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Immediate–early gene induction in hippocampus and cortex as a result of novel experience is not directly related to the stressfulness of that experience

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Abstract

The stressful quality of an experience, as perceived by rats, is believed to be largely represented by the magnitude of a hypothalamic–pituitary–adrenal (HPA) axis response. The hippocampus may be especially important for assessing the stressfulness of psychological stressors such as novel experience. If such is the case then experience-dependent immediate–early gene expression levels within the hippocampus may parallel relative levels of HPA axis activity. We examined this prospect in rats that were placed in four different novel environments (empty housing tub, circular arena, elevated pedestal or restraint tube). Restraint and pedestal produced the largest magnitude of increased ACTH and corticosterone secretion, arena an intermediate level (Experiment 2) and tub the least magnitude of increase. We saw a very similar experience-dependent pattern of relative Fos protein, *c-fos* mRNA and *zif268* mRNA expression in the paraventricular nucleus of the hypothalamus. However, in hippocampus (and select regions of cortex), immediate–early gene expression was associated with the exploratory potential of the novel experience rather than level of HPA axis activity; pedestal and arena elicited the greatest immediate–early gene expression, tub an intermediate level and restraint the least amount of expression. We conclude that the stressfulness of psychological stressors is not represented by the amount of immediate–early gene induction elicited in hippocampus and cortex, nor does there appear to be a general enhancing or depressive influence of acute stress on immediate–early gene induction in those brain regions.

Introduction

A key objective in the study of stress neurobiology is to determine the extent to which there are neural responses specifically associated with stress that constitute a central stress state. For example, are there specific brain neurocircuits that extract 'stressfulness' from an experience and coordinate stress responses? When considering this question, it may be important to distinguish between the neural processing of physical and psychological stressors (Sawchenko *et al*., 1996; Herman *et al*., 2003). Although many physical stressors directly activate homeostasic-related sensors (e.g. nociceptors, osmoreceptors, chemoreceptors and baroreceptors) that have fairly direct projections to the hypothalamic paraventricular nucleus (PVN; Watts, 1996; Herman & Cullinan, 1997; Pacak & Palkovits, 2001), many experiences that do not result in direct activation of these sensors also lead to activation of the hypothalamic–pituitary–adrenal (HPA) axis. These 'psychological stressors' are believed to represent situations that are perceived by the individual as a threat to well-being (Dayas *et al*., 2001).

One strategy that has been widely used to assess the neural processing of stressors is measurement of immediate–early gene (e.g. *c-fos*) expression in brains of animals exposed to various stressors (Sawchenko *et al*., 1996; Kovacs, 1998). These studies have identified stressor-associated increased immediate–early gene expression patterns within a number of brain regions with a focus on a network of subcortical limbic and brainstem structures (Herman & Cullinan, 1997; Senba & Ueyama, 1997; Pacak & Palkovits, 2001). Several of these studies have also noted significant induction of *c-fos* gene expression in the hippocampus (Melia *et al*., 1994; Cullinan *et al*., 1995; Ryabinin *et al*., 1995; Bozas *et al*., 1997; Kovacs, 1998; Chowdhury *et al*., 2000; Figueiredo *et al*., 2002). The hippocampus may be especially important for the higher level neural processing required for mounting a stress response to psychological stressors (Herman *et al*., 2003; Mueller *et al*., 2004). Moreover, there is evidence from some, but not all, lesion studies for the hippocampus to modulate stress-induced HPA axis activity (Magarinos *et al*., 1987; Herman *et al*., 1989; Herman *et al*., 1992; Bradbury *et al*., 1993; Feldman & Weidenfeld, 1993; Tuvnes *et al*., 2003).

To further explore the role of the hippocampus in neural responding to psychological stressors, we examined the extent to which the amount of immediate–early gene expression in hippocampus reflects the stressfulness associated with a novel experience. It is generally believed that the magnitude of HPA axis response may be used as an indicator of the relative stressfulness of an experience (Hennnessy *et al*., 1979; Armario *et al*., 1986; Dallman *et al*., 1987). In this study we placed rats in several different novel situations that we had previously found to elicit a varying amount of plasma ACTH and corticosterone secretion (Pace & Spencer, 2005). We hypothesized that there should be a correlation between the activity levels of brain regions (and associated immediate–early gene expression) involved in processing the stressfulness of these experiences and the resulting levels of HPA axis activity. As a proof of principle, we examined

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immediate–early gene expression (*c-fos* mRNA and *zif268* mRNA) in the neural component of the HPA axis (PVN). We measured concurrent immediate–early gene expression in hippocampal subregions and, for specificity comparison purposes, select regions of cortex.

Materials and methods

Subjects

Subjects were young adult (250–305 g) male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN, USA). For Experiment 1 (see below), rats were pair-housed in hanging wire mesh cages. For Experiment 2, rats were pair-housed in clear polycarbonate housing tubs (47 × 23 × 20 cm) with a woodchip layer on the floor. Rats were housed in sound-attenuated rooms that were maintained on a 12-h light–dark cycle (lights on at 07.00 h). After arrival at the University of Colorado at Boulder animal care facilities, the rats were given a 2-week acclimation period before the onset of experimentation. Rat chow and water were provided *ad libitum* except during novel experience exposure. The experimental treatments took place between 08.00 and 11.00 (i.e. during the light period of the light–dark cycle. All experiments were approved by the University of Colorado Institutional Animal Care and Use Committee.

Novel experiences

Treatments were given in a room adjacent to the rat's home room for both experiments. Rats were exposed to one of four novel experiences (tub, arena, pedestal or restraint) or were left undisturbed in their home cage (no-stress control). Tub exposure consisted of placing a rat in a clean housing tub (47 × 23 × 20 cm) with (Experiment 1) or without (Experiment 2) wood chip flooring. A metal grate lid was secured on the tub to contain the subject, which was otherwise free to move around. Arena exposure consisted of placing a rat on the floor of the test room within a large circular sheet metal enclosure (2-m diameter, 1-m-high wall). A paper mat covered the floor of the arena and the subject was free to move around within the enclosure. Pedestal exposure consisted of placing rats on a linoleum-covered wood surface (27 cm square) that was raised 60 cm off the ground by four wooden legs attached to the underside of each corner of a wood platform. A small wood lip (1 × 1 cm) was placed around the perimeter of the upperside of the wood pedestal. Restraint exposure consisted of placing rats in a well-ventilated, adjustable length, clear Plexiglas tube (6.3 cm in diameter and 15.5 ± 2.5 cm length).

Experiment 1 general procedure

The first experiment examined the effect of four different novel experiences (tub, arena, pedestal or restraint) on HPA axis plasma hormone levels and Fos protein expression (in PVN and hippocampus). Rats were randomly assigned to six treatment groups ($n = 6$–9), two of which served as no-stress control groups and four consisted of different novel experience conditions. For the novel experience groups, rats were exposed to 30 min of one of the four different novel experiences. At the end of this 30 min a blood sample was taken from the tail prior to returning the rat to its home cage. This required briefly (within 1 min) placing the rats that had been exposed to the tub, arena or pedestal into a restrainer. Two hours after the end of novel experience exposure rats were deeply anaesthetized and perfused transcardially with solutions (see Immunohistochemistry procedure). In previous time-course studies we found peak Fos expression in PVN and hippocampus 1.5–2.5 h after restraint onset (Fevurly & Spencer, 2004). One group of control rats was left undisturbed in the home cage until time of perfusion. A second control group was used to examine the extent to which taking a blood sample in otherwise undisturbed rats would affect Fos levels at the time-point examined. Those rats were removed from their home cage for a tail blood sample 2 h before time of perfusion. Experimentation was conducted with three separate cohorts of rats (two or three rats per treatment group per cohort).

