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Stressor categorization: acute physical and psychological stressors elicit distinctive recruitment patterns in the amygdala and in medullary noradrenergic cell groups

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Abstract

It has been hypothesized that the brain categorizes stressors and utilizes neural response pathways that vary in accordance with the assigned category. If this is true, stressors should elicit patterns of neuronal activation within the brain that are category-specific. Data from previous immediate–early gene expression mapping studies have hinted that this is the case, but interstudy differences in methodology render conclusions tenuous. In the present study, immunolabelling for the expression of c-*fos* was used as a marker of neuronal activity elicited in the rat brain by haemorrhage, immune challenge, noise, restraint and forced swim. All stressors elicited c-*fos* expression in 25–30% of hypothalamic paraventricular nucleus corticotrophin-releasing-factor cells, suggesting that these stimuli were of comparable strength, at least with regard to their ability to activate the hypothalamic–pituitary–adrenal axis. In the amygdala, haemorrhage and immune challenge both elicited c-*fos* expression in a large number of neurons in the central nucleus of the amygdala, whereas noise, restraint and forced swim primarily elicited recruitment of cells within the medial nucleus of the amygdala. In the medulla, all stressors recruited similar numbers of noradrenergic (A1 and A2) and adrenergic (C1 and C2) cells. However, haemorrhage and immune challenge elicited c-*fos* expression in subpopulations of A1 and A2 noradrenergic cells that were significantly more rostral than those recruited by noise, restraint or forced swim. The present data support the suggestion that the brain recognizes at least two major categories of stressor, which we have referred to as 'physical' and 'psychological'. Moreover, the present data suggest that the neural activation footprint that is left in the brain by stressors can be used to determine the category to which they have been assigned by the brain.

Introduction

Stimuli which act as stressors elicit a complex centrally co-ordinated response involving changes in mood, cognition, behaviour, autonomic function and endocrine output. A major question facing researchers is whether the brain deals with stressors categorically. Although not universally accepted (e.g. see Pacák *et al*., 1998), the idea that the brain categorizes stressors and uses response pathways that vary according to the category has gained significant support, particularly amongst groups investigating stress-induced hypothalamic–pituitary–adrenal (HPA) axis activation (Sawchenko *et al*., 1996; Herman & Cullinan, 1997; Sawchenko *et al*., 2000). Categorization proponents generally suggest that the brain discriminates between two major types of stressor: (i) stimuli which produce actual disturbances of physiological status that overwhelm specific homeostatic mechanisms, e.g. haemorrhage or infection; (ii) stimuli which threaten the individual's current or anticipated state, e.g. social conflict, aversive environmental stimuli, predator-related cues, failure to satisfy internal drives. Stressors in the first category have been labelled with terms such as physical or systemic; those of the second category have commonly been labelled as psychological, emotional or processive.

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If the brain does deal with stressors categorically, one might predict that stressors should elicit category-specific patterns of neuronal activity in the brain. Retrospective analyses of the many reports that have appeared concerning stress-induced immediate–early gene expression have indeed provided hints that category-specific patterns occur (e.g. see Kovacs, 1998). However, interstudy differences in methods of activity mapping and analysis render conclusions tenuous. A direct, within-study comparison of neuronal recruitment patterns elicited by different stressors from different categories would clearly be more reliable but, to date, only four groups have attempted this (Sawchenko *et al*., 1996; Emmert & Herman, 1999; Abraham & Kovacs, 2000; Sawchenko *et al*., 2000; Thrivikraman *et al*., 2000). Even in these cases, only a single stressor was used to represent each category, thus limiting our confidence in the universality of the results.

In the present study we have compared, in adult male rats, the Fos induction patterns elicited by two stressors tentatively classified as physical (haemorrhage and immune challenge) and two stressors tentatively classified as psychological (noise and restraint). We have also examined the pattern of Fos induction elicited by a fifth stressor, forced swim; this stressor was selected specifically because of the difficulty that even categorization proponents have in classifying it. Most researchers would probably accept a classification of haemorrhage and immune challenge as physical stressors, and noise and restraint as psychological stressors, but general agreement on the

classification of forced swim is unlikely. Some researchers regard forced swim primarily as a physical stressor (Neumann *et al*., 1998); others regard it primarily as psychological (Cullinan *et al*., 1995); yet others regard it as a mixture of both (Harbuz & Lightman, 1989). It was our hope that, if the activity patterns elicited by our putative physical and psychological stressors were sufficiently distinct, the resulting data would settle this dispute by, in effect, allowing the brain itself to reveal its own decision.

Stress-induced neuronal activity patterns were quantified for hypothalamic paraventricular nucleus corticotrophin-releasing factor (CRF) cells, amygdala cells and medullary catecholamine cells. CRF cell responses were taken as a measure of HPA axis activation, which is regarded as a hallmark of the stress response. Our purpose in monitoring this endpoint related to our concern that other results might be skewed if the stressors under study differed significantly in strength. As for amygdala and catecholamine cells, these were chosen for detailed analysis because there is strong evidence that these populations play pivotal roles in generating the response to both physical and psychological stressors (Sawchenko *et al*., 1996; Herman & Cullinan, 1997; Dayas *et al*., 1999; Xu *et al*., 1999; Sawchenko *et al*., 2000; Buller *et al*., 2001).

Materials and methods

All experiments were carried out in accordance with protocols approved by the University of Queensland Animal Experimentation Ethics Committee. Adult male Wistar rats (n = 42, 300–400 g) were maintained under standard laboratory conditions consisting of a 12-h light : 12-h dark cycle (lights on 06.00 h) with food and water available *ad libitum*. One week prior to experimentation, animals assigned to control, haemorrhage or immune challenge groups were anaesthetized (sodium pentobarbitone, 50 mg/kg i.p.) and prepared with an indwelling femoral artery cannula which was routed under the skin and exteriorized at the nape of the neck. Twenty-four hours prior to the experiment, all animals were transferred from their home cage to an individual experimental chamber. In the case of animals prepared with a vascular cannula, the cannula was attached via a polyethylene tube to a syringe located outside the chamber. After overnight habituation to the new cage the experiment was performed the next morning, with stimulus delivery always initiated between 08.00 and 09.00 h to avoid response variations related to circadian cycles.

