

Fos Expression is Selectively and Differentially Regulated By Endogenous Glucocorticoids in the Paraventricular Nucleus of the Hypothalamus and the Dentate Gyrus

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Abstract

We examined the extent to which basal levels of corticosterone, which vary in a circadian fashion, influence the pattern of Fos protein expression in the paraventricular nucleus of the hypothalamus (PVN), the hippocampal formation and three different functional cortical areas. Basal and poststress (1 h of restraint) Fos expression, as determined by immunohistochemistry, was examined in male rats with either no previous surgical manipulation or in rats 5 days after: (i) sham adrenalectomy; (ii) adrenalectomy with no corticosterone replacement; or (iii) adrenalectomy with corticosterone (25 µg/ml) in the drinking water replacement. In adrenal-intact rats, restraint produced similar patterns of Fos expression in the PVN, cortical areas and hippocampus (CA1–CA3), with peak levels of expression attained 60–90 min after restraint onset. Surprisingly, in the dentate gyrus, there was a dissociation between the two blades in the pattern of Fos expression after restraint. In the inner blade (suprapyramidal), there was a delayed induction that occurred between 60 and 90 min after restraint onset and, in the outer blade (infrapyramidal), there was a steady decline in Fos expression after restraint. Adrenalectomy had an effect on Fos expression only in the PVN and dentate gyrus, and the nature of the effect was quite different for both brain regions. In the PVN, adrenalectomy had no effect on Fos expression in unstressed rats, but resulted in an enhanced number of Fos positive cells after restraint. In the dentate gyrus, adrenalectomy resulted in an overall reduction of Fos positive cells in both blades, and this reduction was present in unstressed and stressed rats. Corticosterone replacement normalized the adrenalectomy effect on Fos expression in both brain regions. Thus, Fos expression in the rat brain displays specific patterns of dependency on the permissive effects of glucocorticoids, and this dependency varies between brain regions.

It is well established that basal and stress-induced activity of the hypothalamic-pituitary-adrenal (HPA) axis is constrained by glucocorticoid negative feedback (1, 2). This feedback, as expressed at a systems level, appears to encompass a number of glucocorticoid-dependent molecular actions present at multiple anatomical sites, both intrinsic and extrinsic to the HPA axis. However, the relative importance of these various molecular effects and sites of action for the range of HPA axis systems level operation, remains largely underdetermined. One strategy that may be useful in dissecting glucocorticoid regulatory effects on HPA axis activity is to examine glucocorticoid effects on basal and stress-induced *c-fos* gene expression in relevant brain regions. Extensive characterization indicates that the neuronal expression of *c-fos* mRNA and/or Fos protein primarily reflects increased intercellular/

transynaptic stimulatory input, with very little *c-fos* gene expression evident during basal conditions of neuronal activity (3, 4).

Removal of endogenous glucocorticoids by adrenalectomy results in a dramatic increase in basal and stress-induced adrenocorticotrophic hormone (ACTH) levels (2). An indication that part of this increased activity takes place at the central level of the HPA axis is a concurrent increase in basal and stress-induced corticotropin releasing hormone (CRH) and arginine vasopressin gene expression in the medial parvocellular portion of the paraventricular nucleus of the hypothalamus (PVN) (5–8). Several studies, but not all, find that adrenalectomy also results in increased stress-induced *c-fos* expression in the PVN (9–13). However, the extent to which this adrenalectomy effect is glucocorticoid-dependent

has not been carefully examined. One study found that adrenalectomy combined with low-dose corticosterone pellet treatment produced enhanced Fos protein expression relative to sham adrenalectomized rats (14). Because that study did not include an adrenalectomy alone group, it is not possible to determine whether the corticosterone pellet replacement had any effect. Several other studies found that replacement of adrenalectomized rats with relatively high concentrations of corticosterone in continuous release subcutaneous pellets produced reductions in stress-induced *c-fos* expression that were below sham adrenalectomy levels (10, 13). A corticosterone pellet replacement regimen is limited in the amount of information that it provides concerning the corticosterone conditions responsible for regulating *c-fos* expression. For example, suppressed *c-fos* expression could be a result of either the previous sustained or the concurrent presence of high corticosterone levels.

The present study compared basal and stress-induced Fos protein expression in rats that were either adrenalectomized, adrenalectomized and given corticosterone in their drinking water or sham adrenalectomized. Treatment of rats with corticosterone in the drinking water is an effective means of replacing corticosterone levels in adrenalectomized rats that approximate normal endogenous basal corticosterone levels that fluctuate in a circadian fashion (15–17). With this treatment, there is a fairly large increase in plasma corticosterone during the early portion of the dark period when rats consume the majority of their available drinking water in conjunction with feeding. These relatively high levels of corticosterone clear rapidly due to the short half-life of corticosterone and, throughout the light period, corticosterone levels are barely detectable. Importantly, this treatment regimen insures that there is no phasic increase in corticosterone levels during or following acute stress. Consequently, this treatment procedure allows determination of the extent to which a circadian pattern of basal corticosterone exposure, in the absence of stress-induced phasic increases in corticosterone, is sufficient to normalize adrenalectomy effects on Fos expression.

Previous studies have not examined in detail the extent to which changes in *c-fos* expression as a result of adrenalectomy are selective for the PVN. A comparison between brain regions can help in determining whether glucocorticoids act directly within the PVN to regulate Fos expression or, instead, have a more general widespread effect on Fos expression, perhaps reflecting a change in general neuronal excitability. Thus, in the present study, several other brain regions were included in the analysis, specifically, the hippocampal formation and three cortical regions: (i) the motor cortex; (ii) the somatosensory cortex and (iii) a limbic association cortical region, the retrosplenial cortex. We chose to examine the hippocampal formation in detail because neural activity within the hippocampus is regulated by glucocorticoids (18). This structure may also provide an indirect inhibitory influence on the HPA axis (19, 20), although the hippocampus does not appear to be necessary for normal HPA activity (21). Fos induction in the primary motor and somatosensory cortical areas may represent the general motor and somatosensory neural demands of the restraint experience,

independent of the 'stressful' nature of the experience. By contrast, the retrosplenial cortex is more closely associated with the limbic system (22), and Fos induction in that region may better represent some of the stress-related neural demands of the experience.

