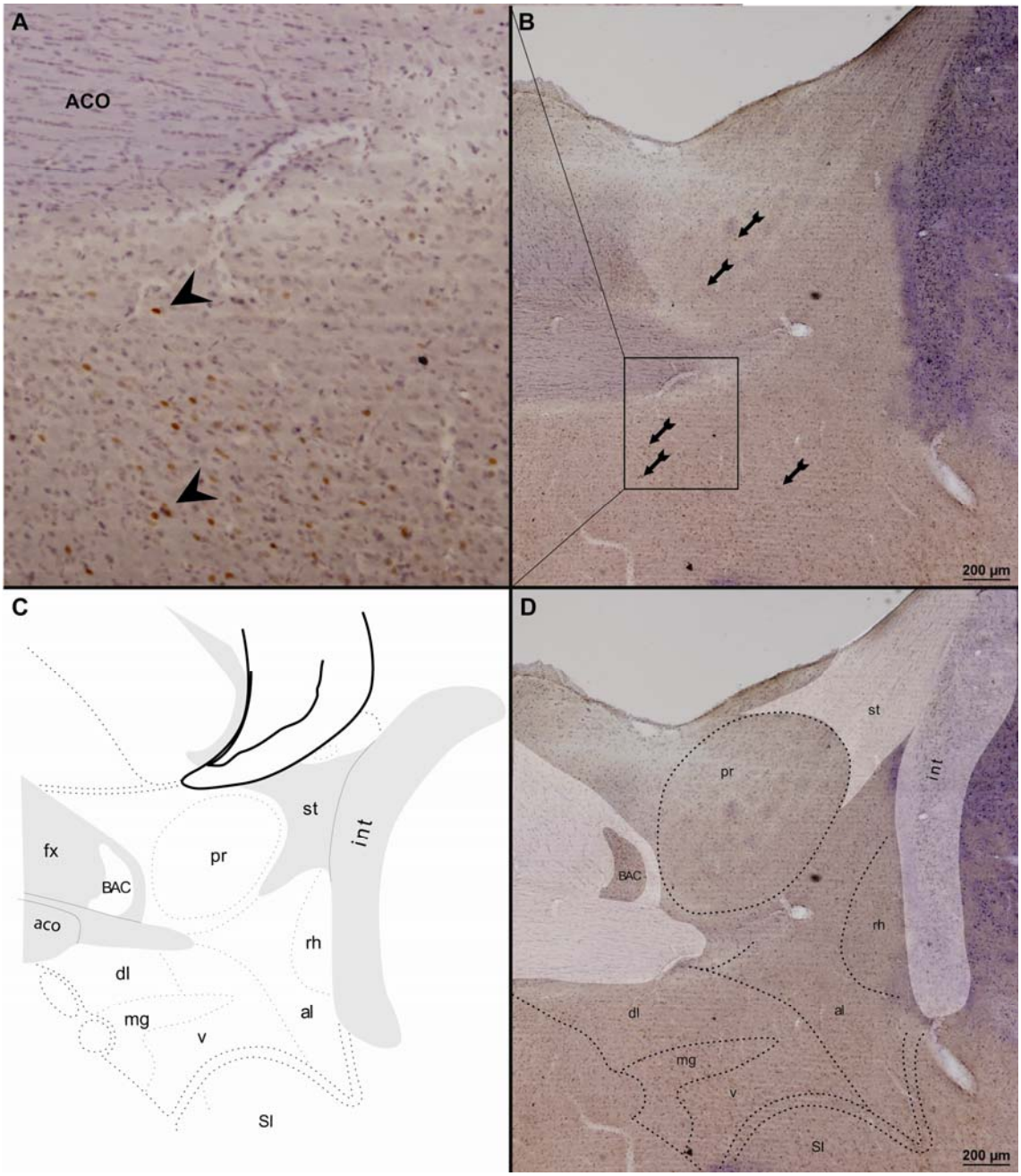

Figure 1. Representative micrographs of immunostained sections of the bed nucleus of stria terminalis for c-fos and delineation of its subnuclei. **A**, detail of Fos-IR neurons (arrowheads); **B**, overview of the BNST showing distribution of Fos-IR neurons (arrows); C, atlas drawing corresponding to the section (Swanson, 1998, Bregma -0.51mm) shown in B; **D**, overlay of atlas drawing on the section. aco, anterior comissure; al, anterolateral nucleus; BAC, bed nucleus of anterior comissure ; dl, dorsolateral nucleus; Fos-IR, c-fos immunoreactive neuron; fx, fornix; int; internal capsule; mg, magnocellular nucleus; pr, principal nucleus; rh, rhomboid nucleus; SI, substantia innominata; st, stria terminalis; v, ventral nucleus.

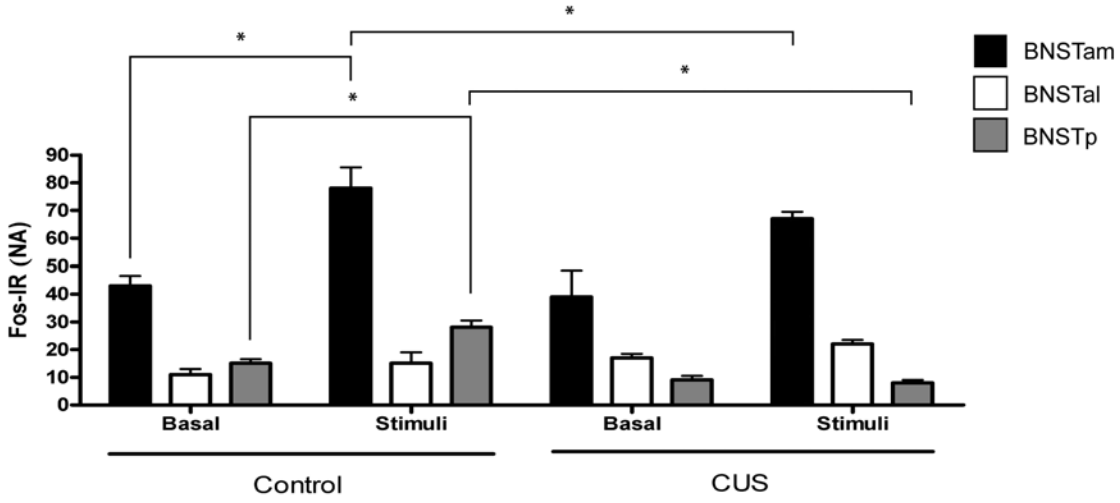

Figure 2. Distribution of Fos-IR in the main divisions of the bed nucleus of stria terminalis, in basal conditions and after anxiogenic stimuli. BNST, bed nucleus of stria terminalis; BNSTal, anterolateral division of the BNST; BNSTam, anteromedial division of the BNST; BNSTp, posterior division of the BNST; Control, control rats; CUS, chronic unpredictable stress treated rats; Fos-IR, c-fos immunoreactive neurons; NA, number per area. * $P < 0.05$.

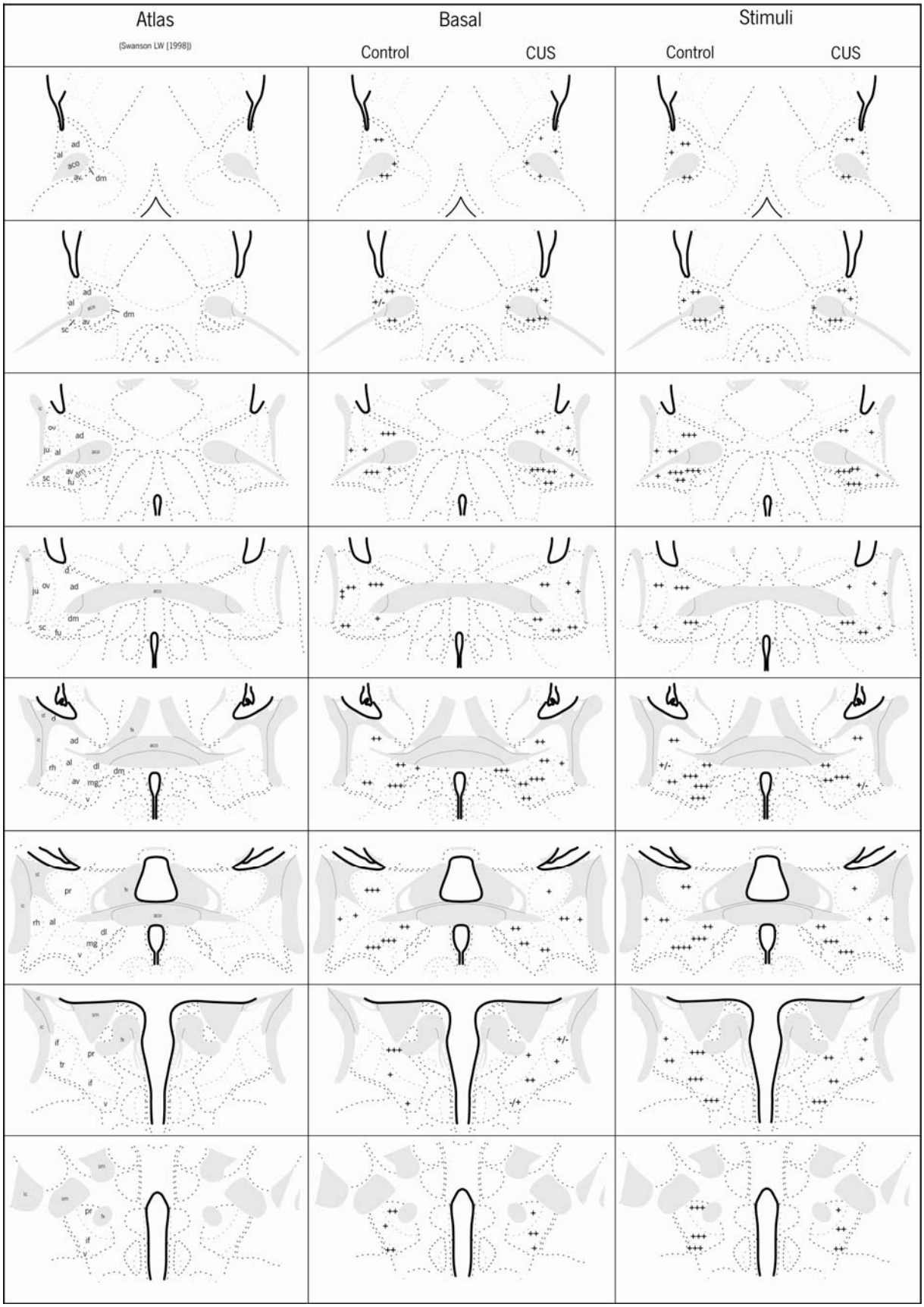
Atlas
(Swanson LW [1998])
Basal
Control
CUS
Stimuli
Control
CUS

Figure 3. Schematic representation of the relative density of Fos-IR neurons in the bed nucleus of stria terminalis of the rat in control and CUS animals, on 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 mm, +0.00 mm, -0.11 mm, -0.26 mm, -0.46 mm, -0.51 mm, -0.60 mm, -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 (<2); + light (2-5); ++, moderate (5-10); +++, dense (10-20); ++++, very dense (>20).

aco, anterior comissure; ad, anterodorsal; al, anterolateral; av, anteroventral; BNST, bed nucleus stria terminalis; control, control animals; CUS, chronic unpredictable stress animals; d, dorsal; dl; dorsolateral; dm, dorsomedial; Fos-IR, c-fos positive cells; fu, fusiform; fx, fornix; if, interfascicular; ju, juxtacapsular; mg, magnocellular; ov, oval; pr, principal; rh, rhomboid; sc, subcomissural zone; sm, stria medularis; st, stria terminalis; tr, transverse; v, ventral.

Chapter 2.3

Pêgo JM, Morgado P, Cerqueira JJ, Almeida OFX & Sousa N

Corticotrophin-releasing factor mediated anxiety correlates with synaptic changes in bed nucleus of the stria terminalis

(Manuscript to be submitted)

(2007)

Rationale of this chapter and on-going work

The findings of structural remodeling and differential activation of the BNST following CUS, highlights the importance of this structure in stress-induced anxiety behavior. The fact that exogenous administration of corticosterone recapitulated structural changes, except spinal sprouting, led us to desing a new study to address the role of central mediators in this process. Adrenalectomized rats were chronically treated with intracerebroventricular administration of vehicle, CRF_1R or CRF_2R ligands, and it was found that CRF_1R ligands induced anxiety behavior that was correlated to spinal sprouting in BNST bipolar neurons without the accompanying dendritic hypertrophy observed in CUS. We hypothesize that the elevation of CRF is a complementary mediator of the stress-induced structural changes in the BNST

This on-going work will be carried on by assessing the expression of synaptic proteins in specific divisions/nuclei of the BNST and also by performing confocal and ultrastructural characterization of these synaptic changes.

Corticotrophin-releasing factor mediated anxiety correlates with synaptic changes in bed nucleus of the stria terminalis

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Number of pages: 30

Number of figures: 4

Number of tables: 1

Supplementary material: 2 tables

Keywords: Stress, anxiety, BNST, stereology

Abstract

Chronic stress is causally implicated in disorders of emotional behavior. Previous studies have indicated a role for the amygdala and bed nucleus of the stria terminalis (BNST) in the neuronal circuitries underlying fear and anxiety, respectively. We recently showed that chronic stress influences BNST structure and function by inducing dendritic remodeling. Exogenous corticosterone (CORT) was found to reproduce most of those changes except for the stress-induced reduction of dendritic spines in BNST neurons, arguing for the involvement of other central mediators of the latter. The BNST is a target of corticotrophin-releasing factor (CRF) and is endowed with both types of CRF receptors (CRF_1R and CRF_2R); moreover, this nucleus is directly involved in the regulation of the hypothalamo-pituitary-adrenal (HPA) axis and, thus, in the neuroendocrine response to stress. In the present study rats, adrenalectomized and supplemented with low doses of CORT, were given intracerebroventricular (icv) infusions of CRF (a ligand of CRF_1R), urocortin (a mixed ligand of CRFRs) and urocortin III (a specific ligand of CRF_2R) over 14 days. Animals were then tested for anxiety in the elevated plus maze (EPM) and for locomotor/exploratory behavior in the open field test. At sacrifice, BNST and amygdala volumes and cell numbers were estimated by stereology and a 3-dimensional analysis of dendritic morphology was performed. Results showed that treatment with CRF_1R, but not CRF_2R, agonists induces hyperanxiety without influencing locomotory/exploratory activity. CRF-mediated anxiety was not correlated with stereological changes in either the BNST or amygdala, but there was a strong correlation between behavior and synaptic remodeling, in the anteromedial area of the BNST. Thus, it is likely that CRF_1R agonists induce hyperanxiety by remodeling synapses in the BNST.

Introduction

Chronic stress is implicated in the genesis of several psychiatric conditions especially mood and affect disorders such as anxiety and depression (Arborelius *et al.*, 1999). Clinical studies have correlated emotional disorders with dysfunction of the hypothalamo-pituitary-adrenal (HPA) axis (Aihara *et al.*, 2007; Shelton, 2007), including hypercortisolemia (Gillespie & Nemeroff, 2005) and elevated levels of corticotrophin-releasing factor (CRF) in the cerebrospinal fluid (Strohle & Holsboer, 2003). Increased anxious-like behavior is observed in animals undergoing chronic stress and in animals experiencing pharmacologically-induced hypercorticalism (File, 1996; Vyas *et al.*, 2002; Anisman & Matheson, 2005; Pêgo *et al,* unpublished data, Chapter 2.1). Both experimental paradigms were also previously reported to trigger changes in the structure of neurons in areas directly related to emotional behavior and the regulation of the HPA axis, such as the bed nucleus of stria terminalis (BNST) (Vyas *et al.*, 2002; Vyas *et al.*, 2003), the prefrontal cortex (PFC) (Wellman, 2001; Cook & Wellman, 2004; Cerqueira *et al.*, 2005b; Cerqueira *et al.*, 2007) and hippocampus (Sousa *et al.*, 1998).

Emotional behavior has been consistently linked to the activity of the amygdala, a deep temporal structure which is richly interconnected with the hippocampus and PFC. Several studies have suggested the importance of this region in the acquisition and expression of fear (Davis *et al.*, 1997; Walker *et al.*, 2003). On the other hand, anxiety is thought to be regulated by the BNST (Davis *et al.*, 1997; Walker *et al.*, 2003). Thus, stress or icv administration of CRF or directly into the BNST induces anxiety-like behavior, whereas intra-amygdaloid injections of CRF do not elicit a hyperanxious state (Lee & Davis, 1997a; b). Interestingly, both chronic stress and exogenous corticosteroids are anxiogenic and produce morphological alterations in the BNST but not the amygdala (Vyas *et al.*, 2002; Pêgo et al., unpublished data, Chapter 2.1). However, the specific role of other central mediators of stress in the manifestation of the behavioral and structural effects is not known.

The 41-amino acid neuropeptide CRF is an important secretagogue of pituitary adrenocorticotrophin (ACTH), and thus plays a pivotal role in the regulation of the endocrine response to stress (Vale *et al.*, 1981). CRF and the related peptides Urocortin (Ucn), UcnII and UcnIII, form a family of peptides. They act through G-protein-coupled receptors, CRF_1R and CRF_2R, which have distinct distribution

patterns and physiological functions (Chen *et al.*, 1993; Chalmers *et al.*, 1995; Lovenberg *et al.*, 1995; Dautzenberg & Hauger, 2002; Reul & Holsboer, 2002; Hauger *et al.*, 2003). While CRF shows selectivity for CRF_1R, Ucn binds to both CRF_1R and CRF_2R (Vale *et al.*, 1981; Dautzenberg & Hauger, 2002). However, the more recently discovered UcnII and UcnIII show high affinity for CRF_2R (Lewis *et al.*, 2001; Reyes *et al.*, 2001). In the brain, CRF is expressed in neurons of the paraventricular nucleus of the hypothalamus (PVN), the cerebral cortex, cerebellum, amygdala and hippocampus; Ucn expression is confined to the Edinger-Westphal, lateral olivary and supraoptic nuclei (Reul & Holsboer, 2002); UcnII is exclusively expressed in subcortical regions related to stress such as PVN, the locus coeruleus, the hypothalamic supraoptic and arcuate nuclei, and several motor nuclei of the brainstem and spinal cord (Reyes *et al.*, 2001; Reul & Holsboer, 2002); finally, UcnIII is expressed in the median preoptic area, rostral perifornical area, BNST, medial nucleus of the amygdala and lateral septum (Lewis *et al.*, 2001; Reul & Holsboer, 2002).

The CRF_1R, strongly implicated in stress-related behaviours and autonomic activation (Liebsch *et al.*, 1995; Heinrichs *et al.*, 1997; Skutella *et al.*, 1998; Liebsch *et al.*, 1999), is robustly expressed in the anterior pituitary, cerebral cortex, cerebellum, amygdala, hippocampus and olfactory bulbs (Dautzenberg & Hauger, 2002; Reul & Holsboer, 2002); this receptor appears to be crucial for the initiation of the pituitary-adrenocortical response to stress although it does not seem to be important for maintaining HPA axis activity under baseline conditions (Reul & Holsboer, 2002). The CRF_2R is predominantly expressed in subcortical areas such as the PVN, lateral septum, amygdala, hippocampus and BNST. The role of CRF_2R is still poorly defined although there are convincing data that suggest that its activation is important for curtailing the adrenocortical response to stress (Bale *et al.*, 2000; Coste *et al.*, 2000). With respect to its role in behavior, some authors have described the anxiogenic effects of activation of central CRF_2R (Radulovic *et al.*, 1999), while others have ascribed an anxiolytic function to the CRF_2R (Valdez *et al.*, 2003; Zhao *et al.*, 2007).

The aim of the present study was to clarify the contribution of receptor-selective CRF-related peptides to chronic stress-induced hyperanxiety. Our approach was to administer CRF receptor agonists chonically via the icv route to adrenalectomized (ADX) rats that were clamped with physiological doses of corticosterone; this paradigm was designed to allow analysis of the role of the CRF-related

petides without interference from fluctuations in endogenous corticosterone secretion. The animals were assessed in anxiety- and exploratory/locomotor-tests, and these behaviors were subsequently correlated with morphometric data derived from analysis of the amygdala and BNST.

Materials and Methods

Animals and treatments

All experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation.

Adult male Wistar rats (Charles River Laboratories, Barcelona, Spain) were housed in groups of 2/3 under standard laboratory conditions with an artificial light/dark cycle of 12/12 hours (lights on at 8 a.m), 22°C room temperature and provided with food and water *ad libitum*. Treatment protocols were initiated when the animals were 10 weeks of age and were continued over a period of 2 weeks. Behavioural tests were performed between days 8 and 14.

Weekly body weight recordings and post-mortem thymus weights provided an index of treatment efficacy. Corticosterone levels were measured in blood serum, obtained between 9:00 and 10:00 a.m., by radioimmunoassay (R & D Systems, Minneapolis, MN).

Male Wistar rats (n = 10 per experimental group) were submitted to stereotaxic surgery for the implantation of brain infusion cannulae (Alzet® Brain Infusion Kit, Alza Corp., Palo Alto, CA) in the lateral ventricle. Rats were anesthetized with ketamine/metomidine (Imalgene®, Agroviseu, Viseu, Portugal, 75mg/Kg; Dormitor®, Agroviseu, 0.5 mg/Kg) and secured in a stereotaxic instrument (Stoelting Co, Ilinois, USA). The skin and connective tissues were removed from the skull, a hole was drilled, and a 28-gauge cannula (Alzet Brain® Infusion Kit) was inserted into the unilateral ventricular space, using stereotaxic coordinates: 0.92 mm posterior to bregma, 1.2 mm lateral, and 5.0 mm below the skull surface at the point of entry.

Chronic treatment was achieved with mini-osmotic pumps (Alzet 2200, Alza Corp.) connected to the cannulae using artificial CSF (aCSF) as vehicle. To blunt the peripheral activation of the HPA axis, animals from all treatment groups were ADX but supplemented with physiological doses of corticosterone (Sousa *et al.*, 1999) before stereotaxic surgery. Rats were randomly assigned to sham (Sham, aCSF, non-ADX), ADX (ADX, aCSF), ADX+human/rat CRF (CRF 1.8 μg/day), ADX+rat urocortin (Ucn, 7 μg/day) or ADX+human urocortin III (UcnIII, 3.5 μg/day) groups (n=10 per group). These ligands were selected due to their binding properties and relative potency; Ucn III was

preferred to UcnII, in light of previous data reporting its expression in the BNST (Lewis *et al.*, 2001; Reul & Holsboer, 2002). All drugs were acquired from Sigma (Sintra, Portugal).

Behavioral analysis included the open field test for locomotion and exploratory activity and the elevated plus maze for anxiety-like behavior; testing was carried out during the daily light phase, between 9 a.m. and 4 p.m.

Following the behavioral tests, animals were deeply anesthetized with pentobarbital (Eutasil®, Ceva, Algés, Portugal) and perfused transcardially with either 4% paraformaldehyde solution for glycolmethacrylate inclusion (n = 5 per group), or saline for Golgi-Cox staining (n = 5 per group). Brains were dissected and post-fixed (4% paraformaldheyde) for at least 30 days, or kept in Golgi-Cox solution until processed for histology.

Elevated plus maze

Animals were tested over 5 min in a black polypropylene "plus"-shaped maze (MedAssociates Inc, St. Albans, USA) at a height of 72 cm above the floor. The maze consisted of two facing open arms (50.8 x 10.2 cm) and two closed arms (50.8 x 10.2 x 40.6 cm). Testing was performed under bright white light.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 EPM are presented as percentage of the total duration of the trial.