Treatment groups

Patterns of Fos induction were examined in the following groups: (i) controls, not exposed to any stressor, but administered 0.2 mL saline via the femoral artery cannula; n = 5; (ii) haemorrhage of 12 mL/kg, with blood removed at a rate of 1 mL/min via the femoral artery cannula; n = 6; (iii) immune challenge, 1 μg/kg bolus of interleukin-1β (IL-1β, R & D Systems Inc, Minneapolis, USA) in 0.2 mL saline delivered via the femoral artery cannula; n = 5; (iv) white noise exposure, 1 h at 100 dB with graded onset and offset over a 2–3-min period to avoid startle; n = 6; (v) restraint, 15 min wrapped in pliable wire mesh, thus avoiding body compression and overheating; n = 10; (6) forced swim, 10 min in 40 cm of 23 °C water; n = 10.

Immunocytochemistry

Two hours after initiation of the stressor, or after femoral artery saline delivery in the case of controls, animals were deeply anaesthetized with sodium pentobarbitone (80 mg/kg i.p.) and then perfused transcardially with 2% sodium nitrite solution followed by 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The brain was then removed, postfixed for 2 h at 4 °C in the same fixative solution and cryoprotected overnight (10% sucrose in 0.1 M phosphate buffer, pH 7.4 at 4 °C). After cryoprotection, serial 40-μm sections of the hypothalamus and amygdala and 50-μm sections of the medulla oblongata were cut in the coronal plane using a freezing microtome. To visualize nuclear Fos-like immunoreactivity in combination with either cytoplasmic CRF or catecholaminergic cell markers [tyrosine hydroxylase (TH); phenyl-N-methyl transferase (PNMT)], sections were subjected to a previously documented dual immunoperoxidase technique (Smith & Day, 1993). Briefly, a 1-in-4 series of forebrain sections was processed for Fos and CRF while two 1-in-5 series of brainstem sections were processed, one for Fos and TH, the other for Fos and PNMT. To ensure that no variation occurred across groups, sections from untreated (control) animals were always processed at the same time as sections from experimentals. Sections were incubated for 48 h in primary Fos antibody (1 : 100 000 rabbit polyclonal, Santa Cruz, USA), then incubated in the secondary antibody biotinylated antirabbit (1 : 300, Jackson ImmunoResearch,West Grove, PA, USA) for 2 h followed by an avidin–biotin–horseradish peroxidase complex solution (ABC, Vector Elite Kit, Burlingame, CA, USA) for a further 2 h. To visualize horseradish peroxidase activity, sections were incubated in nickel diaminobenzidine and the reaction was terminated once an optimal contrast between specific cellular labelling and nonspecific background labelling was reached. This was reached within a time frame that only varied between 25 and 30 min in all brain regions. We have previously observed that allowing the reaction to proceed longer than 30 min does not increase the number of cells that demonstrate Fos-like immunoreactivity, but rather results in non-specific deposition of reaction product that diminishes the contrast between specific and nonspecific background labelling (our unpublished observations). For cytoplasmic antigen detection, forebrain and brainstem sections were then divided into appropriate series and incubated for 36 h at 4 °C in the following antibodies: CRF (1 : 25 000 polyclonal; Peninsula Laboratory Inc. Belmont, CA, USA), TH (1 : 40 000 monoclonal, Incstar, Stillwater, Minnesota, USA) or PNMT (1 : 30 000 polyclonal, Incstar). The sections were incubated for 2 h in biotinylated antimouse (for TH; 1 : 400, Jackson ImmunoResearch) or antirabbit (for PNMT & CRF; 1 : 400, Jackson ImmunoResearch) followed by an avidin–biotin–horseradish peroxidase complex solution (ABC, Vector Elite Kit) for a further 2 h. Finally, to visualize horseradish peroxidase activity, sections were incubated in diaminobenzidine (nickel omitted) and the reaction was terminated once an optimal contrast between specific cellular labelling and nonspecific background labelling was reached. Sections were mounted on chrome-alum subbed slides, dehydrated in a series of alcohols, cleared in xylene and cover-slipped.

Analysis

Fos-positive cells were counted in the paraventricular nucleus, amygdala and medullary catecholamine cell groups by staff blind to treatment. Fos-positive CRF cells of the medial parvocellular paraventricular nucleus (mpPVN) were counted bilaterally over two sections corresponding to the antero-medial and postero-lateral subdivisions of the posterior magnocellular PVN because these levels contain the majority of median eminence-projecting mpPVN CRF cells (Swanson *et al*., 1983; Cunningham & Sawchenko, 1988). In the amygdala almost all Fos-positive cells were confined to the medial nucleus of the amygdala (MeA) and central nucleus of the amygdala (CeA) and these were counted bilaterally at 160-μm intervals over five rostrocaudal levels, their boundaries being

determined by cytoarchitectonic features mapped in corresponding Nissl stained sections. In both the ventrolateral medulla (VLM) and the nucleus of the solitary tract (NTS), overlapping populations of noradrenergic cells and adrenergic cells occur. As PNMT is a unique marker for adrenergic cells, PNMT-positive cells were automatically counted as C1 (VLM) or C2 (NTS) adrenergic cells. As TH is a marker for both noradrenergic and adrenergic cells, estimates of noradrenergic cell numbers were obtained by subtracting numbers of PNMT-positive cells in one section from numbers of TH-positive cells in the adjacent section, as previously described (Buller *et al*., 1998). Using this procedure, counts of Fos-positive adrenergic and noradrenergic cells were made over 19 consecutive levels of the medulla from –2.5 mm caudal to obex to +2.0 mm rostral to obex at 250-μm intervals. To convey some sense of the extent of recruitment within each catecholamine cell group, the percentage of each population expressing c-*fos* was estimated in relation to the total number of A1, A2, C1 and C2 cells that we routinely observe within the 19 sections analysed (i.e. 500 A1, 550 A2, 200 C1 and 80 C2 cells).

Initially raw counts of Fos-positive cells from each neuronal population of interest were tested for equality of variance using Bartlett's test. This test demonstrated that the difference among the standard deviations was significant in all cases. To normalize the data a log transformation was then performed. This was validated by re-testing transformed data using Bartlett's tests. Total counts from the PVN, amygdala and medullary catecholamine cells were compared using a one-way ANOVA followed by Student–Newman–Keuls (SNK) *post hoc* tests. The distributions of Fos-positive catecholamine cells along the rostrocaudal axis of the medulla were compared on the basis of modal level. Thus, the rostrocaudal level containing the greatest number of Fos-positive A1, A2, C1 or C2 cells was determined for each animal and a mean ± SEM then derived for each type of stressor. A one-way ANOVA followed by SNK *post hoc* tests was then used to compare the average modal score for the particular cell groups across the different stressors. In all figures and tables, results are expressed as the mean ± SEM.