Materials and methods

Subjects

Adult male Sprague-Dawley rats were purchased from Harlan (Indianapolis, IA, USA; Facility 218, Prattville, AL, USA) and housed in animal facilities at the University of Colorado. Rats were allowed a 2-week acclimation period after arrival before exposure to experimental procedures. Rats were housed two per cage in polycarbonate tubs (47 × 23 × 20 cm) with wood chip bedding in a temperature (22 ± 2 °C) and humidity controlled room. Lights were maintained on a 12 : 12 h light/dark cycle (lights on 07.00 h). Food and water were continuously available except during the time of experimentation. All procedures were approved by the University of Colorado at Boulder Institutional Animal Care and Use Committee.

Adrenalectomy and corticosterone replacement

Rats were adrenalectomized (ADX) or sham-adrenalectomized (Sham) with use of aseptic surgical procedures. A mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg) was administered i.p. to produce full anaesthesia. After surgery, adrenalectomized rats were provided with 0.9% saline drinking water. Adrenalectomized rats in the corticosterone replacement group (ADX + CORT) were given 0.9% saline drinking water that contained 25 µg/ml corticosterone. Corticosterone was first dissolved in ethanol resulting in a final low level amount of ethanol in the drinking water (0.1%). Rats were given 5 days of recovery after surgery before subsequent experimentation.

Restraint stress

Restraint involved taking rats from the home cage and placing them in adjustable length (15.5 ± 2.5 cm long and 6.3 cm diameter) Plexiglass tubes with air holes in the front, top and back. This stressor is considered to be primarily psychological in nature because it does not produce pain or direct physical insult (23). Restraint took place in a separate room adjacent to the home cage room and was administered during the first third of the light period (between 08.00 and 11.00 h). At the end of restraint, the rats were either immediately anaesthetized for subsequent perfusion or first returned to their home cages, depending on their poststress time-point assignment. Water bottles were not returned to the home cages after restraint.

Experimental procedures

Experiment 1: time course of Fos expression following restraint

The first experiment examined a detailed time-course for Fos induction in response to 1 h of restraint. Rats in this experiment were adrenal-intact and were not subjected to previous surgical procedures. Rats were anaesthetized, transcardially perfused and brains removed for subsequent immunohistochemistry at various time-points after restraint onset (1, 1.5, 2.5 and 3.5 h). An additional group of rats (0 h) was left undisturbed in their home cage until anaesthetization for perfusion. The experiment was conducted with two separate cohorts of rats, resulting in an overall total of six rats per time-point (n = 6) with the exception of the 1-h time-point that was only examined in the second cohort (n = 3).

Experiment 2: effect of adrenalectomy on Fos expression

The second experiment examined the Fos response to restraint in 5-day adrenalectomized (ADX) or sham adrenalectomized (Sham) rats. The same time-points after restraint onset (1, 1.5 and 2.5 h) were examined in this experiment as in Experiment 1, with the exception that the last time-point (3.5 h) was omitted. Again, an additional group of rats (0 h) was left undisturbed in their home cage until anaesthetization for perfusion. The experiment was also conducted with two separate cohorts of rats, resulting in

an overall total of four to five rats per time-point per ADX or Sham treatment condition ($n = 4-5$).

Experiment 3: replacement of corticosterone in ADX rats and its effects on Fos expression

The third experiment examined the reproducibility of the effect of adrenalectomy on Fos expression in the PVN and dentate gyrus seen in the second experiment (see Results). In addition, the third experiment examined whether these effects of adrenalectomy could be prevented by treating adrenalectomized rats with a relatively low dose of corticosterone (25 µg/ml) in the drinking water (ADX + CORT). The same time-points after restraint onset (1, 1.5 and 2.5 h) were examined in Experiment 3 as in Experiment 2, and again a no stress group was included. As with the other two experiments, two separate cohorts of rats were used. There was an overall total of four to five rats per time-point per Sham, ADX or ADX + CORT treatment condition ($n = 4-5$), with the one exception that there were only three rats in the 1-h ADX group.

Tissue preparation and immunohistochemistry

At the appropriate experimental time-point, rats were deeply anaesthetized (75 mg/kg ketamine/15 mg/kg xylazine i.p) and transcardially perfused with 400 ml of heparinized (1 unit/ml) 0.01 M phosphate buffer followed by 400 ml of 4% paraformaldehyde in phosphate buffer. After removal, brains were postfixed in 4% paraformaldehyde phosphate buffer at 4 °C for approximately 48 h. Brains were sectioned (50 µm thick coronal sections) with a vibratome (The Vibratome Company, St Louis, MO, USA).

Fos protein was visualized using a standard avidin-biotin-horseradish peroxidase immunohistochemical procedure. Tissue sections were incubated overnight in a rabbit polyclonal anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 7500 dilution) that does not cross-react with other Fos related gene products (Fos B, Fra-1 or Fra-2). After washing in assay buffer (0.1 M sodium phosphate) sections were incubated in biotinylated goat antirabbit antibody (Vector Laboratories Inc., Burlingame, CA, USA; 1 : 750 dilution) followed by incubation in an avidin-biotin-horseradish peroxidase solution. Diaminobenzidine (DAB, 0.2 mg/ml in the presence of 7 mg/ml nickel ammonium sulphate) was used as chromogen.

To allow for a direct comparison of the number of immunopositive cells between different brains, all brain sections from a given experimental cohort were processed simultaneously in the same pool of each reagent using staining net dishes (Brain Research Laboratories, Boston, MA, USA).