Open field

Animals were individually tested for 5 min each in an OF arena (43.2 x 43.2 cm) that had transparent acrylic walls and a white floor (MedAssociates Inc, St. Albans, USA). Each subject was initially placed in the centre of the arena and horizontal activity and instant position were registered, using a system of two 16-beam infrared arrays connected to a computer. Total distances were used as indicators of locomotor activity. Times and distances in the pre-defined central and peripheral areas were recorded and used to calculate the ratio of time spent in the central area over total time

of the trial, and distance travelled in the central as a function of total area. Number and duration of rearings were recorded. The test room was illuminated with bright white light.

Histological procedures

Brains (n = 5 per group) from the 4% paraformaldehyde-perfused animals (see above) were embedded in glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Werheim, Germany) before sectioning on a microtome, as described previously (Cerqueira *et al.*, 2005a). Every other 30 μm-thick coronal section was placed on a gelatinized slide, stained with Giemsa, mounted with Entellan (Merck, Darmstadt, Germany) and coverslipped. The shrinkage factor was calculated according to Madeira *et al* (1990).

Brains (n = 5 per group) from animals that had been transcardially perfused with 0.9% saline (see above) were processed for Golgi-Cox staining according to a published protocol (Gibb & Kolb, 1998). Briefly, brains were removed and immersed in Golgi-Cox solution (a 1:1 solution of 5% potassium dichromate and 5% mercuric chloride diluted 4:10 with 5% potassium chromate (Glaser & Van der Loos, 1981) for 14 days; brains were then transferred to a 30% sucrose solution (3 days), before being cut on a vibratome. Coronal sections (200 μm thick) were collected in 6% sucrose and blotted dry onto gelatin-coated microscope slides. They were subsequently alkalinized in 18.7% ammonia, developed in Dektol (Kodak, Portugal), fixed in Kodak Rapid Fix (prepared as manufacturer instructions), dehydrated through a graded series of ethanols, and cleared in xylene before being mounted and coverslipped.
Slides were coded before morphometric analysis in both sets.

Regional boundaries

Stereological parameters were analyzed on 6 regions of the amygdaloid complex, including the basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA) and lateral (La) nuclei/regions/areas, as outlined in Swanson (1998). Individual nuclei were discernible according to established criteria and parcellations were recognized as detailed before (Pêgo *et al,* unpublished data, Chapter 2.1, Fig. 1).

The anatomical organization of the BNST has been thoroughly characterized by Swanson and collaborators (Ju & Swanson, 1989; Ju *et al.*, 1989; Swanson, 1998), being subdivided into major anterior and posterior (BNSTp) divisions (relative to stria terminalis main fiber bundles); the former can be further parcelled into dorsal, lateral and ventral areas, relative to the anterior comissure fibers. However, in a subsequent revision of the anatomical, cytoarchitectural and projection studies, the cell groups of the anterior division were arranged into medial (BNSTam) and lateral (BNSTal) groups (Dong *et al.*, 2001). The medial division is characterized by dense projections to hypothalamic regions that are closely associated with neuroendocrine control, and includes the anterodorsal and anteroventral areas (Dong & Swanson, 2006); in contrast, the lateral group of the anterior division (which includes the anterolateral area) is characterized by projections to hypothalamic areas concerned with autonomic control, energy homeostasis and feeding behavior (Dong & Swanson, 2004). The above-mentioned regions were outlined according to anatomical references and recognized on the basis of clear cytoarchitectural differences, namely the density of cells, size of the perikarya and relative position (Swanson, 1998) (Pêgo *et al,* unpublished data, Chapter 2.1, Fig. 2).

Stereological procedures

Volume estimations were performed using StereoInvestigator® software (Microbrightfield, VT, USA) and a camera attached to a motorized microscope (Axioplan 2, Carl Zeiss, Hamburg, Germany).
Cavalieri's principle was used to assess the volume of each level. Briefly, every 4th section was used and the cross-sectional area of each region of interest was estimated by point counting (final magnification x112). We used a test-point system in which the inter-point distance, at tissue level, was 100 μm for the BMp and BLp, 250 μm for the BNST and 150 μm for the remaining nuclei. The volume of the region of interest was determined from the number of points that fell within its boundaries and the distance between the systematically sampled sections.

Average cell numbers were estimated using the optical fractionator method (West *et al.*, 1991). Briefly, a grid of virtual, equally-spaced 3D-boxes (30 μm x 30 μm x 20 μm) (same grid spacing as for volume estimations) was superimposed on every 4th section of the lamina of interest and the number of neurons falling inside the boxes was counted, according to standardized stereological procedures (Gundersen *et al.*, 1999; Coulin *et al.*, 2001). The estimated total number of neurons in

each region was then calculated on the basis of the number of counted neurons, grid spacing, volume of the box, and number of boxes counted. Neurons were differentiated from other cells on the basis of perikaryon size and shape (Peinado *et al.*, 1997).

Dendritic trees analysis

Three-D reconstructions of representative Golgi-impregnated neurons from the BNST were made. The criteria used to select neurons for reconstruction were as follows: (i) full impregnation of the neurons along the entire length of the dendritic tree; (ii) dendrites without truncated branches, except on the most superficial layer; (iii) relative isolation from neighboring impregnated neurons to avoid interference with the analysis; (iv) no morphological changes attributable to incomplete dendritic impregnation of Golgi–Cox stain. We chose neurons for dendritic analysis in the anteromedial area, using the criteria followed by Pêgo *et al* (unpublished data, Chapter 2.1, Fig. 3): (i) presence of transverse anterior comissure; (ii) rostral location to the stria terminalis main bundle; (iii) selection of neurons adjacent to the anterior comissure. These landmarks correspond to the rostral portion of the medial division described by McDonald (1983) as being populated by cells with characteristic ovoid soma and polarized dendritic trees that branch sparingly. These cells are characteristically sparsely to moderate spiny, contrasting with the spine- and dendrite-rich cells of the lateral division

For each selected neuron, all branches of the dendritic tree and the location of all dendritic spines were reconstructed at 600X magnification, using a motorized microscope (Carl Zeiss Axioplan 2, with oil-objectives), attached to a camera (DXC-390, Sony Co., Japan) and Neurolucida software (Microbrightfield). Three-D analysis of the reconstructed neurons was performed using NeuroExplorer software (Microbrightfield). In each brain, 10 neurons of the BNSTam were studied. Several aspects of dendritic morphology were examined. To assess overall changes, total dendritic length and number of dendrites were compared between groups. The total number of spines on dendrites and spine density (total spines divided by dendritic length) were also compared between groups. To assess differences in the arrangement of dendritic material, a 3-D version of a Sholl analysis (Sholl, 1956; Uylings & van Pelt, 2002) was performed; for this, the number of intersections of dendrites with concentric spheres positioned at radial intervals of 20 μm were recorded.

Data analysis

Except for coefficients of error, all results are expressed as group means ± standard error. A repeated measures test was used for evaluating data from the Sholl analysis. ANOVA test was used to compare means between groups for the remainder variables. Post-hoc analysis was perfomed using Tukey test. Statistical significance was accepted for a probability level below 0.05.

Coefficients of error were computed according to the formulas of Gundersen *et al.* (1999) for cell numbers and Gundersen and Jensen (1987) for volume estimates. Coefficients of variation (CV = standard deviation/mean) were determined for the stereological estimates. It is important to note that the low coefficients of error, which did not exceed the biological variance (Supplementary material Table 1 & 2), attest to the validity of the stereological determinations (Coulin *et al.*, 2001).

Results

Plasmatic levels of corticosterone

There were no significant differences in the plasmatic levels of corticosterone among experimental groups (Sham, 45 ± 3 ng/ml; ADX, 50 ± 10 ng/ml; ADX+CRF, 66 ± 6 ng/ml; ADX+Ucn 55 ± 5 ng/ml; ADX+UcnIII 64 ± 8 ng/ml), which demonstrates that the supplement of corticosterone given to adrenalectomized rats was able to restore the basal levels of this hormone.

CRF_1R mediates stress-induced hyperanxiety

Data obtained in the EPM revealed an overall effect of treatment on the percentage of time spent in the open arms ($F_{4,35} = 2.68$, $p < 0.04$). Post-hoc analysis showed that chronic CRF (a CRF_1R ligand) and Ucn (a mixed ligand of CRF receptors) administration induced an anxiety-like phenotype, with both treatments resulting in significantly reduced times spent in the open arms of the EPM (CRF *vs* Sham, $p < 0.035$; *vs* ADX, $p < 0.043$; *vs* Ucn, $p < 0.48$; *vs* UcnIII, $p < 0.47$; and Ucn (Ucn *vs* Sham, $p < 0.007$; *vs* ADX, $p < 0.02$; *vs* CRF, $p < 0.48$; *vs* UcnIII, $p < 0.20$) (Fig. 1). Interestingly, treatment with UcnIII, a CRF_2R ligand, did not alter anxiety-behavior in the EPM.

Importantly, the number of closed arm ($F_{4,35} = 0.40$, $p = 0.80$) entries did not significantly differ between the groups, indicating that exploratory/locomotory activity was preserved in all experimental groups. Measurements in the OF paradigm confirmed that none of the treatments induced significant alterations in locomotor activity or exploratory (rearing) behavior (see Table 1 for details).

Morphological correlates of anxiety-like behavior in the BNST

Volumetric analysis failed to reveal an effect of treatment on individual amygdaloid nuclei (BLa, $F_{4,20} = 1.52$, $p = 0.22$; BLp, $F_{4,20} = 1.88$, $p = 0.14$; BMa, $F_{4,20} = 0.48$, $p = 0.74$; BMp, $F_{4,20} = 2.44$, $p = 0.07$; CeA, $F_{4,20} = 1.83$, $p = 0.15$; La, $F_{4,20} = 2.40$, $p = 0.77$) or BL ($F_{4,20} = 2.02$, $p = 0.12$) and BM ($F_{4,20} = 1.22$, $p = 0.32$) (Fig. 2, A). Additionally, none of the treatments produced any changes in the estimates of total neuron numbers (BLa, $F_{4,20} = 2.62$, $p = 0.06$; BLp, $F_{4,20} = 1.91$, $p = 0.14$; BMa, $F_{4,20}$

= 0.93, $p = 0.46$; BMp, $F_{4,20} = 1.96$, $p = 0.13$; CeA, $F_{4,20} = 0.37$, $p = 0.82$; La, $F_{4,20} = 2.14$, $p = 0.10$; BL, $F_{4,20} = 2.06$, $p = 0.12$; BMp, $F_{4,20} = 1.91$, $p = 0.15$) (Fig. 2, C).

Whereas ANOVA failed to reveal treatment effects on BNST volumes (BNSTam, $F_{4,20} = 1.31$, $p = 0.31$; BNSTal, $F_{4,20} = 0.80$, $p = 0.54$; BNSTp, $F_{4,20} = 0.80$, $p = 0.98$) (Fig. 2, C) and stereological estimates of total neuron numbers in the various sub-divisions of the BNST (BNSTam $F_{4,20} = 2.38$, $p = 0.11$; BNSTal $F_{4,20} = 0.64$, $p = 0.64$; BNSTp $F_{4,20} = 0.21$, $p = 0.92$) (Fig. 2, D), a subsequent 3D morphological analysis of dendritic arborizations in the anteromedial division of the BNST revealed treatment effects on the total number of dendritic spines ($F_{4,20} = 3.07$, $p < 0.02$; Fig. 3, A) and average spine density ($F_{4,20} = 3.43$, $p < 0.01$; Fig. 3, C), but not on total dendritic length ($F_{4,20} = 0.37$, $p = 0.83$; Fig. 3, B). Changes in spine number and density were conspicuously greater in BNST bipolar neurons of CRF-treated rats than in Sham (number of spines 52% increase, $p < 0.03$; spine density 73% increase, $p < 0.04$) or in ADX animals (number of spines 33% increase, $p < 0.05$; spine density 41% increase, $p < 0.05$), but did not differ significantly from other treatment groups (Fig. 3). Interestingly, activation of CRF_2R (UcnIII) did not trigger significant changes in number and density of spines in BNST bipolar neurons.

Sholl analysis of the number of dendritic intersections of BNST bipolar neurons as a function of their distance from the soma (Fig. 4) also failed to show an effect of treatment ($F = 1.20$, $p = 0.32$).

Discussion

Anxiety is defined as excessive worry about events or activities that are difficult for the subject to control (DSM IV of Mental Disorders, American Psychiatric Association). The condition is characterized by a generalized sensation of discomfort and apprehension in response to unconditioned diffuse cues (Koch, 1999). Anxiety triggers behavioral reactions that are slowly activated and sustained over time (Davis *et al.*, 1997; Walker *et al.*, 2003). Importantly, these responses outlast the triggering stimulus, suggesting long-term activation of the neuroanatomical substrate responsible for regulating anxiety (Lee *et al.*, 1994; Lee & Davis, 1997; b). Present evidence indicates that activation of the BNST, rather than that of the amygdala, underlies anxiety (Davis *et al.*, 1997).

Several studies have shown that central administration of CRF_1R ligands elicit anxiety-like responses (Britton *et al.*, 1986; Swerdlow *et al.*, 1986; Dunn & File, 1987; File *et al.*, 1988; Shekhar *et al.*, 2005). Studies based on pharmacological and genetic models have suggested an opposite role of CRF_2R in anxiety (Bale *et al.*, 2000; Valdez *et al.*, 2002; Valdez *et al.*, 2003), although there are also reports to the contrary (Radulovic *et al.*, 1999); in the latter work, activation of CRF_2R in the lateral septal area was found to inhibit the expression of anxiety-like behavior. Consistently, with most published reports, our present observations show that activation of CRF_1R, but not CRF_2R, results in the expression of an anxious phenotype. Interestingly, the CRF_1R agonists proved to be inherently anxiogenic since they produced signs of anxiety in ADX animals receiving a basal dose of exogenous corticosterone, i.e. in animals that could not increase their endogenous corticosterone levels in response to the CRF peptide treatments. However, in contrast to our previous observations in stressed or corticosterone-treated animals (Pêgo *et al,* unpublished data, Chapter 2.1), the display of CRF_1R-mediated anxiety-like behavior was not paralleled by volumetric changes or alterations in the total number of neurons in either the amygdala or BNST.

In accordance with the lack of volumetric changes, subsequent 3D morphological analysis of dendritic trees in the BNST also failed to reveal signs of neuronal hypertrophy/atrophy. However, neurons in the BNSTam responded to CRF_1R activation with a significant increase in number and density of spines, reflecting an increase in their synaptic contacts. Interestingly, these results

complement our previous observations on discriminating the different mediators of stress-induced structural changes in the BNST. Indeed, while chronic stress was accompanied by a hypertrophy and an increased number of dendritic spines of bipolar BNST neurons (Pêgo *et al,* unpublished data, Chapter 2.1), corticosterone treatment reproduced the former, but without affecting spine density or numbers in BNST bipolar neurons. Taken together, these findings highlight a clear distinction between the neuroanatomical manifestations of corticosteroids and CRF_1R agonists, even thought all these experimental procedures triggered anxiety behavior. It is important to mention, however, that sustained activation of CRF_2R failed to alter the behavior phenotype and also the structure of the BNST; as these receptors are known to be present in this region of the brain (Lewis *et al.*, 2001; Reul & Holsboer, 2002), these findings suggest that their role must be related to other function of the BNST.

In summary, our results demonstrate the inherent anxiogenic properties of agonists of the CRF_1R agonists and show that they exert their actions by inducing synaptic rearrangements in the BNSTam. Importantly, together with the results reported previously (Pêgo *et al.,* unpublished data, Chapter 2.1), we show that the actions of corticosteroids and CRF_1R agonists are complementary in determining the behavioral and morphological phenotypes elicited by chronic stress. Our results confirm further that the BNST, rather than the amygdala, represents the neural substrate that regulates stress- and CRF-induced anxiety.

Abbreviations

ACTH - adrenocorticotrophin

ADX – adrenalectomized

aCSF – artificial cerebrospinal fluid

BLa – basolateral anterior amygdaloid nucleus

BLp – basolateral posterior amygdaloid nucleus

BMa – basomedial anterior amygdaloid nucleus

BMp – basomedial posterior amygdaloid nucleus

BNST – bed nucleus of the stria terminalis

BNSTam – anteromedial division of BNST

BNSTal – anterolateral division of BNST

BNSTp – posterior division of BNST

CE – coefficient of error

CeA – Central amygdaloid nucleus

CV – coefficient of variation

CORT – corticosterone

CRF – corticotrophin-releasing-factor

CRFR – CRF receptor

CV – coefficient of variation

EPM – elevated plus maze

HPA – hypothalamus-pituitary-adrenal

icv – intracerebroventricular

La – lateral amygdaloid nucleus

OF – open field

PFC – prefrontal cortex

PVN – paraventricular nucleus of the hypothalamus

SEM – standard error of the mean

Sham – sham operated rats

Ucn - urocortin

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Table 1. Results of open field tests in sham-operated controls and ADX rats, and ADX rats receiving CRF (a CRF_1R ligand), Ucn (a mixed ligand of CRF receptors) and UcnIII (a specific ligand of CRF_2R). Note that all ADX animals were supplemented with exogenous corticosterone (20 μ/ml in drinking water). Means ± SEM are shown.

	Sham	ADX	CRF	Ucn	UcnIII	F ; *p*
Rear Number (n)	25 ± 4.3	17 ± 3.9	19 ± 2.2	19 ± 3.1	26 ± 7.0	1.07 ; 0.39
Rear Time (s)	51 ± 10.5	27 ± 7.8	35 ± 5.9	35 ± 8.0	53 ± 16.0	1.40 ; 0.25
Center Distance (cm)	53 ± 11.3	63 ± 13.5	60 ± 8.2	45 ± 8.5	54 ± 12.1	0.38 ; 0.82
Outer Distance (cm)	470 ± 104	334 ± 67	439 ± 59	393 ± 61	451 ± 42	0.73 ; 0.57
Center/Outer Time Ratio	0.039 ± 0.008	0.056 ± 0.01	0.042 ± 0.007	0.039 ± 0.007	0.043 ± 0.006	2.32 ; 0.07

ADX, adrenalectomized rats; CRF, ADX + CRF treated rats; Sham, sham rats; Ucn, ADX + Ucn treated rats; UcnIII, ADX + UcnIII treated rats.

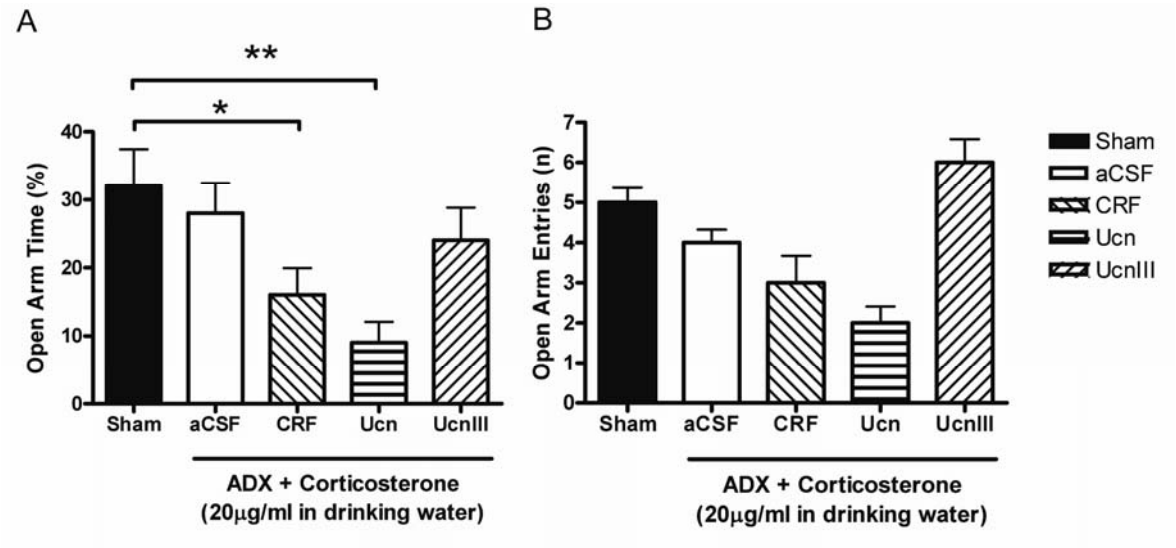

Figure 1 – Anxiety-like behavior, measured in the Elevated Plus Maze test. **A**, Time spent in the open arms given as percentage of total time. **B**, Number of entries in the open arms. ADX, adrenalectomized rats; CRF, CRF treated rats; Sham, sham rats; Ucn, Ucn treated rats; UcnIII, UcnIII treated rats. Results are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.001$.

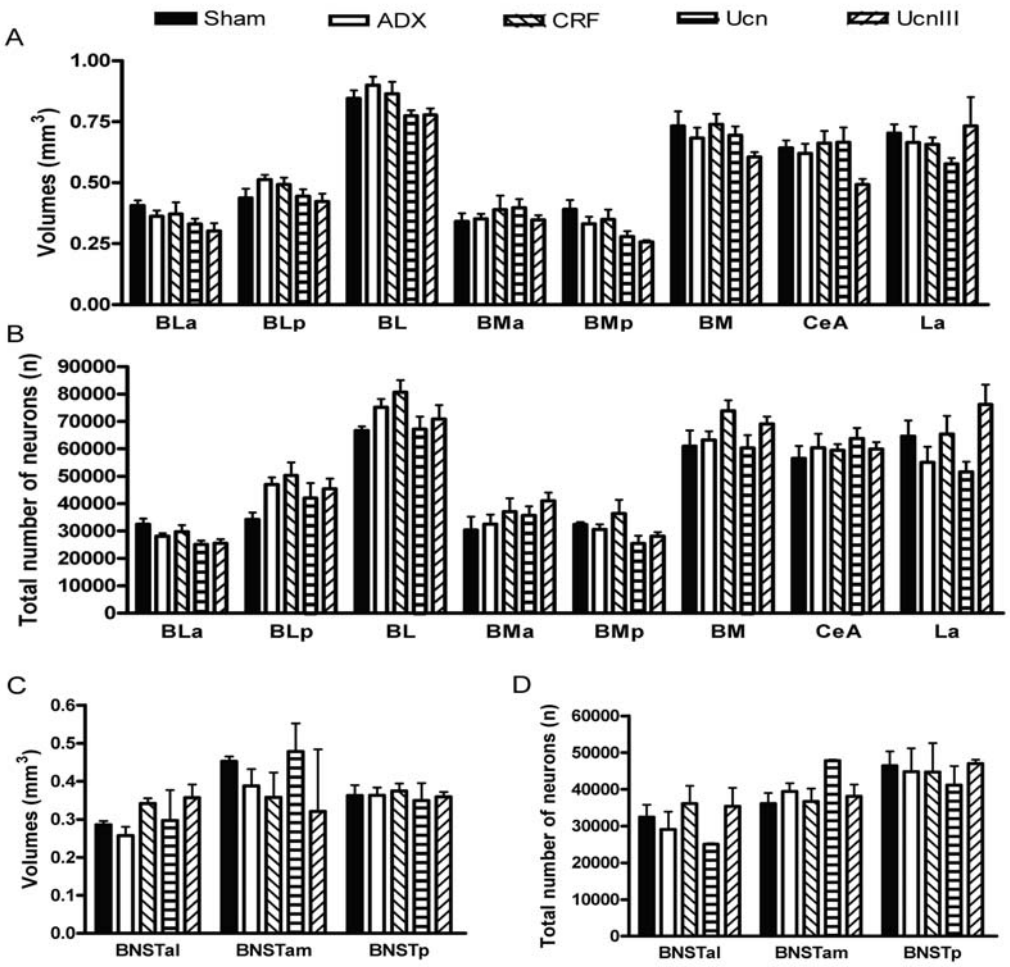

Figure 2 - Volumetric and total number of neuron estimates, obatined by stereological analysis. **A**, **C**, Volumes of amygdaloid nuclei and of the bed nucleus of stria terminalis (BNST). **B**, **D**, Total number of neurons of amygdaloid nuclei and BNST. ADX, adrenalectomized rats; CRF, ADX + CRF treated rats; Sham, sham rats; Ucn, ADX + Ucn treated rats; UcnIII, ADX + UcnIII treated rats. All ADX animals were supplemented with low levels of exogenous corticosterone (20 μ/ml in drinking water). BLa, Basolateral anterior; BLp, Basolateral posterior; BMa, Basomedial anterior; BMp, Basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, Central; La, Lateral. Results are presented as mean ± SEM.