Some of the data presented in this report has been previously described, but not quantitatively analysed in the manner required for the purposes of the present study (Buller *et al*., 1999a, b).

Results

Stressor effects on mpPVN CRF cells

In control animals, which had been cannulated arterially seven days before, transferred to testing chambers one day before, and given an intra-arterial bolus of saline 2 h before being killed, the PVN was virtually devoid of Fos-positive cells. In contrast, substantial numbers of Fos-positive mpPVN CRF cells were apparent in sections taken from animals subjected to either immune challenge, haemorrhage, restraint, noise or forced swim ($F = 226$, $P < 0.0001$; Figs 1 and 2). Notably, there were no statistically significant differences between the stressors in terms of their effect on mpPVN CRF cell recruitment ($F = 2$, $P < 0.14$). Moreover, the level of recruitment of the mpPVN

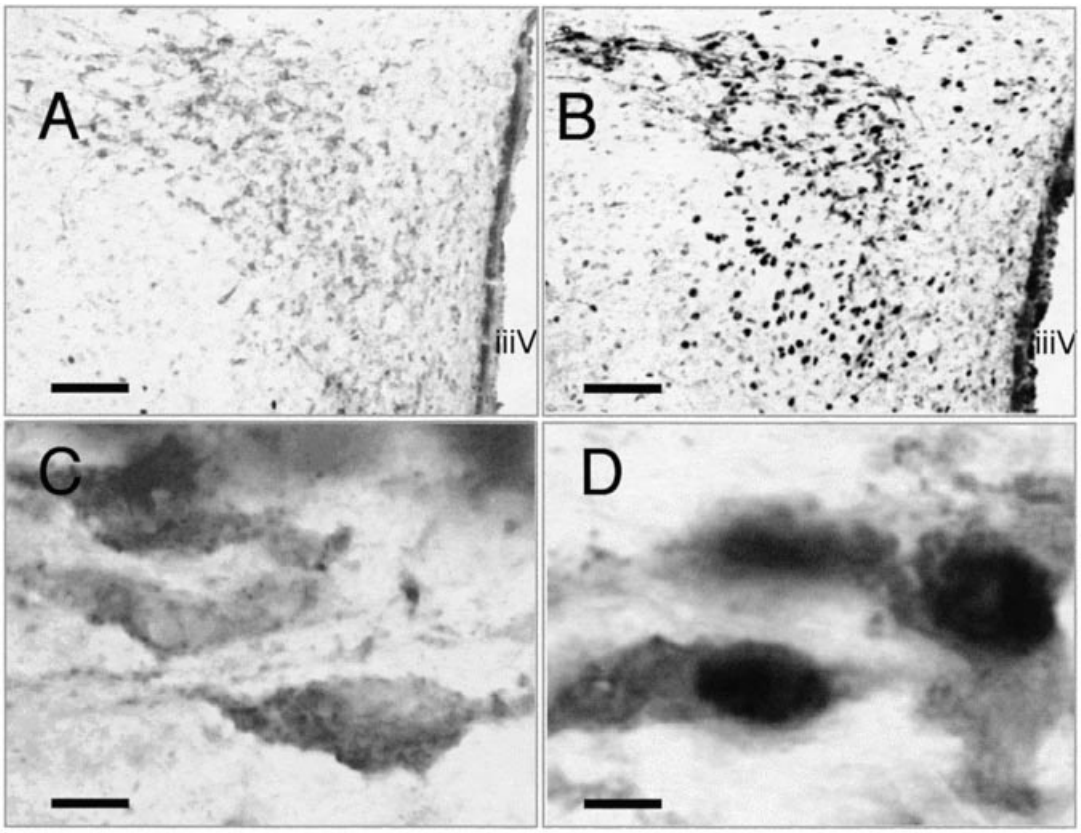

FIG. 1. (A and B) Low and (C and D) high power photomicrographs of coronal PVN sections taken from (A and C) a control animal and (B and D) an animal that had been subjected to restraint. All sections were immunolabelled for Fos and CRF. In controls (A and C), CRF immunoreactivity was apparent in the cytoplasm of many mpPVN cells. In stressed animals (B and D), large numbers of mpPVN CRF cells colocalized Fos immunoreactivity in their nuclei. iiiV, third ventricle. Scale bars, 100 μm (A and B), 5 μm (C and D).

CRF cell population was almost certainly submaximal as Fos-positive CRF cells accounted for no more than one-third of the ≈1200 mpPVN CRF cells normally discernible in the sections selected for quantification.

Stressor effects on amygdala cells

Very few Fos-positive cells were seen in the amygdala of control animals (Fig. 3A and B). In contrast, sections from animals subjected to any of the stressors under investigation contained substantial numbers of Fos-positive cells in the amygdala, although they were largely restricted to the MeA ($F = 70$, $P < 0.0001$) or the CeA ($F = 30.187$, $P < 0.0001$). It was particularly noticeable that CeA activation predominated in animals subjected to haemorrhage or immune challenge, whereas MeA activation predominated in animals subjected to noise, restraint or forced swim (Figs 3C–F and 4). Thus, the number of Fos-positive MeA cells observed in animals subjected to immune challenge or haemorrhage was significantly smaller than seen after noise, restraint or forced swim. Conversely, the number of Fos-positive CeA cells observed in animals subjected to immune challenge or haemorrhage was significantly greater than observed after noise, restraint or forced swim.

Because PVN and amygdala sections were processed together, we routinely visualized CRF as well as Fos in the CeA. However, it was very rare to find Fos colocalized in any of the CRF cells located in that region, regardless of the stressor used (Fig. 3).

