EJN fens European Journal of Neuroscience

European Journal of Neuroscience, Vol. 36, pp. 3396–3406, 2012 doi:10.1111/j.1460-9568.2012.08262.x

NEUROSYSTEMS

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

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

Abstract

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

Introduction

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

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

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

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Received 2 April 2012, revised 10 July 2012, accepted 18 July 2012

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

Materials and methods

Animals and treatments

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

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

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

In Experiment 1 we determined the activation pattern of the BNST and paraventricular nucleus of the hypothalamus (PVN) in basal conditions and after exposure to an anxiogenic stimulus. At 24 h after the last exposure to stress or handling, some of the animals ($n = 4$/group) were killed, whereas the remainder ($n = 4$) were submitted to one session of acoustic startle (anxiogenic stimuli) and killed 30 min later. Animals were previously habituated to the startle apparatus for 5 days to decrease the effect of novelty. For killing, animals were deeply anesthetised with pentobarbital (0.5 mg/kg) and perfused transcardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate-buffered solution (PBS). Brains were dissected, post-fixed (4% paraformaldehyde) for 4 h, immersed in 8% sucrose in 0.1 M PBS (2 days at 4 °C) and then further processed for cFOS immunohistochemistry and morphological analysis.

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

Anxiety behavior

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

Elevated plus maze

At the end of 4 weeks of treatment (Cont or CUS), animals were tested over 5 min in a black polypropylene 'plus'-shaped maze (ENV-560, MedAssociates Inc., St Albans, VT, USA) as previously reported (Pêgo *et al.*, 2008). The times spent in the open arms, junction area and closed arms, as well as the number of entrances and explorations in each section were recorded using a system of infrared photobeams, the crossings of which were monitored by computer. The times spent in each of the compartments of the elevated plus maze are presented as a percentage of the total duration of the trial.

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

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

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

Acoustic startle as an anxiogenic stimulus

An acute state of anxiety was induced by the acoustic startle reflex paradigm, using a startle response apparatus (SR-LAB, San Diego Instruments, San Diego, CA, USA) as previously reported (Pêgo *et al.*, 2008). Animals were habituated to the apparatus (5 min daily) for 2 days before the actual trial. Rats were placed in the startle chamber and allowed to acclimate to the chamber for 5 min. They were then presented with 20 anxiogenic startle stimuli (50 ms pulse of white noise at 120 dB) at a randomly assigned interstimulus interval ranging from 10 to 20 s. The procedure lasted 15 min in total. Animals were then returned to a resting cage for 30 min before killing. Trials on individual animals were conducted sequentially. Between tests, the chambers and acrylic holders were thoroughly cleaned (10% ethanol) to eliminate residual olfactory cues.