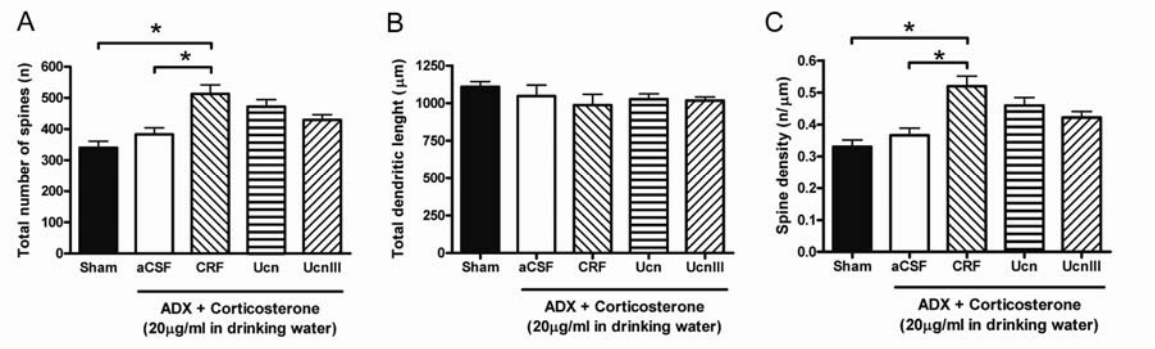

Figure 3 – Morphometric analysis of 3D reconstructions of bipolar neurons of the bed nucleus of stria terminalis (BNST). **A**, Total number of spines. **B**, Total dendritic length. **C**, Spinal densities. ADX, adrenalectomized rats; ADX + CRF, CRF treated rats; Sham, sham rats; ADX + Ucn, Ucn treated rats; ADX + UcnIII, UcnIII treated rats. Results are presented as mean ± SEM. * $P < 0.05$.

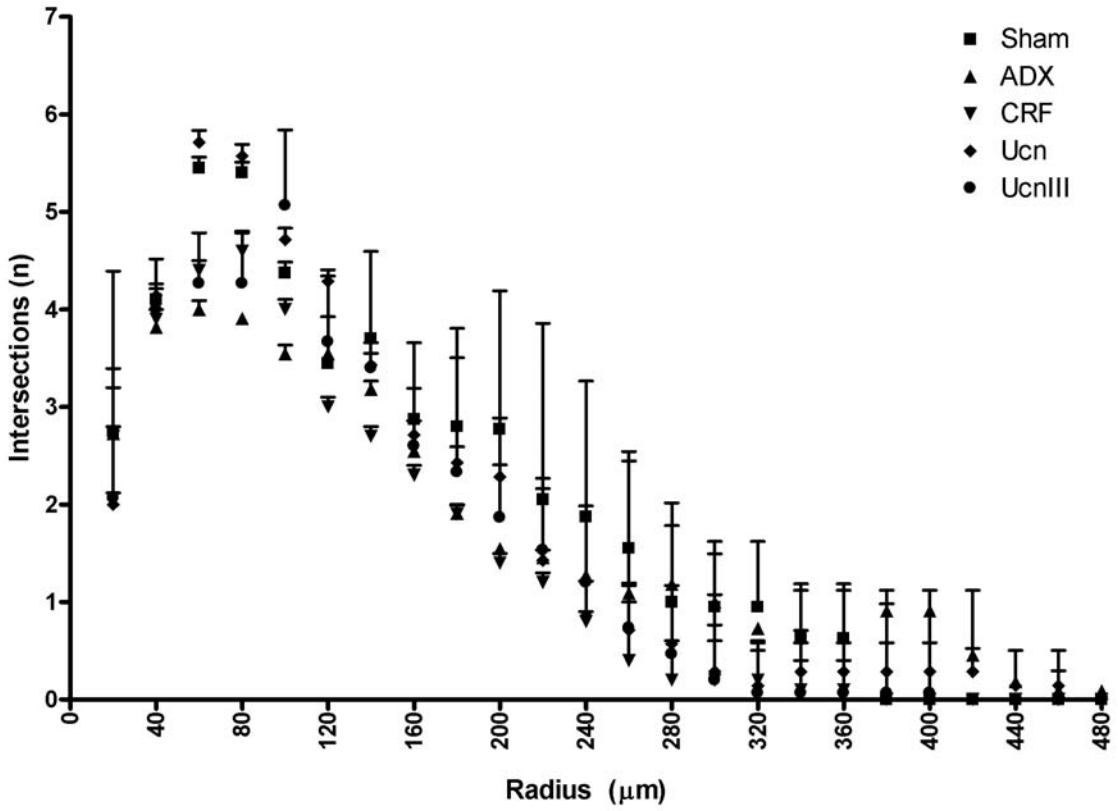

Figure 4 - Sholl analysis data of the dendritic trees of bipolar neurons in the bed nucleus of stria terminalis (BNST). ADX, adrenalectomized rats; CRF, ADX + CRF treated rats; Sham, sham rats; Ucn, ADX + Ucn treated rats; UcnIII, ADX + UcnIII treated rats. All ADX animals were supplemented with low levels of exogenous corticosterone (20 μ/ml in drinking water). Results are presented as mean ± SEM.

Supplementary material

Table 1. Coeffients of error and biological variance of volumetric estimates of individual amygdaloid nuclei and of the bed nucleus of stria terminalis (BNST).

	Sham		ADX		CRF		Ucn		UcnIII	
	CE	**BV**	**CE**	**BV**	**CE**	**BV**	**CE**	**BV**	**CE**	**BV**
BLa	0.08	0.12	0.06	0.15	0.04	0.31	0.1	0.17	0.06	0.21
BLp	0.03	0.21	0.05	0.10	0.03	0.14	0.04	0.16	0.04	0.15
BL	-	0.10	-	0.09	-	0.14	-	0.07	-	0.07
BMa	0.06	0.23	0.07	0.17	0.04	0.36	0.06	0.20	0.06	0.10
BMp	0.07	0.23	0.06	0.25	0.06	0.29	0.05	0.20	0.06	0.06
BM	-	0.20	-	0.14	-	0.14	-	0.13	-	0.06
CeA	0.07	0.12	0.08	0.18	0.05	0.19	0.05	0.22	0.06	0.09
La	0.04	0.12	0.06	0.28	0.04	0.11	0.03	0.10	0.05	0.28
BNSTam	0.06	0.07	0.06	0.20	0.08	0.31	0.05	0.27	0.04	0.14
BNSTal	0.09	0.09	0.07	0.15	0.04	0.17	0.08	0.47	0.07	0.16
BNSTp	0.08	0.18	0.08	0.10	0.08	0.09	0.11	0.23	0.06	0.06

ADX, adrenalectomized rats; CRF, ADX + CRF treated rats; Sham, sham rats; Ucn, ADX + Ucn treated rats; UcnIII, ADX + UcnIII treated rats. All ADX animals were supplemented with low levels of exogenous corticosterone (20 μ/ml in drinking water). Coefficient of error (CE) and biological variance (BV) are presented for volumes of individual nuclei. BL, basolateral; BLa, basolateral anterior; BLp, basolateral posterior; BMa, basomedial anterior; BMp, basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, central; La, lateral.

Table 2. Coefficients of error and biological variance of volumetric estimates of individual amygdaloid nuclei and of the bed nucleus of stria terminalis (BNST).

	Sham		ADX		CRF		Ucn		UcnIII	
	CE	BV	CE	BV	CE	BV	CE	BV	CE	BV
BLa	0.10	0.13	0.12	0.11	0.08	0.20	0.14	0.14	0.11	0.12
BLp	0.05	0.15	0.07	0.16	0.06	0.21	0.06	0.32	0.06	0.16
BL	-	0.04		0.11	-	0.12	-	0.17		0.14
BMa	0.08	0.32	0.12	0.28	0.10	0.29	0.09	0.21	0.11	0.14
BMp	0.07	0.05	0.08	0.17	0.08	0.30	0.08	0.28	0.08	0.10
BM	-	0.16		0.13	-	0.10	-	0.17		0.08
CeA	0.06	0.16	0.10	0.22	0.09	0.09	0.07	0.15	0.09	0.09
La	0.07	0.18	0.09	0.29	0.09	0.25	0.09	0.18	0.09	0.16
BNSTam	0.13	0.18	0.12	0.10	0.12	0.16	0.11	0.12	0.11	0.12
BNSTal	0.15	0.28	0.12	0.29	0.12	0.23	0.14	0.22	0.13	0.24
BNSTp	0.12	0.15	0.14	0.25	0.13	0.30	0.12	0.10	0.12	0.13

ADX, adrenalectomized rats; CRF, ADX + CRF treated rats; Sham, sham rats; Ucn, ADX + Ucn treated rats; UcnIII, ADX + UcnIII treated rats. All ADX animals were supplemented with low levels of exogenous corticosterone (20 μ/ml in drinking water). Coefficient of error (CE) and biological variance (BV) are presented for total number of neurons of individual nuclei. BL, basolateral; BLa, basolateral anterior; BLp, basolateral posterior; BMa, basomedial anterior; BMp, basomedial posterior; BNSTam, anteromedial division of BNST; BNSTal, anterolateral division of BNST; BNSTp, posterior division of BNST; CeA, central; La, lateral.

Chapter 2.4

Pêgo JM, Morgado P, Cerqueira JJ, Almeida OFX & Sousa N

Mismatch between anxiety status and morphometric parameters in the amygdala and bed nucleus of the stria terminalis

Behavioural Brain Research 173:320-325

(2006)

Available online at www.sciencedirect.com
ScienceDirect
Behavioural Brain Research 173 (2006) 320–325
BEHAVIOURAL BRAIN RESEARCH
www.elsevier.com/locate/bbr

Short communication

Mismatch between anxiety status and morphometric parameters in the amygdala and bed nucleus of the stria terminalis

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Received 14 April 2006; received in revised form 22 June 2006; accepted 26 June 2006
Available online 10 August 2006

Abstract

Aging is associated with behavioral changes, including increased anxiety. In this study we confirmed a hyperanxious status in aged animals, measured in the elevated-plus maze and in the acoustic startle. Subsequently, we searched for age-related changes in the volume and cell numbers in the amygdala or in the bed nucleus of the stria terminalis, but failed to detect gross structural changes in these two brain areas, both implicated in emotionality.

Keywords: Aging; Amygdala; Anxiety; BNST; Stereology

1. Introduction

Normal aging in humans and animals may be accompanied by certain behavioral abatements, the neurobiological bases of which remain unknown. Changes in emotional behaviour, including a heightened level of anxiety, are prominent in apparently healthy aged humans and animals [3,17,19,21,22]. Importantly, there is evidence that increased anxiety is correlated with amygdala hypertrophy [16,19].

A number of brain areas have been implicated in the genesis of fear and anxiety. While earlier studies suggested activation of the amygdala to be the primary trigger for these behaviors [15], more recent work has shown the involvement of the bed nucleus of the stria terminalis (BNST) in the generation of these emotional states [7]. In fact, the BNST is considered to be part of the so-called 'extended amygdala'; it closely resembles the central nucleus of the amygdala in terms of neurotransmitter content and cell morphology [1]. In addition, both areas receive highly processed sensory information from the basolateral nucleus of the amygdala, and send efferents to various hypothalamic and brainstem target areas that are involved in specific signs and symptoms of fear and anxiety [7]. On the other hand, it is now apparent that the BNST and amygdala may play differential roles in the regulation of fear and anxiety: whereas the BNST responds to signals that trigger anxiety, the central nucleus of the amygdala is responsible for generating a state of fear [7].

In this study, we hypothesized that age-related changes in the morphology of the amygdala and BNST might underlie increased anxiousness in older subjects. Having confirmed that 24-month old rats show more anxiety-like behavior than 4-month old animals, we sought a correlation between emotional state and various morphometric parameters in the anterior amygdaloid area, basolateral, basomedial, cortical, medial, lateral, posterior and central amygdaloid nuclei and BNST, using an unbiased stereological approach.

2. Materials and methods

2.1. Animals

Experiments were conducted in accordance with local regulations (European Union Directive 86/609/EEC) and NIH guidelines on animal care and experimentation. Two groups of male Wistar rats, aged 4 and 24 months (Charles

Abbreviations: BNST, bed nucleus of stria terminalis; AAA, anterior amygdaloid area; BLa, basolateral anterior; BLp, basolateral posterior; BMa, basomedial anterior; BMp, basomedial posterior; CeA, central; CO, cortical; La, lateral; MEa, medial anterior; MEp, medial posterior; PA, posterior

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doi:10.1016/j.bbr.2006.06.035

River Laboratories, Barcelona, Spain; $n = 8$ per group) were housed under standard laboratory conditions.

2.2. Behavioral procedures

2.2.1. Elevated-plus maze

Animals were tested during 5 min in a black polypropylene "plus" shaped maze (ENV-560, MedAssociates Inc.) elevated 72.4 cm above the floor. The maze consisted of two opposite open arms (50.8 cm × 10.2 cm) and two enclosed arms (50.8 cm × 10.2 cm × 40.6 cm). Time spent in open arms, junction area and closed arms and the number of entrances and explorations were recorded using an infrared photobeams system connected to a computer. This data was used to calculate the ratio of time spent in open arms over closed arms time and the percentage of open arm entries. White light was used in the testing room.

2.2.2. Acoustic startle

Startle reflexes were measured in two identical startle response systems (SR-LAB, San Diego Instruments, San Diego, CA, USA), each consisting of a non-restrictive Plexiglas cylinder (i.d. 8.8 cm, length 22.2 cm), mounted on a Plexiglas platform and placed in a ventilated, sound-attenuated chamber. Cylinder movements were detected and measured by a piezoelectric element mounted under each cylinder. A dynamic calibration system (San Diego Instruments) was used to ensure comparable startle magnitudes across the two devices. Startle stimuli were presented through a high frequency speaker located 33 cm above the startle chambers. Startle magnitudes were sampled each millisecond (ms) during a period of 200 ms beginning at the onset of the startle stimulus. A startle response is defined as the peak response during this 200-ms period. Animals were habituated to the apparatus 5 min daily 2 days before the testing period.

2.2.3. Startle response as a function of stimulus intensity

Rats were taken from their home cage and placed in the test chamber. The chamber was then sealed and each animal allowed to acclimatize to the startle chamber for a period of 5 min. Additionally, background white noise, with an intensity of 63 dB, was maintained to minimize the impact of acoustic stimuli outside of the chamber environment. Once the habituation period had elapsed, each animal was presented five baseline startle stimuli (50-ms pulse of white noise at 120 dB) at an interstimulus interval of 30 s. The purpose of these baseline trials was to familiarize the animal with the startle stimulus in order to facilitate more accurate measurement of the animal's overall startle amplitude. After the final baseline trial had been delivered, each animal was then randomly presented 60 startle stimuli, each of which was 50 ms in duration, but varied in intensity from 70 to 120 dB, in 10-dB increments. Once the 70 randomized startle trials had terminated, each animal was removed from the startle chamber and returned to its home cage. Following this, the acrylic holder, as well as all components within the startle chamber, was wiped down with alcohol (70%) to minimize residual olfactory cues.

2.3. Histological procedures

After the last testing session, rats were perfused transcardially with fixative (4% paraformaldehyde), under deep pentobarbital anesthesia. After approximately 4 weeks in fixative, brains were split into two hemispheres by a mid-sagittal section, before being processed. Each hemisphere was then included in glycolmethacrylate (Tecnovit 7100, Heraeus Kulzer, Werheim, Germany) and sectioned on a microtome as described in detail elsewhere [14]. Every other 30 μm thick coronal section was collected on a gelatinized slide, stained with Giemsa, mounted with Entellan-New (Merck) and coverslipped. The shrinkage factor (SFv) was calculated has previously described [5]. Stereological procedures were performed "blind" to the observer.

2.3.1. Regions and layer boundaries

We analysed stereological parameters on 11 regions of the amygdaloid complex including anterior amygdaloid area (AAA), basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA), cortical (CO), lateral (La), medial anterior (MEa), medial posterior (MEp) and posterior (PA) and of the bed nucleus of stria terminalis (BNST) has a whole (Fig. 1). The above-mentioned regions were outlined according to the atlas of Swanson [20] and based on noticeable citoarchitectural differences, namely density of cells and size of the perikarya.

2.4. Stereological procedures

Volume and neuronal number estimations were performed using StereoInvestigator® software (Microbrightfield, VT, USA) and a camera attached to a motorized microscope (Axioplan 2, Carl Zeiss, Germany).

The Cavalieri's principle was used to assess the volume of each level. Briefly, every 4th section was used and the cross-sectional area was estimated by point counting (final magnification 112×). We used a test point system in which the interpoint distance, at the tissue level, was 350 μm for BNST, 300 μm for CeA and La and 150 μm for the remainder nuclei. The volume of the region of interest was then calculated from the number of points that fell within its boundaries and the distance between the systematically sampled sections. The volume of each brain hemisphere was calculated by delineation of the ipsilateral total supratentorial parenchyma.

Average cell numbers were estimated using the optical fractionator method [23]. In summary, a grid of virtual 3D-boxes (30 μm × 30 μm × 20 μm) equally spaced (using the same grid spacing as for the volume estimations) was superimposed on every 4th section of the lamina of interest and we counted the number of neurons falling inside them, according to well defined "stereological" rules. The estimated total number of neurons on that region was then calculated from the number of counted neurons, the grid spacing, the volume of the box and the number of boxes counted. Neurons were differentiated from other cells on the basis of the size and shape of their perikarya.

Coefficients of error (CE) were automatically computed by the software according to the formulas of Gundersen et al. [12] for cell numbers and Gundersen and Jensen [11] for volume estimations.

2.4.1. Data analysis

Results are expressed as group means ± standard error. Biological variance (BV = S.D./mean) was determined for the stereological estimates. Data were analyzed using *t*-test for EPM parameters, volumes and stereological estimates of individual nuclei. The behavioural data in the acoustic startle was analysed by repeated measures with stimulus intensity as within-group variables. Correlation between behavioural and morphological variables was tested using Pearson's correlation coefficient. Differences were considered to be significant if $p < 0.05$.

3. Results

3.1. Behavioral data

3.1.1. Elevated-plus maze

Increased signs of anxiety (measured by open arm time and entries) were found in aged animals. Aged animals spent significantly less time ($p < 0.014$) and had less entries ($p < 0.001$) in the open arms of the EPM than younger rats (Fig. 2). No changes were detected in locomotor behavior between ages as no significant differences were found in the number of closed arm entries between the age groups.

3.1.2. Acoustic startle

The startle response varied as a function of the age of rat × startle intensity interaction ($F = 7.566$, $p < 0.002$). There was a significant trend for the startle amplitudes of the aged rats to increase more quickly as a function of stimulus intensity. Comparison between groups revealed that in comparison to young rats, the startle amplitude of aged rats was elevated at the 80, 90 and 100 dB and decreased at 110 and 120 dB intensities (see Table 1).

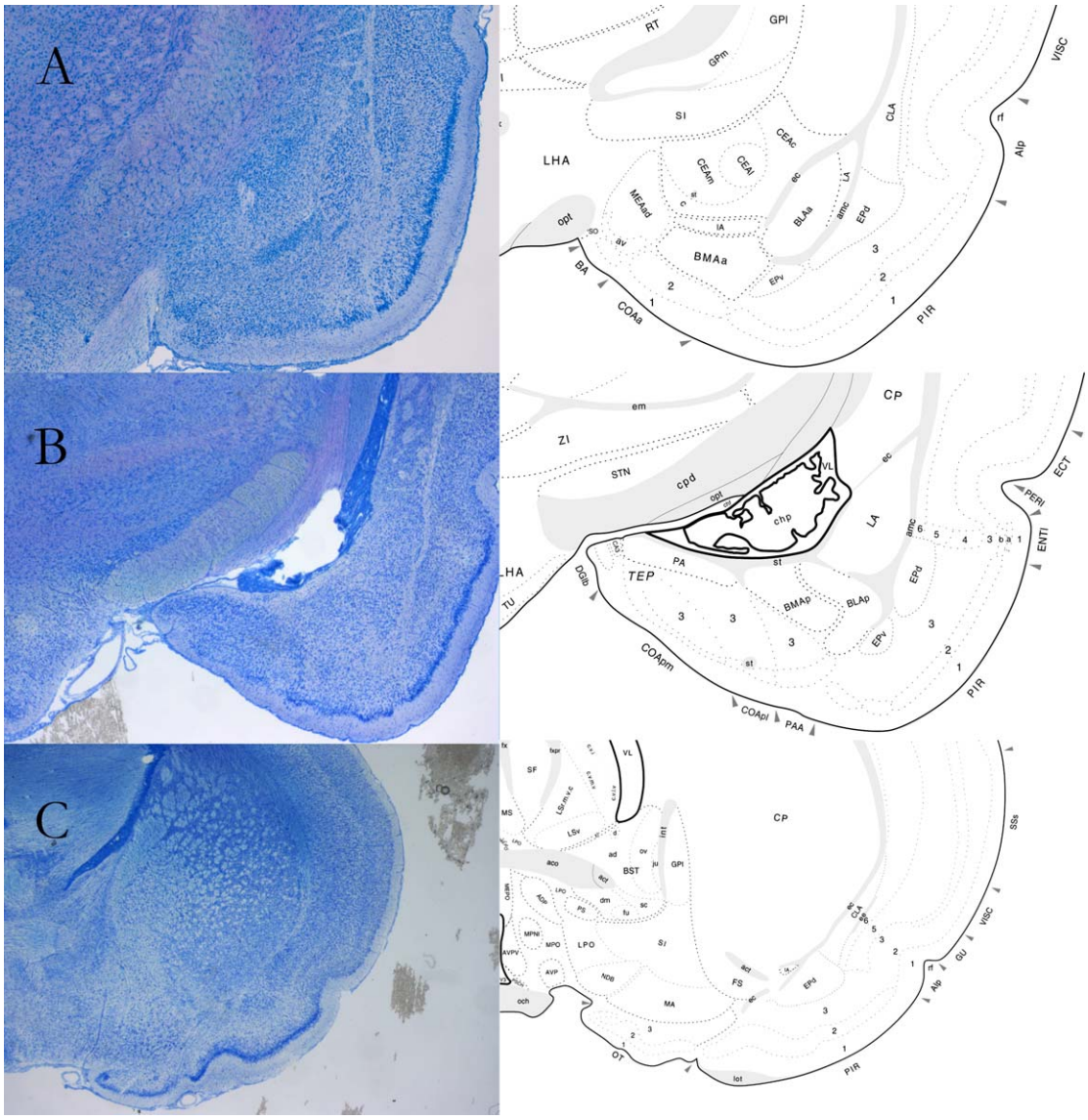

Fig. 1. Low magnification micrographs of glycolmethacrylate-embedded coronal section of temporal lobe of the rat stained with Giemsa and the corresponding atlas schemes used for delineation of different amygdaloid nuclei and BNST: (A) section of the anterior region of the amygdala; (B) section of the posterior region of the amygdala; (C) section of the BNST. Anterior amygdaloid area (AAA), basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA), cortical (CO), lateral (La), medial anterior (MEa), medial posterior (MEp) and posterior (PA), bed nucleus of stria terminalis (BNST).