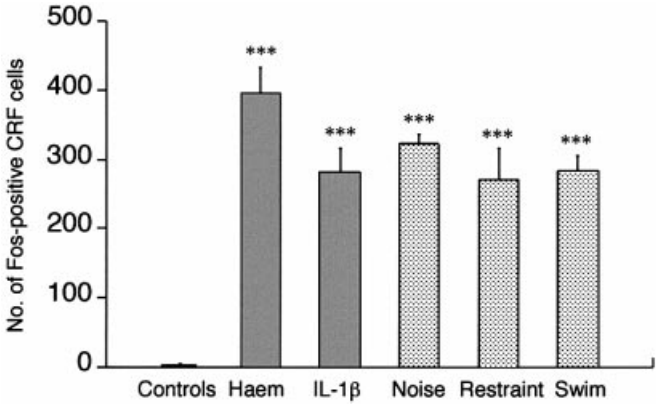

FIG. 2. Effect of haemorrhage (Haem), immune challenge (IL-1β), noise, restraint and forced swim on numbers of Fos-positive CRF cells in the medial parvocellular division of the PVN. All groups subjected to a stressor displayed significantly higher counts than controls (***$P < 0.001$; SNK *post hoc* tests) but there were no significant differences between stressors.

Stressor effects on medullary catecholamine cells

In control animals, the medulla oblongata contained almost no Fos-positive A1 noradrenergic, A2 noradrenergic, C1 adrenergic or C2 adrenergic cells. In contrast, stressed animals displayed substantial numbers of Fos-positive catecholamine cells in both the ventrolateral medulla (A1, $F = 30$, $P < 0.0001$; C1, $F = 36$, $P < 0.0001$) and dorsomedial medulla (A2, $F = 13$, $P < 0.0001$; C2, $F = 10$, $P < 0.0001$; Table 1, Fig. 5). In absolute numbers, the recruitment of noradrenergic cells exceeded the recruitment of adrenergic cells, the C2 cell population usually displaying particularly small numbers of Fos-positive cells. Nevertheless, with the single exception of C2 cells in noise-stressed animals, numbers of Fos-positive A1 noradrenergic, A2 noradrenergic, C1 adrenergic and C2 adrenergic

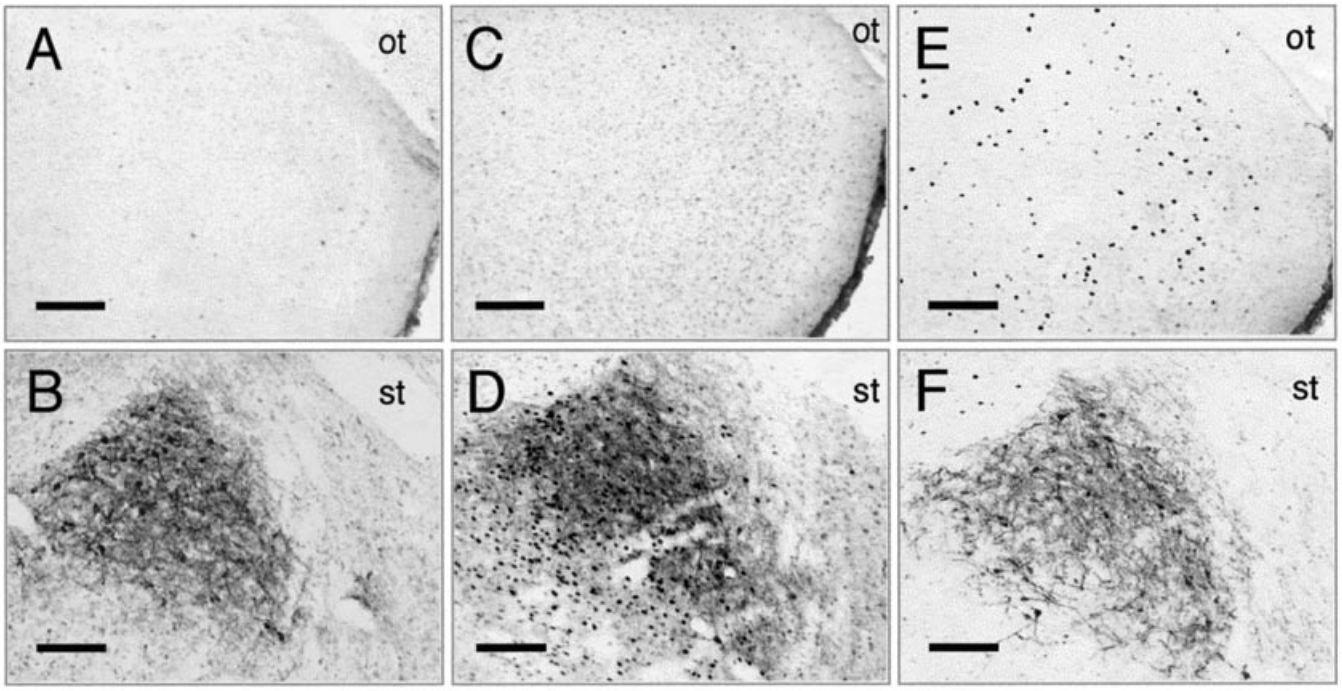

FIG. 3. Coronal sections through the left hand side of (A, C and E) the medial and (B, D and F) the central amygdala of (A and B) a control, (C and D) an animal subjected to an immune challenge and (E and F) an animal subjected to restraint. All sections were immunolabelled for Fos and CRF, the latter revealing the well-known population of CRF fibres and cell bodies within the CeA (B, D and F). Few if any Fos-positive cells were observed in (A) the MeA or (B) CeA of control animals. (D) Immune challenge elicited c-*fos* expression primarily in the CeA, although it should be noted that none of the Fos-positive cells were also CRF-positive. Restraint elicited c-*fos* expression primarily in (E) the MeA rather than (F) the CeA. ot, optic tract; st, stria terminalis. Scale bars, 100 μm.

cells were all significantly increased relative to controls, regardless of stressor type. None of the catecholamine cell groups displayed evidence of response saturation; the largest proportion of a particular cell population displaying Fos-like immunoreactivity was 30% in the case of A1 cells after forced swim or C1 cells after an immune challenge.

By far the most novel aspect of the data obtained in relation to stress-induced recruitment of catecholamine cells was revealed only by examining the distribution of Fos-positive catecholamine cells along the rostrocaudal axis of the medulla. In animals subjected to immune challenge or haemorrhage, Fos-positive A1 ($F = 30$, $P < 0.0001$) and A2 ($F = 24$, $P < 0.0001$) noradrenergic cells were located at significantly more rostral levels than in animals subjected to noise, restraint or forced swim (Table 1 and Figs 5 and 6). However, no such trend was apparent in the case of C1 ($F = 0.86$, $P = 0.43$) and C2 adrenergic cells ($F = 2.2$, $P = 0.13$; Table 1 and Fig. 7).