Fos-positive cell counts

The number of Fos positive cells was counted using a computerized image analysis system (Olympus Microsuite Analysis 3.2; Soft Imaging System Corp., Lakewood, CO, USA). A threshold grey level for positive cells was chosen that was approximately 50% of the maximum grey level signal over background. For a given brain region, four to six separate counts taken from separate sections/hemispheres were conducted for each brain and were then averaged to yield a final value for that brain. The brain sections utilized were first carefully matched between brains for the same relative rostral-caudal location, and were then coded so that cell count analysis was performed without concurrent knowledge of the treatment group assignments. Six brain regions were examined in detail. For the PVN, immunopositive cells were counted within the entire nucleus including both parvocellular and magnocellular neurones. On the same coronal sections that contained the PVN, counts were made within three cortical regions: (i) the retrosplenial granular cortex; (ii) the primary motor cortex and (iii) the primary somatosensory cortex-barrel field (24). The retrosplenial cortex was divided into two layers (inner and outer) to distinguish between more granular layers from more diffuse layers, respectively. Within the hippocampus, cell counts were performed over the pyramidal cell body layer extending from subregions CA1-CA3. Within the dentate gyrus, separate cell counts were performed over the granule cell body layer in the inner (suprapyramidal) blade and the outer (infrapyramidal) blade. To control for slight differences in brain region cross-sectional area/length, Fos positive cell counts were expressed as the number of cells per square micrometer of the region of interest (PVN and cortical areas) or the number of cells per linear micrometer of the length of the respective structure (hippocampus and dentate gyrus).

Determination of pyknotic cells in dentate gyrus

The presence of pyknotic cells in the inner and outer blades of the dentate gyrus was analysed via cresyl violet staining of sections from the Sham, ADX and ADX + CORT no-stress rats in Experiment 3 ($n = 4$). Pyknotic cells were identified and counted manually examining tissue sections through a $\times 40$ objective (Olympus BX61 microscope). The primary criteria for pyknotic cell designation was light or absent cytoplasm and the pronounced presence of condensed granule chromatin (visible as dark, separated clumps). A total of four separate counts taken from different tissue sections/hemispheres were performed for each blade per brain. The number of pyknotic cells was divided by the cross-sectional area for that blade and the resulting densities were averaged for each brain.

Statistical analysis

Fos expression and the number of pyknotic cells were analysed by analysis of variance (ANOVA), followed by Fisher's least significant difference post-hoc test (Fisher's LSD). For Experiment 1, a single-factor (time after restraint onset) ANOVA was conducted. For Experiments 2 and 3, a two-factor (surgery/replacement condition and time after restraint onset) ANOVA was conducted. $P < 0.05$ was considered to be statistically significant. Data are presented as means \pm SEM.

Results

Experiment 1: time course of Fos expression following restraint stress

As described in many other studies, restraint produced a large induction of Fos immunopositive cells in a number of brain regions. All of the brain regions examined, with the exception of the dentate gyrus, exhibited a similar time-course with peak levels of Fos protein evident by 1-1.5 h after restraint onset, and considerable, but not complete return to basal levels by 3.5 h after restraint onset. Thus, there was an overall significant effect for time after restraint onset within the PVN [$F(4,22) = 7.24$, $P < 0.01$], hippocampus CA1-CA3 [$F(4,22) = 3.55$, $P < 0.05$], inner layers of the retrosplenial cortex [$F(4,22) = 2.77$, $P = 0.05$], outer layers of the retrosplenial cortex [$F(4,22) = 5.19$, $P < 0.01$], primary motor cortex [$F(4,22) = 4.70$, $P < 0.01$] and primary somatosensory cortex [$F(4,22) = 7.82$, $P < 0.01$] (data not shown).

Within the hippocampus, we observed a robust increase in the number of Fos positive cells throughout the CA1-CA3 hippocampal subfields that was primarily restricted to the pyramidal cell body layer (Fig. 1A). However, it should be noted that only a small subset of the total number of cell bodies within this layer were Fos positive, even at the peak of the response (an average of 215 cells per hemisphere per tissue section). Within the dentate gyrus, Fos positive cells were similarly localized to the granule cell body layer and were also relatively sparse (Fig. 1A). Interestingly, the effect of restraint on Fos positive cells in the dentate gyrus revealed a unique temporal and qualitative pattern relative to the other brain regions examined. Within the inner blade (Fig. 1B), there was a delayed (no increase at 60 min), but subsequent rapid induction (maximal at 90 min) of Fos, resulting in an overall significant effect of time after restraint onset [$F(4,22) = 2.80$, $P = 0.05$]. Surprisingly, in the outer blade, restraint produced a significant decrease in Fos positive cells that was evident by 1 h after restraint onset and remained reduced throughout the 3.5 h time-course [$F(4,22) = 7.89$, $P < 0.01$].

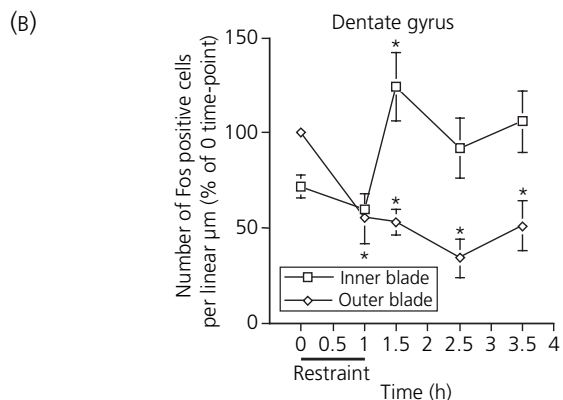
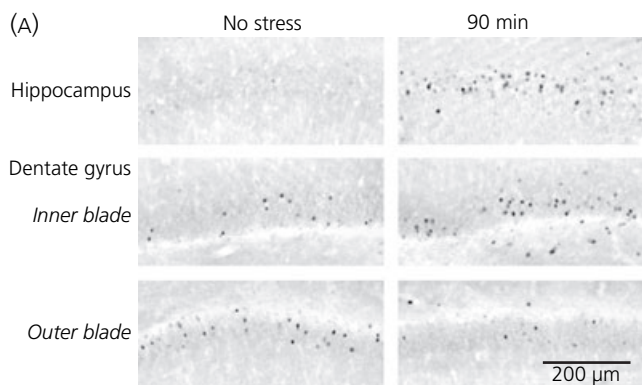


Fig. 1. Fos expression after restraint stress in hippocampus (CA1), and dentate gyrus of adrenal-intact rats. (A) Example photomicrographs are shown for rats that were not exposed to restraint (No stress) or exposed to 60 min of restraint and killed 30 min later (90 min Stress). Photomicrographs are centred over the pyramidal cell body layer of the hippocampus or the granule cell layer of the dentate gyrus. (B) Time-course for changes in Fos expression in the dentate gyrus. Data are expressed as a percent of the outer blade no-stress group's mean value. *Significant difference from time 0 group within the same blade, $P < 0.05$ (Fisher's LSD; $n = 6$, with exception of the 1-h time-point that had three rats).