Experiment 2 general procedure

The second experiment examined the effect of three different novel experiences on HPA axis plasma hormone levels and immediate–early gene expression (*c-fos* mRNA and *zif268* mRNA) in PVN, hippocampus and several cortical brain regions. Rats were randomly assigned to 10 treatment groups ($n = 8$ per group). A control group of no-stress rats were killed immediately after removal from their home cage. The remaining rats were exposed to either 15, 30 or 60 min of one of three different novel experiences (tub, arena or restraint). The experiment was conducted with two separate cohorts of 40 rats and all treatment groups were counterbalanced between cohorts.

Blood sample procedures and plasma hormone measures

Blood samples were collected into EDTA-coated tubes either from the tip of the tail (Experiment 1; tail clip method, 300 μL samples) or from the trunk following decapitation (Experiment 2). Plasma samples were stored at −80 °C.

Plasma corticosterone and ACTH were measured by radioimmunoassay (Ginsberg *et al.*, 2003). The corticosterone assay measures total (bound and unbound) plasma corticosterone and has an assay sensitivity of 0.5 μg/100 mL for a 20-μL sample. Within- and between-assay coefficients of variability were < 5%. The ACTH assay has an assay sensitivity of 25 pg/mL for a 25-μL sample. Within- and between-assay coefficients of variability were 10 and 13%, respectively.

Fos immunohistochemistry

Rats from Experiment 1 were deeply anaesthetized with an intraperitoneal injection of ketamine (75 mg/kg) and xylazine (15 mg/kg) and were then perfused transcardially via the ascending aorta. Rats were first perfused with 400 mL of 0.01 M phosphate-buffered saline and then 400 mL of 4% paraformaldehyde in 0.1 M phosphate buffer. Perfusion solutions were kept at 4 °C during the perfusion procedure. Brains were removed and stored at 4 °C in 4% paraformaldehyde–phosphate buffer solution for 48–72 h. Brains were sectioned (50 μm coronal sections) using a vibratome (The Vibratome Company, St Louis, MO, USA). Brain sections were incubated overnight in rabbit anti-Fos antibody (sc-52; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactivity was visualized using the ABC method (Vector Laboratories, Inc.). Diaminobenzidine tetrahydrochloride (0.2 mg/mL), with nickel ammonium sulphate (6 mg/mL) intensification, was used as chromogen. Normal goat serum (1.5%) (Vector) and 0.3% triton-X were included in the incubations to limit nonspecific binding of antibodies and increase membrane permeability, respectively.

The number of Fos-immunoreactive cells in the PVN (≈ 1.8 mm posterior to bregma) and dorsal hippocampus (≈ 3.3 mm posterior to

bregma) were manually counted from digitized images viewed on a computer monitor with the aid of a computerized image analysis program (NIH Image v1.62). PVN cell counts were performed on the entire PVN (both medial parvicellular and lateral magnocellular regions); however, the majority of the positive immunostaining was localized to the medial parvicellular portion of the PVN (see Results).

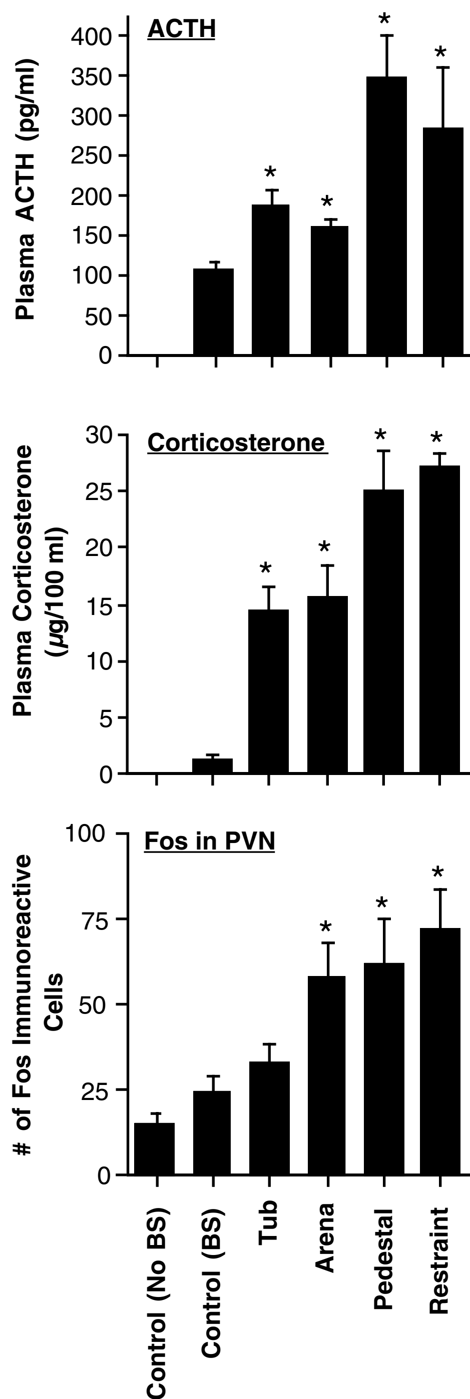

FIG. 1. Effect of four novel experiences on HPA axis hormone levels and Fos protein expression in PVN. Blood samples were taken immediately after 30 min of novel experience (tub, arena, pedestal or restraint) exposure. A blood sample (BS) was also taken from one of the no-stress control groups. Two hours after the end of novel experience exposure rats were perfused with fixative for subsequent Fos immunoreactivity measures. *$P < 0.05$ vs. no-stress control rats with blood sample (FLSD; $n = 6$–9).

Hippocampal cell counts were performed on hippocampal subdivisions (CA1-CA2, CA3, inner blade of dentate gyrus and outer blade of dentate gyrus) using the rat brain atlas of Paxinos & Watson (1998) as a visual guide. Only cells located within the principal cell body layer of each hippocampal subdivision were included in the counts. Four sections per brain region were analysed from each animal. Separate counts were performed for each hemisphere, and the eight independent estimates per brain were averaged. Sections were analysed in a pseudorandom order and the individual performing the cell counts was not aware of the treatment group assignment.

In situ *hybridization for c-fos mRNA and zif268*

Rats from Experiment 2 were rapidly decapitated by guillotine. Whole brains were rapidly removed, frozen in chilled isopentane (−35 °C) and then stored at −80 °C. Brains were sectioned (10 μm thick) using a cryostat (Leica, Nussloch, Germany). Sections were saved for subsequent analysis from the rostral–caudal level of the PVN (≈ 1.8 mm posterior to bregma), dorsal hippocampus (≈ 3.3 mm posterior to bregma) and ventral hippocampus (≈ 5.8 mm posterior to bregma). Brain sections were thaw-mounted on polylysine-coated slides and then stored at −80 °C for subsequent *in situ* hybridization analysis.

Sections from all three rostral–caudal levels of each brain were assayed for *c-fos* mRNA expression. Adjacent sections from each brain at the level of the PVN were also assayed for *zif268* (also known as *NGFI-A*, *Krox-24* and *Egr-1*) mRNA expression. Radiolabelled (35S-UTP, 35S-CTP) antisense riboprobes complementary to *c-fos* mRNA (Dr Tom Curran, St Jude Children's Research Hospital, Memphis, TN, USA) or *zif268* mRNA (Dr James Herman) were generated in an *in vitro* transcription assay. Tissue was fixed in 4% paraformaldehyde, acetylated (0.1 M triethanolamine, pH 8.0, and 0.25% acetic anhydride) and dehydrated prior to exposure to riboprobe. The selected radioactive riboprobes were diluted in hybridization buffer (Campeau *et al*., 1997) and 65 μL was placed on each slide (1–2 × 10^6 c.p.m. per slide) and then covered with a glass coverslip. Slides were then placed in sealed plastic incubation chambers lined with chromotography paper dampened with 50% formamide and incubated overnight at 55 °C. After overnight incubation cover slips were removed and the slides washed and then incubated in RNAse A (200 μg/mL) for 1 h at 37 °C. Slides were then subjected to a high stringency incubation (15 mM sodium chloride, 1.5 mM sodium citrate, pH 7.4) for 1 h at 70 °C. After drying, slides were exposed to X-ray film (Kodak XAR) for 1–3 weeks.