Discussion

Even amongst researchers who consider it likely that the brain deals with stressors according to category, there has been considerable disagreement as to how such categories should be defined and labelled. However, such debates can obscure the fact that what is important is how the brain categorizes stressors, not how researchers categorize them. The definition and naming of stressor categories by researchers amounts to little more than the initial formulation of an hypothesis as to how the brain is organized. The significance of the present data derive from the fact that they offer strong support for the hypothesis that the brain recognizes at least two major categories of stressor and that the neural pathways activated by these stressors vary accordingly. Moreover, the present data suggest that the neural activation footprint that is left by stressors can be used to determine the category to which they are assigned by the brain.

Effects of stressors on HPA axis CRF cells

The CRF cells of the mpPVN constitute the functional apex of the HPA axis and previous work from this laboratory has shown a strong correlation between plasma adrenocorticotrophic hormone levels and the number of Fos-positive mpPVN CRF cells in individual animals subjected to a stressor (Buller *et al*., 1998). Consistent with their designation as stressors, all five treatments applied in the present

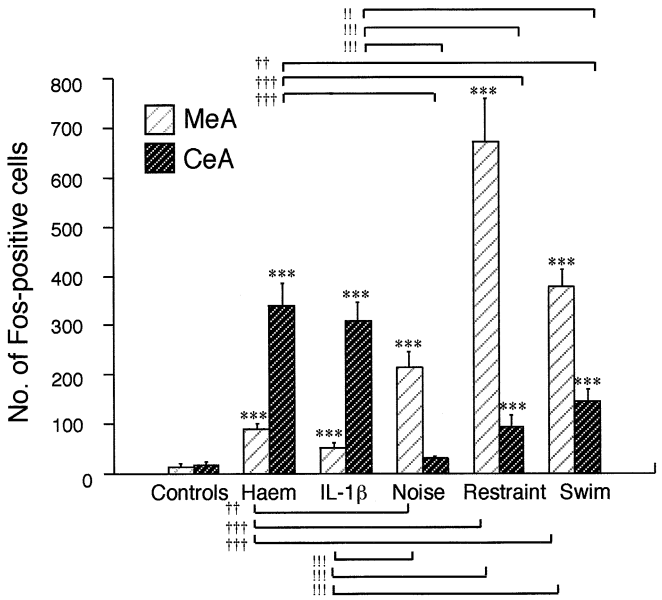

FIG. 4. Effect of haemorrhage (Haem), immune challenge (IL-1β), noise, restraint and forced swim on numbers of Fos-positive cells in the medial and central amygdala. In the case of the physical stressors, haemorrhage and immune challenge, the main effect was on CeA cells. The reverse was true for all other stressors. ***$P < 0.001$ vs. controls; $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. haemorrhage; $^{!!}P < 0.01$, $^{!!!}P < 0.001$ vs. immune challenge, (SNK *post hoc* tests).

TABLE 1. Recruitment of medullary noradrenergic (A1, A2) and adrenergic (C1, C2) cells by exposure of animals to immune challenge, haemorrhage, noise, restraint or forced swimming

Cell population	Controls	Haemorrhage	Immune challenge (IL-1β)	Noise	Restraint	Forced swimming
Ventrolateral medulla						
Total A1 cells (mean ± SEM)	1 ± 1	123 ± 38***	80 ± 10***	57 ± 10***	60 ± 13***	150 ± 16***
A1 cells expressing c-*fos* (%)	0	25	17	11	12	30%
A1 cells, rostrocaudal level	NA	0.4 ± 0.1	-0.1 ± 0.2	–1.3 ± 0.4†††‡‡	–1.5 ± 0.1†††‡‡	–1 ± 0.0†††‡
Total C1 cells (mean ± SEM)	1 ± 1	48 ± 4***	60 ± 9***	8 ± 2***†††‡‡‡	26 ± 4***	31 ± 8***
C1 cells expressing c-*fos* (%)	0	24	30	4	13	16%
C1 cells, rostrocaudal level	NA	0.5 ± 0.1	0.3 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.9 ± 0.1
Dorsomedial medulla						
Total A2 cells (mean ± SEM)	1 ± 1	42 ± 11**	112 ± 9***	102 ± 11***††	101 ± 16***†	109 ± 10***††
A2 cells expressing c-*fos* (%)	0	7	20	19	18	20%
A2 cells, rostrocaudal level	NA	0.1 ± 0.1	0.0 ± 0.1	–1 ± 0.2†††‡‡‡	–0.7 ± 0.1†††‡‡‡	–1 ± 0.1†††‡‡‡
Total C2 cells (mean ± SEM)	1 ± 1	20 ± 3***	7 ± 2**	3 ± 1†††	5 ± 1**†	8 ± 2**††
A2 cells expressing c-*fos* (%)	0	25	9	3	6	10%
A2 cells, rostrocaudal level	NA	0.7 ± 0.1	1.0 ± 0.2	0.8 ± 0.1	0.6 ± 0.1	1.0 ± 0.1

For each cell group and each stressor three parameters are shown: the average number (± SEM) of Fos-positive cells, the approximate proportion of the cell group that was Fos-positive (based on the total population usually detectable in these sections), and the rostrocaudal level relative to obex at which peak numbers of Fos-positive cells were observed (see Materials and methods for descriptions of how these figures were derived). Significance of difference (SNK post-hoc tests) compared to controls: *$P < 0.05$, **$P < 0.01$, < ***$P < 0.001$. Significance of difference (SNK post-hoc tests) compared to haemorrhaged animals: †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$. Significance of difference (SNK post-hoc tests) compared to immune challenged animals: ‡$P < 0.05$, ‡‡$P < 0.01$, $^{‡‡‡}P < 0.001$.