Experiment 2: effect of adrenalectomy on Fos expression

In the dentate gyrus, sham rats displayed a very similar pattern of Fos expression to that seen in Experiment 1 (Fig. 2). In the inner blade, there was a sharp increase in Fos positive cells between the 60 min and 90 min time-points [$F(3,22) = 8.20$, $P < 0.01$], whereas, in the outer blade, there was a steady decrease in Fos positive cells over the 2.5 h after restraint onset [$F(3,22) = 3.44$, $P < 0.05$].

Adrenalectomy had no effect on basal or restraint-induced Fos expression in the cortical regions or hippocampus CA1–CA3 (data not shown). On the other hand, in the PVN, there was a strong trend ($P = 0.07$, Student's two-tailed t -test) for a greater level of Fos positive cells 60 min after restraint onset in ADX rats compared to Sham rats (Fig. 2). Interestingly, in the dentate gyrus, there was an overall reduction in Fos expression in both the inner [$F(1,22) = 13.17$, $P < 0.01$] and outer blades [$F(1,22) = 10.69$, $P < 0.01$] of ADX rats (Fig. 2). This reduction was seen in the no-stress rats as well as at all time-points following restraint, such that there was no treatment-by-time after stress onset interaction.

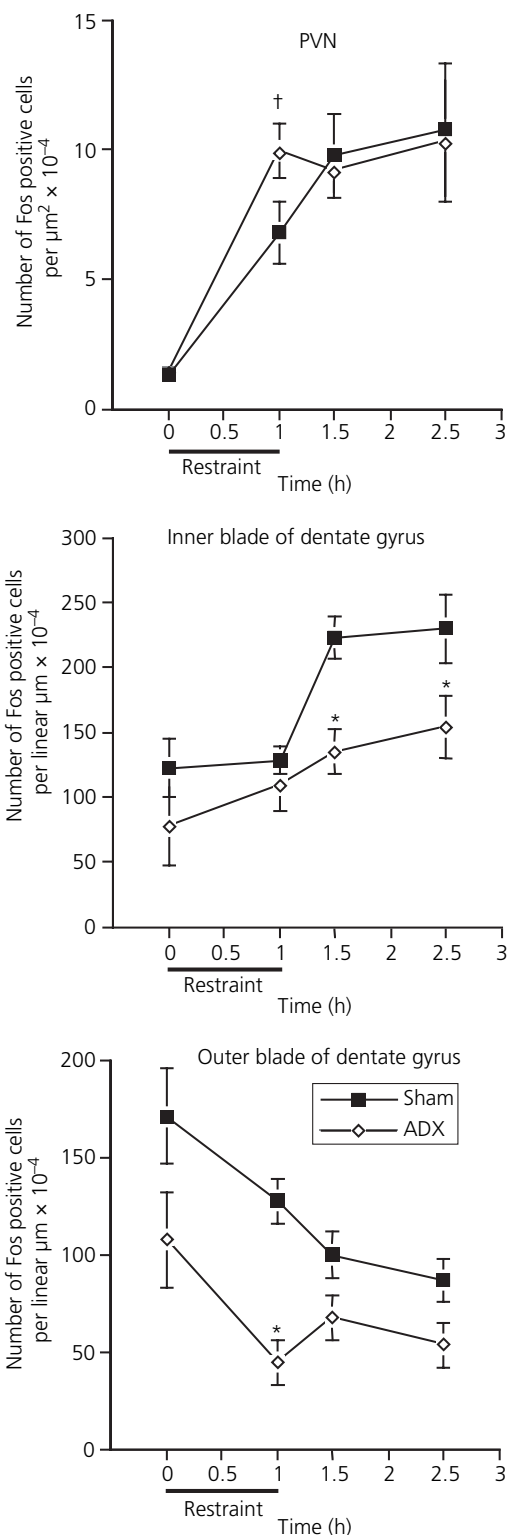


Fig. 2. Differential effect of adrenalectomy on basal or stress-induced Fos expression in the paraventricular nucleus (PVN) and dentate gyrus. Fos expression was examined in unstressed rats (Time 0) or rats at various time-points after 60 min of restraint. Rats were either adrenalectomized (ADX) or sham adrenalectomized (Sham) 5 days earlier. *Significant difference between ADX and Sham rats at the same time-point, $P = 0.05$ (Fisher's LSD; $n = 4$, with exception of the 1-h Sham group that had five rats). †Statistical comparison between ADX and Sham rats at the 1-h time-point in PVN, $P = 0.07$ (Student's two-tailed t -test).

Experiment 3: replacement of corticosterone in ADX rats and its effects on Fos expression and pyknosis

The effects of ADX very closely matched those seen in Experiment 2, with selective, but differential effects of ADX in the PVN and dentate gyrus. Within the PVN, there was a more pronounced effect of ADX in the PVN than in Experiment 2, with an enhanced level of stress-induced Fos expression 1.5 and 2.5 h after restraint onset (Figs 3 and 4). Replacement of ADX rats with corticosterone in the drinking water completely normalized the Fos response. Thus, there was an overall effect of surgery/replacement condition on Fos expression in the PVN [$F(2,37) = 6.10$, $P < 0.01$]. Collapsed across time, levels of Fos were significantly different between ADX and ADX + CORT ($P < 0.01$) and between ADX and Sham groups ($P < 0.01$; Fisher's LSD).

For the dentate gyrus, the opposite stress-induced change in Fos expression between the outer and inner blades was seen for the third time (Figs 3 and 5). As in Experiment 2, there was an overall lower level of Fos expression at all time-points in both the inner and outer blades of ADX rats, and this effect was normalized in ADX + CORT rats. Thus, in the inner blade of the dentate gyrus, there were significant effects of surgery/replacement condition and time after restraint onset [$F(2,37) = 19.50$, $P < 0.01$ and $F(3,37) = 16.04$, $P < 0.01$, respectively]. Similarly, for the outer blade, there were also significant effects of surgery/replacement condition and time after restraint onset [$F(2,37) = 10.26$, $P < 0.01$ and $F(3,37) = 11.06$, $P < 0.01$, respectively].

