Dissociation between c-fos mRNA in the paraventricular nucleus and corticosterone secretion in rats with adjuvant-induced arthritis

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Abstract

Increased c-fos mRNA or fos immunoreactivity within the central nervous system has been used as a marker of neuronal activation. Acute stress and acute immune challenge result in an increase in c-fos mRNA in corticotrophin-releasing factor (CRF)-containing neurons in the paraventricular nucleus (PVN). It has often been implied that an increase in fos in the PVN can be equated to an increase in the activity of CRF itself, although there is some evidence to suggest these events are not linked. In the present study we have used the rat model of adjuvant-induced arthritis (AA), in which, despite the activation of the pituitary–adrenal system associated with inflammation, there is a paradoxical decrease in CRF mRNA and CRF peptide release. AA rats are unable to mount a hypothalmo–pituitary–adrenal (HPA) axis response to stress. They are, however, able to mount a response to acute immune stimulation, e.g. lipopolysaccharide injection. Despite the lack of HPA axis response to stress, there is an increase in c-fos mRNA to these challenges in AA. This suggests that the increase in c-fos mRNA in response to acute stress is not related to a subsequent increase in CRF mRNA in this model. We can conclude that under these conditions, c-fos mRNA is not a good marker of HPA axis activation and independent estimation of the involvement of CRF in the stimulation of the HPA axis should always be obtained. The AA model may prove useful for the comparison of the relationship between immediate early genes and heteronuclear RNAs in response to acute stress and immune stimuli with which to tease apart the molecular mechanisms underlying the control of releasing factor activation at the level of the PVN.

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Introduction

Increased expression of the immediate early gene c-fos has been used in numerous studies as a marker of neuronal activation. Exposure to a variety of acute stressful stimuli results in an increase in c-fos mRNA and/or fos immunoreactivity in the parvicellular cells of the paraventricular nucleus (PVN) of the hypothalamus. Increased fos has been reported in response to restraint, i.p. hypertonic saline (HS), acute immune challenge and many other acute stimuli (Ceccatelli et al. 1989, Imaki et al. 1992, Kononen et al. 1992, Rivest et al. 1992, Covenas et al. 1993, Harbuz et al. 1993a, Senba et al. 1994). Double-labelled immunohistochemistry has revealed fos immunoreactivity in corticotrophin-releasing factor (CRF)-containing cells (Ceccatelli et al. 1989, Ju et al. 1991, Rivest et al. 1992, Covenas et al. 1993, Veening et al. 1993). These observations provide compelling evidence for the involvement of fos in mediating the stress-induced stimulation of CRF neurons, CRF itself and hence of the hypothalamo–pituitary–adrenal (HPA) axis. In general there has been good agreement that following acute stimuli where there is increased HPA activity there is also an increase in CRF mRNA, c-fos mRNA and/or fos peptide in the PVN.

There have, however, been a number of studies that question the relationship between CRF and c-fos. First, the CRF gene does not appear to contain the AP-1 binding site through which fos regulates transcription of target sites (Seasholtz et al. 1988, Chan et al. 1993, Hoffman et al. 1993). Secondly, it appears that during the stress-hyporesponsive period in neonates while there is only a minimal stress response, the c-fos mRNA response in the PVN is intact (Smith et al. 1997). Finally, there is evidence that expression of heteronuclear (hn) CRF can precede that of c-fos (Herman et al. 1992, Kovacs & Sawchenko 1996), suggesting that c-fos is not a requirement for increased CRF. However, this latter observation may be dependent on the type of stress used (Imaki et al. 1996, Kovacs & Sawchenko 1996).

Despite the observations suggesting a dissociation between fos and CRF mRNA in the PVN in response to acute stimuli, many authors have linked increased fos and HPA axis activity to increased CRF activity. In many
cases these reports contain data supporting an increase in CRF activity by measuring hnCRF RNA or CRF mRNA directly; however, in others this relationship is implied (Lee et al. 1994, Laflamme et al. 1996, Callahan & Pickut 1997, Campeau & Watson 1997, Larsen et al. 1997, Buller et al. 1998).

We have previously reported that rats with adjuvant-induced arthritis (AA) are unable to mount an HPA axis response to acute stress (Harbuz et al. 1993b, 1999a, Aguillera et al. 1997). We have been unable to detect increases in either CRF mRNA in the PVN or plasma corticosterone in response to the predominantly psychological stressors of restraint or noise, or to the physical stress of i.p. HS. However, these animals are able to mount a response of the HPA axis to acute injection of lipopolysaccharide (LPS) with increases in CRF mRNA centrally and in plasma levels of adrenocorticotrophin (ACTH) and corticosterone (Harbuz et al. 1999b).