Regional boundaries

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

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

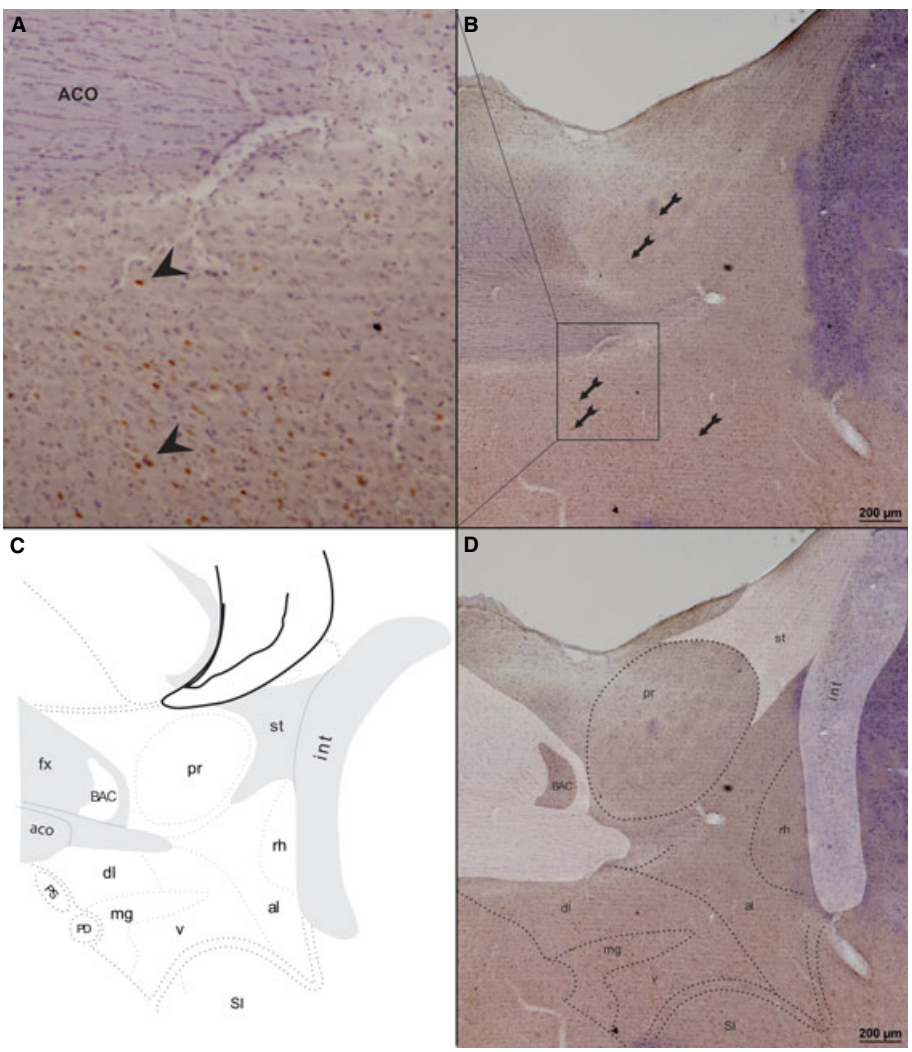

FIG. 1. Representative micrographs of immunostained sections for cFOS of the BNST and delineation of its subnuclei. (A) Detail of cFOS-immunoreactive (Fos-IR) neurons (arrowheads). (B) Overview of the BNST showing distribution of Fos-IR neurons (arrows). (C) Atlas drawing corresponding to the section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

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

Immunohistochemistry and quantification procedures

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

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

Laser microdissection and quantitative real-time polymerase chain reaction analysis

Coronal cryostat sections (20 μm) were mounted on Molecular Machines & Industries membrane-coated slides (Olympus), immersed in 70% isopropanol (1 min), rinsed in Diethylpyrocarbonate-treated water, and stained with hematoxylin, before final immersion in 100% isopropanol (2 min). After air-drying, sections were ready for laser microdissection (Microdissector CellCut, Olympus) of three subnuclei of the BNST [dorsomedial ($BNST_{dm}$), fusiform ($BNST_{fu}$) and principal ($BNST_{pr}$) nucleus], based on data obtained from Experiment 1 (Fig. 2).

RNA from the microdissected samples was extracted using the RNeasy Plus Micro Kit (Qiagen) and frozen at −80 °C until use. One RNA aliquot was used to assess the quantity and quality of the RNA (Experion RNA HighSense Analysis kit, Bio-Rad) (Table 2). RNA was amplified with the SuperScript RNA Amplification System (Invitrogen) following the manufacturer's instructions, and cDNA was subsequently synthesised using the SuperScript First Strand Synthesis for RT-PCR kit (Invitrogen). qRT-PCR analysis was used to measure the levels of mRNA encoding the following proteins: CRHR1 (*Crhr1*; Fw, CCTTAGGGCTTCTTTGTG; Rw, GGACTGCTTGATGCTGT GAA), CRHR2 (*Crhr2*; Fw, TTTTCCTAGTGCTGCGGAGT; Rw, AGCCTTCCACAAACATCCAG), CRH (*Crh*; Fw, GCTAACTT TTTCCGCGTGTT; Rw, GGTGGAAGGTGAGATCCAGA), GA-BA_A receptor, subunit alpha 3 (*Gabra3*; Fw, TGGTCATGTTG TTGGGACAG; Rw, TGGCAAGTAGGTCTGGATGA), glutamic acid decarboxylase 1 (*Gad1*; Fw, TCGGTTTTTCAACCAGCTCT; Rw, AACAAACACGGGTGCAATTT), glutamic acid decarboxylase 2 (*Gad2*; Fw, GCCAACTCTGTGACATGGAA; Rw, GGTAGGAA GCATGCATCTGG), and *N*-methyl-D-aspartate receptor subunit 2B (*Nr2b*; Fw, GCATGCCTACATGGGAAAGT; Rw, GTTGAGCA CAGCTGCATCAT). Levels of the house-keeping gene hypoxanthine guanine phosphoribosyl transferase mRNA (*Hprt*; Fw, GCAGA CTTTGCTTTCCTTGG; Rw, TCCACTTTCGCTGATGACAC) were also monitored and used for normalisation.