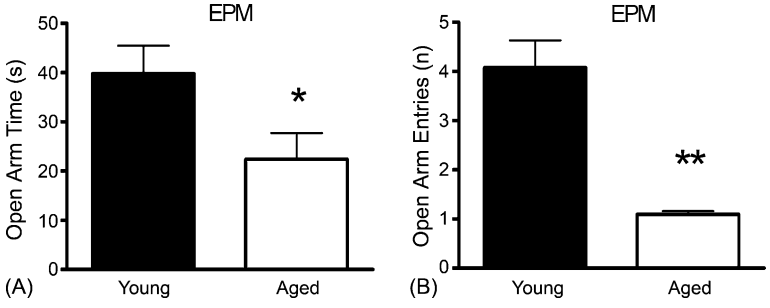

Fig. 2. Elevated-plus maze results: (A) time spent in the open arms; (B) number of entries in the open arms. Results are presented as mean ± S.E.M. $^{*}p = 0.014$, $^{**}p < 0.001$.

Table 1
Acoustic startle data

Noise (dB)	Startle amplitude (arbitrary units)	
	Young	Aged
70	21.57 ± 2.60	33.17 ± 6.17
80	45.23 ± 4.17	98.83 ± 26.36*
90	192.56 ± 33.56	671.80 ± 108.28*
100	954.67 ± 212.30	1911.97 ± 111.21*
110	6223.07 ± 473.25	4219.80 ± 664.88*
120	6477.19 ± 443.10	4613.79 ± 363.66*

Startle amplitude in response to acoustic stimulus. Results are presented as mean ± S.E.M.
* $p < 0.05$.

3.2. Stereological data

The shrinkage factor calculated was 1.07 for young group and 1.09 for aged animals. The absolute volumes of the anterior amygdaloid area (AAA), basolateral (BL; anterior and posterior), basomedial (BM; anterior and posterior), cortical (CO), medial (ME; anterior and posterior), lateral (La), posterior (PA) and central (CeA) amygdaloid nuclei and BNST did not differ between the younger and older groups of animals (Fig. 2 and Table 2). Likewise, neuronal numbers in the different amygdaloid nuclei and BNST did not differ significantly between the two groups (Fig. 3 and Table 3). There was no correlation between these morphometric parameters and the age-related changes in emotional status (Table 4).

Table 2
Statistical data on volumetry measurements

	Young		Aged		Absolute volume, p	Relative volume, p
	CE	BV	CE	BV		
AAA	0.07	0.285	0.11	0.331	0.775	0.886
BLa	0.04	0.223	0.05	0.265	0.789	0.882
BLp	0.03	0.092	0.03	0.127	0.208	0.406
BMa	0.05	0.230	0.05	0.165	0.114	0.165
BMp	0.03	0.115	0.03	0.061	0.213	0.109
CeA	0.03	0.150	0.02	0.108	0.181	0.381
CO	0.03	0.077	0.03	0.134	0.216	0.366
La	0.02	0.145	0.02	0.155	0.727	0.992
MEa	0.07	0.125	0.06	0.237	0.162	0.274
MEp	0.04	0.129	0.06	0.182	0.237	0.060
PA	0.03	0.173	0.03	0.147	0.966	0.750
BNST	0.05	0.107	0.05	0.162	0.503	0.213

Coefficient of error (CE) and biological variance (BV) are presented for absolute volumes of individual nuclei. Relative volume represents the ratio between absolute volume of each individual nuclei vs. total hemispheric volume. Anterior amygdaloid area (AAA), basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA), cortical (CO), lateral (La), medial anterior (MEa), medial posterior (MEp) and posterior (PA), bed nucleus of stria terminalis (BNST).

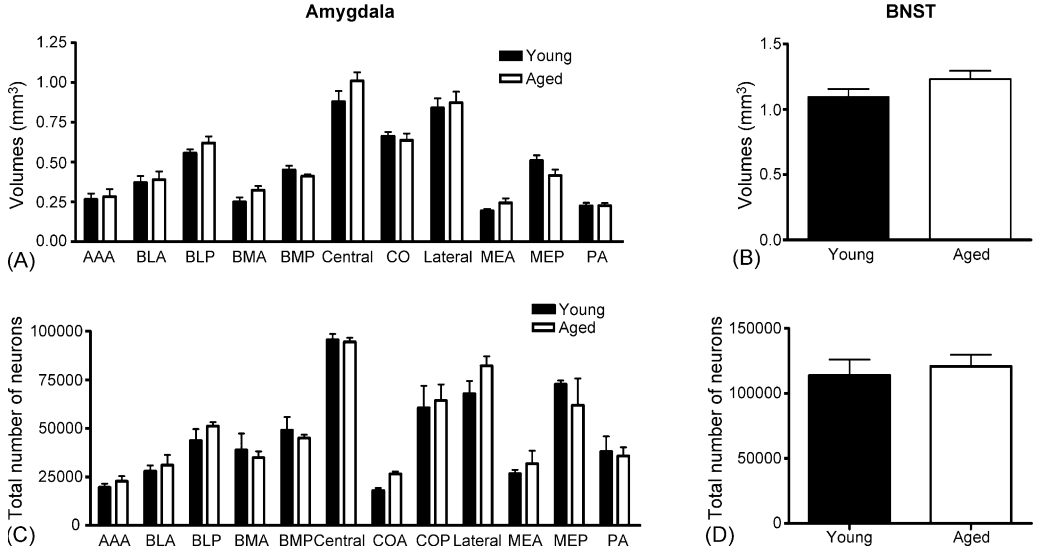

Fig. 3. Stereological data: (A) volumes of amygdaloid nuclei; (B) volumes of the bed nucleus of stria terminalis (BNST); (C) total number of neurons in the different amygdaloid nuclei; (D) total number of neurons in the bed nucleus of stria terminalis (BNST). Results are presented as mean ± S.E.M. Anterior amygdaloid area (AAA), basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA), cortical (CO), lateral (La), medial anterior (MEa), medial posterior (MEp) and posterior (PA), Bed nucleus of stria terminalis (BNST).

Table 3
Statistical data on stereological estimates of total number of neurons in individual nucleus of the amygdala

	Young		Aged		Neuronal pop
	CE	BV	CE	BV	*p*
AAA	0.09	0.16	0.11	0.20	0.390
BLa	0.07	0.18	0.09	0.29	0.629
BLp	0.06	0.23	0.07	0.07	0.296
BMa	0.07	0.38	0.08	0.16	0.694
BMp	0.06	0.24	0.06	0.07	0.600
CeA	0.05	0.06	0.05	0.06	0.785
CO	0.07	0.27	0.12	0.17	0.468
La	0.11	0.20	0.12	0.14	0.131
MEa	0.08	0.13	0.09	0.36	0.488
MEp	0.05	0.05	0.07	0.38	0.481
PA	0.07	0.35	0.09	0.22	0.806
BNST	0.10	0.21	0.10	0.17	0.645

Coefficient of error (CE) and biological variance (BV) are presented for total number of neurons in each individual nuclei. Anterior amygdaloid area (AAA), basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA), cortical (CO), lateral (La), medial anterior (MEa), medial posterior (MEp) and posterior (PA), bed nucleus of stria terminalis (BNST).

Total hemispheric volume was not significantly different between young and aged animals ($p = 0.389$).

The ratio between absolute volumes of BNST and each amygdaloid nucleus versus total hemispheric volume was then calculated (Table 2); no significant difference was found between different ages.

In order to assess stereological validity, biological variance was calculated and compared to coefficients of error; data is presented in Tables 2 and 3, for volumes and neuronal numbers, respectively.

4. Discussion

The present study shows that age-associated increases in anxiety in the rat [2,4] cannot be accounted for by changes in the volume of, or number of neurons in, the amygdala and BNST. To the best of our knowledge, this work represents the first determination of total volumes and neuronal numbers in 'extended amygdala' in which subject age has been considered as a variable. It is important to note that stereological tools used were unbiased and reliable, as indicated by the low coefficients of error which did not exceed the biological variance [6].

Our results are particularly remarkable in light of clear age-related increases in anxiousness and the strong implication of the amygdala and BNST in the genesis of anxiety. Indeed, the behavioral data clearly establishes a state of increased anxiousness in aged subjects both when assessing anxiety in the elevated-plus maze and in the acoustic startle. Importantly, data gathered in the elevated-plus maze, the "gold standard test" for measuring anxiety behavior in rats [8–10,13,18] confirms that aged animals spent less time in the open arms of the maze, despite preserving a normal locomotion pattern; interestingly, age-associated declines in open arm entries, another marker of anxiety, were even more obvious. In the acoustic startle, we also found an increased startle response in the aged group, demonstrating increased reactivity to anxiogenic acoustic stimuli [2,7]. This effect was more pronounced with increasing levels of noise, exception made for those in the higher extreme of rage, which could be explained by a plateau effect in the aged animals for hearing function. Taken together, the present data shows a general hyperanxious in elderly animals that cannot rely merely on cortical functions as the acoustic startle is mediated by a reflex neuronal circuitry.

The present results, together with previous observations that hippocampal neuronal numbers do not correlate with age-related impairments in learning and memory, raise an important question: do age-related alterations in emotional or cognitive behavior have a structural basis? The answer is clearly 'no' if one simply relies on measurements based on the limited resolution afforded by volumes and neuronal numbers, even though there is a trend towards an increased volume in both the amygdala (as a whole) and the BNST (4.4% and 6.9%, respectively). This becomes particularly relevant when considering previous reports, both in animal models and in humans, suggesting a link between increased emotionality and amygdala hypertro-

Table 4
Statistical correlation (r; p) of behavioural variables (open arm time [OA] and acoustic startle at 80 dB [V_{max}]) with stereological parameters

	Total number of neurons		Absolute volume	
	OA time (r; p)	V_{max}, 80 dB (r; p)	OA time (r; p)	V_{max}, 80 dB (r; p)
AAA	−0.138; 0.653	0.129; 0.675	−0.385; 0.141	0.125; 0.645
BLa	0.182; 0.552	−0.115; 0.708	−0.357; 0.432	−0.126; 0.641
BLp	0.222; 0.466	−0.043; 0.890	0.271; 0.557	−0.152; 0.574
BMa	−0.216; 0.478	0.198; 0.516	0.178; 0.510	0.182; 0.499
BMp	−0.067; 0.827	0.178; 0.561	−0.221; 0.411	0.393; 0.132
CeA	−0.140; 0.648	0.176; 0.566	0.072; 0.790	−0.467; 0.069
CO	0.007; 0982	0.102; 0.739	−0.195; 0.468	0.354; 0.179
La	0.282; 0.350	−0.126; 0.682	0.312; 0.240	0.278; 0.297
MEa	−0.111; 0.719	−0.179; 0.558	0.129; 0.635	−0.456; 0.76
MEp	0. 019; 0.952	0.334; 0.265	−0.065; 0.811	0.323; 0.223
PA	−0.066; 0.829	0.218; 0.475	−0.044; 0.870	0.116; 0.669
BNST	−0.441; 0.131	0.146; 0.633	−0.221; 0.411	−0.297; 0.264

Anterior amygdaloid area (AAA), basolateral anterior (BLa), basolateral posterior (BLp), basomedial anterior (BMa), basomedial posterior (BMp), central (CeA), cortical (CO), lateral (La), medial anterior (MEa), medial posterior (MEp) and posterior (PA), bed nucleus of stria terminalis (BNST).

phy [17,19,21,22]. Interestingly, although present results fail to demonstrate significant changes in volumes and neuronal number in the amygdala and BNST, there was a trend for an increased number of cells in the lateral nucleus of the amygdala in aged animals, which fits data previously reported by Salm et al. [19] in a rat model of anxiety. At this stage, however, it is important not to exclude fine structural changes (e.g. in dendritic morphology and synaptic numbers) and the neurochemical adaptations (e.g. transmitter and receptor content) that may go hand-in-hand with the morphological changes, as potential underlying causes of the altered behavioral states.

Acknowledgements

We are thankful to Professor Pedro Oliveira for statistical consultation. This study was supported by a grant from Gulbenkian Foundation (JG 0495).

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Chapter 3

Discussion

3. DISCUSSION

The present work, by discerning specific aspects of anxiety and fear, has characterized how stress and age influence emotional behavior. The neuroanatomical substrates of stress-induced anxiety (but not fear behavior) have also been focused on these studies. Subsequently, the specific contribution of corticosteroids (by administration of exogenous corticosteroids) and CRF (by icv administration) to the stress-induced hyperanxious phenotype have been assessed. Finally, a first approach to correlate anxiety behavior with activation of the BNST, in particular with its anteromedial division, was attempted using immediate early-gene expression. In summary, the results add new information on the contribution of stress to anxiety disorders.

3.1. Animal models and experimental considerations

The primary aim of the present work was to thoroughly characterize emotional behavior in the context of increased allostatic load using a variety of paradigms that ranged from physiological and pharmacological stress models to aging (as a factor of increased burden to homeostasis).

Particular care was given to the several experimental factors, in order to develop an animal model that best represented the conditions observed in the clinical settings. The first factor to emphasize relates to the duration of the experimental protocol, an aspect central to the expression of a fully developed phenotype. Importantly, most of the previous studies addressing the topic of the impact of stress/corticosteroids/CRF in anxiety behavior were performed in acute/subacute conditions. This represents a major pitfall since most deleterious effects of stress exposure occur in a chronic setting. In fact, the inadequate response to stressful challenges that characterizes chronic stress is the end-result of the exhaustion of several imediate stress-responsive systems and is time-dependent (Cerqueira *et al.*, 2007a; McLaughlin *et al.*, 2007). Our experimental observations have shown that most biological markers associated with chronic stress (e.g., fur degradation, gastric ulcers, weight gain impairment, etc) do not overtly develop before 2/3 weeks of exposure; the same happens with the typical stress-induced anxious phenotype. Indeed, although using stress paradigms similar to ours, others researchers have failed to find typical behavioral traits when using shorter experimental procedures (Vyas *et al.*,

2002). Importantly, a similar temporal window of installation of anxiety disorders is observed in the wards; In fact, some clinical conditions present a late onset after the triggering episode (e.g. post-traumatic stress disorder [PTSD], specific phobias) and others require a prolonged period of sensitization (e.g. generalized anxiety disorder). Therefore, we have chosen to use prolonged periods of exposure to stress in our experimental work.

The (un)predictable nature of stressful insults acts as a factor of amplification, reinforcing the disruption of coping mechanisms. Animals that are given the possibility to choose between predictable or unpredictable aversive stimuli prefer the predictable event, even when that event is unavoidable and inescapable (Gliner, 1972). Additionally, biological markers of stress response (e.g. gastric ulcers) seem to be attenuated if the event is predictable, providing evidence that the magnitude of stress response is partially related to the feeling of control over the course of events. On the other hand, the presentation of random and unpredictable stimuli induces a state of permanent alertness, required for mounting a prompt response, which can only be supported by the continuous activation of the HPA axis. This in turn, will induce most of the deleterious effects observed after exposure to chronic stress. Again, one can establish parallels with the clinical settings, where anxiety prevails as an underlying condition. For instance, patients suffering from PTSD or obsessive-compulsive disorders report feelings of anxiety in response to daily life events (that would otherwise not trigger anxiety in a healthy individual). This illustrates a diminished threshold of alertness that these patients try to dampen by avoiding the triggering events or by creating mechanisms of controlled environment (e.g. rituals). Coherently, the anxiogenic nature of a particular situation (e.g. high-speed driving) can be weakened by training (e.g. professional race driver) or motivational values (e.g. monetary prizes, emergency car staff). This points out that, at least partially, anxiety behavior is modulated by learning/motivation.

Interindividual variability was observed in all experimental models used in these studies revealing that different subjects have different resilience to allostatic load. It is especially striking that particular animals behaved differently in response to stressors; some subjects would consistently be more relentless to counteract in aversive conditions. Several factors like social dominance (Razzoli *et al.*, 2006), housing conditions (e.g. number of animals per cage; relative position to light) (Izidio *et al.*, 2005; Reiss *et al.*, 2007), prenatal stress (Weinstock *et al.*, 1998; Oliveira *et al.*, 2006), maternal care (McPherson *et al.*, 2007; Renard *et al.*, 2007) can influence how a particular individual reacts to challenging events. This reflects the importance of past experiences

in shaping the coping mechanisms acquired throughout life (Earls, 1998; Heim & Nemeroff, 1999; Heim *et al.*, 2002; Anda *et al.*, 2006) and may explain why individuals show different susceptibilities to the development of anxious behavior with age (C. Heim *et al.*, 2002). Such findings are consonant with the hypothesis that aging results from the accumulation of factors leading to increased or decreased HPA reactivity (Landfield & Eldridge, 1994b; McEwen, 2002; Landfield *et al.*, 2007). This hypothesis postulates that once the emotionality and the reactivity of the adrenocortical system are established by early life events, the subsequent activation of the stress response (including the HPA axis) during life will likely contribute to the rate and "quality" of aging of the brain and the body. In fact, rats with increased HPA reactivity show early decline of cognitive functions but also an emotional shift towards anxiety (Dellu *et al.*, 1996; Vallee *et al.*, 1997; Bessa *et al.*, 2005). In contrast, rats that were submitted to neonatal handling show lower HPA reactivity to stressors during lifetime, with subsequent slower cognitive deterioration and less anxiety behavior (Vallee *et al.*, 1997; Vallee *et al.*, 1999).

The view of cumulative load (allostasis) to the organism can be subdivided in four basic states: *i)* exposure to repeated challenges; *ii)* failure to habituate with repeated challenges; *iii)* failure to shut off the response after the challenge disappears; *iv)* failure to mount an adequate response. Two of these (states *i* and *iii*) have been recognized, in the hippocampus and PFC, and act through mechanisms that involve the release of excitatory neurotransmitters. Under stressful conditions, rats show increased extracellular levels of glutamate in the hippocampus and PFC (Levine *et al.*, 1967; Moghaddam *et al.*, 1994; Moghaddam, 2002) and this increase will lead to dendritic remodeling in these brain regions (Cerqueira *et al.*, 2007a). Excessive glutamate will result in increased calcium mobilization, via activation of NMDA (N-methyl-D-aspartic acid) and AMPA (α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid) receptors and second messengers overactivation, leading to a series of events that are believed to impair information storage mechanisms, such as long-term potentiation (LTP) (Bliss & Collingridge, 1993; Bennett, 2000; Goosens & Maren, 2002; 2004) and long-term depression (LTD) (Diamond *et al.*, 2005). As the reuptake and rebuffering of calcium ions is an active process (Choi, 1988; Keller & Mattson, 1998), whenever these processes are not rapid and efficient, free-radicals, formed as by-products of lipid peroxidation, will accumulate and produce allostatic load upon neuronal structures (Mattson, 1996; Keller & Mattson, 1998). Stress increases the production of free radicals in the brain (Liu *et al.*, 1996), apparently through a direct effect of corticosteroids. Indeed, GCs appear

to potentiate the increase in extracellular levels of excitatory amino acids under stress (Moghaddam *et al.*, 1994; Bagley & Moghaddam, 1997).

Animal models demonstrate that calcium homeostasis undergoes progressive changes with age. L-type calcium channels activity increases with age which results in increased after-hyperpolarization (AHP) and decreased neuronal survival (Landfield & Eldridge, 1994a; Porter *et al.*, 1997; Brewer *et al.*, 2007). In rodent hippocampal pyramidal neurons, repetitive discharges are followed by a slow AHP as a result of activation of a $Ca2^{+}$-dependent K^{+} current. Modulations of the AHP have been shown to be closely associated with synaptic plasticity, learning, and aging processes (Disterhoft *et al.*, 1993). Interestingly, increased AHP phenomena are associated with altered LTP and LTD in hippocampal neurons (Norris *et al.*, 1998). These changes have a putative role in cognitive impairment observed in the aged rat. Additionally, GCs enhance calcium channels activity and AHP (Landfield & Eldridge, 1994b; McEwen, 2002). In young rats, glucocorticoid actions are decreased by down-regulation of GR following repeated-stress, while with increasing age this protective mechanism is impaired or lost (Kerr *et al.*, 1991). Therefore, aged rats seem more vulnerable to increased levels of GCs and the consequent dysregulation of calcium channels and free radicals formation, which will ultimately damage brain cells (Sapolsky *et al.*, 1986).

Pharmacological modulation, by dissecting the different components of the stress response, has helped clarify how stress modulates emotional behavior. The mechanisms that regulate the activation of the HPA axis are intricately regulated in a series of negative feedback loops. Activation of stress-sensitive areas will lead to increased release of CRF by neurosecretory neurons in the PVN. The subsequent release of the ACTH by the pituitary gland and its action on adrenal glands results in the release of corticosteroids. These, in turn, are the mediators of the response to stress both peripherally and centrally. Along this cascade of events, different mechanisms have evolved to self-regulate and limit the magnitude of the stress response. Such mechanisms allow mounting an adequate energetic response while avoiding the deleterious actions of corticosteroids when chronically elevated. One of the main shut-off mechanisms is the downstream regulation exerted by corticosteroids, which readily cross the blood-brain barrier and put forward an inhibitory influence on the hypothalamus (Makino *et al.*, 2002). Additionally, corticosteroids act upon stress-sensitive areas that influence the tonic release of CRF by the PVN, namely the hippocampus and PFC. In fact, structural remodeling of these brain areas has been

correlated to HPA overactivation and is thought to be the end result of corticosteroid-dependent insults to the neuronal structure. The structural remodeling, in turn, has a putative role in the perpetuation of the vicious cycle of HPA activation.

Animal models of physiological stress closely resemble the settings found in clinical conditions. Overactivation of the HPA axis has been demonstrated by altered expression of CRF in the brain and elevated levels of circulating corticosteroids, both biological hallmarks that characterize chronic stress (Lightman & Harbuz, 1993; Cullinan *et al.*, 1995; Herman *et al.*, 1995; Cullinan & Wolfe, 2000; Cerqueira *et al.*, 2005). By introducing different pharmacological treatment paradigms we were able to discern how central and peripheral mediators act to shape the structure of the amygdala and the BNST. Exogenous administration of corticosterone was particularly helpful in demonstrating the hormone-dependent effects of stress. Most of the systemic effects, like weight gain impairment, gastric ulcers, thymic atrophy and immunological impairment, muscle mass loss, etc., can be ascribed to increased levels of circulating corticosteroids (Sapolsky *et al.*, 1986; Dallman *et al.*, 1987; Sapolsky *et al.*, 2000). On the other hand, most of the structural remodeling found in the brain of stressed rats (Sousa *et al.*, 1998b; 1999; Sousa *et al.*, 2000; Cerqueira *et al.*, 2005; Cerqueira *et al.*, 2007b; Sousa *et al.*, 2007) was replicated by the administration of exogenous corticosteroids. This provides a robust model that reproduces most of the physiological markers found in chronic stress. However, the major pitfall of this model is the inhibitory influence of exogenous corticosteroids over the HPA axis which leads to decreased secretion of CRF. By using a sustained administration of CRF receptors agonists, we further elucidated the specific role of these factors in the stress-associated behavior. Because CRF receptors have distinct expression patterns and biological actions, the use of specific agonists discriminates between the roles of different receptors in the structural remodeling associated with chronic stress. However, it is crucial that the peripheral response that follows HPA activation is blunted; otherwise it would be impossible to dissociate the influence of central mediators from the hormonal response that follows. In order to achieve that goal, adrenalectomy was performed in animals treated with CRF ligands. Importantly, these animals were supplemented with the administration of basal levels of corticosterone, as the absence of corticosteroids has a deleterious effect on the brain in itself.

Therefore, the present work addresses the central questions of this thesis using rodent models of chronic stress paradigms and advanced age. The experimental work was designed in order to

reproduce physiological stress and decompose the stress response in its central and peripheral components. Specifically, the experimental work aimed to:

1. Analyze the influence of stress on emotional behavior using the CUS paradigm and in the senescent rodent (Chapters 2.1 and 2.4).

2. Correlate the behavioral changes observed with structural parameters in the amygdala and the BNST (Chapters 2.1 and 2.4).

3. Discriminate the relative contribution of corticosteroids and CRF agonists to the behavioral and structural changes using pharmacological models. Exogenous administration of corticosterone and intracerebroventricular administration of CRFR agonists were used, respectively. (Chapters 2.1 & 2.3).