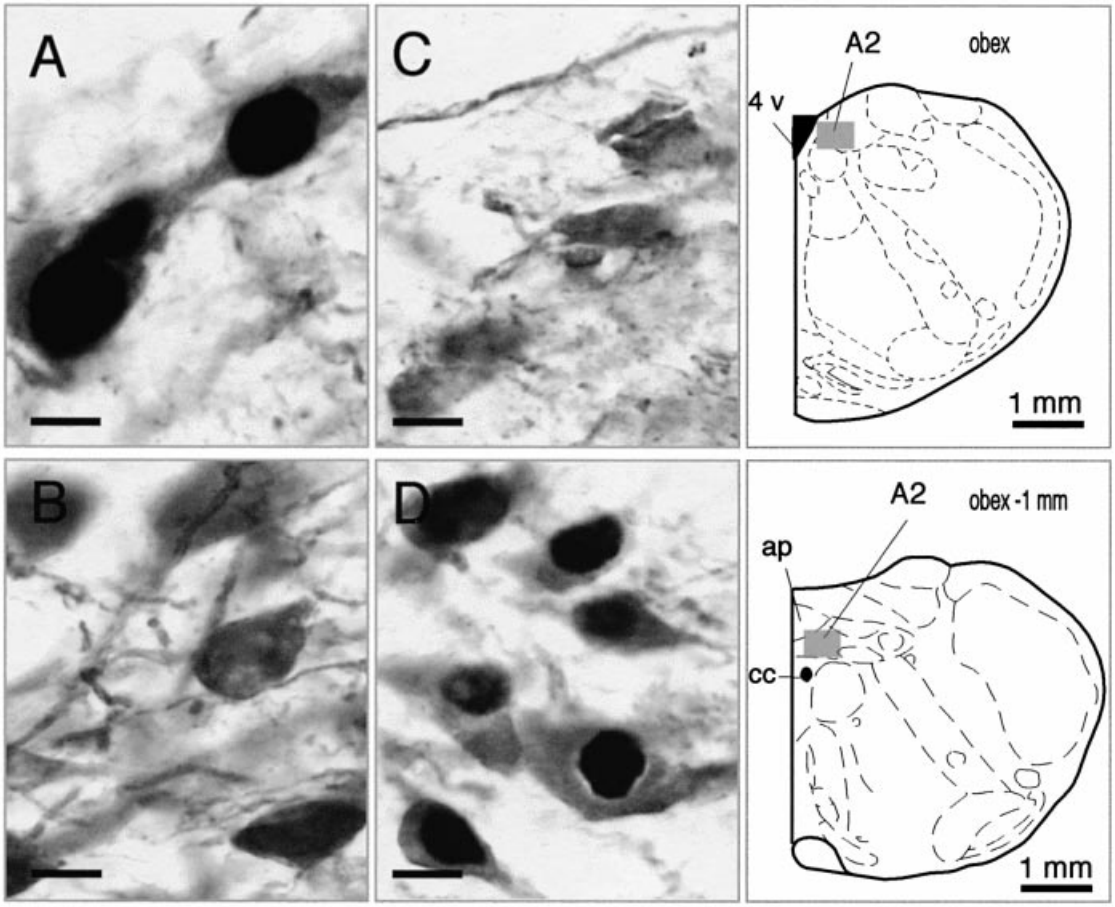

FIG. 5. Photomicrographs illustrating the differing effects of (A and B) immune challenge and (C and D) restraint on A2 noradrenergic cell c-*fos* expression, (A and C) at the level of the obex and (B and D) 1.0 mm caudal to the obex. All sections were immunolabelled for Fos and tyrosine hydroxylase. Adjacent line drawings at the right hand side provide a low power representation of the levels corresponding to these coronal sections. Immune challenge elicited peak c-*fos* expression amongst A2 cells the level of the obex (A) whereas restraint elicited peak A2 cell c-*fos* expression more caudally (D). ap, area postrema; cc, central canal; 4v, fourth ventricle. Scale bars, 10 μm.

study elicited c-*fos* expression in approximately one third of the mpPVN CRF cell population. Although HPA axis activation is just one of the endpoints of the stress response, these data do suggest that the stressors were of comparable strength, yet still submaximal.

Contrasting patterns of amygdala cell recruitment

The present study provides strong evidence that physical stressors (e.g. immune challenge and haemorrhage) preferentially activate CeA cells while psychological stressors (e.g. restraint and noise) preferentially activate MeA cells, confirming impressions gained from a retrospective analysis of previous reports concerning stress-induced c-*fos* expression in the amygdala (Arnold *et al*., 1992; Honkaniemi *et al*., 1992; Ericsson *et al*., 1994; Li & Dampney, 1994; Chen & Herbert, 1995; Cullinan *et al*., 1995; Traub *et al*., 1996; Bonaz & Tache, 1997; Campeau & Watson, 1997; Bhatnagar & Dallman, 1998; Buller *et al*., 1998; Li & Sawchenko, 1998; Martinez *et al*., 1998; Xu *et al*., 1999; Dielenberg *et al*., 2001). The differential sensitivity of CeA and MeA cell populations to physical vs. psychological stressors is also supported by the handful of previous reports in which stressors presumed to represent the different categories were directly compared. Thus, Emmert & Herman (1999) reported that although exposure to ether fumes had no effect on MeA c-*fos* expression, open field exposure triggered a substantial rise. Thrivikraman *et al*. (2000) reported greater CeA activation after haemorrhage than after air puff. Sawchenko *et al*. (1996, 2000) reported that immune challenge recruited fewer MeA cells but more CeA cells than did footshock, while Abraham & Kovacs (2000) found that ether exposure recruited fewer MeA cells but more CeA cells than did restraint. It must be noted, however, that the latter two groups provided qualitative rather than quantitative data.

Contrasting patterns of catecholamine cell recruitment

Previous studies have clearly established that medullary catecholamine cells are recruited in considerable numbers by both physical and psychological stressors. Reports on the effects of psychological stressors on catecholamine cell c-*fos* expression (Ceccatelli *et al*., 1989; Pezzone *et al*., 1993; Senba *et al*., 1993; Chen & Herbert, 1995; Palkovits *et al*., 1997) have been less numerous than those concerning effects of physical stressors, but it has generally been held that there is relatively little difference in the patterns of medullary catecholamine recruitment that they elicit; indeed, one group has referred to the patterns as 'essentially indistinguishable' (Li *et al*., 1996). This view is perfectly consistent with our own initial impressions of the present data when analysis was restricted to a consideration of the overall