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

Data analysis

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

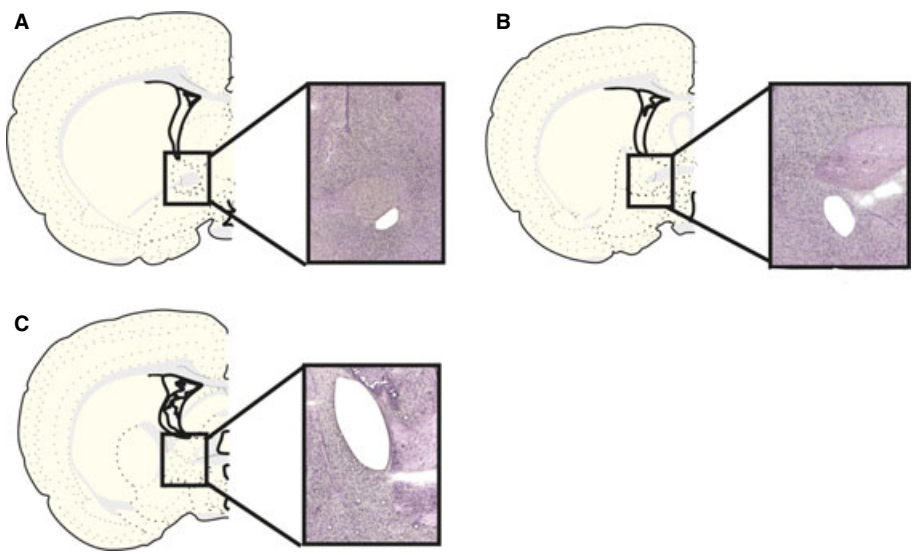

FIG. 2. Schematic representation of sections according to the atlas of Swanson (1998) and representative pictures of the microdissected nuclei of the BNST. (A) $BNST_{dm}$; (B) $BNST_{fu}$; (C) $BNST_{pr}$.

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

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

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

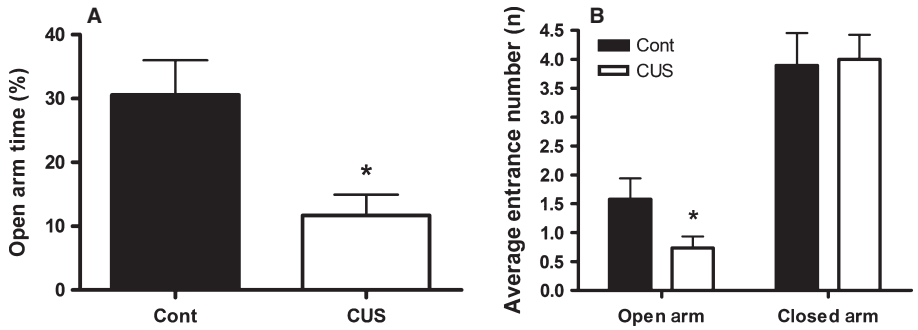

FIG. 3. Results from the elevated plus maze test. (A) Time spent in the open arms given as percentage of total time. (B) Number of entries in the open and closed arms. Results are presented as mean SEM. *$P < 0.05$.

Results

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

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

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

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

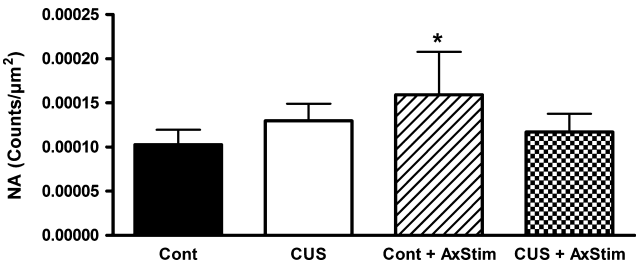

FIG. 4. cFOS expression in the PVN. Stress induced a significant effect on cFOS expression in the PVN. Cont+AxStim, control rats submitted to anxiogenic stimuli; CUS+AxStim, CUS rats submitted to anxiogenic stimuli. NA, number of cells per area. Results are presented as mean SEM. *$P < 0.006$ Cont vs.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