4. Assess the functional activation/inactivation of the BNST after CUS using immunohistochemical analysis of the expression of c-fos (Chapter 2.2).

3.2. Behavioral measurements of anxiety

In these studies, it is shown that the increased allostatic load of aging or stress paradigms triggers a hyperanxious phenotype as measured in the elevated-plus maze (EPM). The EPM test is based on the natural conflict of rodents to explore a novel environment and their innate aversion to open, elevated and brightly lit spaces. This paradigm is considered to be the most reliable and coherent test of anxiety behavior in rodents (Pellow *et al.*, 1985; Lister, 1987; Rodgers & Dalvi, 1997; File, 2001). Validation of a behavioral test in animal models requires fulfillment of several conditions, which has been the case for EPM (Ohl, 2005). First, the behavioral response of an animal model to a threatening stimulus should be comparable to the response known for humans (face validity). Such findings have been consistently demonstrated in the EPM by several studies (Papp *et al.*, 1993; Peskind *et al.*, 1998; Adamec *et al.*, 2004; Chotiwat & Harris, 2006; Imanaka *et al.*, 2006). Second, it should display reduced anxiety when treated with drugs known to trigger anxiolysis (predictive validity). Indeed, drugs that have anxiolytic effects in humans have shown a therapeutic effect in rodents tested in the EPM (Pellow *et al.*, 1985; Pellow & File, 1986; Lister, 1987; Dawson & Tricklebank, 1995), by increasing the relative time that animals spend in the open arms, confirming that the maze tests

anxiety rather than exploratory behavior. And third, the mechanisms underlying anxiety as well as the psychological causes should be identical (construct validity). This has been demonstrated in several paradigms that resemble the putative mechanisms underlying human anxiety disorders like stress and aging (Vyas *et al.*, 2002; Bessa *et al.*, 2005). The validity of the test has been so consistent that it is now vital for a new anxiolytic drug to be screened in the elevated test maze to be considered for clinical trials in humans.

The nature of the EPM implicates that multisensorial inputs are processed and then compiled in a locomotory response that is not reflexive but rather is modulated by complex aspects of behavior (Dawson & Tricklebank, 1995; File, 2001). In fact, in all experimental protocols tested in these studies, stressed animals behaved in a very cautious and hyperreactive manner reflecting a diminished threshold to anxiogenic situations. The fact that the innate aversive conditions of the maze (File, 1993; Hogg, 1996) are unconditioned and not triggered by specific cues suggests that the underlying neuronal pathways are activated by diffuse (like the BNST) and not by specific cues (like the amygdala). To confirm this hypothesis we have used, whenever possible (this test could not be reliably applied to animals previously submitted to stereotaxic procedures), the acoustic startle paradigm (Koch, 1999). In the acoustic startle the animal is presented to loud noises and its reflex changes are recorded in one or more measures of autonomic (heart rate, blood pressure, pupilary diameter, etc) and motor responses (muscle tension, movement, etc). Additionally, the reflexive nature of the acoustic startle is a valuable assertion that autonomic and motor responses are the outputs of involuntarily activated circuitries and not the result of complex processing. Hence, the influence of environmental variables (the experimenter handling the animal; the position of the home cage; behavioral state of the animal immediately before the test [arousal or rest]; overcrowding) (Izidio *et al.*, 2005; Reiss *et al.*, 2007) that may interfere with the anxiogenic status assessed with the EPM are greatly abolished. As a result, the changes observed are likely to reflect the functional impairment of specific (and simple) neuronal circuitries and not the conjugated response of polissensorial processed inputs. Importantly, because the nature of the triggering stimuli (conditioned vs unconditioned) is what confers a distinctive characteristic activation pattern of brain structures (Davis, 1998; 2006), the phenotypic expression of unconditioned emotional behavior in the acoustic startle is also a consequence of the activation of either BNST or the amygdala (through the CeA) (Walker *et al.*, 2003). However, while the BNST is activated by long-lasting and unconditioned stimuli typical of anxiogenic situations, the amygdala is triggered by discrete short-

lasting stimuli with a clear offset which characterize fear. By introducing different protocols of stimulation in the acoustic startle paradigm, it was possible for us to discriminate the behavioral correlates of stress and to clearly demonstrate that stress/age affects anxiety but not fear behavior.

In contrast to the influence that stress produces on anxious behavior, fear-conditioning was unaltered throughout the experiments. Fear behavior is closely related to anxiety, as it shares behavioral and neural pathways. Fear is characterized by a behavioral pattern that resembles anxiety behavior. Autonomic and neuroendocrine activation are set in motion by common neural pathways; however, the nature of the triggering events and the persistence of symptoms define most of the dissimilarities of these two aspects of emotional behavior. Fear is triggered by stimuli that are specific but brief, and present an imminent threat to the individual. The bodily activation that follows is aimed at providing a rapid response to the threat and is terminated as soon as the threat disappears. On the contrary, anxiety is triggered by more diffuse and prolonged cues, and induces a state of hyperesponsiveness that outlasts the presentation of triggering events. The arousal that succeeds provides the individual with readily available resources that allow a prompt response to eventual threats while searching for its source. The neuroanatomical framework of such dichotomy has been thoroughly studied by Davis and collaborators (Davis *et al.*, 1997; Davis, 1998; Walker *et al.*, 2003). The BNST and CeA present similar projections to brainstem neuroendocrine and autonomic nuclei that are activated in anxiety and fear responses, respectively. It is the distinct set of inputs that reach these nuclei and how their activity is modulated that distinguishes the relative importance to different aspects of emotional behavior. The observation that fear behavior was unaffected by stress discloses that the neural pathways that underpin fear (amygdala and related areas) were unaffected, at least from the functional point of view. It further reinforces that stress-induced anxiety is correlated to dysfunction of extra-amygdalar areas like the BNST.

3.3. Anxiety correlates with structural changes in the BNST but not in the amygdala

One of the most surprising observations in the structural studies herein presented is the relative preservation of the morphology of the amygdala after prolonged exposure to stress. These results may appear paradoxical, in light of current knowledge that attribute a putative role of the

amygdala in the integration, processing, and modulation of emotional behavior both in animals and in humans (LeDoux, 2000; Garakani *et al.*, 2006; Lang & Davis, 2006; Dolan, 2007; Ledoux, 2007). The amygdala is involved in the recognition of emotional value of context, conditional learning, and modulation of the function of other mnesic (e.g. hippocampus), rewarding (e.g. nucleus accumbens) and processing (e.g. prefrontal cortex) systems. Therefore, it would be reasonable to believe that emotional disorders that accompany stress would translate into structural changes of this area. Moreover, recent research has focused on the role of the amygdala in processing of other emotionally driven functions like reward, maternal, sexual, aggressive and ingestive behaviors (Ledoux, 2007); however, the role of the amygdala in the setting of these emotional behaviors is not so straightforward. In addition to its role in emotion, the amygdala is also involved in the regulation of cognitive functions, such as attention, perception and explicit memory. It is generally accepted that these functions are modulated by the processed output of polissensorial information arriving at the amygdala and that attribute an emotional significance to context. Such modulation can be exerted through direct connections to cognitive centers or the influence (mediated by the BNST) over the HPA axis.

The absence of structural changes in the amygdala after chronic stress is in good accordance with our observation that chronic stress did not induce changes in fear-acquisition. The meaning of such findings must be taken into consideration together with the relevance of the activity of the amygdala for the individual's survival. In fact, by playing a key role in the learning and long-term storage of fearful memories, the amygdala is essential to establish strong associations between overtly threatening cues (or context) and an aversive condition that must be avoided. This process represents a specific form of learning that provides biological advantage, in as much as it diminishes the probability of harmful/threatening encounters. However, if this process was triggered by unspecific, daily life stressors, which characterize allostatic load, survival would be jeopardized by an overactive "all-or-nothing" system. Subsequently, the resilience shown by the amygdala in stress paradigms becomes logical from an evolutionist perspective.

The amygdala is composed by masses of neurons organized in subareas or nuclei. There is still some debate about how the amygdala should be parcelled according to anatomical, histochemical, embryological and functional characteristics of the different nuclei (Swanson & Petrovich, 1998; Ledoux, 2007). Regardless of such questions, it is consensual that the amygdala consists of an evolutionary primitive division related to the olfactory system (cortico-

medial regions) and an evolutionary newer division related to the neocortex (basolateral region). The cortico-medial region comprises the cortical, medial and central nuclei and receives inputs from the olfactory and accessory olfactory bulb; it processes olfactory, pheromonal and viscero-sensorial stimuli and is involved in the expression of social, reproductive, ingestive behavior and modulates neuroendocrine control. The basolateral region is the most thoroughly investigated area and is involved in the acquisition and storage of fearful memories. It receives polyssensorial inputs from auditory, visual, somatossensorial, olfactory and taste information that are processed in order to establish associations between events and aversive conditions. The intricate network of connections that links most amygdalar nuclei converge processed information to the central nucleus. Therefore, the main output pathways is relayed by the central nucleus that projects to several brainstem and neuroendocrine nuclei controlling emotional reactions, like freezing in the presence of a predator.

The BNST, on the other hand, is known to be involved in specific aspects of anxiety behavior (Walker *et al.*, 2003). Consequently, the structural changes that we observed in the BNST in the context of aging and chronic stress are likely to correlate with the behavioral changes observed. Anxiety is characterized by a feeling of apprehension and restlessness that recapitulates much of the systemic effects of autonomic activation. Its biological significance is related to the anticipatory activation of response systems in the presence of unfamiliar aversive contexts. Such behavioral changes can prove helpful in acute situations by prompting the individual to a state of readiness. In fact, anxiogenic situations trigger autonomic activation (File *et al.*, 1988) and acutely activate the HPA, which results in an elevation of circulating corticosteroids. This arousal of the organism to face new situations is an advantage if self-limited. However, if there is a sustained activation of these systems, including the prolonged activation of the HPA, the risk for deleterious effects in the structure of stress-responsive systems is increased and may, ultimately, disrupt the normal shut-down of these anxiogenic systems.

The anatomical organization of the BNST has been revised by several authors and is still under debate. The division into medial/lateral divisions proposed by Krettek and Price (1978) based on pure cytarchitectural characteristics has been classically accepted and revised by several authors (Moga *et al.*, 1989; De Olmos *et al.*, 2004). Subsequently, a posterior/anterior division was identified on developmental grounds (Bayer, 1987), and this view was elaborated into a comprehensive anatomical organization based on cyto- and chemoarchitectonic and

developmental evidence by Swanson and collaborators (Ju & Swanson, 1989; Ju *et al.*, 1989; Swanson, 1998). In this parcellation, the anterior division of the BNST was separated into anterodorsal, anterolateral, and anteroventral areas with somewhat ill defined borders and, embedded within these areas several cell nuclei were identified. Specifically, juxtacapsular, oval, rhomboid, and fusiform nuclei are found in the anterolateral area, along with a subcommissural zone; and dorsomedial, dorsolateral, magnocellular, and ventral nuclei are found in the anteroventral area. However, a new organization has been proposed, based on recent data from connectional and projection studies; the cell groups of the anterior division have been rearranged into medial and lateral groups (Dong *et al.*, 2000; Dong *et al.*, 2001a; Dong *et al.*, 2001b; Dong & Swanson, 2003; 2004b; a; 2006a; b; c). The medial group of the anterior division is characterized by inputs from the MeA and basomedial anterior amygdalar nuclei and projects densely to regions of the hypothalamus closely associated with the neuroendocrine system; it includes the anterodorsal and anteroventral areas. In contrast, the lateral group of the anterior division is characterized by inputs from the CeA and by projections to autonomic-related parts of the hypothalamus and lower brainstem, and includes the anterolateral area and its four clear differentiations (the juxtacapsular, oval, rhomboid, and fusiform nuclei). The posterior BNST division has at least five distinguishable regions: the dorsal, principal, interfascicular, and transverse nuclei, and the cell-sparse zone. This division is characterized by inputs from the medial nucleus of the amygdala and projections to the medial nuclei of the hypothalamus.

The putative role of the BNST in modulating the activity of the HPA through its inhibitory influence on PVN is of paramount importance for the explanation of the structural findings of the present work. Stress-induced hypertrophic alterations were largely confined to the anteromedial aspects of the BNST (BNSTam), an area that has a pivotal role in neuroendocrine and autonomic control (Dong & Swanson, 2006a; b; c), which strongly implicates the BNST in the genesis of stress-induced anxiety. Such evidence stems from several aspects: *i)* BNSTam projects directly to specific brainstem and hypothalamic nuclei, integrating cerebral hemisphere neuroendocrine, autonomic and behavioral aspects of energy balance (Dong & Swanson, 2006a); *ii)* neurons lying closest to the anterior comissure appear to densely innervate the hypothalamic periventricular region (Dong & Swanson, 2006a); *iii)* CRF-immunoreactive cells are found in the neuronal groups composing the anteromedial area (Ju *et al.*, 1989) and have a putative role in regulating the hypothalamic function; and *iv)* electrical stimulation of the anteromedial aspects of the BNST is associated with increased corticosteroid secretion (Dunn, 1987). These facts support the

hypothesis that allostatic load, triggered by aging or by sustained exposure to stress, acts upon the structure and function of the BNST. In parallel, the structural remodeling of the BNSTam may have another major implication: trigger a hypercorticalismic state by disturbing the inhibitory tone over the PVN.

The stereological evaluation of the BNST reveals that hypertrophic changes are not due to cell proliferation. This finding excludes major structural alterations in terms of cell fate. However, volumetric changes raise questions about the nature of the expansion in that area. It is important to note that, contrary to what is usually observed in the brain after exposure to neuronal insults, we are facing an accretion to the tissue, rather than its destruction/loss. The subsequent analysis of the BNST neuronal dendrites revealed a remarkable reshaping of neurons with enlarged dendrites and increased number of spines. Such findings indicate that the processes underlying stress-induced anxiety are related to disturbance of normal synaptic activity, which have implications in the functional connectivity of this structure.

Remodeling of neuronal networks by stress (Sousa *et al.*, 2000; Fuchs *et al.*, 2006; Cerqueira *et al.*, 2007b) is a well described process. It is now consensual that this phenomenon is the basis of most behavioral impairments and not the cell fate processes as was initially postulated (Sapolsky *et al.*, 1986). Indeed, most post-mortem studies in humans have failed to show significant differences in total number of cells or neurodegenerative processes (in stress-sensitive areas like the hippocampus), in depressed patients (known to have associated hypercortisolemia) or in those submitted to prolonged corticotherapy (Muller *et al.*, 2001). In fact, the rationale for the functional recovery that is observed in both experimental animal studies (Sousa *et al.*, 2000) and after effective treatment in clinical settings (Starkman *et al.*, 1992; Starkman *et al.*, 2003), is based on the hypothesis that neuroadaptation comprehends reversible plasticity events rather than fatal losses. Neural plasticity is absolutely necessary for adequate functioning of an individual in the continuously changing environment. However, as demonstrated by the altered structure and functions in the brains of patients with mood disorders, it becomes clear that neural plastic changes are not always beneficial. Additionally, in animal models, the effects of increased allostatic load have been demonstrated in limbic areas that modulate cognitive, mnesic and emotional behavior and that are involved in HPA regulation. Remodeling of dendritic trees has been demonstrated in the hippocampal formation (Sousa *et al.*, 2000; Vyas *et al.*, 2002), prefrontal cortex (Wellman, 2001; Cerqueira *et al.*, 2007b) and amygdala (Vyas *et al.*, 2002;

Vyas *et al.*, 2003; Rubinow *et al.*, 2007), in the senescent rodent or following chronic stress paradigms and corticosteroid treatment. These effects on dendrite structure were, however, different in the various studied brain regions. While the hippocampal (Sousa *et al.*, 2000; Vyas *et al.*, 2002) and prefrontal cortex neurons (Wellman, 2001; Cerqueira *et al.*, 2007b) showed, in general, atrophy of dendritic trees, pyramidal neurons (putatively excitatory) in the amygdala have shown hypertrophic responses to repetitive immobilization stress (Vyas *et al.*, 2002; Vyas *et al.*, 2003) and with aging (Rubinow *et al.*, 2007).

The underlying mechanisms that explain these phenomena are not, yet, fully understood. Shrinkage of excitatory glutamatergic neurons in the hippocampus and in the prefrontal cortex has been hypothesized as a reactive response to increased corticosteroid levels or excitotoxicity due to enhanced glutamatergic transmission (Moghaddam *et al.*, 1994; Sapolsky, 1999; Roy & Sapolsky, 2003). The by-products of calcium metabolism are thought to induce dendritic/spinal pruning that are seen in atrophic neurons in stressed animals and contribute to cognitive impairment, as has been addressed in section 3.1 of this chapter. There is evidence for the involvement of excitatory amino acids and NMDA receptors, as well as serotonin, in the hippocampal dendritic remodeling (Magarinos & McEwen, 1995; Magarinos *et al.*, 1999; McEwen, 1999), which are modulated by glucocorticoid levels. In the amygdala and BNST, detailed aspects of molecular mechanism of neuronal reshaping are still missing, although recent studies have clarified specific aspects. Fear learning is thought to occur in the amygdala through similar processes to the hippocampus and evidence supports a role for NMDA mediated glutamatergic transmission (Matus-Amat *et al.*, 2007). In addition, electrophysiological studies have found striking correlates between LTP formation in the amygdala and fear conditioning. Indeed, this phenomenon was shown to occur in neuronal networks known to mediate fear conditioning (Weisskopf & LeDoux, 1999) and anxiety (Egli & Winder, 2003). However, LTP formation in the amygdala (Weisskopf *et al.*, 1999) and BNST (Egli & Winder, 2003) show distinct electrophysiological proprieties of hippocampal LTP. In these structures, LTP was independent of NMDA receptors, despite their presence at these synapses, and instead was dependent on L-type voltage-gated calcium channels. Activation of voltage-gated calcium channels (Weisskopf *et al.*, 1999) is thought to induce the phosphorylation of several kinases in the post-synaptic neuron which, subsequently, will lead to the expression of proteins involved in the strengthening of synaptic transmission and structural remodeling of neurons (Ledoux, 2007). Such evidence may explain differences in spatiotemporal dynamics of intracellular calcium that

have been postulated to underlie contrasting patterns of dendritic remodeling between the amygdala and hippocampus, following stress-induced anxiety (Vyas *et al.*, 2002).

The present work does not reveal major differences in the arborizations of neurons in the amygdala. Although such observations may seem divergent with previous reports (Vyas *et al.*, 2002), they highlight the stimulus-dependent specificity of the neuronal circuitry responsible for generating anxiety (Gewirtz *et al.*, 1998; Rosen *et al.*, 1998; Dagnino-Subiabre *et al.*, 2005; Miracle *et al.*, 2006). The inescapability associated with the chronic immobilization stress paradigm used in the Vyas (2002) study triggers an emotional phenotype that results in the expression of fear responses rather than anxiety; this interpretation would explain the stress-induced structural changes in the amygdala observed by those authors, who actually failed to reproduce them whenever applying a chronic unpredictable stress paradigm (Vyas *et al.*, 2002; Mitra *et al.*, 2005).

3.4. Functional evidence of the role of BNST in anxiety behavior and HPA axis regulation

Although the behavioral-structural correlations above-described strongly suggests a role of BNST in anxiety behavior and in disruption of the HPA axis, we considered of interest to search for a functional evidence of this link. Thus in another study (Chapter 2.2) we analyzed the pattern of activation of BNST neurons by immunohistochemical analysis of the expression of c-fos, an immediate-early gene that is expressed in recently activated cells (Curran *et al.*, 1985). Two sets of animals were studied: i) in one set we compared the basal expression of c-fos in animals that were submitted to CUS to that of controls; ii) in a second set, CUS and control animals were challenged with 20 bursts of loud noise in the acoustic startle chamber, prior to the sacrifice, in order to characterize the functional changes in response to anxiogenic stimuli.

The results obtained indicate that the activation of BNSTam neurons in chronically stressed animals was impaired. In fact, on basal conditions c-fos expression in the BNSTam was decreased in stressed animals compared to controls. Stochastic analysis of activation patterns in the BNST revealed imbalances in the excitatory/inhibitory influence of specific nuclei over the PVN. Importantly, such differences were further enhanced following an anxiogenic stimulus. When analyzing these data together with the structural results, the ensuing picture indicates that

stress induces structural remodeling of the BNSTam neurons, affecting synaptic activity and functional activation of this area. Functional impairment of the BNST will in turn lead to disruption of the inhibitory tone over the PVN and autonomic centers, which constitute the basic framework of neuroendocrine and behavioral derangements that characterize stress behavior. This hypothesis is coherent with current knowledge of the role of BNST in relaying brainstem and limbic inputs that ultimately lead to the activation of the HPA and descending pathways setting up the corporeal alterations that characterize anxiety in the theatre of emotions.

3.5. Stress mediators: The role of corticosteroids and CRF receptors

After characterizing the effects of stress on anxiety behavior and on the structure of the extended amygdala, a subsequent aim of this study was to the search for the mediators exerting these effects (Chapters 2.1 and 2.3). For discriminating the role of corticosteroids and CRF we designed the following experimental procedures: i) in intact animals, the hormonal limb of the stress response was mimicked by administration of exogenous corticosterone for 28 days; ii) in adrenalectomized rats, central administration of different CRF receptors agonists was achieved through the use of osmotic pumps, which directly delivered the neuromediators in the lateral ventricle for 14 days partially mimicking the central response to stress. In the adrenalectomized animals, replacement therapy with oral corticosterone was used to replace basal levels in order to avoid the deleterious effects of the absence of corticosteroids.

Most of the structural reshaping of neurons was reproduced by exogenous corticosteroids, which leaves a smaller role for centrally administered CRF receptors (CRFR) agonists. In fact, experimental work with exogenous corticosteroids could recapitulate most of the behavioral and morphological changes in the BNST that were induced by chronic stress. Close scrutiny of the results, however, showed that some synaptic changes were not reproduced by corticosterone administration. Such finding raised the issue of the role of CRFR agonists upon spinal sprouting that was observed after chronic stress. It is known that CRF may be capable of up-regulating CRF_1R, enhancing its own biosynthesis in the PVN in a paracrine or autocrine manner (Makino *et al.*, 2002). Such phenomenon may represent one mechanism of persistent activation of CRF neurons in the PVN during stress. Therefore, similar mechanisms may underpin the fine structural changes observed in BNSTam neurons.

Prolonged occupancy of CRF_1R receptors also triggered a hyperanxious behavior; this result fits with several previous reports in the literature (Heinrichs *et al.*, 1997; Reul & Holsboer, 2002; Heinrichs & Koob, 2004). A major finding of our study relates with the observation of an increase in the total number of spines in bipolar neurons of the BNST. The enhanced spinogenesis may reflect increases in individual neuronal synaptic activity. In fact, a previous study (Bowers *et al.*, 1998) reported upregulation of two isoforms of glutamic acid decarboxylase (GAD), GAD65 and GAD67, in the BNST, after stress. Of interest, acute stress only increased GAD67, a marker of acute activity, while chronic stress induced a marked increase in GAD67 and GAD65, which is present in presynaptic terminals. Similar findings were encountered in basolateral anterior amygdaloid nucleus (BLa) pyramidal neurons following prolonged immobilization stress (Mitra *et al.*, 2005). Interestingly, in that study, it was found that the structural remodeling of pyramidal neurons was initially confined to spinogenesis and subsequently progressed to dendritic hypertrophy with prolongation of treatments (Mitra *et al.*, 2005). These resemblances to our results illustrate the consistency of mechanisms underlying amygdalar and BNST remodeling. On the other hand, the absence of structural remodeling in the BLa neurons in CUS may reflect methodological specificities, as discussed above.

There is now further increasing evidence that CRFRs are involved in structural plastic changes. Indeed, it has been demonstrated that CRF promotes growth of neuronal processes in noradrenergic neurons of locus coeruleus (Swinny & Valentino, 2006). This process involves protein kinase A and mitogen-activated protein kinase, both relevant for the phosphorylation of proteins of relevance to synaptic transmission, and Rac1, a member of the Rho family of GTPases that regulates actin and microtubules. Although still not known, a similar process may underpin the structural findings that were observed in bipolar neurons of stressed animals.