TABLE 1. Experiment 1: Pearson correlation coefficient (r) between the number of Fos-positive cells and plasma corticosterone or ACTH for PVN and hippocampal subregions

	Pearson correlation coefficient (r)	
Region	Fos and corticosterone	Fos and ACTH
PVN	0.53***	0.45**
CA1-CA2	0.24	−0.02
CA3	−0.10	0.03
DG		
Inner blade	−0.11	−0.05
Outer blade	−0.27	−0.29

Correlation coefficients were calculated using data from all rats in Experiment 1 except the no-blood sample control rats ($n = 39$). ***$P < 0.001$, **$P < 0.01$.

Images from autoradiograms were digitized and analysed with the aid of image analysis software (NIH Image v1.62). Analysis was performed without concurrent knowledge of the treatment group assignment for each image. Four brain slices were analysed for each brain and separate measures were performed for each hemisphere yielding eight independent estimates per brain per brain region. The average of these eight estimates was used as the final value for each brain. Data are expressed as either integrated grey level (Campeau *et al*., 2002) or, in cases where the signal was restricted to a narrow cell layer (hippocampus, piriform cortex, cortical amygdaloid), average uncalibrated optical density within regions of interest. Quantification of the primary somatosensory cortex (barrel field), primary motor cortex, piriform cortex and cortical amygdaloid were performed on the same sections that contained the PVN of the hypothalamus. Separate sections were collected, assayed and analysed at the level of the dorsal hippocampus (no ventral hippocampus present) or ventral hippocampus (ventral hippocampus prominent). All six cell layers were included in analysis of the primary somatosensory and motor cortex. Only the densest layer was analysed within the piriform and cortical amygdaloid. For hippocampal analysis, the principal cell layer was subdivided into the CA1-CA2 and CA3 subregions and the inner and outer blades of the dentate gyrus. Approximate locations of brain regions were determined based on visual comparison of autoradiograms with images in the rat brain atlas of Paxinos & Watson (1998).

Statistical analysis

ANOVA was used to determine whether the experimental treatments produced an overall statistically significant ($P < 0.05$) effect on the dependent measures. Fisher's least significant difference test (FLSD) was used for *post hoc* comparisons of specific group differences. A between-groups one-way ANOVA followed by FLSD was used to analyse data from Experiment 1. For Experiment 2, a one-way ANOVA followed by FLSD was used to determine which novel-experience treatment groups differed from the no-stress control group. A two-way ANOVA (Novel Experience × Time of Exposure) was then used to compare the response to the different novel experiences. The correlation (Pearson correlation coefficient r) between Fos protein expression (Experiment 1) or *c-fos* and *zif268* mRNA expression (Experiment 2) and plasma hormone levels was also determined. For Experiment 2, separate correlations were determined for each

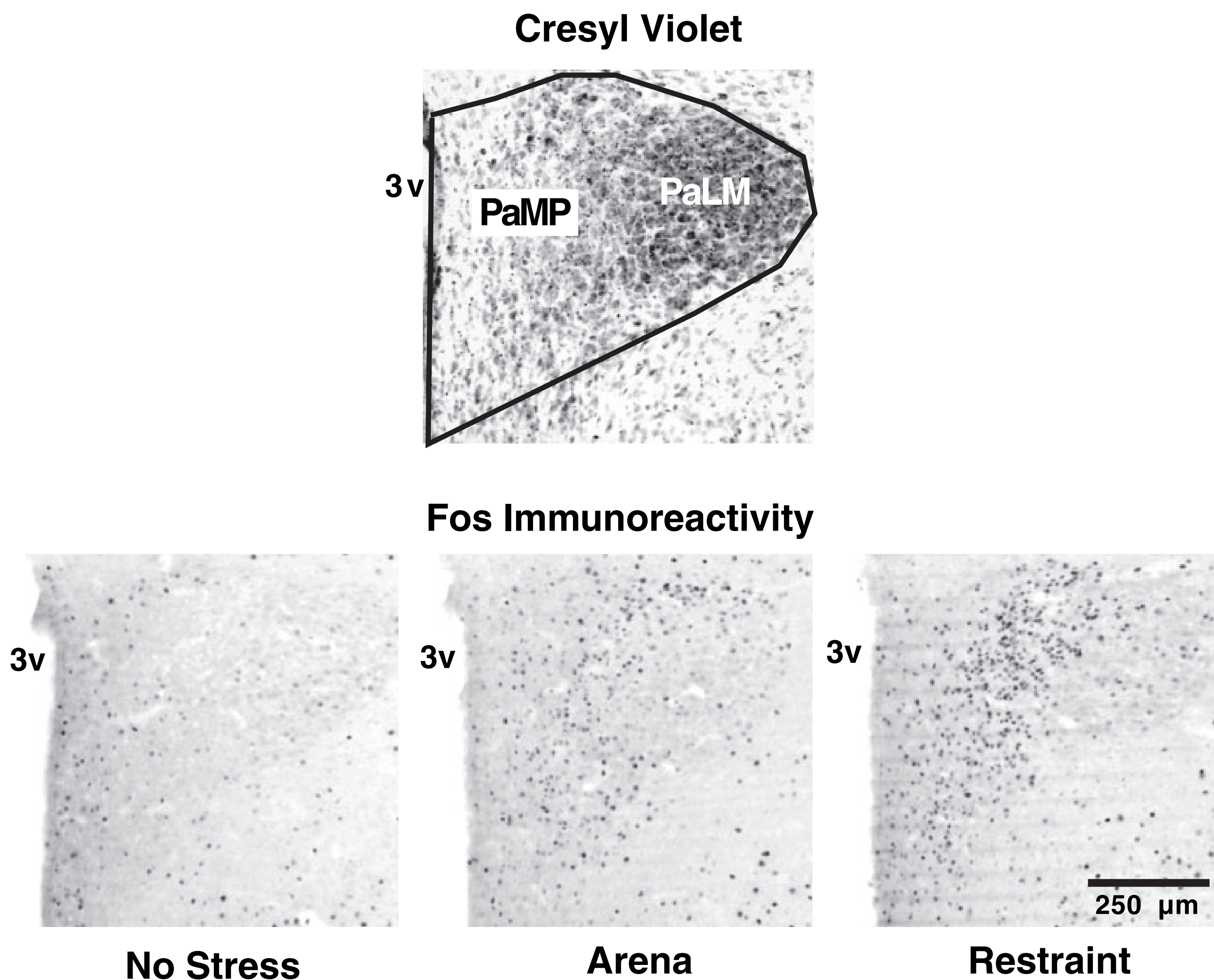

FIG. 2. Sample photomicrographs of Fos immunoreactivity in the PVN. The top photomicrograph shows one hemisphere of PVN as revealed by Cresyl Violet staining (PaLM, lateral magnocellular region; PaMP, medial parvicellular region; 3v, third ventricle). The solid line shows a representative region of interest for Fos-immunoreactive cell counts within the PVN. Note that Fos-immunoreactive cells within the PVN after arena or restraint exposure are primarily localized within the PaMP.

experience duration time-point and, in each case, values from the no-stress control group were included. All data figures show the mean ± SEM for the various treatment groups.

Results

Experiment 1: effects of novel experience on plasma hormone and Fos protein levels

Plasma ACTH and corticosterone

There was an overall effect of novel experience on plasma ACTH ($F_{4,34} = 4.1$, $P < 0.01$) and corticosterone ($F_{4,34} = 24.9$, $P < 0.001$). All experiences produced a significant increase in ACTH and corticosterone levels compared to the no-stress with blood sample control group (FLSD). On a relative basis, pedestal and restraint exposure produced similar strong increases in plasma ACTH and corticosterone levels whereas tub and arena produced moderate increases in both hormones levels (Fig. 1).