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

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

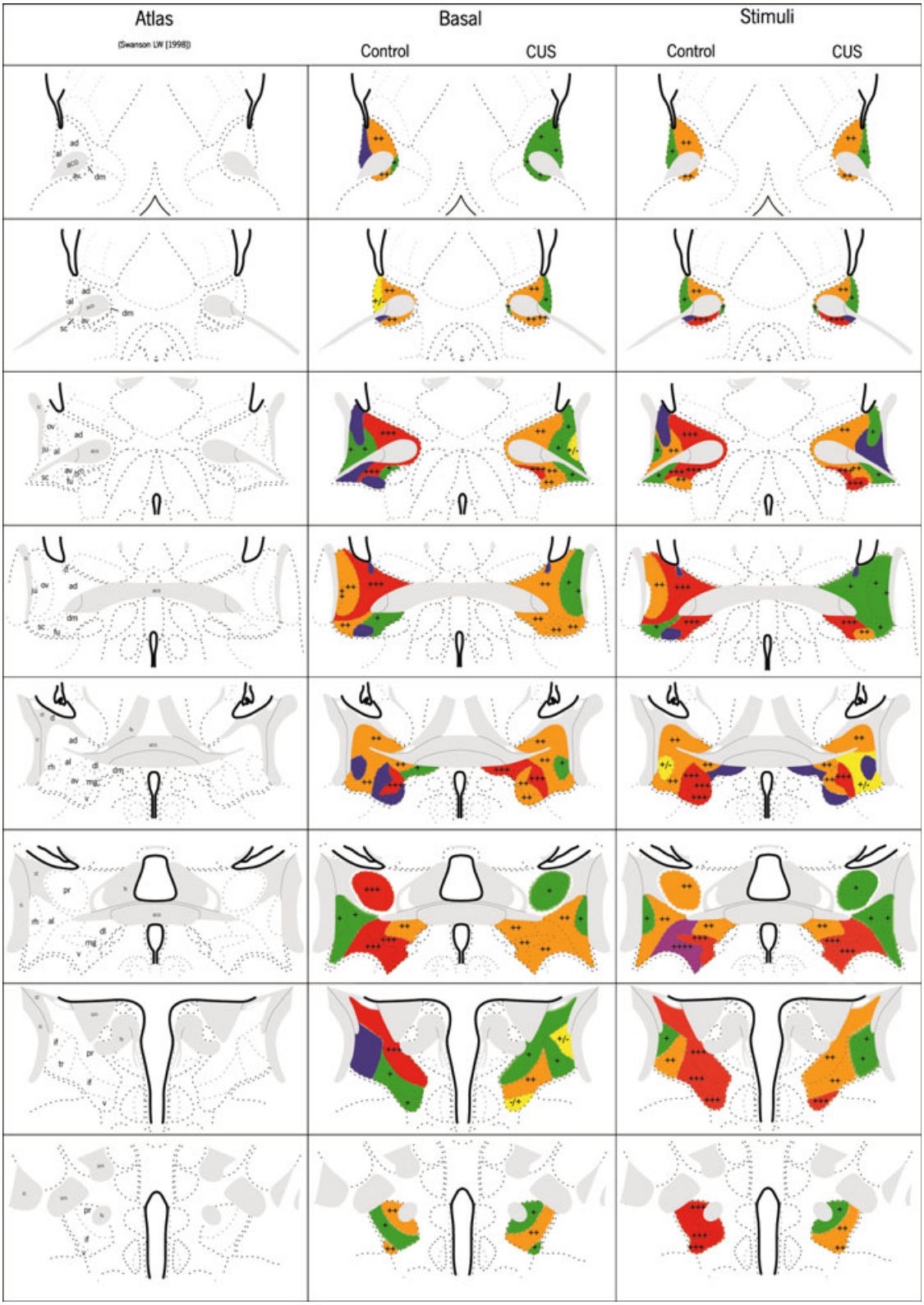

FIG. 5. Schematic representation of the relative density of cFOS-immunoreactive (Fos-IR) neurons in the BNST of the rat in control and CUS animals, in basal conditions and after exposure to anxiogenic stimuli. Left panels: schematic representation of the sections according to the atlas of Swanson (1998).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