There were no significant effects of ADX on Fos expression in the hippocampus CA1–CA3 or the cortical regions examined (Fig. 6). Interestingly, in ADX + CORT rats, there was significantly greater Fos expression 1 h after restraint onset in many of the brain regions examined (retrosplenial cortex, somatosensory cortex, hippocampus and both blades of the dentate gyrus).

The number of pyknotic cells present in the dentate gyrus was counted for a subset of brains from Experiment 3 (all brains from no-stress groups). Very few pyknotic cells were present in the inner or outer blades of Sham rats or ADX + CORT rats (Table 1). On the other hand, many more pyknotic cells were present in the dentate gyrus of ADX rats. Although pyknotic cells were evident in both blades of ADX rats, the density was greater in the inner blade. Even within the inner blade, the number of pyknotic cells was sparse compared to the total number of normal appearing granule cells.

Discussion

This study demonstrates a role for basal endogenous corticosterone in regulating Fos protein expression in the brain. However, this role is not only brain region selective, but also the nature of the role varies between brain regions. Specifically, we observed a similar time-course for Fos induction after acute restraint stress in the PVN, hippocampus CA1–CA3 and several cortical regions. However, adrenalectomy resulted in an enhanced stress-induced Fos expression only in the PVN. This enhanced Fos expression was not present in adrenalectomized rats treated with a low dose of corticoster-

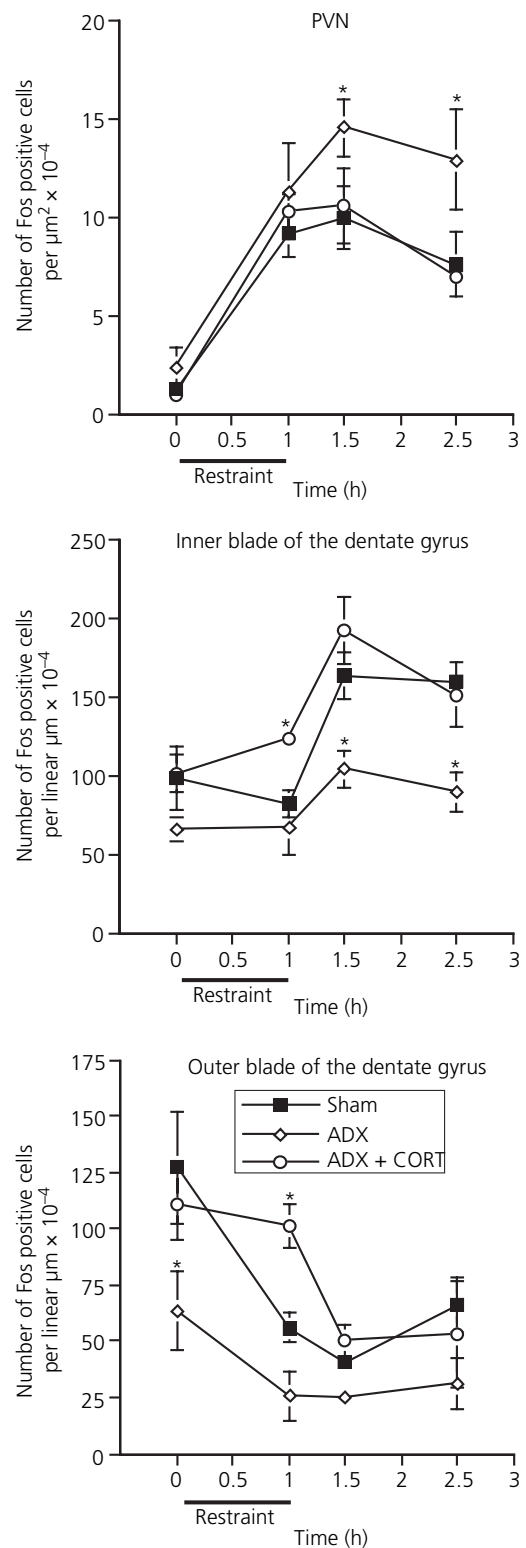


Fig. 3. Corticosterone replacement normalizes adrenalectomy effect on Fos expression in the paraventricular nucleus (PVN) and dentate gyrus. Fos expression was examined in unstressed rats (Time 0) or rats at various time-points after 60 min of restraint. Rats were either sham adrenalectomized (Sham), adrenalectomized (ADX), or adrenalectomized and provided with corticosterone (CORT) (25 $\mu\text{g}/\text{ml}$) in their drinking water (ADX + CORT) 5 days earlier. *Significant difference compared to Sham rats at the same time-point, $P = 0.05$ (Fisher's LSD; $n = 4-5$, with exception of the 1-h ADX group that had three rats).