It is evident that a variety of acute stimuli are able to increase c-fos mRNA in CRF-containing neurons in the PVN. This implies an increase in the activity of CRF systems but does not confirm it, as CRF is co-localised with numerous other releasing factors (Hökfelt et al. 1983, Whitmull et al. 1985, 1987, Hisano et al. 1987, Sakanaka et al. 1989, Merchenthaler 1992). In the present study we wished to test the hypothesis that c-fos mRNA is a reliable marker of an increase in CRF mRNA and/or the HPA axis. We have used the AA model to compare the increase in c-fos mRNA in the PVN in response to acute stress (i.p. injection of HS), and to an acute immune stimulus (i.p. injection of LPS). Plasma corticosterone was measured to confirm changes in the HPA axis in response to these stimuli. CRF mRNA in the PVN following these challenges has previously been reported (Harbuz et al. 1999b, 1999b).

Materials and Methods

Male Piebald–Viral–Glaxo rats (8–9 weeks of age) were housed four to a cage under standard lighting conditions with free access to food and water. On day 0 the rats were given an i.d. injection (0·1 ml) of vehicle (paraffin oil), or a suspension of ground, heat-killed Mycobacterium butyricum (Difco Laboratories, East Molesey, Surrey, UK) in paraffin oil (10 mg/ml) into the tail base. Fourteen days after adjuvant injection the animals received either (i) an i.p. injection of isotonic saline (IS) or HS (9% saline, 1·8 ml/100 g as acute stress) or (ii) an i.p. injection of IS or LPS (250 µg/0·5 ml saline; E. coli, 055B5; Sigma Chemical Co., Poole, Dorset, UK). Only animals with clear visual signs of hind-paw inflammation were included in the study. Animals were decapitated (i) at 30 and 90 min and (ii) at 90 min. Trunk blood was collected into heparinised tubes, centrifuged and plasma stored at −20 °C prior to RIA for corticosterone. The brains were quickly removed, frozen on dry ice and stored at −80 °C prior to in situ hybridisation (ISH). All studies were carried out in full accordance with UK Home Office guidelines.

ISH was performed using a 48-mer oligonucleotide complementary to part of the exonic mRNA sequence coding for c-fos, as previously described (Young et al. 1986, Harbuz & Lightman 1989). Briefly, 12 µm-thick sections were taken on a cryostat and thaw-mounted onto gelatin-coated slides, dried and stored at −80 °C prior to ISH. On the day of the assay, sections were transferred to room temperature, air dried and fixed in 4% formaldehyde for 5 min, washed twice in 1× PBS and transferred into 0·25% acetic anhydride in 0·1 M triethanolamine/0·9% saline for 10 min. Sections were then dehydrated through graded ethanol washes, delipidated in chloroform for 5 min and rehydrated in ethanol. All control and experimental sections were hybridised in the same hybridisation reaction. Probes were labelled with 35S-ATP using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) to a specific activity of 1×1018 d.p.m./mol. Forty-five microlitres of hybridisation buffer (50% formamide, 4× saline sodium citrate (SSC), 0·5 mg/ml sheared single-stranded salmon sperm DNA, 0·25 mg/ml yeast tRNA, 0·5× Denhardt’s solution, 10% dextran sulphate), containing labelled probe were added to the slides and coverslipped. All control and experimental sections were hybridised in the same reaction. After hybridisation overnight in a humid chamber at 37 °C with 105 c.p.m./slide, coverslips were removed and slides washed four times at 55 °C in 1× SSC, twice at room temperature in 1× SSC, and after two brief dips in distilled water, to remove salts, they were dried and exposed to Amersham HyperfilmMP (Amersham International, Amersham, Bucks, UK) together with 14C standards for 14 days. The autoradiographic images of probe bound to the medial parvicellular region of the PVN were analysed as described previously (Harbuz & Lightman 1989), using Image 1·22 software developed by Wayne Rasband (NIH, Bethesda, MD, USA) and run on an Apple Macintosh computer. The results are presented as the mean percentage change from control with a standard error about the mean.

RIA

Total plasma corticosterone was measured directly in plasma (1 µl diluted in 100 µl buffer) using antiserum kindly supplied by G Makara (Institute of Experimental Medicine, Budapest, Hungary). The tracer was 125I-corticosterone (ICN Biomedicals, Irvine, CA, USA) with a specific activity of 2–3 mCi/µg. The intra-assay variation was 9%.

Statistics

Statistical comparisons were made using the Fisher protected least significant difference test following one-way
ANOVA (n=5–8 per group). P<0·05 was considered significant.

Results

In response to HS stress there was a significant increase in plasma corticosterone in the control animals at both 30 min (P<0·05) and 90 min (P<0·01) compared with the levels seen in the IS-injected control rats (Fig. 1a). In contrast, in the AA animals corticosterone levels were not increased by HS at either time-point compared with either IS-injected AA or control rats. Levels in control+HS rats were significantly (P<0·05) elevated compared with those in AA+HS rats at both the 30 and 90 min time-points.