Fos in PVN

There was also an overall effect of experience on the number of Fos-immunoreactive cells in the PVN ($F_{5,41} = 6.8$, $P < 0.001$). The rank order across experiences for the mean number of Fos-positive cells and the mean level of plasma corticosterone was identical (Fig. 1). This overall positive relationship between Fos expression in the PVN and HPA axis activity is reflected in a significant correlation between the number of Fos-positive cells in the PVN and plasma corticosterone or plasma ACTH (Table 1). There was a small, statistically nonsignificant, greater number of Fos-positive cells in the control rats from which a blood sample was taken than in the control rats from which a blood sample was not taken. Upon visual inspection, the majority of Fos-immunoreactive cells were localized to the medial parvocellular portion of the PVN (Fig. 2).

Fos in hippocampus

There was also an overall increase in the number of Fos-positive cells in the CA1-CA2 ($F_{5,41} = 7.4$, $P < 0.01$) and CA3 ($F_{5,41} = 14.2$, $P < 0.01$) subdivisions of the hippocampus as well as in the inner blade of the dentate gyrus ($F_{5,41} = 5.7$, $P < 0.01$) after novel experience exposure (Figs 3 and 4). On the other hand, all novel experiences led to a significant reduction in Fos-positive cells in the outer blade of the dentate gyrus ($F_{5,41} = 6.9$, $P < 0.05$). Interestingly, the between-experience pattern of Fos expression in CA1-CA2, CA3 and the inner blade of the dentate gyrus differed from that in the PVN (Figs 1–4). Whereas restraint resulted in the greatest mean amount of Fos expression in the PVN for the four experiences examined, it produced the least mean amount of Fos expression in these hippocampal subregions. Moreover, Fos expression in these hippocampal subregions after restraint was no greater than in the control group that was subjected to a brief blood sample. Consequently, in contrast to the PVN, there was not a significant correlation between hippocampal Fos expression and hormone levels (Table 1).

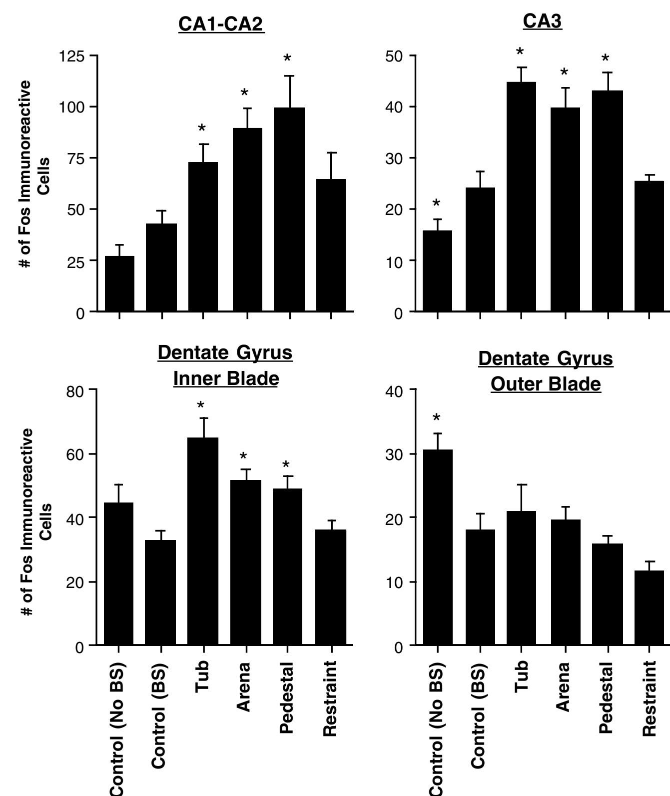

FIG. 3. Effect of four novel experiences on Fos protein expression in dorsal hippocampus. Rats were exposed to 30 min of novel experience (tub, arena, pedestal or restraint). Control rats remained undisturbed in their home cage (Control No BS) or were briefly removed from their home cage for a blood sample (Control BS). Two hours after the end of novel experience exposure rats were perfused with fixative for subsequent Fos immunoreactivity measures. *$P < 0.05$ vs. no-stress control rats with blood sample (FLSD; $n = 6$–9).

Experiment 2: effects of novel experience on plasma hormone, c-fos mRNA and zif268 mRNA levels

In order to examine further the dissociation in the relative amounts of Fos expression produced by restraint compared to other novel experiences in the PVN and hippocampus, a follow-up experiment was conducted. This experiment examined the time-course for *c-fos* mRNA induction in response to tub, arena and restraint exposure. Due to the relatively large number of treatment groups and subjects resulting from the addition of the time variable we omitted from this second experiment the pedestal experience. In an attempt to examine three different experiences that differed in their magnitude of HPA axis activity, we decided to drop the pedestal experience as in the first experiment it produced very similar HPA axis activity as restraint. We retained the restraint experience because it has been widely utilized in stress research. Expression patterns for *c-fos* mRNA were examined in the PVN and hippocampus. In addition, in order to determine the specificity of experience-dependent *c-fos* expression patterns in the hippocampus, we also examined immediate–early gene expression in neocortical and allocortical brain regions present on brain sections at the rostral–caudal level of the PVN. Finally, to determine the extent to which the differential pattern of *c-fos* mRNA expression observed generalizes to other markers of general neuronal activity, *zif268* mRNA expression was also evaluated for some of these brain regions (PVN, primary somatosensory cortex and primary motor cortex).

Plasma ACTH and corticosterone

There was an overall effect of novel experience exposure on plasma ACTH ($F_{9,70} = 22.9$, $P < 0.01$) and corticosterone levels ($F_{9,70} = 46.4$, $P < 0.01$). There was a graded increase in corticosterone levels for the three experiences, with a small, moderate and large increase produced by tub, arena and restraint exposure, respectively (Fig. 5). The mean level of ACTH secretion collapsing across the three