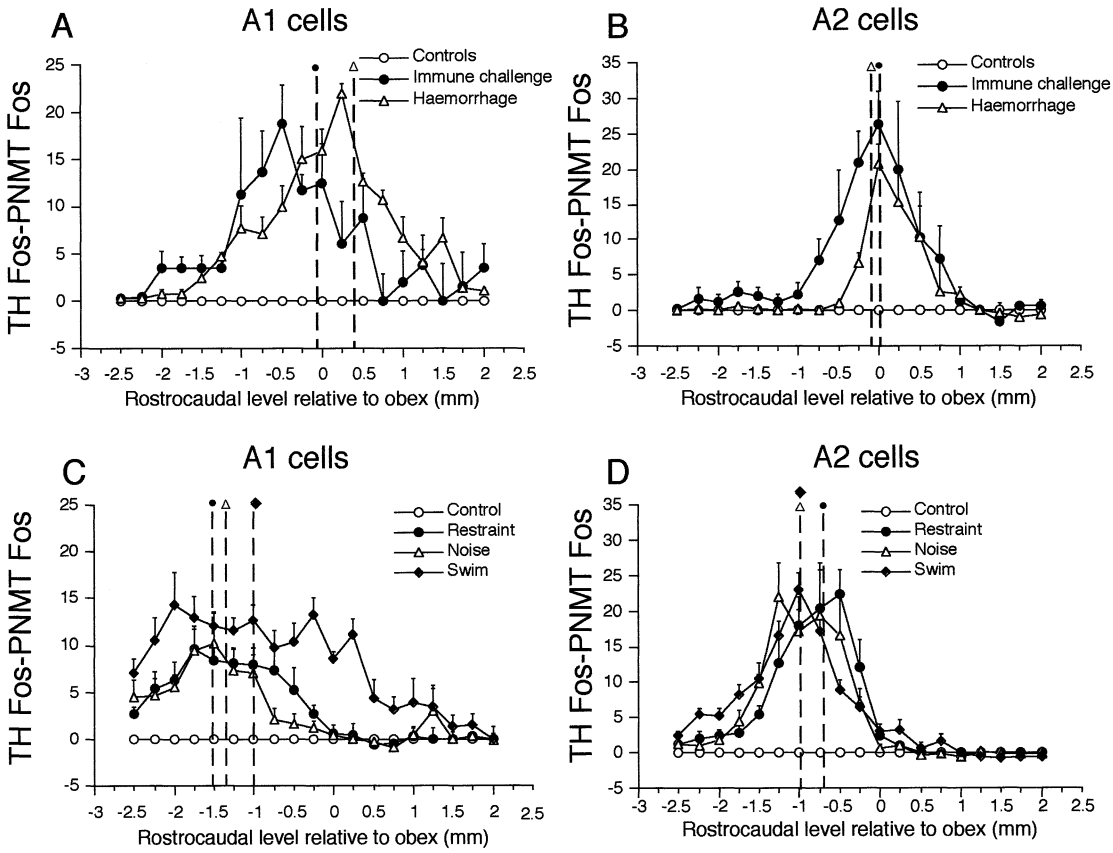

FIG. 6. Plots of Fos-positive noradrenergic (A and C) A1 cell and (B and D) A2 cell numbers in animals subjected to physical stressors (A and B, immune challenge or haemorrhage) or psychological stressors (C and D, restraint, noise or forced swim). Vertical bars indicate the average rostrocaudal level at which the greatest numbers of Fos-positive cells were seen for that particular stressor category. Physical stressors elicited c-*fos* expression in significantly more rostral populations of A1 and A2 cells than did the other stressors tested.

number of Fos-positive cells; all of the stressors recruited significant numbers of A1, A2 and C1 cells, and smaller numbers of C2 cells. However, upon analysing the rostrocaudal distribution of Fos-positive catecholamine cells, a striking category-specific difference in recruitment pattern became apparent; immune challenge and haemorrhage were found to recruit significantly more rostral populations of A1 and A2 noradrenergic cells than noise or restraint.

The idea that physical and psychological stressors might recruit different subpopulations of medullary catecholamine cells is in keeping with evidence concerning catecholaminergic control of stress-induced HPA axis activation. Thus, medullary catecholamine cells are thought to contribute to HPA axis responses to both physical and psychological stressors, but only in the case of physical stressors does this involve a direct input to PVN CRF cells (Chuluyan *et al*., 1992; Li *et al*., 1996; Buller *et al*., 2001; Dayas *et al*., 2001). A plausible explanation for this finding is that categorically separate stressors recruit catecholamine cell populations that differ in their connection with mpPVN CRF cells. At the current time, however, we have no means of further testing this proposal. Both physical stressors, such as immune challenge and haemorrhage (Chan & Sawchenko, 1994; Ericsson *et al*., 1994; Buller *et al*., 2001), and psychological stressors, such as footshock and restraint (Li & Sawchenko, 1998; Dayas *et al*., 2001), recruit PVN-projecting medullary catecholamine cells; however, there is no way of determining whether they differ in their synaptic connection with mpPVN CRF cells.

Forced swim: assignment to a category or placement within a spectrum?

In previous reports, forced swim has been variously categorized as physical, psychological or a mixture of both (e.g. see Harbuz & Lightman, 1989; Cullinan *et al*., 1995; Neumann *et al*., 1998). In the present study, forced swim produced c-*fos* expression patterns that were very similar to those produced by noise and restraint, and significantly different from those elicited by immune challenge and haemorrhage. Thus, in the amygdala, forced swim elicited a response pattern in which MeA activation predominated over CeA activation; in the medulla, the average peak of A1 and A2 noradrenergic cell recruitment along the rostrocaudal axis was located well caudal to obex. We interpret these results as indicating that the brain places forced swim in the same category as noise and restraint, stressors that we tentatively classified as psychological. However, we acknowledge that such an interpretation entails the assumption that the brain assigns stressors to mutually exclusive categories rather than placing them along a spectrum ranging from purely physical to purely psychological. Some readers might argue that, because forced swim tended to elicit CeA and A1 cell activation patterns that were intermediate between that produced by our representative physical