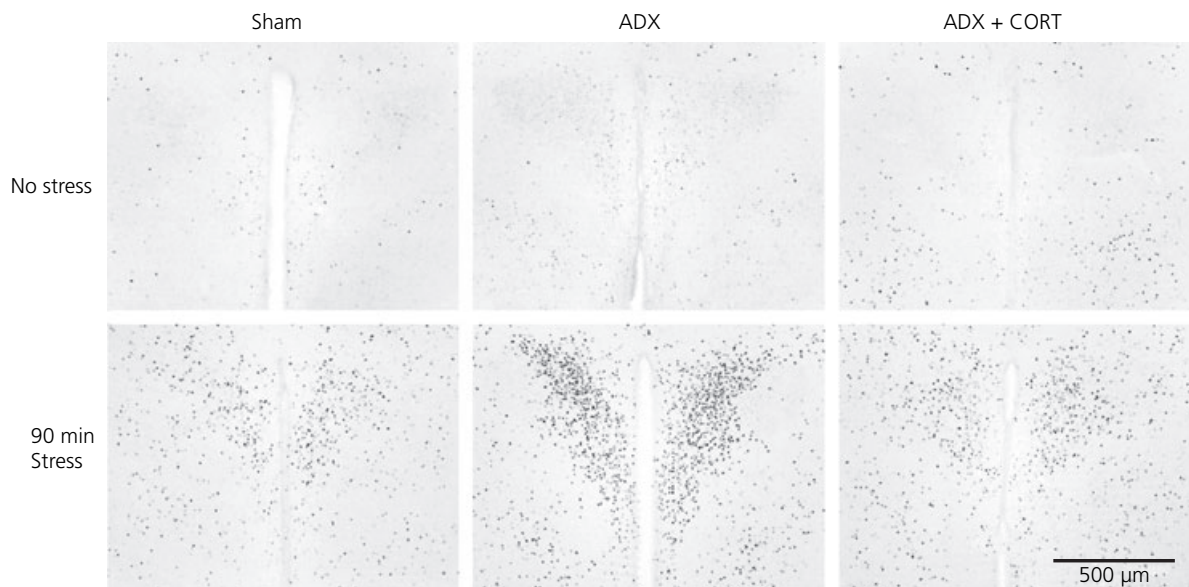


FIG. 4. Representative photomicrographs of Fos expression in the paraventricular nucleus (PVN) of rats in Experiment 3. Example photomicrographs are shown for rats that were not exposed to restraint (No stress) or exposed to 60 min of restraint and killed 30 min later (90 min Stress). In addition, rats were either sham adrenalectomized (Sham), adrenalectomized (ADX), or adrenalectomized and provided with corticosterone (CORT) (25 $\mu\text{g}/\text{ml}$) in their drinking water (ADX + CORT) 5 days earlier. The third ventricle is visible in the centre of each photomicrograph.

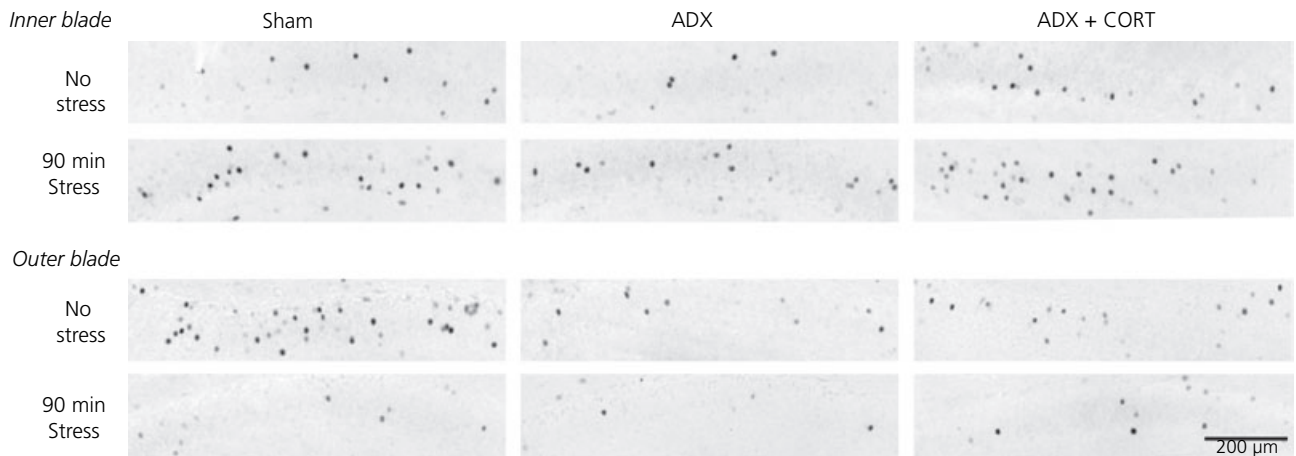


FIG. 5. Representative photomicrographs of Fos expression in the inner and outer blades of the dentate gyrus of rats in Experiment 3. Example photomicrographs are shown for rats that were not exposed to restraint (No stress) or exposed to 60 min of restraint and killed 30 min later (90 min Stress). In addition, rats were either sham adrenalectomized (Sham), adrenalectomized (ADX), or adrenalectomized and provided with corticosterone (CORT) (25 $\mu\text{g}/\text{ml}$) in their drinking water (ADX + CORT) 5 days earlier. Photomicrographs are centred over the granule cell layer of the dentate gyrus.

one in the drinking water. These studies also revealed a unique temporal and qualitative pattern of Fos expression in the dentate gyrus, which was also affected by adrenalectomy, but in a manner different than in the PVN. Within the dentate gyrus, restraint produced a delayed induction of Fos in the inner (suprapyramidal) blade compared to the other brain regions examined. Unexpectedly, we found a reproducible decrease in Fos expression in the outer (infrapyramidal) blade of the dentate gyrus following restraint. For both blades, the total number of Fos positive cells, regardless of stress condition, was reduced in adrenalectomized rats. As was the case for the PVN, treatment of adrenalectomized rats with corticosterone in the drinking water normalized the Fos

expression in the dentate gyrus. However, it should be emphasized that the effect of adrenalectomy in the PVN was to increase stress-induced Fos expression whereas, in the dentate gyrus, the effect of adrenalectomy was to reduce basal and poststress levels of Fos expression.

Glucocorticoid regulation of stress-induced Fos in PVN

Although glucocorticoids appear to not regulate basal Fos expression in the PVN, in our study, adrenalectomy produced an increased level of Fos in the PVN after restraint. This effect was more pronounced in the third experiment than the second, but given other reports of enhanced stress-induced