Chronic stress induced neurite growth and enhanced spinogenesis in bipolar neurons of the BNST; the same hypertrophic response was observed after corticosteroid treatment, while spinogenesis was recapitulated by CRF administration. Despite most evidence in the literature suggesting a detrimental effect of stress/corticosteroids (Sapolsky *et al.*, 1986; Sousa *et al.*, 1998a; Sousa *et al.*, 2000; Wellman, 2001; Nacher *et al.*, 2004; Cerqueira *et al.*, 2005; Cereseto *et al.*, 2006; Cerqueira *et al.*, 2007b; Sousa *et al.*, 2007), recent evidence has demonstrated that steroids may have additive effects on neuronal cytoskeleton (Fontaine-Lenoir *et al.*, 2006). In this vein, hyperplastic changes may result from variation in neuronal

transmission. One important mechanism is the extracellular proteolysis, a mechanism through which axons could remodel their synaptic connections. Proteases can facilitate axonal plasticity by degrading extracellular matrix proteins (Baranes *et al.*, 1998), interacting with membrane receptors (Nicole *et al.*, 2001) or activating latent growth factors (Nicole *et al.*, 2001). One likely candidate for such a molecule is tissue plasminogen activator (tPA), a plasticity-related serine protease. Interestingly, for the context of the present studies is the observation that tPA is expressed in the amygdala and BNST and is essential to the expression of anxiety behavior (Matys *et al.*, 2004; Matys *et al.*, 2005). Importantly, tPA expression was found to be involved in neuroplasticity in the amygdala (Pawlak *et al.*, 2003) and to be upregulated by stress and CRF in the BNST (Matys *et al.*, 2005).

The interpretation of structural results must be cautious but clarifies the relative importance of corticosteroids and central mediators in the stress-induced changes observed in the BNST. Increased levels of corticosteroids induce major structural remodeling of neuronal arborizations highlighting the influence of the corticosteroid milieu in the reshaping of neuronal cytoskeleton, and perpetuating the cycle that viciously activates the HPA axis. Prolonged occupancy of CRF_1R led to plastic changes that reflect altered synaptic activity, irrespective of HPA activation, and suggests that this is a parallel step leading to anxiety-behavior in the context of chronic stress.

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Chapter 4

Conclusions

4. CONCLUSIONS

The present work, by thoroughly characterize the structural transformations associated to altered emotional behavior, has pinpointed the neural substrates of how allostatic load, triggered by aging or exposure to stress, impacts on anxiety behavior. By doing so, we have documented potential targets for pharmacological manipulation in the clinical settings.

In summary, the results of the present work indicate that:

1) The amygdala seems resilient to modulation by stressful insults, while the BNST is highly sensitive to them. Interestingly, this specific vulnerability is not surprising in light of the biological significance of these regions: while the amygdala is involved in fear learning and long-term storage, the BNST is directly implicated in anxiety behavior.

2) The increased allostatic load of aging and chronic stress induces a hyperanxious phenotype.

3) The hyperanxious phenotype associated to aging and chronic stress correlates with structural changes in the BNST, but not in the amygdala. These changes were largely dependent on plastic hyperplasic changes in the dendritic morphology of bipolar neurons of the BNST

4) Most of the effects induced by chronic stress were reproducible by exogenous corticosterone administration, indicating that corticosteroids are able to mediate the gross anatomical changes of stress.

5) The spinal sprouting observed in bipolar neurons of the BNST in chronically stressed animals was, however, only replicated by sustained CRF_1R occupancy.

6) Chronic stress significantly impairs activation of the BNST; subsequently, the loss of inhibitory tone over the HPA provides an anatomical basis for deregulated closure of this neuroendocrine loop under conditions of chronic stress.

Chapter 5

Future perspectives

5. FUTURE PERSPECTIVES

Taken together, the conclusions withdrawn from this work show that the BNST is a target of aging and stress. However, new questions arise from these observations. It remains to answer whether the BNST is a primary target of peripheral and central mediators or the structural reshaping is the end-result of changes occurring in other stress-sensitive targets that are connected to this brain region.

Future work should be directed at establishing clear physiological and functional correlates of the neuroanatomical changes observed. Additionally, neurochemical studies characterizing particular neurotransmitter systems affected by the burden of allostatic load are needed in order to identify the specific neuronal networks and pathways involved in the impaired regulation of the HPA axis.

These goals can be achieved using several strategies:

1) Baseline electrophysiological studies in the BNST would establish a clear association between functional impairment and anatomical changes. Alterations in the firing rate or in the ability to induce LTP or LTD would explain differences in the control of the HPA axis in response to anxiogenic situations.

2) Specific aspects of the regulatory loops that control the activity of the BNST over the HPA could further be explored by stimulating specific sites and recording the activity in the BNST. Due to the key position of the BNST in the relay of limbic inputs into the HPA, the amygdala, prefrontal cortex and hippocampus become preferential sites of interest. These have particular interest in light of its putative role in perception of cognitive aspects of stressful situations and the structuring of complex stress responses.

3) Histochemical studies of the BNST in stressed animals characterizing specific neurotransmitter systems would establish differences in the expression of particular mediator systems. The inhibitory GABAergic system would be a preferential target of such research as it is the main neurotransmitter in the BNST and is involved in the regulatory influence over the HPA.

4) The expression of specific molecules in particular areas of the brain, and even in individual cells, is now possible using laser microdissection tools and genetic expression analysis. This

would be of special interest in the search for differences in the expression of receptors for specific mediators; CRF and vasopressinergic receptors, in particular, are a preferential target due to its key role in the regulation of the HPA.

5) The observed changes in dendritic morphology and spine number of BNST neurons suggest that synaptic transmission may, primordially, be affected by stress. Studies clarifying this issue should look for differences in the expression of adhesion molecules and synaptic proteins involved in synaptic transmission.

6) Finally, studies combining tracers and electronic microscopy would be of interest to further characterize the synaptic influence of BNST on CRF-producing neurons in the PVN.

Chapter 6

Annexes

Chapter 6.1

Lu J, Némethy Zs, Pêgo JM, Cerqueira JJ, Sousa N & Almeida OFX

Cellular and molecular analysis of stress-induced neurodegeneration - methodological considerations

Handbook of stress and the brain, Vol 15 – Part I: The neurobiology of stress

Steckler T, Kalin NH & Reul JMHM eds, Elsevier (2005)

Handbook of Stress and the Brain, Vol. 15
ISSN 0921-0709

CHAPTER 5.6

Cellular and molecular analysis of stress-induced neurodegeneration – methodological considerations

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Abstract: Evidence that chronic hypercorticalism induces a broad spectrum of deleterious cellular effects in the brain has accumulated over the last two decades. These principal effects of hypercorticalism include neuronal atrophy, neuronal death and glial responses. Importantly, these changes, which may occur interdependently and/or concomitantly, lead to neurodegeneration. While there has been a significant expansion of the number of techniques available for examining effects of chronic stress in the brain, the cellular and molecular mechanisms underpinning stress-induced neurodegeneration are still only partially known. This article appraises the major current methodologies available for analyzing stress-induced neurodegeneration, and considers the advantages and limitations of each of these methods.

What do we understand by stress?

Stress refers to the organism's attempt to mount an 'adaptive' (beneficial) response to aversive stimuli in order to maintain or restore homeostasis. Different sensory and motor systems are differentially activated depending on the quality and intensity of the stressful (aversive) stimulus, and the magnitude and duration of the response are influenced by the "context" of the stimulus (experience, mood, age, environmental factors) (Herman and Cullinan, 1997). Thus, extreme caution is necessary before generalizing about the effects of one particular "stress"; it is fair to say that much of the confusion existing in the field is a consequence of the false presumption that elevated corticosteroids mimic stress and/or one stressor is a representative of every stressor. Another important point to be noted is that prolonged elevations in corticosteroid secretion are a crucial accompaniment of the chronic response to stress (Sapolsky et al., 2000). Briefly, in the event that adequate adaptive mechanisms cannot be recruited, chronic stress will result in a state of chronic hypercorticalism and, as a consequence, deleterious effects, including immune suppression and a variety of mental disturbances will emerge (Sapolsky et al., 2000).

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Forms of neurodegeneration

Numerous studies have demonstrated that chronic hypercorticalism induces a broad spectrum of deleterious cellular effects in the brain, which can be conveniently categorized as neuronal atrophy, neuronal death, and glial responses. These changes, which may occur interdependently and/or concomitantly constitute neurodegeneration. The neurodegenerative changes associated with hypercorticalism are by far less-marked than the damage seen in the so-called

neurodegenerative diseases (Parkinson's, Alzheimer's, etc.) and it would therefore, probably be more prudent to consider them as representative of the selective vulnerability of given brain regions. Most of the examples given in this review relate to the responses of hippocampal cells to stress (and pharmacological hypercorticalism). The hippocampus has been the most extensively studied brain region in this respect; its particular vulnerability to corticosteroids most probably reflects its high concentrations of corticosteroid (mineralocorticoid and glucocorticoid) receptors.

Neuronal atrophy

An important notion to be kept in mind when referring to neuronal degeneration is that it does not necessarily imply the death of neurons. Indeed, most events perceived by a living organism (either positive or negative) are believed to modulate the structure of neuronal networks rather than lead to changes in neuronal number (Segal, 2002; Erickson et al., 2003).

To evaluate dendritic arborizations one can use the Golgi technique, which selectively impregnates single neurons with silver chromate (Camillo Golgi, 1843–1924). This method has provided indispensable information about the way in which sets of neuronal elements contribute to the gross structure of the neuropil and tracts. Impregnations show up as black, purple or reddish-brown against a pale yellow background; it is essentially a stochastic technique, the exact chemical mechanism of which remains unclear. This approach allows impregnated neurons and boundaries in any region of interest to be traced and reconstructed from successive serial sections. Two-dimensional (2D) analysis can be performed from traces obtained using a drawing tube attached to a light microscope. This type of analysis does not require any sophisticated equipment and has been widely used in the past. However, it has one major disadvantage: converting a 3D probe into a 2D probe results in a loss of information on the suppressed dimension. To achieve 3D reconstructions, cell bodies, dendritic arborizations, and boundaries of the region of interest should be drawn (under 25–100 × oil immersion objectives) and plotted in 3D using a video computer system (e.g., Neurolucida from MicroBrightField, Inc.). Three-dimensional models of neurons can be visualized using appropriate software. Three-dimensional reconstructions of neurons can be rotated around any of the *x*-, *y*-, and *z*-axes to allow the best visualization of the dendritic trees. Total dendritic lengths, number of segments/bifurcations, Sholl analysis (which provides an estimate of dendritic densities, based on the number of intersections between concentric circles centered in cell soma and the dendritic segments) and spine densities are just some of the parameters this analysis provides.

Importantly, the use of these techniques allowed the pioneers of neuroanatomy to recognize the organization of neuronal networks, and to eventually demonstrate the occurrence of remarkable alterations in dendritic trees and synaptic contacts following neuronal insults. Indeed, such knowledge existed long before the description of different forms of neuronal death.

Several studies in the 1990s demonstrated that hypercorticalism (pharmacological or stress-induced) induces alterations in cytoplasm organelles (Miller et al., 1989) and, ultimately, atrophy of CA3 pyramidal cell dendrites in the hippocampal formation; (Watanabe et al., 1992; Magarinos and McEwen 1995a); subsequent work confirmed these results in this neuronal population but also observed similar alterations in all the other major subdivisions of the hippocampal formation (Sousa et al., 2000). Furthermore, the later studies noted a marked loss of synapes in at least one of the links of the intrinsic hippocampal circuitry (the mossy fiber–CA3 connection). It may therefore be concluded that elevated corticosteroids trigger structural responses within cytoplasmic organelles, dendrites, axons, and their synaptic contacts; importantly, such changes do not necessarily involve the irreversible loss of neurons (Sousa et al., 2000).

Neuritic alterations of the type described above correlate with behavioral deficits and would appear to serve as the neuroanatomical basis of adaptive mechanisms underlying learning and memory (Erickson et al., 2003). The cellular basis of learning and memory has long been believed to include alterations in dendrites (mainly in spines) and in the number and structure of synapses (Cajal, 1893). The validity of this notion was explored in a number

of quantitative light and electron microscopic studies, which, in the majority of cases, showed that the richness of dendritic arborizations and the numerical density of synapses increases as a consequence of learning of novel behaviors. More recent studies have also shown that, despite numerical changes in dendritic spines and synapses, the cellular mechanisms of hippocampus-dependent associative learning include the remodeling of existing hippocampal synapses; these changes most likely reflect an involvement of signal transduction proteins and the transformation of silent postsynaptic synapses into active ones (Rusakov et al., 1997; Stewart et al., 2000). In light of these robust correlations between neuritic (dendritic spine and synapse) changes and cognitive performance, it seems warranted to conclude that perturbations of the former will result in impaired performance in hippocampus-dependent learning tasks.

Most interestingly, although the neuritic atrophy and synaptic loss referred to above would be expected to provoke some degree of functional impairment, together with the fact that these paradigms are not necessarily associated with neuronal cell loss, it seems more than likely that neuronal reorganization (regrowth of dendrites and axons and establishment of new synapses) of damaged neuronal circuits is an important mechanism allowing recovery from insults (McEwen, 1999). The above proposition appears to be valid insofar that studies in rats have shown that, whereas no significant structural reorganization occurs during or immediately after the termination of elevated corticosteroid levels (by pharmacological means or after the imposition of stressors), significant reorganization does occur within one month of withdrawal from the damaging stimulus (Sousa et al., 2000). This so-called "reactive synaptogenesis" occurs throughout the hippocampal formation and is commensurate with restoration of spatial learning and memory to levels found in control animals. Thus, the more recent findings match well with older observations that hypercorticalism-induced cognitive impairment is a reversible phenomenon.

Importantly, regeneration of dendritic, axonal, and synaptic elements does not seem to be compromised in conditions when profound neuronal loss has occurred, e.g., in the dentate granule cell layer after adrenalectomy, a manipulation accompanied by marked collapse of the mossy fiber inputs to the CA3 pyramidal layer. Administration of low doses of corticosterone to adrenalectomized rats can, at least partially, restore the total dendritic length of granule cells and the volume and surface area of the mossy fiber terminals (Sousa et al., 1999a). In addition, substitution therapy with corticosterone results in complete recovery of the volume of the suprapyramidal bundle, number, and surface area of mossy fiber–CA3 synapses, and the surface area of dendritic excrescences (Sousa et al., 1999a). These observations on the fine structural adjustments fit with results of other work showing that behavioral functions impaired by adrenalectomy can be partially reinstated by the administration of corticosterone (McCormick et al., 1997).

The evidence summarized above firmly indicates that alterations of the corticosteroid milieu can induce profound, but largely reversible, changes in the ultrastructural organization of the hippocampal formation; these bidirectional alterations, more than changes in neuron viability, may represent the neuroanatomical correlation of hippocampus-dependent learning and memory. Presently, there is no clear data available as to what neurochemical mechanisms might underlie the fine structural observations described above. However, NMDA and serotonin (5-HT) receptors appear to be key players since the administration of either NMDA antagonists or serotonin reuptake inhibitors have been shown to abrogate CA3 dendritic atrophy (Watanabe et al., 1992; Magarinos and McEwen, 1995b). Growth factors also seem to be likely mediators, as suggested by data showing that elevated corticosteroid levels (including those produced in response to stress) attenuate hippocampal brain-derived growth factor (BDNF) and nerve growth factor (NGF) levels (Smith et al., 1995; Hansson et al., 2000) and that adrenalectomy results in significant alterations in the levels of neurotrophin-3 and fibroblast growth factor-2 (FGF-2) (Barbany and Persson, 1992; Hansson et al., 2000). Finally, it seems highly plausible that neurotrophins play a major role in the neuritic regrowth seen after recovery from exposure to high corticosteroid levels because the recovery phase is characterized by an increase in their synthesis (Smith et al., 1995).

Neuronal death

Neuronal death can occur through one of the two basic mechanisms – necrosis or apoptosis (see Fig. 1 and for review Majno and Joris, 1995). Necrosis is the unexpected death of cells resulting from "external damage," usually mediated via destruction of the integrity of plasma membrane and/or the trophic support of the cell. Morphologically, there is lysis of the plasma membrane of the swollen necrotic cell, which leads to release of cytoplasmic components into the surrounding tissue spaces. Inflammatory cells, attracted by the necrotic debris, trigger tissue destruction. Necrosis of isolated cells can occur, although necrosis usually affects large clusters. Consequently, there is significant tissue inflammation (with subsequent repair and scarring), with permanent alteration of architecture and function. Since necrosis usually ensues from cytotoxins, the process is completed rapidly within seconds-to-minutes.

Apoptosis differs from necrosis in that it involves the triggering of specific, sequentially occurring, events. Although the term apoptosis was originally coined to describe a specific morphological sequel, it is now known that apoptosis depends on activation of a genomic program; as such, the term apoptosis is frequently used synonymously with the term programmed cell death (Fig. 2). It should be mentioned that most current methods for the detection of apoptosis can only detect late stages of the process, and that some programmed cell death may not involve the mechanisms of apoptosis (e.g., oncosis – the term oncosis (derived from onkos, meaning swelling) was proposed in 1910 by von Recklinghausen precisely to mean cell death with swelling; oncosis leads to necrosis with karyolysis and stands in contrast to apoptosis, which leads to necrosis with karyorhexis and cell shrinkage). In contrast to necrosis, apoptosis is a much slower process; depending on the initiating stimulus, apoptosis requires from a few hours to several days for its complete manifestation. Conceptually, this form of cell death is analogous to "suicide," inasmuch as death results from the activation of the dying cell's own death machinery. Apoptosis, first recognized by embryologists, has now come to be recognized as being important for maintaining tissue homeostasis and to constitute a major component of many pathological responses, including neurodegenerative diseases. It is important to note that the genetic program for apoptosis can be triggered by both intrinsic (e.g., during histogenesis) and extrinsic factors, including stressful stimuli and exogenous corticosteroids, although the intracellular signaling cascades and morphological changes are essentially the same in both situations.

As already mentioned, apoptosis is now known to be important during embryogenesis/histogenesis but

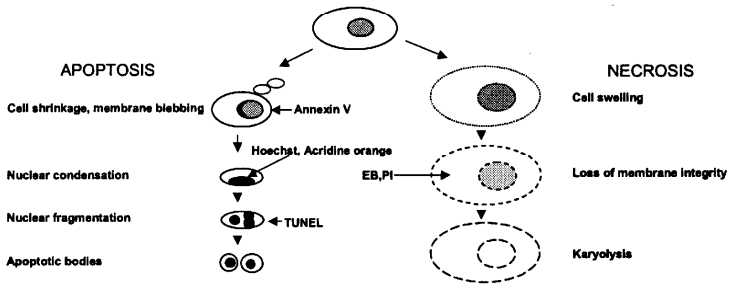

Fig. 1. Comparision of morphological changes in apoptosis and necrosis. Apoptosis, characterized by cell shrinkage, membrane blebbing, nuclear condensation, nuclear fragmentation, and apoptotic bodies developed in different stages of injury is shown on the left-side. As described in the main text, apoptotic cells can be identified in a variety of ways, some of which (annexin V-binding, Hoechst, acridine orange, and TUNEL staining) are indicated here. Note that the majority of methods are based primarily on changes in the properties of the cell membrane and nucleus. Necrosis, characterized by cell swelling, loss of membrane integrity, and karyolysis is shown on the right. Membrane-impermeable markers such as ethidium bromide (EB) and propidium iodide (PI) can be used to identify necrosis.

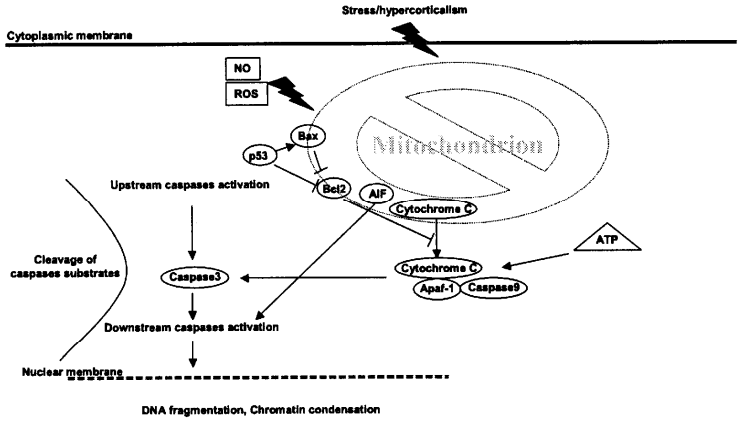

Fig. 2. Signal pathways in apoptosis. The mitochondrion as the integrator of apoptotic signals from stress or other factors such as nitric oxide (NO) or reactive oxygen species (ROS), can release cytochrome *c* and apoptosis-inducing factor (AIF); cytochrome *c*, together with Apaf1, Caspase9, and ATP activate caspase3, which, in turn, activates downstream caspases for DNA cleavage. Note that Bcl2 from the mitochondrial membrane can prevent mitochondrial pore formation, which antagonizes cytochrome *c* activity; Bax can increase mitochondrial permeability. Separation of living/apoptotic cells by flow cytometry. For flow cytometric analysis, ethanol-fixed cells are washed in phosphate–citrate buffer and stained with propidium iodide. As cells pass in front of a laser, they absorb, diffract, refract and reflect incident light, and emit fluorescence. The scattered light is focused by a lens into a photomultiplier, the emitted fluorescent signal is optically filtered through dichroic mirrors, and subsequently processed by wide bandpass filters selected to optimize the various fluorescent emissions; signals are detected by photomultiplier tubes, and based on fluorescence intensity profiles, living and apoptotic cells can be distinguished.

also in the course of normal tissue turnover. Of course, the mature brain is traditionally not regarded as an organ where cell and tissue turnover occurs, but with the increasing number of reports that, besides glial cells, neurons can also be generated in certain regions, the original concept does not seem to be strictly correct. Furthermore, it is being increasingly recognized that apoptosis makes a significant contribution to neural cell loss in pathological conditions, e.g., in neurodegenerative diseases such as Alzheimer's and Parkinson's disease (Honig and Rosenberg, 2000; Friedlander, 2003). It is also pertinent to mention that a revisionist view with respect to the distinctive roles and mechanisms of necrosis and apoptosis has emerged since the late 1990s; according to these authors it is now accepted that virtually any insult just below the threshold to induce necrosis results in an apoptotic response (McConkey, 1998). The cellular response becomes relevant in this process in that, in contrast to the situation in necrosis, apoptosis involves active processes within the dying cell and does not merely depend on the insult itself.

In contrast to most other cells, neurons have elaborate morphologies with complex neuritic arborizations that often extend long distances from the perikarya. It is in fact the richness of complex contacts between neurons that results in the establishment of functional networks. With this concept in mind, it is not difficult to accept that neuronal degeneration does not necessarily imply neuronal death; neuronal atrophy and synaptic loss also represent forms of nervous tissue degeneration. It was recently shown that the biochemical cascades leading to apoptosis can be activated locally in synapses and dendrites (Mattson, 2000), indicating a much more complex role for apoptosis than previously envisaged, i.e., in synaptic loss and dendritic remodeling.

Glial response

Glia mediate neuroendocrine and neuroimmune functions that are altered in the face of a number of neuronal insults, including prolonged stress.