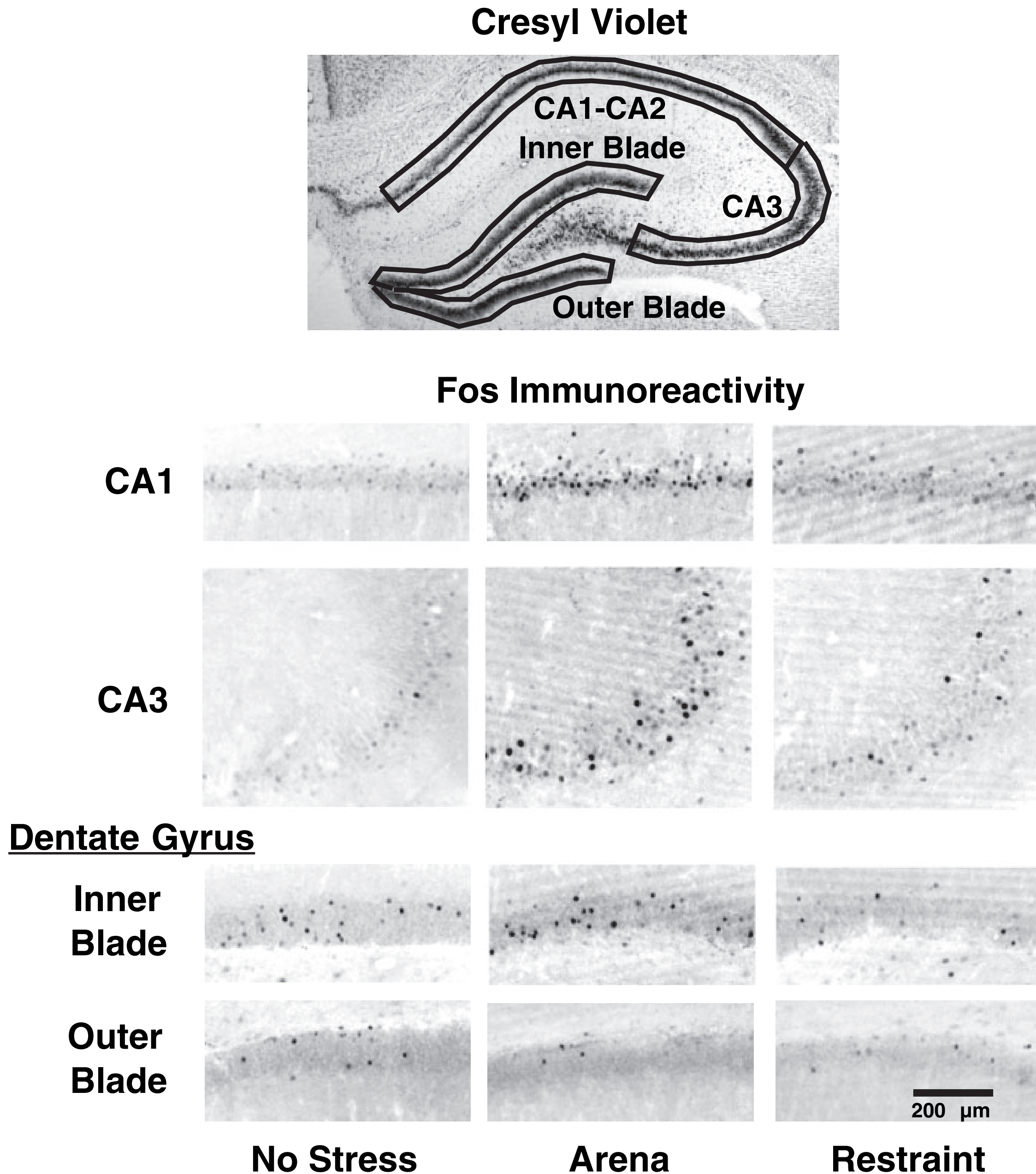

FIG. 4. Sample photomicrographs of Fos immunoreactivity in the dorsal hippocampus. The top photomicrograph shows one hemisphere of hippocampus as revealed by Cresyl Violet staining. The solid lines show representative regions of interest for Fos-immunoreactive cell counts within the various hippocampal subregions. Samples of Fos immunoreactivity from three of the treatment groups (no stress without blood sample, arena exposure and restraint exposure) are shown for a portion of the indicated hippocampal subregions.

time-points also exhibited the same rank order for the three experiences.

c-Fos mRNA in PVN

There was an overall significant induction of *c-fos* mRNA in the PVN in response to novel experience exposure ($F_{9,70} = 10.8$, $P < 0.01$). As was the case for ACTH levels, restraint produced a large increase in *c-fos* mRNA in the PVN whereas there was a much smaller, but statistically significant, increase in response to arena and tub (Figs 5 and 6). The amount of *c-fos* mRNA expression in the PVN for each experience duration time-point (including the no-stress values) was positively correlated with plasma corticosterone and plasma ACTH (Table 2).

c-Fos mRNA in hippocampus

Analysis of the expression of *c-fos* mRNA in the hippocampus was performed separately for the CA1-CA2 and CA3 hippocampal subregions and the inner and outer blades of the dentate gyrus taken from the dorsal hippocampus (Fig. 6) and the ventral hippocampus. The relative patterns of results were very similar and did not differ statistically between the dorsal and ventral hippocampus. For economy of presentation purposes we show only the dorsal hippocampal

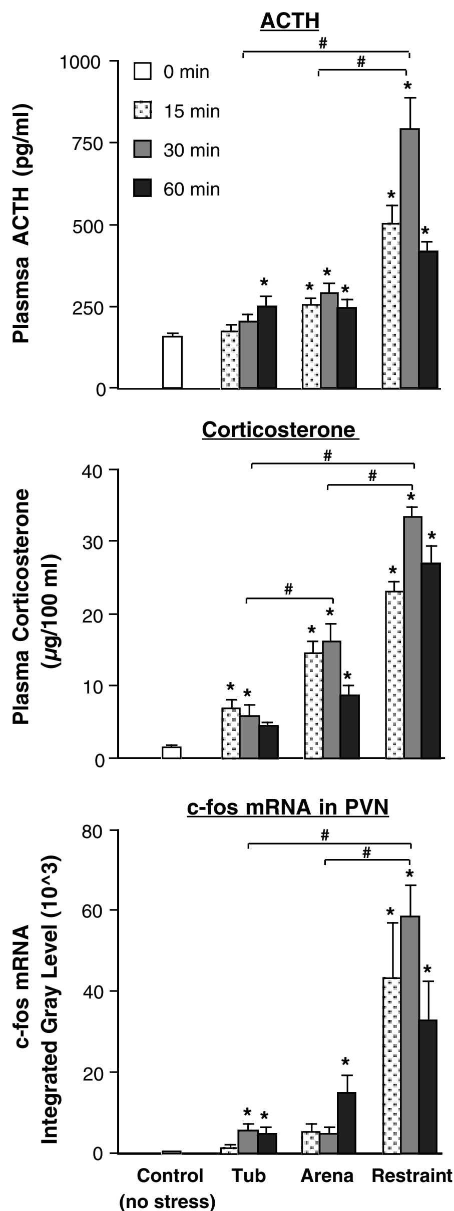

FIG. 5. Effect of three novel experiences on HPA axis hormone levels and *c-fos* mRNA in PVN. Blood samples and brain tissue were collected from rats immediately after 15, 30 or 60 min of novel experience (tub, arena or restraint) or from no-stress (0 min) control rats ($n = 8$). *$P < 0.05$ vs. no-stress control rats (FLSD). $^{\#}P < 0.05$ between novel experiences when collapsing across time (FLSD).

data. In all cases, except the outer blade of the dentate gyrus, there was a significant overall effect of novel experience ($F_{9,70}$ ranged from 3.1 to 7.2, $P < 0.01$). Consistent with the results seen in Experiment 1, restraint produced the least amount of *c-fos* mRNA in the hippocampus compared to the response to the other experiences (Figs 6 and 7). There was not a significant correlation between *c-fos* expression and plasma hormone levels for any hippocampal subregion or experience duration time-point (Table 2).

c-Fos mRNA in cortical regions

The expression of *c-fos* mRNA was examined in four cortical areas: primary somatosensory cortex (barrel field), primary motor cortex and two limbic association cortical areas (cortical amygdaloid and piriform cortex; Fig. 6). In each cortical region there was a substantial increase in *c-fos* mRNA after novel experience ($F_{9,70}$ ranged from 12.8 to 30.7, $P < 0.01$), and the pattern of relative expression levels between experiences was very similar for each of these cortical areas and the hippocampus (Figs 7 and 8). *Post hoc* tests collapsing across time indicate that in each cortical region there was a greater level of *c-fos* mRNA in response to arena exposure than tub exposure, both of which produced greater levels than restraint exposure. With one exception, there was not a significant correlation between *c-fos* expression and plasma hormone levels for any cortical subregion or experience duration time-point (Table 2). The one exception was a small positive correlation between *c-fos* mRNA in the somatosensory cortex and plasma corticosterone at the 15-min time-point ($r = 0.38$, $P = 0.03$).

Zif268 mRNA in PVN, primary motor and somatosensory cortex

The relative pattern of *zif268* mRNA expression in the PVN was very similar to that seen for *c-fos* mRNA (Fig. 9). There were barely detectable levels of *zif268* mRNA in control rats and progressively greater increases for the three novel experiences, tub, arena, and restraint, respectively ($F_{9,70} = 27.2$, $P < 0.01$). There was a positive correlation between PVN *zif268* mRNA and plasma corticosterone for each experience duration time-point (15-min, $r = 0.78$, $P < 0.0001$; 30-min, $r = 0.87$, $P < 0.0001$; 60-min, $r = 0.87$, $P < 0.0001$). There was a similar positive correlation between PVN *zif268* mRNA and plasma ACTH (15-min, $r = 0.75$, $P < 0.0001$; 30-min, $r = 0.84$, $P < 0.0001$; 60-min, $r = 0.77$, $P < 0.0001$).