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

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

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

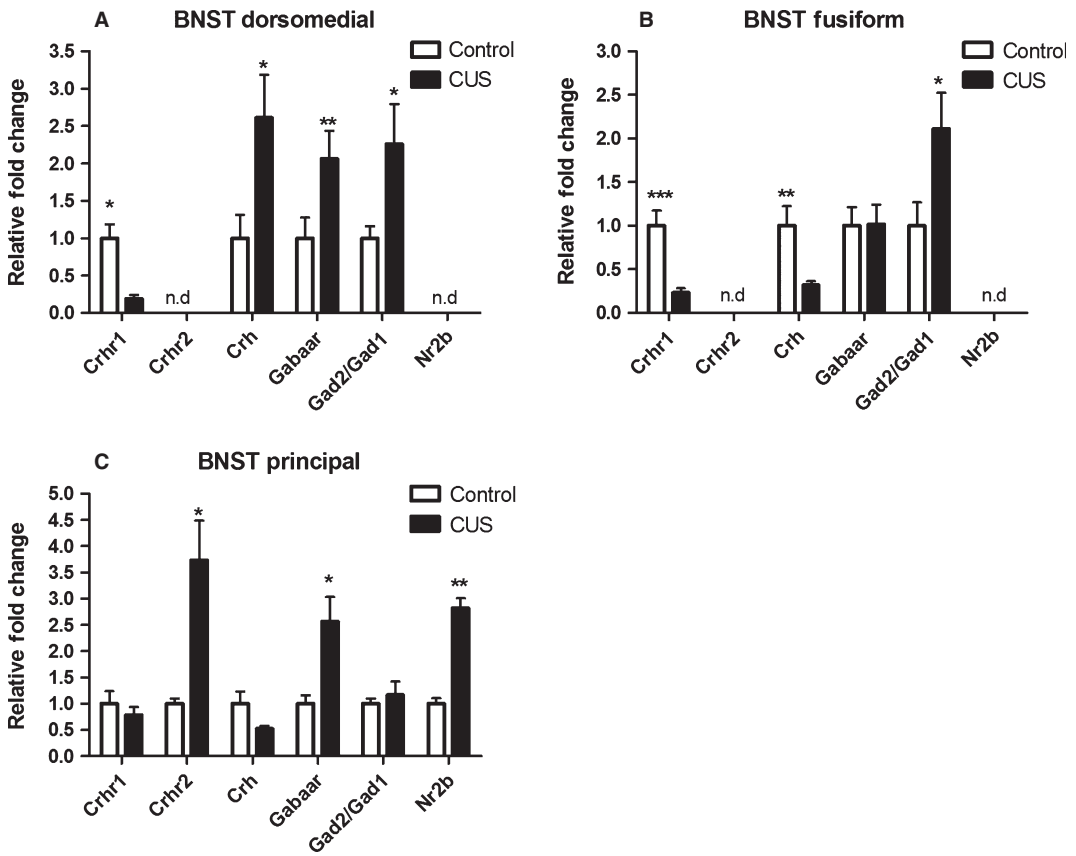

FIG. 6. Gene expression analysis of the BNST in response to stress. Expression levels for (A) dorsomedial nucleus of the BNST; (B) fusiform nucleus of the BNST; (C) principal nucleus of the BNST. CUS leads to decreased *Crh1* expression in the dorsomedial and fusiform nucleus and also to an increase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

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

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

Molecular correlates of stress-induced anxiety

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

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

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

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

Discussion

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

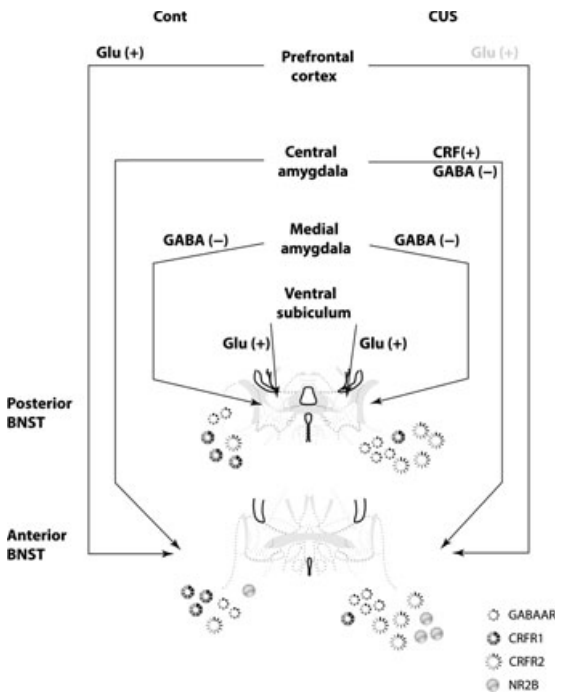

FIG. 7. Schematic representation of the changes occurring after stress exposure (right side) when compared with baseline (left side), which define the molecular signature of stress in the BNST. CRF, corticotrophin-releasing factor; Glu, glutamate.

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

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

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

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

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

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

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

Acknowledgements

A.P.V.-S., A.J.R., F.M. and A.R.M. were supported by Fundação para a Ciência e Tecnologia (FCT) grants. The authors declare no conflicts of interest.

Abbreviations

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

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