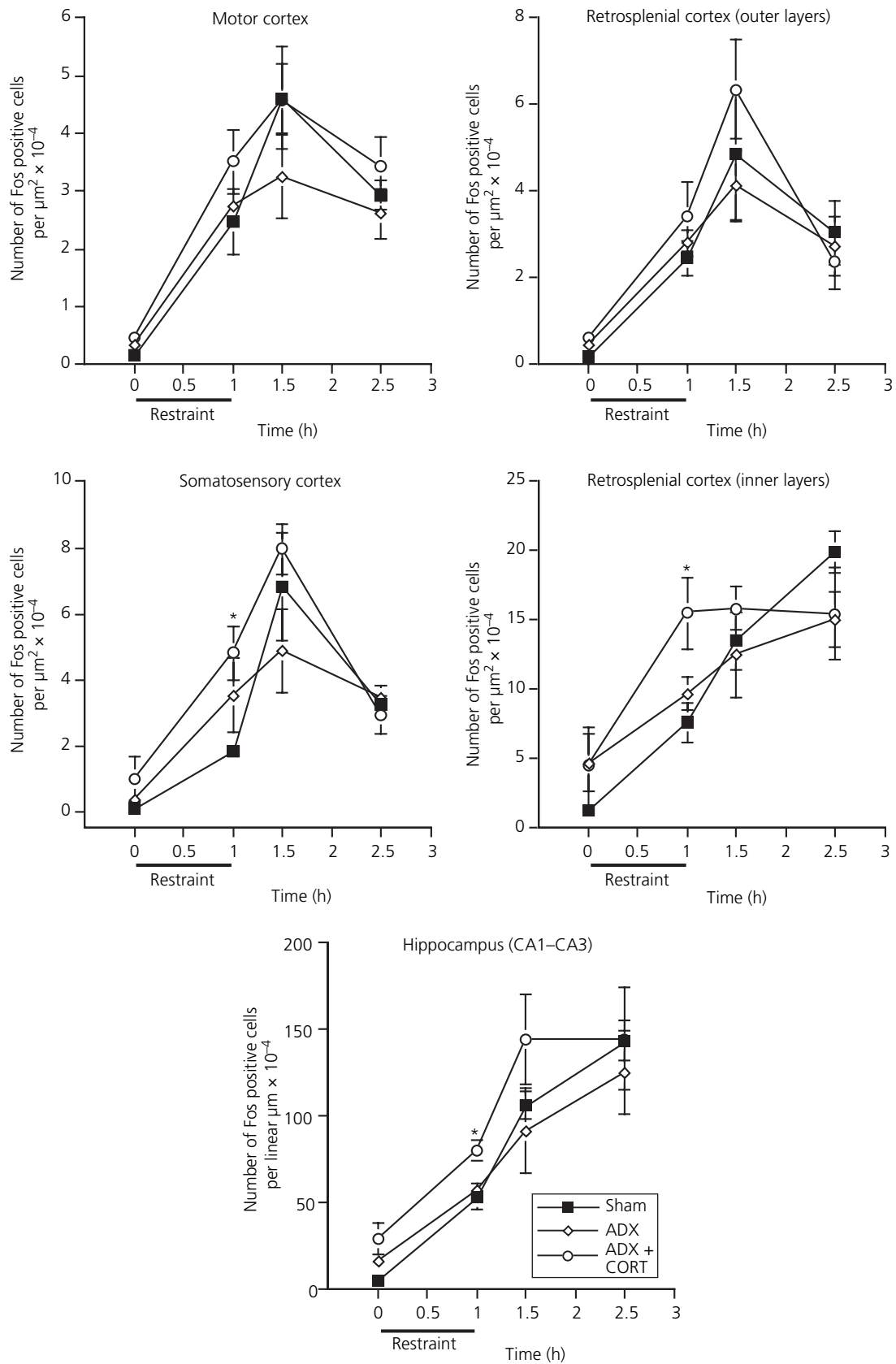


FIG. 6. Lack of effect of adrenalectomy on basal or stress-induced Fos expression in cortex and hippocampus (CA1-CA3). Fos expression was examined in unstressed rats (Time 0) or rats at various time-points after 60 min of restraint. Rats were either sham adrenalectomized (Sham), adrenalectomized (ADX), or adrenalectomized and provided with corticosterone (CORT) (25 µg/ml) in their drinking water (ADX + CORT) 5 days earlier. *Significant difference compared to Sham rats at the same time-point, $P = 0.05$ (Fisher's LSD; $n = 4-5$, with exception of the 1-h ADX group that had three rats).

TABLE 1. Density of Pyknotic Cells (Number of Cells per $\mu\text{m} \times 10^{-4}$) in the Dentate Gyrus.

Condition	Inner blade	Outer blade
Sham	5.84 \pm 2.79	2.01 \pm 1.16
ADX	254.22 \pm 91.94*	80.40 \pm 14.95*
ADX + CORT	2.38 \pm 1.89	1.99 \pm 0.81

*Significant difference from the Sham group, $P < 0.05$ (Fisher's LSD; $n = 4$). ADX, Adrenalectomized; CORT, corticosterone.

c-fos mRNA levels in the PVN of adrenalectomized rats, this effect appears to be reliable (11, 12). Importantly, in our study, treatment of adrenalectomized rats with corticosterone in the drinking water was sufficient to normalize PVN Fos expression. This is in contrast to a study by Li and Sawchenko (14) in which adrenalectomized rats replaced with a low-dose corticosterone pellet (25 mg s.c.) exhibited an increased number of Fos positive cells in the PVN after foot-shock compared to sham adrenalectomy. The low-dose corticosterone pellet used in the study by Li and Sawchenko (14) produced plasma corticosterone levels of approximately 3.5 $\mu\text{g}/100\text{ ml}$, which is a level believed to occupy the majority of mineralocorticoid receptors but very few glucocorticoid receptors (25). In our study, we did not collect blood for plasma corticosterone measures on the test day because we did not want the stress of blood collection to confound our Fos measures. Because our replacement regimen (CORT in the drinking water) typically results in very low plasma corticosterone levels during the light period (15–17), it is likely that there was very little occupancy of the lower affinity glucocorticoid receptor for a number of hours before restraint onset in our study. Whether there was a reduced occupancy of the higher affinity mineralocorticoid receptor is harder to predict. Certainly, there was a complete absence of a phasic change in mineralocorticoid receptor and glucocorticoid receptor occupancy during restraint. If the normalization of Fos expression that we saw is due to a direct effect of corticosterone on cells in the PVN, then glucocorticoid receptor occupancy is probably necessary because these cells appear to express very few mineralocorticoid receptors (26). It appears that either a circadian variation in plasma corticosterone and/or significant glucocorticoid receptor occupancy sometime in the previous 24 h is necessary to maintain normal Fos induction to acute stress in the PVN. This pattern of glucocorticoid dependency is reminiscent of the ability of corticosterone in the drinking water, but not low-dose corticosterone pellets, to normalize the timely shut-off of poststress ACTH levels in adrenalectomized rats (16, 27). The ability of glucocorticoids to maintain a normal Fos response to restraint in the absence of concurrent phasic changes in glucocorticoid secretion is representative of what has traditionally been referred to as permissive or proactive effects of glucocorticoids (28–30).