There were relatively high constitutive levels of zif268 mRNA expression in primary somatosensory and motor cortex of the no-stress control rats (Fig. 9). *Zif268* mRNA levels increased significantly in the somatosensory cortex ($F_{9,70} = 4.8$, $P < 0.01$) and motor cortex ($F_{9,70} = 12.5$, $P < 0.01$) with novel experience exposure. As was the case for c-fos mRNA expression, the experience-dependent *zif268* mRNA expression levels in the primary somatosensory and motor cortex differed from the PVN. In these cortical regions restraint produced a lower amount of induction than in tub and arena, and in the case of the motor cortex the increase was not statistically significant. There was not a statistically significant correlation between *zif268* mRNA levels in the somatosensory or motor cortex and plasma hormone levels for any experience duration time-point (r ranged between −0.23 and 0.26, $P > 0.1$).

Discussion

In this study we examined the relationship between hippocampal *c-fos* gene expression induced by four different novel experiences (empty housing tub, circular arena, elevated pedestal or restraint tube) and the stressfulness of those experiences as represented by HPA axis activity. As none of these experiences are believed to directly activate homeostatic-related sensors, the stressfulness of these novel experiences must somehow be derived from integration of the primary exteroceptive sensory information associated with each experience (Herman *et al*., 2003). Restraint and pedestal reliably produced the largest magnitude of increased ACTH and corticosterone secretion, arena an intermediate level (Experiment 2) and tub the least magnitude of increase. There does not appear to be any simple stimulus dimension such as feature novelty or enclosure size that can account for the differential HPA axis response to these various experiences. Consequently, the stressfulness of the experience may depend on high-level neural integration of the situation. Whatever feature processing is involved, this study illustrates a remarkably consistent gradation of HPA axis response to the various experiences across individual rats.

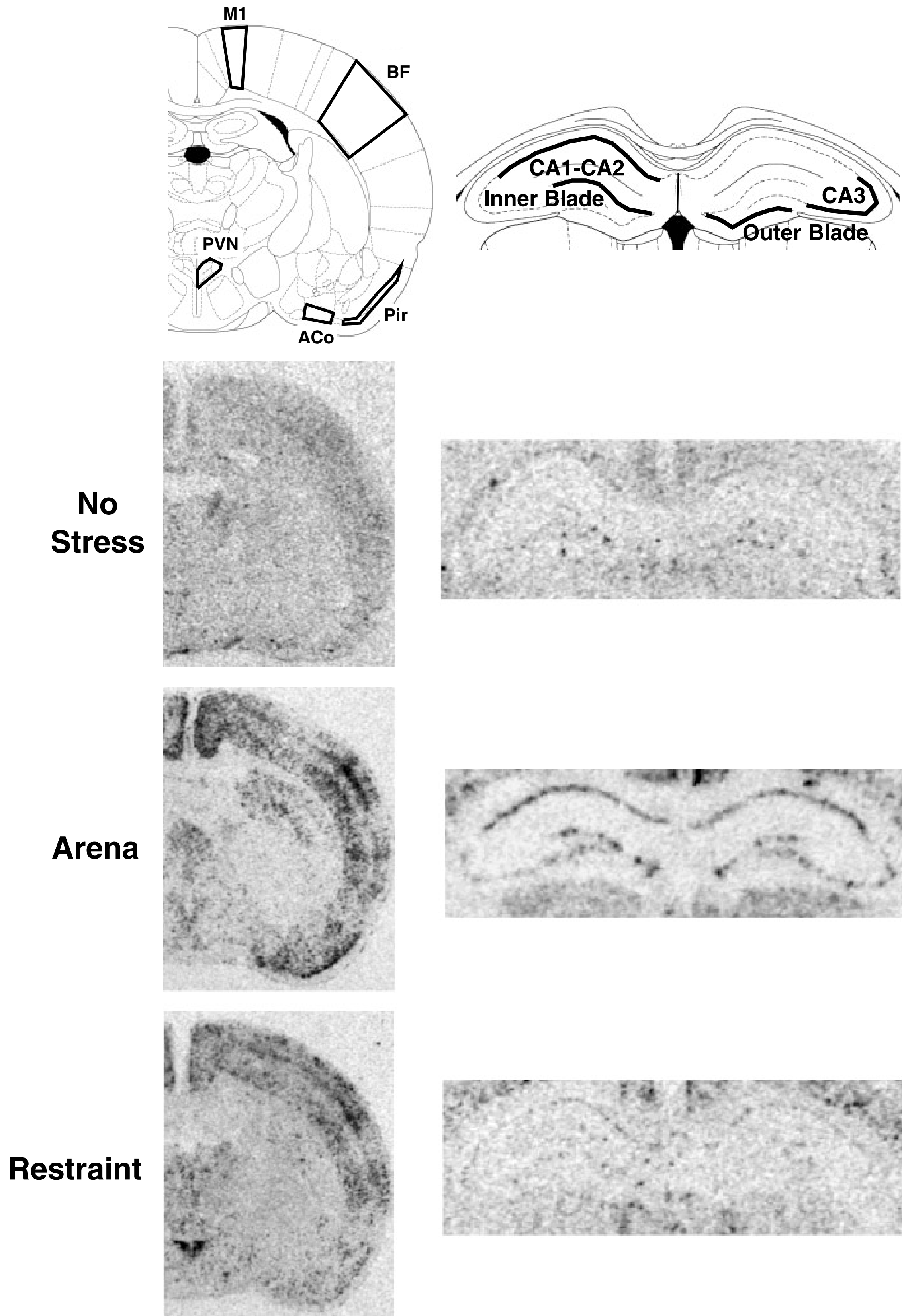

FIG. 6. Sample autoradiograms of *c-fos* mRNA *in situ* hybridization at the rostral–caudal level of the PVN or in the dorsal hippocampus. The top diagrams (adapted from Paxinos & Watson, 1998) depict in bold outline regions of interest that were used for quantification of relative *c-fos* mRNA levels (M1, primary motor cortex; BF, barrel field of primary somatosensory cortex; Pir, piriform cortex; ACo, cortical amygdaloid). Sample autoradiograms are presented from rats exposed to 60 min of arena or restraint, and from a control no-stress rat.

TABLE 2. Experiment 2: Pearson correlation coefficient (r) between *c-fos* mRNA and plasma corticosterone or ACTH for PVN, hippocampal and cortical subregions

Region	Pearson correlation coefficient (r) after onset of novel experience					
	At 15 min		At 30 min		At 60 min	
	c-fos + CORT	*c-fos* + ACTH	*c-fos* + CORT	*c-fos* + ACTH	*c-fos* + CORT	*c-fos* + ACTH
PVN	0.65***	0.76***	0.80***	0.75***	0.57***	0.57***
CA1-CA2	0.10	−0.13	−0.03	−0.13	−0.20	−0.11
CA3	0.13	−0.07	−0.10	−0.18	−0.12	−0.01
DG						
Inner blade	0.06	−0.15	−0.06	−0.21	−0.09	−0.04
Outer blade	0.07	0.08	−0.11	−0.04	−0.15	−0.09
Somatosensory cortex	0.38*	0.03	0.17	−0.01	−0.03	0.12
Motor cortex	0.18	−0.11	0.29	0.16	−0.09	0.12
Cortical amygdaloid	0.33	−0.03	0.26	0.07	0.02	0.12
Piriform cortex	0.32	−0.05	0.25	0.05	−0.06	0.06

CORT, plasma corticosterone. Separate correlation coefficients were calculated for the c-fos mRNA and hormone levels present in rats 15, 30 and 60 min after onset of novel experience. In each case the values for control (no-stress) rats were included ($n = 32$). ***$P < 0.001$, *$P < 0.05$.