The biological functions of glia involve changes in shape, interactions with neurons and other glia, and gene expression. Glia cells become activated in the presence of ongoing neurodegeneration and progress to produce what is termed "reactive gliosis" (Nichols, 1999; Liu and Hong, 2003). Since good markers to distinguish normal from reactive glia are not commonly available, most researchers currently depend on well-defined morphological criteria. In several neurodegenerative conditions, astrocytes exhibit hypertrophy and signs of metabolic activation, and astrocytic processes begin to entwine neurons. Microglia also become activated and subsets of these cells increase in number and may enter the phagocytic or reactive stage. Glial markers of brain aging and glial activation include glial fibrillary acidic protein (GFAP) and transforming growth factor (TGF)-β1, which are increased in astrocytes and microglia, respectively (Nichols, 1999). Interestingly, steroids (Laping et al., 1994), such as those produced in the adrenals (Melcangi et al., 1997), regulate the interactions between glia and neurons, and glial gene expression, including GFAP and TGF-β1.

Despite the recognized relevance of the biological functions of glia, little is known about the effect of stress on hippocampal glial cells. Anecdotal evidence suggests an increase in glial cell number and signs of cytoplasmic transformation of astrocytes and microglia in areas of the brain implicated in stress-induced disorders (namely the prefrontal cortex and the hippocampus) (Ramos-Remus et al., 2002). Based on these findings, it appears that the hippocampal glial response to chronic stress may be similar to that found in endangered or challenged hippocampal environments, such as in ischemia.

A different line of evidence on the glial response to imbalances in the corticosteroid milieu has come from studies in surgically lesioned animals (Vijayan and Cotman, 1987). Animals with surgical entorhinal lesions concomitantly treated with hydrocortisone demonstrated more astrocytes and fewer nonastrocytes in the dentate outer molecular layer compared with untreated animals. Glia in the treated animals also showed a decrease in average optical density of cytoplasmic acid phosphatase staining. These findings suggest that hydrocortisone treatment prior to, and following, an entorhinal lesion accelerates lesion-induced migration of astrocytes to the outer molecular layer, and reduces the increase in microglial number resulting from the lesion. The observed effect on microglia may result from direct hormonal inhibition of local proliferation of microglia or from the well-known systemic anti-inflammatory action of glucocorticoids on monocytes, the putative precursors of brain microglia. In light of these findings it has been suggested that glucocorticoid hormones significantly alter the response of nonneuronal cells to neural tissue damage. Lending support to this view is the observation that adrenalectomized animals show induction of GFAP immunoreactivity, which occurs contemporaneously with neurodegeneration (Trejo et al., 1998). Although no variation in the total number of glial cells is found, signs of astroglial activation can be observed in the adrenalectomized group: astroglial cells change in size and shape, and their processes in the molecular layer, which normally show unipolarity become randomly organized (Sousa et al., 1997). Both effects are confined to the dentate gyrus and mossy fiber zone. The degeneration and astroglial reaction become more pronounced with increasing duration after adrenalectomy, and both can be prevented by placing animals on corticosterone replacement therapy. Results such as these illustrate the close relationship between the glial response and neuronal degeneration in the dentate gyrus following adrenalectomy, in terms of both, time and space (Sousa et al., 1997).

What are the neural targets of stress-mediated degeneration?

Corticosteroids are secreted distal to their brain targets but distribution maps of their receptors serve as reliable indicators of their sites of action. In a landmark study on the rat brain, Reul and de Kloet (1985) reported that radioactively labeled corticosterone binds with differing affinities to two distinct receptors, and that the hippocampus showed the highest signal retention for both receptors; subsequent cloning studies revealed significant homologies between the high-affinity and low-affinity central and peripheral corticosteroid receptors: mineralocorticoid (MR) and glucocorticoid (GR) receptors, respectively. In vitro studies showed that the high-affinity binding site in brain can also bind aldosterone;

in practice however, the endogenous production of aldosterone only reaches concentrations sufficient to activate renal mineralocorticoid receptors (Funder, 1996); thus, cerebral MR show promiscuity in that, like GR, they bind corticosterone; however, since they have a ca. 10-fold greater affinity for corticosterone as compared to GR, MR are predominantly occupied during periods when corticosteroid levels are low, whereas GR only become occupied when corticosteroid secretion increases above a certain threshold (e.g., during stress or in pathological conditions). Further, the presence of two isoforms of the pre-receptor enzyme 11ß-hydroxysteroid dehydrogenase, involved in the interconversion of corticosteroids to active and inactive forms, contribute to the selective access to intracellular receptors (Yau and Seckl, 2001).

While GR are widely distributed throughout the brain, but are particularly concentrated in the hippocampus, hypothalamus, and lower brainstem, MR are almost exclusively confined to the hippocampus and other limbic structures such as the septum, central nucleus of the amygdala, the olfactory nucleus, and some hypothalamic nuclei (Van Eekelen et al., 1988; Ahima and Harlan, 1990; Ahima et al., 1991). Within the hippocampal formation, subfield-specific differences in MR and GR concentration profiles have been described: MR levels are high in CA1 pyramidal layer $\approx$ granule cell layer (dentate gyrus) > CA3 pyramidal layer, and GR are concentrated in the CA1 $\approx$ dentate gyrus $\gg$ CA3 (Van Eekelen et al., 1988). The functional significance, if any, of these differential patterns of receptor distribution may be inferred from the known functions of the particular brain nuclei displaying high levels of MR and GR expression and/or ligand binding. At this juncture, it is important to point out that while the described patterns of MR and GR occurrence in the various hippocampal subdivisions may serve as eventual predictors of function, they do not necessarily reflect the receptor repertoire of individual cells in any region; further, it is still not known to what extent receptor composition (concentration of individual receptors or co-localization of MR and GR in the same cell) determines the fate of a particular cell (e.g., survival vs. death) in response to changes in the corticosteroid milieu.

Experimental paradigms for examining stress-mediated degeneration

Designing models of stress implies a clear definition of the question under study; more specifically, if one wants to determine the effect of stress upon a specific region of the brain, several issues need to be considered. One of them is adaptation; if a single stressor is applied for a prolonged period, then the organism tends to adapt to that stressor and the stress response gets blunted. A second issue to consider is unpredictability; even when applying different stressors, care must be taken to avoid adaptation, e.g., by applying a battery of stressors at different clock times and in random order. A final point to consider is that stressors vary in quality; for example, physical and psychological stressors activate different regions of the brain, with the former depending on perception by brain stem centers as opposed to the latter, which depends on the activation of higher regions of the brain (in particular the limbic system). Obviously, comparisons between different experimental paradigms (and the results therefrom) must also take into account factors such as intensity and duration/chronicity.

A commonly used approach in evaluating the cellular effects of stress involves decomposition of the effectors of these actions, e.g., by mimicking the endocrine response to stress by administering high doses of corticosteroids, a paradigm that does not exactly reproduce the physical, behavioral/emotional, and neurochemical manifestations of stress. Nevertheless, our current understanding of the actions mediated by the two corticosteroid receptors has largely benefited from the exploitation of the high selectivity of aldosterone (the prototypic MR agonist) and dexamethasone or RU28362 (prototypic GR agonists) as well as the antagonists spironolactone and RU28318 (for blocking MR effects) and RU38486 (for blocking GR effects). Further insights into the biological actions of MR and GR are now being gained from MR and GR gain- and loss-of-function mouse models (Muller et al., 2002). The use of such models has proved particularly useful in proving and understanding the importance and role of these receptors in stress-mediated neuronal damage, and neuronal disorders influenced by stress such as anxiety, depression, and dementia.

While in vivo models are necessary for the evaluation of stress effects, in vitro models are indispensable for understanding the cellular and molecular mechanisms underlying those effects. The latter approach is particularly amenable to analysis at the molecular level, but the major caveat here is that in vitro observations do not necessarily apply to the whole organism whose ultimate response to the same stimulus reflects an integration of a plethora of adaptive and signaling pathways emanating from cells with diverse properties, e.g., the liver can substantially influence the response of the brain to endogenous and exogenous stress hormones. As a result, a neurotoxic stimulus in vitro might just happen to be protective or to have no effect in the living organism.

Use of stereology in analyzing neurodegeneration

Another extremely relevant issue to consider when designing an experiment is the sensitivity and specificity of the methodological procedures employed to test the hypothesis. Obviously, the analysis of stress-induced neurodegeneration also follows this rule. A common first analytical approach is to make observations on histological sections. Histological sections define the normal appearance of tissue and organs, and detect natural or induced alterations in structure. Histological descriptions often include terms such as "large," "small," "many," "few," "absent," or "present." Helpful as these terms are for the description of basic features, they are often open to subjectivity and, being qualitative, they do not allow statistical evaluation of the effects of a particular treatment, e.g., stress exposure. Quantitative data can take several forms, but all basically depend on counting cells in a section. One modern approach, superior to previous methods (Abercrombie, 1946; Weibel et al., 1966) is that of stereology, which is given detailed consideration below. Using stereology, one can obtain estimates of object volumes and derive numbers of objects from this data increasing the precision and relevance of data (Gundersen et al., 1988; West, 1999).

The principle behind stereology is to recreate or estimate the properties of geometrical objects in space. Its application to tissue or organ sections allows relatively precise estimation of the geometrical properties of the objects in a given section. As space has three dimensions, objects within it have properties for each possible number of dimensions, and objects within a given space can be defined in terms of their volumes (3 dimensions), surfaces (2 dimensions), lengths (1 dimension) and numbers. Each of these properties can be estimated by stereological methods, usually a two-step procedure involving: sampling and subsequently measuring.

A characteristic of many tissues and organs is that they contain a large number of the objects of interest, but too many to be measured individually. Producing a good sample is an essential step in stereological methods. Errors incurred during sampling can result in difficulties in obtaining meaningful stereological estimates later. Sampling usually starts before the investigator has any predictions as to the study outcome and even before the investigator has thought about applying stereological methods. To avoid later regrets, it is advisable to sample correctly from the very beginning; however, the researcher can be consoled by the fact that stereology-based sampling methods are compatible with all other types of analysis.

One usually wants to make statements about a structure (e.g., the hippocampus) or a cellular population (neurons) by sampling only a part of the structure or population. If such statements are to be valid for the entire structure or population, the sample must be a representative one. Selecting representative samples requires: (i) access to the entire structure or population; (ii) ability to recognize and/or define the entire structure or population; and (iii) that all parts of the structure or population contribute equally to the sample.

These pre-requisites can be met by random sampling in one of two ways: (i) Random independent samples – This is the most obvious approach in which one selects an initial location at random. After measuring the objects of interest, subsequent locations for measurement are chosen independent of the first. When a sufficient number of locations has been sampled, the individual measurements are averaged. Despite providing reliable and reproducible estimates, this method is, however, not an efficient sampling procedure. (ii) Uniform random systematic (URS) samples – In URS sampling, a random starting point is selected and samples are drawn at regular (or uniform systematic) intervals. Choosing

a random starting point means that all areas to be analyzed have an equal chance to contribute to the final sample measure. By eliminating sample clustering, the URS sampling procedure, on average, yields a more accurate estimate than the random independent sampling approach, and is the recommended method of choice. In practice, sections are selected using the URS sampling procedure at the time of tissue sectioning. If, for example, every fifth section is collected, the only requirement is to assure compliance with the need to randomly select the initial section in the series.

The next step in stereology is 'measuring' which involves relatively easy, routine work depending on identification of the object of interest and based on a simple set of rules. Curiously, the volume, surface, length, and number of objects are such basic parameters that it may be surprising to realize that methods for their accurate measurement only became available in the 1980s and did not enter widespread use until the 1990s. Stereological tools have now virtually replaced the earlier error-prone methods, which all suffered from the assumption that histological sections are two-dimensional images from which three-dimensional measurements were nevertheless attempted. The traditional methods involved certain well-grounded assumptions about the "missing dimension" in two-dimensional images. Inherently, the proximity to the true values achieved using such assumption-based methods, depended largely on how good the assumptions were.

In modern stereology, design-based methods have replaced assumption-based ones. These newer approaches involve measurements on a series of sections which in fact do have three dimensions. Therefore, information about the third dimension is based on fact, rather than assumption; obtaining precise measurements then depends on one other factor – the availability of good probes to apply to the sample. The selection of the adequate probe ultimately determines the precision of the estimation (West, 1999).

Estimation of volumes

Estimating volumes using points is conceptually the easiest of all stereological methods and was first described by the Italian mathematician Bonaventura Cavalieri (1598–1647). The "Cavalieri Principle" holds that, if one places a grid of regularly spaced points over an object of interest, the measured surface area will be a function of the number of points falling within it. To calculate the volume of an object (in our case, section), one simply has to multiply the average areas of different sections by the thickness of each section. The point-counting principle can, theoretically, also be applied to very small objects like cells, but this would require very thin sections in order to reduce error and the ability to identify the object in consecutive sections. Other approaches, like the nucleator – in which a point associated with a small particle (e.g., a nucleolus within a cell) is identified and from which rays are extended until the intersection of particle's boundaries to allow the estimation of its profile area and, subsequently, the absolute volume of the particle – have been developed for this purpose (Gundersen et al., 1988).

Estimation of total cell numbers

Measuring neuronal loss has preoccupied many neuroscientists interested in the effects of stress and glucocorticoids on the brain, in particular, the hippocampus. Such information can be generated by simply counting the number of cells in a given section and comparing the values obtained with those for sections from an anatomically matched area in control (e.g., nonstressed) subjects. This procedure yields a probable estimate of cell number per unit area (N_A); to date, this is probably the most widelyused method to count neurons. The precision (relevance) of such an estimate relies entirely on how similar the sections being compared are. A serious (but common) error of such estimates is the "reference trap," which refers to how variation in the volume of reference can affect the final result. This can be illustrated by considering the fact that because the N_A of granule cells in the hippocampus in two different sections from different experimental groups is similar, it does not necessarily follow that the total number of cells in each section is the same; this is because the volume (derived from the third dimension, which is not taken into account in deriving the N_A value) of the dentate granule cell

layer might differ significantly between individual sections and subjects. Design-based methods (e.g., estimating neurons using volumes within a probe) help avoid the introduction of such biases (West, 1999).

Essentially, estimating numbers within a volume is just the corollary of estimating volumes with points. One takes two adjacent sections that are thinner than the diameter of the object (e.g., nuclei) to be counted; the objects visible in the second, but not first, section are counted. Then, the number of objects in the volume represented by the two sections will, on average, correspond to the number of objects counted in the second. This approach is called the (physical) dissector because the counting principle is based on a comparison of two sections. Application of this technique provides the numerical density (N_v) of objects (e.g., neurons) within a region of interest. Now, if the total volume of the structure (e.g., hippocampus) is known, the total number of objects (e.g., neurons) can be derived from the product of N_v and the total volume.

The optical fractionator is another means for obtaining the total number of objects in a given 3D structure. It is based on the combination of systematic sampling (which yields an estimate of the fraction of the tissue – fractionator) and the dissector in thick optical sections that intrinsically have three dimensions.

Detection of cell death

The application of stereological methods to histological sections (e.g., stained with Nissl, Giemsa) can certainly provide information of neuronal loss based on the comparison of total number of surviving neurons between experimental groups (West et al., 1991; Sousa et al., 1999b). However, this approach can also be applied in combination with markers of neuronal death to directly determine the number of dying cells at a particular time-point. Several specific staining methods for detecting neurodegeneration have been developed but their use has not yet been generalized. The earliest markers of neuronal degeneration were based on silver-impregnation methods that provide unspecific indications of degeneration in neuronal soma and neurites.

More recently, the use of two anionic fluorescein derivatives have proved very useful for the simple and definitive localization of neuronal degeneration in brain tissue sections. Initial work on the first generation fluorochrome, Fluoro-Jade, demonstrated the utility of this compound for the detection of neuronal degeneration induced by a variety of well-characterized neurotoxicants, including kainic acid, 3-nitropropionic acid, isoniazid, ibogaine, domoic acid, and high doses of dizocilpine maleate (MK-801) (Schmued et al., 1997). After validation, the tracer was used to reveal previously unreported sites of neuronal degeneration associated with other neurotoxicants. Preliminary findings with a second-generation fluorescein derivative, Fluoro-Jade B, suggest that this tracer is a specific and selective marker for the identification of neurons undergoing degeneration (both apoptotic and necrotic) (Eyupoglu et al., 2003); Fluoro-Jade B also provides improved staining and can stain the distal portion as well as the proximal portion of the dissected axon (the so-called anterograde and retrograde degeneration after axotomy). Furthermore, Fluoro-Jade tracers can be combined with other histologic methods, including immunofluoresence that can help in discriminating different types of neurodegeneration to obtain information on the neurochemical identity of the affected cells (Schmued and Hopkins, 2000); recent preliminary findings on a number of specialized applications of Fluoro-Jade include the detection of apoptosis, amyloid plaques, astrocytes, and dead cells in tissue culture.

An early observation concerning apoptosis was that cells entering apoptosis showed dramatic and characteristic changes in nuclear shape and organization (Fig. 1) (see for review Kerr et al., 1972; Wyllie, 1980; Ucker, 1991). It is still probably correct to say that the characteristic change in nuclear morphology is the most accurate indicator of the involvement of apoptosis in the death of a cell. This is true even in light of the apparently paradoxical observation that nuclear fragmentation per se is not essential for apoptosis; enucleated cells can still undergo other changes characteristic of apoptosis. This unequivocally demonstrates that the effectors of the apoptotic machinery are located in the cytoplasm. However, under normal conditions, changes in nuclear morphology remain an early and relatively unequivocal

hallmark of apoptosis, with such changes occurring at an early point in the series of morphological events, usually soon after the onset of surface blebbing.

Apoptosis is an ATP (energy)-dependent process (Reed and Green, 2002). Since ATP levels fall to a point where the cell can no longer perform basic metabolic functions, the cell will die. Apoptotic cells exhibit significant reductions in their ATP levels, which can serve as an early marker of cell death. Depletion of energy pools is, however, not specific to apoptosis. Either exposure to toxic agents (secondary necrosis) or metabolic damage (primary necrosis) can also induce drops in ATP levels, albeit rapid ones (Leist et al., 1999), followed by necrotic cell death. The change in both ATP and ADP levels (ADP/ATP ratio) has been used to differentiate apoptosis from necrosis (Bradbury et al., 2000). In contrast, cell proliferation and growth arrest can both be recognized by increased levels of ATP and decreased levels of ADP. Determination of the ADP/ATP ratio offers highly consistent results and with excellent correlation to other markers of apoptosis (e.g., TUNEL-based techniques and caspase assays) (Bradbury et al., 2000).

c-Jun N-terminal kinase (JNK) is one of the main MAP kinase groups identified in mammals. Recent evidence suggests that activation of JNK plays an important role in neuronal apoptosis and other physiological and pathological processes (Ham et al., 2000). For measuring JNK activity easily in a large number of samples, one can use an assay that utilizes an N-terminal c-Jun fusion protein bead to selectively "pull down" JNK from cell lysate; c-Jun phosphorylation is then measured using a phospho-c-Jun-specific antibody. Alternatively, one might analyze JNK-specific activity by determining the phosphorylation of c-Jun by Western blotting using a phospho-c-Jun-specific antibody. Given the involvement of JNK in signaling pathways, which may not be directly related to apoptosis, care needs to be exercised in interpreting results obtained with such methods.

One of the first questions to resolve whenever searching for neurodegeneration, whether necrotic or apoptotic, is the ability to distinguish if the cells undergoing degeneration are neurons or glial cells. For this, immunohistochemistry is the most convenient and commonly used approach. Using specific antibodies for each cell population, one can easily identify the lineage of dying cells. Numerous neural cell type-specific (neurons, astroglia, oligodendrocytes, etc.) markers are currently available. For example, one may use antiGFAP to label astrocytes, antidoublecortin to identify neuroblasts (stem cells), antiNeuN to mark mature, differentiated neurons, or antiTuJ1 to study fibers.

As mentioned already, apoptosis is a genetically programmed phenomenon. A complex network of genes (Steller, 1995; Lossi and Merighi, 2003), in particular encoding members of the Bcl-2 family of proteins, play a central role in the regulation of apoptosis. Here, we focus on Bcl-2 family members as these have received most attention in the context of this article. The Bcl-2 family of proteins comprises death-inducer (proapoptotic) molecules such as Bax and Bcl-x_S and death-repressor (antiapoptotic) molecules such as Bcl-2 and Bcl-x_L. These various proteins, which can homo- or heterodimerize with each other, are activated by physiological or injurious stimuli, and appear to operate upstream of events leading to the final execution phase of the apoptotic process; the latter results from the activation of cysteine proteases, the caspases.

Caspases convey the apoptotic signal in a proteolytic cascade, with caspases cleaving and activating other caspases that then degrade other cellular targets that lead to cell death (Friedlander, 2003). Caspase activation can directly initiate the permeability transition of the mitochondrial membrane, resulting in the release of several mitochondrial proteins (see Fig. 2 for a simplified scheme). The large number of products developed to study caspases and their substrates is indirect testimony to their importance; because of space constraints, the authors here only review a few of these. Caspase-3 is a key protease that becomes activated during the early stages of apoptosis. Synthesized as an inactive proenzyme, the activated form cleaves and activates other caspases, in addition to cleaving specific targets in the cytoplasm and nucleus (e.g., DNA and nuclear membrane fragmentation). Once activated, caspase-3 serves as a marker for cells undergoing apoptosis. Several biotin- or FITC-tagged anti-active caspase 3 antibodies are available, facilitating their routine use (Gown and Willingham, 2002). Because caspase

activity is likely to be the most specific indicator of the apoptotic process, the assay of caspase activity through the detection of specific cleavage products in target proteins represents a theoretically valid approach for measuring apoptosis. Recently, antibodies to the caspase-generated cleavage products of cytokeratin 18 have appeared on the market, with several studies demonstrating their utility, especially in cell culture, but probably also in fixed tissue sections (Leers et al., 1999). However, cytokeratin 18 is expressed only in certain cell types and this antibody is not broadly applicable to all cell types. The use of antibodies specific for more generally distributed cleaved substrates of caspases, such as the cleaved form of caspase 3, would have more general applicability (Srinivasan et al., 1998). Owing to their cell-permeable nature, a new line of cell-permeable fluorogenic caspase substrates enables the visualization of intracellular protease activities by standard fluorescence microscopy or multiparameter flow cytometry (see below) in living cells. The substrates, designed for caspase-1, caspase-6, caspase-8 (the caspase-3 processing enzyme), and caspase-9, detect early events in the apoptotic pathway before DNA degradation has started (Davis et al., 1998; Komoriya et al., 2000). Recently, these caspase substrates have been used to demonstrate that the pattern of caspase activation is not only dependent on the apoptosis-inducing agent employed, but also on the cell type (Komoriya et al., 2000). Events occurring downstream of caspase-3 activation include cleavage of poly(ADP-ribose) polymerase (PARP), an enzyme implicated in DNA damage and repair mechanisms. Cleavage of PARP from the native 116 kDa to 85 kDa is considered a hallmark of apoptosis (Sallmann et al., 1997). The availability of FITC-tagged anti-PARP antibodies therefore, provide another useful marker of apoptosis.

In healthy cells, cytochrome *c* is located in the space between the inner and outer mitochondrial membranes. An apoptotic stimulus triggers the release of cytochrome *c* from the mitochondria into the cytosol where it binds to Apaf-1. The cytochrome *c*/Apaf-1 complex activates caspase-9, which then activates caspase-3 and other downstream caspases. Cytochrome *c* released from the mitochondria into the cytosol can be detected by Western blotting using antibodies directed against cytochrome *c*. The procedure is simple, straightforward, and provides an effective means for detecting cytochrome *c* translocation from mitochondria into cytosol during apoptosis (Jemmerson et al., 1999).