As described above, the results of our study and others indicate that endogenous glucocorticoids constrain the magnitude of *c-fos* mRNA and protein expression in the PVN after acute stress. There is also some evidence for tonic high levels of glucocorticoids to further suppress *c-fos* gene expression. Treatment of adrenal-intact or adrenalectomized

rats with moderate- to high-dose corticosterone pellets (50–400 mg) attenuates or dramatically reduces *c-fos* gene expression in response to acute challenge (10, 13, 31, 32). On the other hand, there is little evidence for an acute elevation of glucocorticoids to suppress stress-induced *c-fos* gene expression. We, and another group, have found that pretreatment of rats 1 h before restraint with high-dose corticosterone or a selective glucocorticoid receptor agonist (RU28362 or dexamethasone) had no effect on *c-fos* gene expression in the PVN (33, 34). Thus, it appears that the inhibitory effect of glucocorticoids on stress-induced Fos expression requires more than 1 h to develop, and persists for a number of hours, even when circulating glucocorticoids are very low or undetectable (ADX + CORT treatment group in this study). This temporal pattern of regulation supports the prospect that corticosterone tonically regulates the level (or state) of some protein(s) whose functional levels change slowly in response to corticosterone elevation and removal. In addition, this corticosterone-dependent protein has a modulatory effect on stress-induced Fos expression in the PVN.

It remains to be determined whether the tonic inhibitory influence of glucocorticoids on stress-induced Fos expression in the PVN is a result of a direct effect of glucocorticoids on PVN cells or an indirect effect on the net excitatory input to those cells. However, it is unlikely that glucocorticoids have a general tonic inhibitory influence on neuronal excitability throughout the brain because we saw no effect of adrenalectomy on basal or stress-induced Fos expression in three different functional cortical regions or in the hippocampus CA1–CA3. Other studies that have observed an effect of adrenalectomy or high-dose corticosterone treatment on Fos expression also noted that the effects are largely restricted (although not exclusively) to the PVN (13, 14, 32, 35).

Fos expression in the dentate gyrus of adrenal-intact rats

The opposing effect of stress on Fos expression in the two blades of the dentate gyrus was a surprising result. First, we are unaware of other examples of an acute reduction in Fos positive cells within a brain region in response to restraint or other experiences considered to have a stressor component. Second, we cannot find examples in the literature of a divergence in the activity of inner and outer blade cells within the dentate gyrus. Although we are not sure that the Fos positive cells within the dentate gyrus are granule neurones, the location is consistent with granule cell phenotype. The innervation of the inner and outer blades comes predominantly from the entorhinal cortex and from other portions of the hippocampal formation (hippocampal association and commissural connections). Reviews of the dentate gyrus do not mention a difference in innervation or intrinsic properties of the two blades (36–39). The differential effect of restraint on the number of Fos positive cells in the two blades does not appear to be a result of a differing response to stress-induced corticosterone secretion because the pattern was still evident in ADX rats. Thus, the differential response apparently reflects a difference in the neural input to the two blades resulting from the stimulus of restraint, or a different response of some cells within those two blades to that input.

Glucocorticoid regulation of Fos in hippocampal formation

As noted above, Fos expression in the dentate gyrus also varied with the glucocorticoid manipulations; however, the nature of the glucocorticoid dependence was different from that observed in the PVN. In the case of the dentate gyrus, the glucocorticoid dependence was primarily on the basal number of Fos positive cells, with less cells in the absence of corticosterone. However, as was the case for the PVN, the adrenalectomy effect was normalized with corticosterone in the drinking water replacement.

It is perhaps unexpected that this glucocorticoid dependence was restricted to the dentate gyrus and not expressed in the hippocampus CA1–CA3. There is considerable characterization supporting glucocorticoid regulation of pyramidal cell excitability in the hippocampus (18). Furthermore, as part of the trisynaptic circuit, hippocampal pyramidal cells are downstream from the dentate gyrus. However, this is not the only example for a selective dependence of the dentate gyrus relative to the hippocampus CA1–CA3 on the permissive effects of corticosterone. Survival of granule cells in the dentate gyrus, but not hippocampal pyramidal cells, requires basal corticosterone (specifically mineralocorticoid receptor activation) (40, 41). Furthermore, neurogenesis and granule cell turnover in the dentate gyrus is regulated by glucocorticoids (42). One related possibility that we considered was that, in our experiments, the reduced number of Fos positive cells present in ADX rats was secondary to an overall loss of granule cells after ADX. However, we believe that this is unlikely because of the relatively short interval after ADX (5 days) in which we measured Fos expression. In addition, we performed counts of cells displaying apoptotic-like features (pyknosis) in adjacent tissue sections stained for cresyl violet taken from brains in our third experiment. As seen in several other studies (40, 43), there was a significant increase in pyknotic cells in the dentate gyrus of ADX rats, which was completely blocked by corticosterone in drinking water replacement. However, using estimates for the total number of granule cells present (44), we calculate that the number of pyknotic cells that we observed in both blades of the dentate gyrus within one hemisphere of a 50- μ m tissue section (average of 80) was a small percentage of the total number of granule cells present (approximately 1%), whereas we saw a 50% reduction in the number of Fos positive cells in the dentate gyrus of ADX rats compared to sham rats. Thus, it appears that apoptosis was not a factor in the reduced number of Fos positive cells present in the dentate gyrus of ADX rats. However, we cannot rule out the intriguing possibility that a high proportion of the granule cells destined for death in the absence of corticosterone were also the ones that showed basal and stress-induced changes in Fos expression.

In conclusion, the results of this study indicate that basal levels of endogenous glucocorticoids have distinct permissive effects on cellular activity in the brain as expressed by basal and/or stress-induced Fos protein expression. These effects are restricted to select brain regions, and perhaps to specific subpopulations of cells, such as the CRH secreting neurones of the PVN and the granule cells of the dentate gyrus.

Moreover, the nature of these permissive glucocorticoid effects can vary between cell populations. In the PVN, glucocorticoids appear to restrict the responsiveness of the entire population of CRH neurones to stress-related activation. In the dentate gyrus, glucocorticoids appear to increase the overall number of granule cells that actively express Fos at any one time.

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