Discrimination between the complex stimuli used in this study and detection of their novelty undoubtedly involves hippocampal processing (Squire *et al*., 2004). If the hippocampus contributes to assessing the stressfulness of a novel experience, then perhaps the overall neural activity (and associated immediate–early gene expression) of that brain region correlates closely with the magnitude of the HPA axis

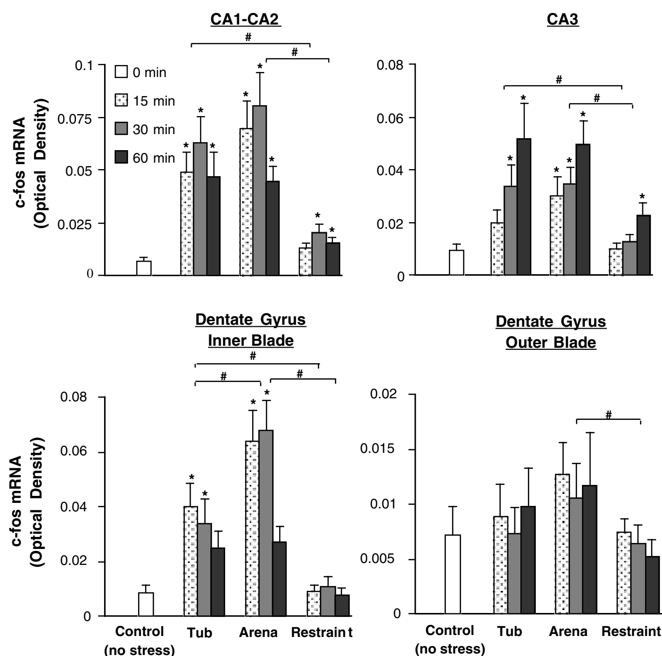

FIG. 7. Effect of three novel experiences on *c-fos* mRNA levels in dorsal hippocampus. Brain tissue was collected from rats immediately after 15, 30 or 60 min of novel experience (tub, arena or restraint) or from no-stress (0 min) control rats ($n = 8$). *$P < 0.05$ vs. no-stress control rats (FLSD). $^{\#}P < 0.05$ between novel experiences when collapsing across time (FLSD).

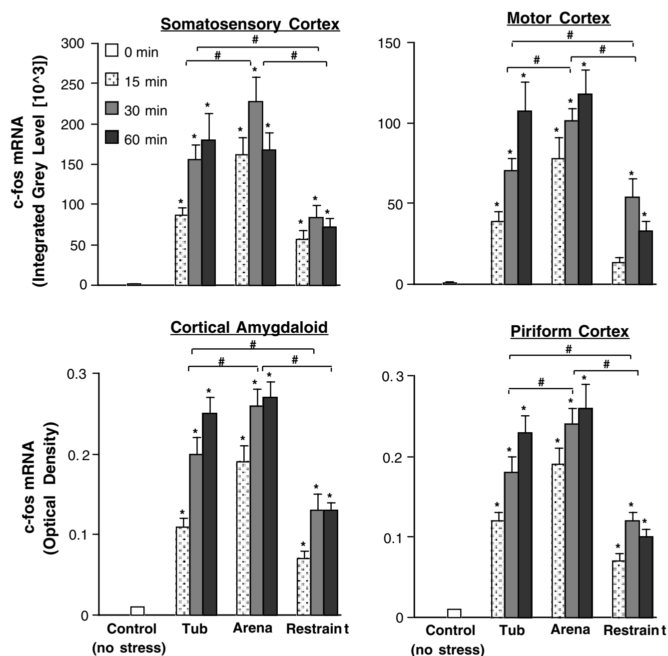

FIG. 8. Effect of three novel experiences on *c-fos* mRNA levels in cortex. Brain tissue was collected from rats immediately after 15, 30 or 60 min of novel experience (tub, arena or restraint) or from no-stress (0 min) control rats ($n = 8$). *$P < 0.05$ vs. no-stress control rats (FLSD). $^{\#}P < 0.05$ vs. between novel experiences when collapsing across time (FLSD).

response. As a validation of this approach, we examined the relative levels of immediate–early gene expression in the PVN for each of our novel experiences. We found that there was a good relationship between the amount of Fos protein or *c-fos* mRNA that was induced in the PVN and the magnitude of the hormonal response. This result is consistent with other studies finding that Fos expression in brain regions that receive direct sensory input varies with stimulus intensity (Campeau & Watson, 1997). Within the PVN, this relationship may not be maintained when comparing responses between physical and psychological stressors (Emmert & Herman, 1999).

As the rapid *c-fos* induction in the PVN primarily reflects the increased transynaptic excitatory input to this brain region (Morgan & Curran, 1991), similar relative levels of *c-fos* expression may be seen in other brain regions that are responsible for the relative magnitude of HPA axis activation. Interestingly, in the first experiment we found a marked dissociation in the relative levels of Fos expression across the four experiences in hippocampus compared to PVN. Specifically, whereas restraint produced strong Fos induction in the PVN it produced the least Fos induction in the hippocampus. We saw the same general pattern in all hippocampal subregions, with the exception of the outer blade of the dentate gyrus in which there was an experience-dependent decrease in Fos. This unusual pattern of a decrease in dentate gyrus Fos expression after acute experience (restraint) has been reported before (Chowdhury *et al*., 2000; Fevurly & Spencer, 2004).

When reflecting on the overall low level of Fos induction in the hippocampus after restraint, we considered the possibility that this might be a result of one important distinction between the restraint experience and the other experiences. Although each experience was novel, restraint was the only experience in which the rat couldn't engage in active exploration of the novel enclosure. Given the important role of the hippocampus in processing novel spatial information (Olton *et al*., 1979; Knight, 1996; Jenkins *et al*., 2004;

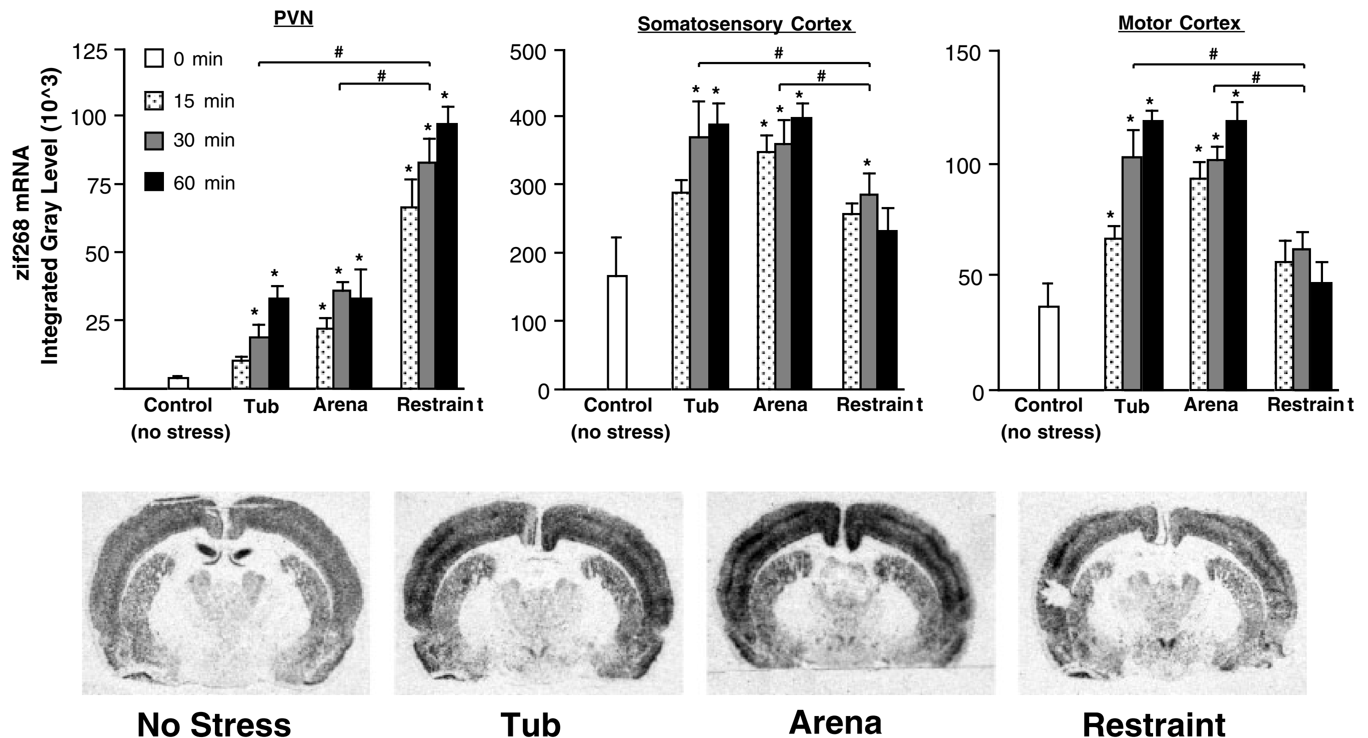

FIG. 9. Effect of three novel experiences on *zif268* mRNA levels in PVN, somatosensory cortex and motor cortex. Brain tissue was collected from rats immediately after 15, 30 or 60 min of novel experience (tub, arena or restraint) or from no-stress (0 min) control rats ($n = 8$). A sample autoradiogram of *zif268* mRNA *in situ* hybridization from each treatment group is presented below the graphs. $^{*}P < 0.05$ vs. no-stress control rats (FLSD). $^{\#}P < 0.05$ between novel experiences when collapsing across time (FLSD).