As already alluded to, Bcl-2 family proteins form complexes, these complexes can enter the mitochondrial membrane where they regulate the release of cytochrome *c* and other proteins. When Bax, for example, localizes to the mitochondrial membrane, it acts to increase mitochondrial permeability, induces the release of cytochrome *c* and other mitochondrial proteins, leading to apoptosis ultimately. In contrast, Bcl-2 and Bcl-x_L prevent mitochondrial pore formation and therefore, block apoptosis. Antibodies (applications include immunocytochemistry, Western blotting) and gene probes (for Northern blotting, in situ hybridization histochemistry, and polymerase chain reaction analysis) are now available for measuring most key members of the Bcl-2 family in a variety of species, including humans, rats, and mice. Such studies have shown that Bcl-2 levels in the brain decline rapidly after birth, except for those areas displaying postnatal neurogenesis such as the dentate gyrus of the hippocampus. Further, numerous studies have shown that Bcl-2 expression can be induced in the adult brain, including the hippocampus, upon experience of various noxious stimuli. Unlike that of Bcl-2, the expression of Bcl-X_L occurs in neurons from early development through to senescence. The proapoptotic protein Bax is expressed through all life stages whereas the smaller proapoptotic splice variant of Bcl-2, Bcl-X_S is only barely detectable in the mature brain. To date, there is no evidence that corticosteroids, which represent the endocrine response to stress, can directly regulate or interact with any members of the *bcl-2* gene family. Rather, corticosteroids appear to influence the pro- and antiapoptotic gene expression and activity by interacting with p53, a ubiquitously distributed tumor suppressor protein, which has been shown to induce and repress the transcription of *bax* and *bcl-2*; glucocorticoids were recently shown to enhance the transactivation potential of p53 (Crochemore et al., 2002). Although measurements of gene or protein expression of Bcl-2 family members might be reasonably expected to correlate with apoptosis, recent studies have shown that absolute levels of these molecules do not reflect the actual viability of

neurons in situ. Rather, the ratio of expression of pro-apoptotic (e.g., Bax) to antiapoptotic (e.g., Bcl-2) molecules factor has proven to be the factor determining neuronal survival (Almeida et al., 2000) insofar that this derivative correlates with the incidence of apoptosis measured by histochemical techniques such as TUNEL (see below).

Disruption of the mitochondrial transmembrane potential is one of the earliest events after apoptosis induction. Normally, cellular energy generated by mitochondrial respiration accumulates in the transmembrane space as an electron gradient called the mitochondrial transmembrane potential $\Delta\Psi_m$. Disruption of the mitochondrial transmembrane potential occurs following the onset of apoptosis. Using a fluorescent lipophilic cation as a mitochondrial activity marker, one can measure differences in the fluorescence displayed by healthy cells versus apoptotic cells: in healthy cells, the dye accumulates and aggregates in the mitochondria, producing a bright red fluorescence, while in apoptotic cells the fluorescence cation cannot aggregate in the mitochondria because of the altered transmembrane potential, thus remaining in its monomeric (green fluorescent) form within the cytoplasm. These fluorescent signals are analyzed by flow cytometry using the FITC channel for green monomers and the propidium iodide (PI) channel for red aggregates. Additionally, apoptotic and healthy cells can be viewed simultaneously by fluorescence microscopy using a wide-band pass filter. Some kits combine detection of disrupted mitochondrial transmembrane potential with changes in the composition of the plasma membrane.

Flow cytometry can be summarized as a method for measuring physical and biochemical features of cell components on a cell-by-cell basis, primarily by optical means. Fluorescent dyes or fluorophore-conjugated antibodies are used to report the quantities of specific cellular components, density of cellular markers and receptors – or even activation state of various enzymes. Put simpler, flow cytometers are highly sophisticated fluorescence microscopes, where fixed or living cells are not attached to a well-defined surface, but rather travel one by one, by continuous flow of a stream of the suspension past a sensor. Each cell scatters some of the excitation laser light, and the labeled cells emit fluorescent signals from the dye. These two parameters are sensed by photodetectors, data are collected, and processed by a computer.

The term 'FACS' is Becton-Dickinson's registered trademark and is an acronym for "Fluorescence-Activated Cell Sorter." FACS is therefore, a machine that can rapidly separate cells in a suspension, based on the size and color of their fluorescence. (Note: not all flow cytometers are necessarily able to separate cells into different vials, but all can analyze the distribution of cell size and/or physical or biochemical cellular properties). These particular features of flow cytometric methods allow the identification and quantification of apoptotic cells as well as possible mechanisms of cell death.

The main flow cytometric approaches that can be used to identify apoptotic cells may be summarized as follows: (1) Apoptosis-associated changes in cell size and granularity can be detected by analysis of laser light scattered by the cell. (2) Using annexin V in combination with propidium iodide (PI), it is possible to differentiate between healthy, early apoptotic, and necrotic cells on the basis of the distribution of plasma membrane phospholipids as well as changes in membrane integrity. (3) Fluorochromes like Rhodamine 123 (Rhod123) or 3,3′-dihexiloxadicarbocyanine ($DiDOC_6$) reveal decreases in the mitochondrial transmembrane potential ($\Delta\Psi_m$) that occurs early during apoptosis. (4) Apoptotic cells can be recognized by their fractional DNA content, or by the presence of DNA strand breaks using fluorochrome-labeled nucleotides attached to the 3′-OH termini in a reaction catalyzed by exogenous terminal deoxynucleotidyl transferase (TdT). As regards the identification of putative mechanisms of cell death, after labeling with primary and fluorescent secondary antibodies, one can detect and measure: (a) cellular levels of death-related proteins (members of Bcl-2 family, proto-oncogenes like c-myc and ras, tumor-suppressor genes such as p53, etc.) or (b) study particular cell functions, such as mitochondrial metabolism, in the context of cell sensitivity to apoptosis.

The main virtue of flow cytometry lies in the possibility of multiparametric, correlated analysis of a multitude of cell attributes and markers, thus addressing problems of cellular heterogeneity. Flow cytometry also provides more effective data acquisition as compared to fluorescence microscopy

(which has similar capabilities, but in which the sample size is limited up to a few hundred cells. Flow cytometry can easily measure 10,000–100,000 cells per sample!). There are, however, certain difficulties associated with this technique, which have to be taken into consideration when using flow cytometry in general. With respect to cell death detection, a major problem is that the single parameter on which the identification of apoptotic or necrotic cells relies on, may be absent, when apoptosis is atypical. Moreover, in the case of nonfixed, living cells, the dissociation procedure may damage the plasma membrane, resulting in PI (a widely used cell viability dye) to enter and label the cells as if they were dead. Clumping of cells may also pose technical difficulties. Since high-speed FACS machines use high pressure to achieve rapid acquisition rate, limitations such as cell type and viability must also be considered.

Externalization of phosphatidylserine (PS) and phosphatidylethanolamine are hallmarks of changes in the cell surface during apoptosis. Annexin V binds to PS with strong avidity and can be used as a marker of PS externalization using either microscopy or flow cytometry (when fluorescent-labeled annexin V is applied). Importantly, annexin V-binding cannot be applied to tissue sections or adherent cells, and when flow cytometric analysis is used on cell suspensions. PS are phospholipids only present on the cytoplasmic face of the plasma membrane and other internal membranes, and it remains unclear as to why certain subpopulations of cells (< 30%) entering apoptosis externalize PS at a very early stage of the process, just after the fragmentation of the nucleus begins. However, since inhibition of caspase activity blocks PS externalization, a role for caspases is indicated. It is important to keep in mind that, during necrosis or the terminal lytic steps of apoptosis, PS that are actually localized on the inner face of the membrane might be accessed by annexin V, giving rise to false positives. When combined with a vital dye such as the red fluorescent DNA-binding compound PI, FITC-annexin V labeling can be used to distinguish necrotic from apoptotic cells; this is because PI does not penetrate live cell membranes or cells in the early phases of apoptosis but only cells that have lost membrane integrity as a result of necrosis or very late apoptosis.

The permeability of the plasma membrane is substantially different in necrotic and apoptotic cells, a fact that can be taken advantage of in the distinction between these forms of cell death. Thus, it is possible to distinguish between stages of apoptosis mainly on the basis of the plasma membrane permeability changes. Large-molecular weight DNA-binding dyes, such as PI or the homodimer of ethidium bromide (EB), cannot enter intact cells because of their large size and, without permeabilization treatments, do not label apoptotic cells until the final stage of cell lysis. On the other hand, in ethanol-fixed cells, which have been subsequently washed in phosphate–citrate buffer, extraction of the low-molecular weight DNA from apoptotic cells takes place, and apoptotic cells appear to the left of the normal G1 peak after PI staining (Fig. 3). This is a fast, simple, but not very specific, method for detecting apoptosis, and has the disadvantage that apoptosis of cells in late S phase or from G2 may be missed. Smaller dyes that can attach to DNA (such as DAPI, Hoechst 33342 or 33258), are furthermore able to enter, and differentially label apoptotic and healthy cells based on the condensation and subsequent fragmentation of the chromatin, which occurs early during apoptosis. Using flow cytometry, for instance, one can distinguish between healthy, apoptotic and dead cells by the simultaneous use of the blue-fluorescent Hoechst 33342 dye (which stains the condensed chromatin of apoptotic cells more brightly than that of normal cells) and PI, which labels dead cells (Pollack and Ciancio, 1991). Acridine orange (AO) (another cell-permeant nucleic acid-binding dye that emits green fluorescence when bound to double-stranded DNA, and red fluorescence when bound to single-stranded DNA or RNA) is another useful probe for identifying apoptotic cells, because its metachromatic fluorescence is sensitive to DNA conformation. Careful combination of fluorescent dyes, furthermore, allows even more accurate determination of different stages of the apoptotic process: for example, 7-aminoactinomycin D (7-AAD) can be used alone or in combination with Hoechst 33342 to separate populations of live, early apoptotic, and late apoptotic, cells (Schmid et al., 1994). In mixed cell populations, however, identification of cell types is necessary, and has also to be taken into consideration. For instance,

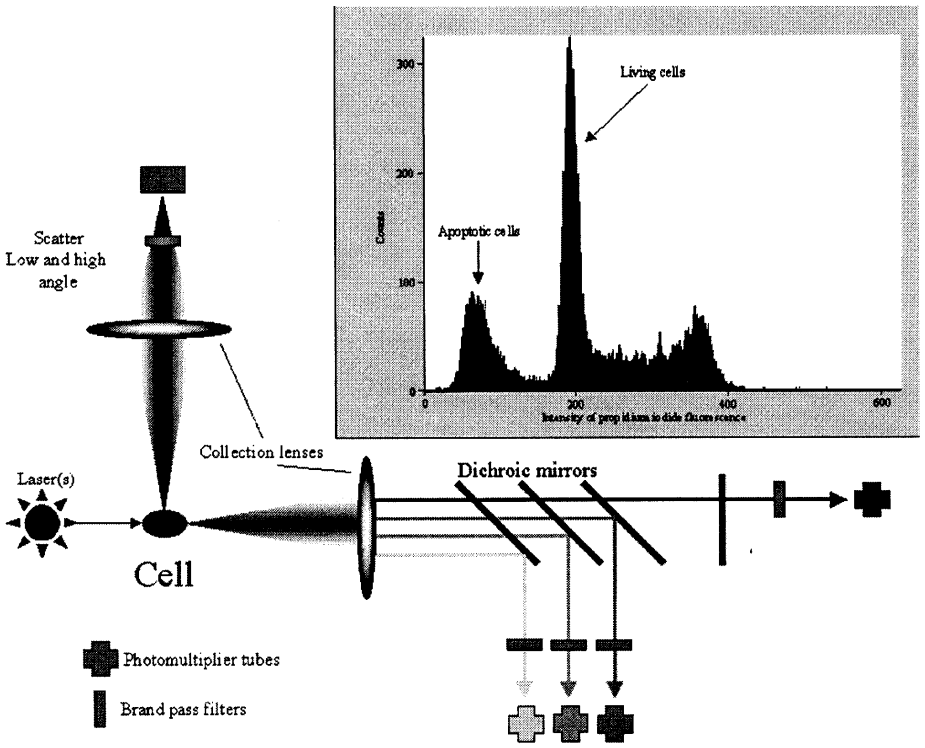

Fig. 3. Separation of living/apoptotic cells by flow cytometry. For flow cytometric analysis, ethanol-fixed cells are washed in phosphate–citrate buffer and stained with propidium iodide. As cells pass in front of a laser, they absorb, diffract, refract, and reflect incident light, and emit fluorescence. The scattered light is focused by a lens into a photomultiplier, the emitted fluorescent signal is optically filtered through dichroic mirrors, and subsequently processed by wide-band pass filters selected to optimize the various fluorescent emissions; signals are detected by photomultiplier tubes, and based on fluorescence intensity profiles, living and apoptotic cells can be distinguished.

the combination of acridine orange and ethidium bromide (AO/EB) is useful to accurately differentiate between healthy, early apoptotic, late apoptotic, and necrotic cells (Liegler et al., 1995), but cannot be used for phenotypic analyses due to the broad emission spectrum of AO and EB. Certain techniques, like DNA strand-break labeling by terminal deoxynucleotidyl transferase (TdT) can overcome this problem, but are technically very demanding. TdT adds biotinylated or digoxygenin-labeled nucleotides to the strand-breaks in the DNA of apoptotic cells – apoptotic cells therefore, can be detected by using fluorochrome-labeled streptavidin conjugates or fluorochrome-labeled anti-digoxygenin antibodies in flow cytometric analysis. By combining this procedure with phenotypic markers tagged to other dyes, it is even possible to obtain cell cycle profiles in cells of a given phenotype (Li et al., 1996).

As mentioned earlier, changes in plasma membrane permeability are signs of late phases of cell lysis. Changes in mitochondrial membrane permeability, however, occur much earlier during apoptosis, and are considered to be a distinctive feature of early programmed cell death. The mitochondrial permeability transition (MPT) is intimately linked to the opening of a "megachannel," the permeability transition pore (PTP). Ionic equilibration through the PTP results in disruption of the mitochondrial

transmembrane potential ($\Delta\Psi_m$), uncoupling of the respiratory chain, and release of cytochrome *c* into the cytoplasm. Of all these features, changes in the mitochondrial permeability can be relatively easily followed by application of fluorescent dyes (e.g., $DiOC_6$), while the subsequent ionic and electrical fluctuations can be investigated by patch-clamp techniques or certain fluorophores. While certain drugs, like the green-fluorescent calcein (which is produced from the nonfluorescent calcein-AM form within the cell itself) are used to indicate PTP opening, and, subsequently, the taking up of the dye into the mitochondrial matrix, others (like JC-1, JC-9, or $DiOC_6$) do not just simply accumulate in the mitochondria, but also indicate changes in $\Delta\Psi_m$ in single-cell imaging or flow cytometric assays. Other dyes, like MitoTracker® Red CMXRos can be fixed by aldehyde-based fixatives and can thus be used for other subsequent analytical procedures such as immunocytochemistry, DNA end-labeling, in situ hybridization, or counterstaining. Ionic concentrations in the mitochondria can be monitored using patch-clamp techniques or fluorescent dyes like the Ca^{2+}-sensor Rhod-2.

Loss of DNA integrity is characteristic of apoptosis (Collins et al., 1997). When DNA extracted from apoptotic cells is analyzed using gel electrophoresis, a characteristic internucleosomal "ladder" of DNA fragments (typically, 180–200 bp in length) is revealed (Compton and Cidlowski, 1986; Walker et al., 1999); larger DNA fragments have also been seen at earlier stages in apoptotic cell cultures. Although these electrophoretic methods are commonly used in apoptosis detection, the results they provide can present interpretational difficulties. Also, these methods cannot be easily applied, requiring extraction of DNA from large numbers of cells undergoing apoptosis in a relatively synchronous way; however, such synchrony is not always present, especially in tissues (Collins et al., 1997), and as noted above, apoptosis is a relatively rare event, occurring in only a subset of cells within a given structure, thus raising problems of sensitivity.

A widely used method that has contributed much to our knowledge of stress- and corticosteroid-induced apoptosis in the brain is also based on the detection of DNA strand-breaks. This approach detects 3′-OH ends of single-stranded DNA after the addition of labeled nucleotides to the open ends of DNA in a procedure known as in situ end-labeling (ISEL). The latter may be achieved using either *E. coli* polymerase (or its Klenow fragment) in a method called in situ nick-translation (ISNT), or terminal transferase in a method referred to as terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) (Modak and Bollum, 1972; Gavrieli et al., 1992; Jin et al., 1999). These methods allow the cytochemical demonstration of free DNA strand openings.

TUNEL staining is now widely used for the detection of apoptotic cells in tissue sections and cells in culture. Despite its apparent simplicity, unless used optimally, this technique may lack sensitivity and, worse, specificity. For example, TUNEL can reportedly label both apoptotic and necrotic cells, and potentially, proliferating cells also, although these problems are less-frequently encountered in tissue sections than when cultured cells are stained. Moreover, as already noted in the main text, apoptotic cells can be easily recognized on the basis of their unambiguous morphological characteristics. With regard to mitotic cells, it deserves mentioning that although chromatin condensation at telophase may mimic apoptosis, the greatest analogy between mitotic and apoptotic aspects occurs in abortive mitosis, a form of cell division that leads to active cell death (sometimes named "mitotic catastrophe").

The major problems associated with the TUNEL technique, especially in tissues, can be summarized as follows: (i) without pretreatment, TUNEL sensitivity is poor and can lead to false negatives; (ii) established pretreatments (proteinase K, microwaves) can easily result in labeling of morphologically normal nuclei; and (iii) the method depends on good fixation and can prove problematic when large tissue blocks are used (outside–inside gradients of penetration of fixative). Other considerations include: (i) the DNA breaks, which are targeted by TUNEL, are less accessible than intact DNA; (ii) besides apoptosis, DNA recombination, replication, repair or compaction–relaxation during mitosis, tissue electrocoagulation, autolysis, fixation, paraffin embedding, cutting, and pretreatments with H2O2, detergent, proteinase K, and microwaves can all result in DNA breaks; and (iii) DNA compaction

(a hallmark of apoptosis) and protein cross-linking and precipitation induced by fixation can mask the 3-OH recessed ends.

Despite the above caveats, TUNEL is still regarded as a reliable marker of the DNA fragmentation, which typically occurs in apoptosis. The key to distinguishing between apoptotic and nonapoptotic DNA is the cautious use of "break disclosure" reagents (detergents, proteases, microwaves). Extensive tests have led some authors to propose that optimal staining results from qualitative adaptations of retrieval techniques rather than retrieval reinforcement; for example, quite different pHs are necessary to obtain specific labeling in formalin- versus Bouin-fixed tissues. When fixation is controlled (e.g., homogeneous and light) as is usually the case in prospective studies, proteinase K alone may be sufficient for all cross-linking aldehyde fixatives (paraformaldehyde, formalin, B5). Proteinase K and microwave treatment may be necessary when tissues are fixed for too long and/or in precipitating solutions (Bouin's).

Nonspecific (background) staining can also present a problem, even when optimal pretreatments are applied. This can be overcome by optimizing the detection system, e.g., dilution of the enzyme-coupled antibody, choice of enzyme, careful monitoring of color development. Absence of standardization of color reaction implies suboptimal quantification of those cells which might otherwise show morphological signs of apoptosis. Also to be remembered is that all labeled cells, irrespective of intensity of labeling, should to be counted as long as they show morphological features of apoptosis.

Another method for detecting these single-strand ends is the use of monoclonal antibody reactive with single-stranded DNA (Naruse et al., 1994; Frankfurt et al., 1996). Since preservation/fixation procedures can have dramatic effects on the detection of single-stranded DNA (Labat-Moleur et al., 1998; Tateyama et al., 1998), careful consideration must be given to this issue and optimized for each cell type or tissue. The investigator should also keep in mind that in cases of overfixation, for example, open DNA strands will be inaccessible to assay reagents (Nakamura et al., 1997). This can be overcome by introducing protease treatments prior to ISNT or TUNEL procedures. Proteases must be used cautiously – protease treatments can mimic the actions of endogenous caspases, thus leading to artefactual DNA strand-breaks. Here, it is also important to note that, depending on permeabilization and fixation protocols, some methods detect so-called preapoptotic nuclei in which strand breaks are detected in the absence of apoptosis-like changes in the morphology of the nucleus. Alternatively, positively labeled strand-breaks may not correlate with nuclear fragmentation in individual cells, or DNA strand-breaks may only become detectable at relatively late stages of the apoptotic process (Collins et al., 1997). Recently, a number of authors have indicated reservations about the use of the TUNEL and ISNT assays for detecting apoptosis. It has become apparent that single-stranded DNA ends are not necessarily specific for apoptosis since they may also occur in necrotic cells (Kockx et al., 1998; Mizoguchi et al., 1998). Therefore, although these methods have been, and remain, very useful (their major advantage being that they can be applied directly to intact tissue sections, thus providing good anatomical resolution), the results they yield must be treated with extreme care; for example, in our studies (e.g. Hassan et al., 1996), we only consider TUNEL-positive cells as apoptotic if they simultaneously display the typical morphological characteristics of apoptotic cells; positively stained cells, which have a clearly defined nucleus and cell body are

Table 1. Dyes commonly used for quantifying apoptosis by flow cytometry

Marker dye	MW	Absorption max.	Emission max.
DAPI	350.25	358	461
PI	668.4	535	617
$DiOC_6$	572.73	484	501
Rhod 123	380.83	507	529
JC-1	652.23	514	529
Annexin V conjugates	Depends on fluorescent conjugate		
AO	301.82	500	526
7-AAD	1270	546	647
MitoTracker Red® CMXRos	531.52	578	599
Rhod-2, AM	1123.96	550	571
Hoechst 33342, 33258	623.96	352	461

excluded, as are cell fragments and endothelial cells; further stringency is added by ensuring that the person performing the cell counts is unaware of the treatments.

Concluding remarks

The main objective of this article was to provide a brief overview of the methodologies available to study the cellular and molecular basis of stress-induced neurodegeneration. While our coverage is by no means exhaustive, we aimed to review each of the major approaches in current use, both in brain tissue and cell culture, and to discuss each of the methods in terms of their advantages and inherent drawbacks; it should become obvious to the reader that no single method can be considered to be definitive by itself, and investigators are encouraged to confirm results obtained one method with that from an alternative technique whenever feasible, in order to avoid from misinterpretation of results. We also attempted to discuss certain important aspects of experimental design in the hope that the use of standardized procedures will contribute to our increased understanding of stress-induced neuronal damage.

List of abbreviations

5-HT	serotonin
7-ADD	7-aminoactinomycin D
AIF	apoptosis-inducing factor
AO	acridine orange
APAF-1	apoptotic protease activating factor 1
BDNF	brain-derived nerve factor
CA	field of hippocampus
DAPI	4′-6-diamidino-2-phenylindole
$DiDOC_6$	3,3′-dihexiloxa-dicarbocyanine
EB	ethidium bromide
FACS	fluorescence-activated cell sorter
FGF-2	fibroblast growth factor
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
ISEL	in situ end-labeling
ISNT	in situ nick-translation
JC-1	5,5′,6,6′-tetra-chloro-1,1′,3,3′-tetraethyl benzimidazolyl-carbocyanine iodide
JNK	c-Jun N-terminal kinase
MK-801	dizocilpine maleate
MPT	mitochondrial permeability transition
MR	mineralocorticoid receptor
N_A	number per unit area
NeuN	neuronal-specific nuclear protein
NGF	nerve growth factor
NMDA	*N*-methyl-*D*-aspartic acid
NO	nitric oxide
N_V	number per unit volume
PARP	poly(ADP-ribose) polymerase
PI	propidium iodide
PS	phosphatidylserine
PTP	permeability transition pore
Rhod	rhodamine
ROS	reactive oxygen species
TdT	terminal deoxynucleotidyl transferase
TGF	transforming growth factor
TuJ1	neuron-specific class III beta-tubulin
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling
URS	uniform random systematic
$\Delta\Psi_m$	mitochondrial transmembrane potential

Acknowledgements

This article was written under the auspices of the German–Portuguese cooperation – Gabinete de Relações Internacionais da Ciência e do Ensino Superior (GRICES) and the German Academic Exchange Service (DAAD). Zs. Némethy was supported by Marie Curie Indivdual Fellowship from the European Commission (QLK6-CT-2001-51072). Many of the methods described here were established in the authors' laboratories by past and present colleagues who are duly thanked.

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