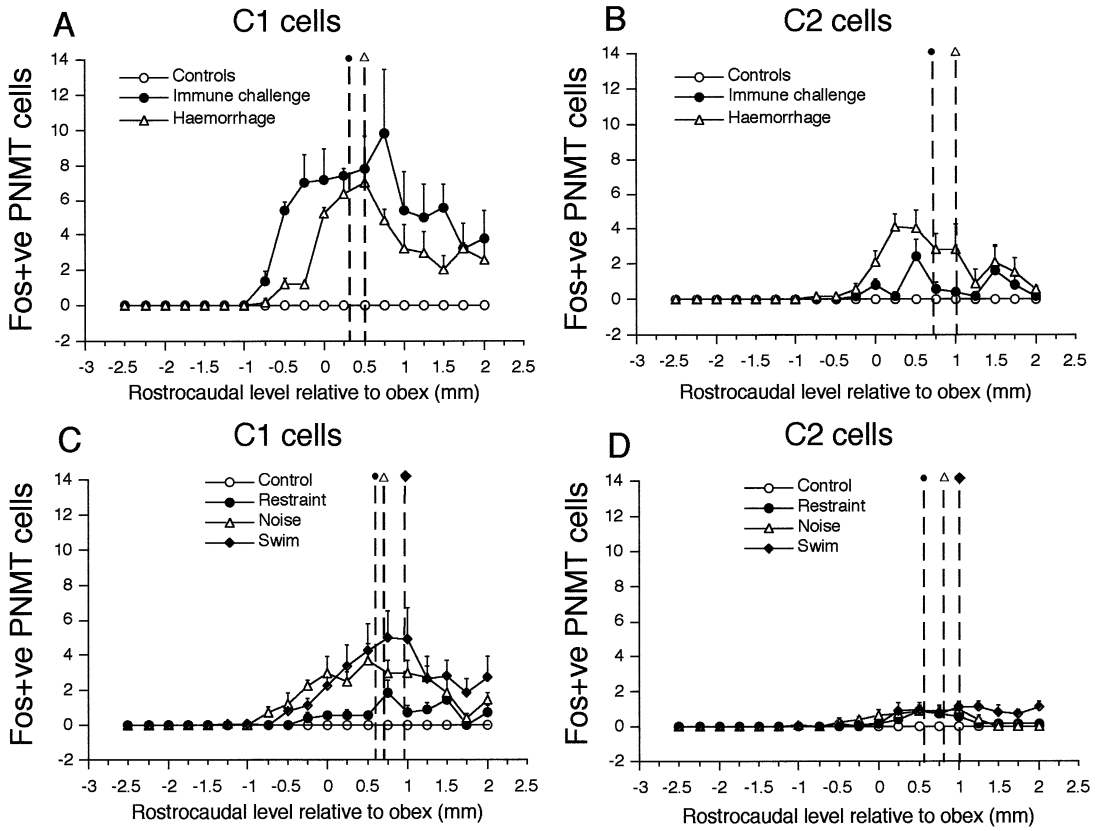

FIG. 7. Plots of Fos-positive adrenergic (A and C) C1 cell and (B and D) C2 cell numbers in animals subjected to physical stressors (A and B, immune challenge or haemorrhage) or psychological stressors (C and D, restraint, noise or forced swim). Vertical bars indicate the average rostrocaudal level at which the greatest numbers of Fos-positive cells were seen for that particular stressor category. The were no significant differences between stressors in terms of the rostrocaudal level at which peak adrenergic cell recruitment occurred.

and psychological stressors, forced swim might best be considered as a mixture of physical and psychological, i.e. it sits part way along a spectrum, albeit closer to the psychological end. We reject this argument primarily because the statistical analysis shows that, despite certain apparent trends, forced swim elicits patterns of both CeA and A1 activation that are significantly different from immune challenge and haemorrhage, just as is the case for noise and restraint. Moreover, the view that the brain assigns stressors to mutually exclusive categories receives additional support from recent findings concerning the role of the amygdala and PVN catecholamine afferents in mediating HPA axis responses to restraint and immune challenge. Thus, in relation to the amygdala, it has been reported that lesions of MeA but not CeA suppress restraint-induced HPA axis activation, whereas lesions of CeA but not MeA suppress immune challenge-induced HPA axis activation (Dayas *et al*., 1999; Xu *et al*., 1999). In relation to PVN catecholamine afferents, destruction of these afferents suppresses immune challenge-induced HPA axis activation, but not restraint-induced HPA axis activation (Chuluyan *et al*., 1992; Buller *et al*., 2001; Dayas *et al*., 2001). Given that the activation patterns elicited by both restraint and immune challenge are partially overlapped (e.g. restraint does produce some CeA activation, and immune challenge does produce some MeA activation), we view the outcomes of these studies as validating an approach whereby the predominant amygdala (CeA vs. MeA) and medullary noradrenergic (rostral vs. caudal) activation patterns can be used as a defining sign of categorization of a stressor by the brain. It may well be that activity patterns in other areas can also be used in this way and clearly there are several areas, such as the lateral septum (Sawchenko *et al*., 1996, 2000) and the peri-aqueductal grey (Bandler *et al*., 2000) that merit close attention.

Conclusions

Category-specific patterns of stress-induced amygdala or medullary catecholamine cell activation have hitherto gone largely unremarked in the literature. This is likely to be at least partly due to researchers being cautious in adopting conclusions based on retrospectively collated multisourced evidence. Such caution is well-founded, for two reasons. Firstly, reports of stress-induced activation patterns are frequently subjective because many groups do not quantify their data. Secondly, only a very small number of the existing reports directly compare, in the same study, the activation patterns elicited by stressors thought to be representative of different categories. The latter point is particularly important because it is well known that the immediate–early gene detection procedures employed by different laboratories, or even in the one laboratory over a period of time, can vary considerably, both in selectivity and sensitivity. For example, some reports show substantial Fos immunolabelling even in control subjects, making patterns of specific, stress-induced c-*fos* expression more difficult to discern. Within this context, the present findings can be seen as relatively reliable and unambiguous; our Fos detection procedures were such that control animals were virtually devoid of Fos-immunolabelling, suggesting that the patterns we have observed

are stressor-specific; we carefully quantified the stress-induced patterns of cellular activation and we simultaneously compared several different stressors. Accordingly, we believe that the present data provide important and reliable evidence that psychological and physical stressors leave distinctive cellular activity 'footprints' within the brain. Moreover, the conclusion that the brain utilizes category-specific pathways to drive stress responses has significant implications for the design of experiments directed at understanding the neural basis of stress.

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Abbreviations

CeA, central nucleus of the amygdala; CRF, corticotrophin-releasing factor; HPA, hypothalamic–pituitary–adrenal; MeA, medial nucleus of the amygdala; mpPVN, medial parvocellular paraventricular nucleus; NTS, nucleus of the solitary tract; PNMT, phenyl-N-methyl transferase; SNK, Student–Newman–Keuls; TH, tyrosine hydroxylase; VLM, ventrolateral medulla.

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