Lee *et al*., 2005), the different amounts of exploration associated with each experience may largely account for the corresponding relative hippocampal Fos expression. Further support for this notion is the observation that there was a greater amount of Fos expression in response to placement in an arena or on a pedestal than in a relatively small housing tub. It appears that *c-fos* gene induction in the hippocampus primarily reflects hippocampal processing of the various stimulus features of a novel experience rather than stressfulness of that experience. A study by Campeau & Watson (1997) illustrates the importance of using carefully selected comparison conditions to separate the pattern of *c-fos* induction due to the primary sensory processing of loud auditory stimuli from processing of the stressfulness of that stimuli. Whether or not stress can modulate the experience-dependent magnitude or pattern of immediate–early gene induction in the hippocampus remains to be determined. From our experiment we can conclude that the low amount of Fos expression in the hippocampus of rats placed in a restrainer is not due to some general inhibitory influence of stress or corticosterone on hippocampal immediate–early gene induction. If such were the case then pedestal exposure, which produced high levels of corticosterone secretion similar to those present during restraint, should also have been associated with low levels of hippocampal Fos expression.

Our second experiment examined further the differential pattern of Fos expression in the PVN and hippocampus in response to these novel experiences. This experiment determined whether this difference would be manifest at the level of *c-fos* mRNA, and it examined the time-course of the *c-fos* induction. The *c-fos* mRNA expression patterns parallelled very closely the Fos protein expression patterns observed in the first experiment. Again, of the three novel experiences examined in the second experiment (tub, arena and restraint), restraint produced the largest induction of *c-fos* mRNA in the PVN and the smallest induction in each hippocampal subregion.

In order to determine whether the relative pattern of *c-fos* expression across the three novel experiences was unique to the hippocampus, we also examined *c-fos* expression in several functionally distinct regions of cortex. For this comparison we examined two cortical areas, piriform cortex and the associated anterior cortical nucleus of the amygdala (Sah *et al*., 2003), that exhibit strong *c-fos* induction with stressor exposure (Kovacs, 1998; Figueiredo *et al*., 2002). These allocortical regions may be important for integrating emotional tone with olfactory information (Majak *et al*., 2004). The pattern of greater *c-fos* induction with tub and arena exposure than with restraint was present in both of these cortical regions.

We also examined a portion of the primary motor cortex and a region of primary somatosensory cortex that receives the primary sensory afferents from vibrissae (barrel field). Interestingly, we saw very similar patterns of *c-fos* expression in primary motor and primary somatosensory cortex, with restraint producing a relatively small increase in *c-fos* mRNA and tub or arena exposure producing much greater increases. It is notable that these differences were already pronounced by 15 min after experience onset, indicating that the relative *c-fos* expression patterns were initiated very early after experience onset. Such clear differences between each experience in immediate–early gene induction in these primary cortical areas was unexpected as the absolute amount of somatosensory input and motor activity appears to be similar for each experience, especially shortly after experience onset. Rats placed in a restraint tube are initially very active as they attempt to back out, turn around and generally struggle to escape the restrainers. During this time rats also continuously rub

against the walls of the restrainer, undoubtedly activating somatosensory receptors on the surface of their body and those associated with their vibrissae. Perhaps the relative amount of *c-fos* induction within the primary somatosensory cortex is more closely associated with how the sensory information is used than with the absolute amount of sensory input. Similarly, within the primary motor cortex the relative amount of *c-fos* induction may depend more on the higher level coordination of motor activity than on the absolute level of motor activity. A common feature of neuroimaging studies is that greater neural activity is elicited by stimuli during trials in which a more active rather than passive response is required from the subject. These task-dependent differences in neural activity level are often present in primary sensory cortical regions, and have been interpreted as an example of top-down neural regulation of early cortical processing of sensory information (Shulman *et al*., 1997). The amount of experience-dependent *c-fos* induction in cortical regions seen in this study may not simply reflect the general amount of neuronal activity within that brain region (Labiner *et al*., 1993; Hoffman & Lyo, 2002). Instead, the *c-fos* induction may better reflect the specific neural plastic demands of that experience. In either case, the amount of cortical *c-fos* induction was substantially greater for experiences that allowed for active exploration of the novel environment.

In the second experiment we also examined the expression of another immediate–early gene, *zif268*. The *zif268* gene has higher levels of constitutive expression in cortex than c-fos and its expression levels, at least in visual cortex, have been shown to change more dynamically with ongoing steady-state levels of neural activity (Kaplan *et al*., 1996). Within the PVN, there were barely detectable levels of *zif268* mRNA in brains from no-stress rats, perhaps reflecting the low intrinsic activity of these neurons during the trough of the circadian cycle of HPA axis activity (Dallman *et al*., 1987). There was a significant and rapid induction of PVN *zif268* mRNA with each experience, and the relative increases closely paralleled those seen for *c-fos* mRNA in the PVN, with restraint producing the largest increase. Although there were relatively high levels of *zif268* expression in the primary somatosensory and motor cortex of no-stress rats, the levels increased further with each experience and, as was the case for *c-fos*, there was greater induction in response to tub and arena than to restraint. This result indicates that the experience-dependent and brain region-specific pattern of *c-fos* mRNA seen in this study was not unique to the cellular activity reported by *c-fos* gene expression but was also evident with another widely expressed immediate–early gene.

It is not likely that the intensity coding of psychological stressors is determined within the PVN as the corticotropin-releasing hormone motor neurons receive limited direct input from sensory cortical brain regions (Sawchenko *et al*., 1996). Consequently, those neurons do not receive the necessary afferent information to discriminate between experiences such as placement in a tub, arena, pedestal or restraint tube. The relative levels of *c-fos* expression in the PVN probably reflect relative levels of net excitatory input to those cells. If this excitatory input comes primarily from some other population of cells, one would expect that those afferent cells would also have greater levels of activity and *c-fos* expression with more intense stressors than with less intense stressors. Although the hippocampus does not satisfy this condition, it may be fruitful to look for other brain regions whose activity levels in response to various psychological stressors correlates closely with HPA axis activation. Such brain regions may be key integrators of the stressfulness of an experience and may serve as generators of a stress state. In support of this prospect, Dayas *et al*. (2001) have found that restraint and other psychological stressors produce a subregional differential pattern of Fos induction in the amygdala and medulla compared to some physical stressors (haemorrhage and immune challenge).

In summary, the amount of immediate–early gene induction in the PVN was positively correlated with the magnitude of the HPA axis response to an acute psychological stressor. In contrast, the pattern of differential immediate–early gene expression throughout cortex and hippocampus was associated more with the extent of active exploration available with each experience than level of HPA axis activation. This latter result also indicates that the amount of immediate–early gene expression in hippocampus and cortex wasn't largely dependent on nonspecific levels of arousal as one would expect greater levels of arousal with more intense stressors.

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Abbreviations

ACTH, adrenocorticotropic hormone; FLSD, Fisher's least significant difference test; HPA, hypothalamic–pituitary–adrenal; PVN, paraventricular nucleus.

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