Injection of IS did not result in an increase in c-fos mRNA in the PVN in control rats. Levels of c-fos mRNA were also below the limits of detection in AA rats and were not elevated in response to IS (Fig. 1b). Following HS, c-fos mRNA was noted in both control and AA rats at both 30 and 90 min after injection. There was no significant difference in c-fos mRNA between the treatment groups.

LPS injection resulted in a similar increase in plasma corticosterone in both AA and control animals at 90 min (Fig. 2a). These levels were significantly (P<0·01) elevated compared with the respective vehicle-injected rats. Levels of c-fos mRNA were below the limit of detection in vehicle-injected control and AA rats. LPS resulted in a marked increase in c-fos mRNA in control rats at the

Figure 1 Plasma corticosterone levels (a) and c-fos mRNA levels (b) 30 and 90 min following i.p. injection of either IS or HS in non-arthritic controls or rats with AA. Values represent means ± S.E.M. for n=4 or 5 per group. nd=not detected. **P<0·01 compared with control/IS values at 30 min.

Figure 2 Plasma corticosterone levels (a) and c-fos mRNA levels (b) 90 min following i.p. injection of either saline or LPS in non-arthritic controls or rats with AA. Values represent means ± S.E.M. for n=6 per group. **P<0·01 compared with (a) respective saline-injected rats, or (b) control+LPS-injected rats.
90 min time-point. LPS injection in AA rats resulted in a further significant (P<0.01) increase in c-fos mRNA over levels seen in control rats given LPS (Fig. 2b).

**Discussion**

Plasma corticosterone was not increased in response to acute HS stress in rats with AA, confirming previous observations that these animals are unable to mount a response to acute stress (Harbuz et al. 1993b). In addition to the lack of a corticosterone response we have previously reported a failure to elevate CRF mRNA in the PVN in response to HS stress (Table 1), and other acute stress stimuli (Harbuz et al. 1993b, 1999a, Aguilera et al. 1997).

Despite this lack of involvement of central CRF and subsequent HPA axis activation, we have shown that the c-fos mRNA response in the PVN remains intact in these animals, suggesting a dissociation between the elevation in c-fos mRNA and CRF activity.

In contrast to the inability of AA rats to respond to acute stress, the response to LPS remains intact with a significant increase in plasma corticosterone in both control and AA rats. This is reflected at the level of the PVN by an increase in c-fos mRNA which was further elevated in the AA rats over the levels seen in control animals. We have recently reported that unlike the lack of response to acute stress, stimulation of the HPA axis in response to acute immune stimuli such as LPS occurs at all levels of the axis with an increase in plasma ACTH, pro-opiomelanocortin mRNA in the anterior pituitary and CRF mRNA in the PVN (Table 1; Harbuz et al. 1999b). In response to a single exposure to LPS there is HPA axis activation. However, following repeated LPS injections ACTH and corticosterone responses are markedly attenuated and c-fos mRNA remains below the limit of detection. Despite the lack of increase in c-fos mRNA, CRF mRNA levels remain elevated suggesting a possible discrepancy in these mRNAs in this repeated immune-stimulus paradigm (Takemura et al. 1997).

**Table 1** CRF mRNA in the parvocellular cells of the PVN of adjuvant-injected (AA) rats with hind-paw inflammation 4 hours following: (i) i.p. injection of isotonic saline (IS) or hypertonic saline (HS), or (ii) in a separate study, vehicle or lipopolyasaccharide (LPS). Values (means ± S.E.M.) are expressed as the percentage change from control, non-arthritisic animals (non-AA), assigned a value of 100%

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<tr>
<td>Non-AA</td>
<td>100 ± 6.0</td>
<td>100 ± 11.4</td>
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<tr>
<td>AA+IS</td>
<td>57 ± 6.1*** n=14</td>
<td>32 ± 6.7*** n=5</td>
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<tr>
<td>AA+HS</td>
<td>43 ± 4.7*** n=13</td>
<td>100 ± 15.9## n=7</td>
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**P<0.01** compared with non-AA rats. **##P<0.01** compared with corresponding AA + vehicle animals.

Increased c-fos mRNA and/or fos protein has been used to map neuronal pathways through the central nervous system in response to a variety of stimuli. However, the precise nature of the relationship between fos and its subsequent effects on gene expression remain to be determined, particularly in respect to the stress response. Following acute stress, increased c-fos mRNA in CRF-containing neurons in the medial parvocellular region of the PVN has resulted in the general assumption of a direct relationship between these events preceding increased activity in the HPA axis. This assumption has been challenged by reports of a temporal dissociation following ether stress with an increase in hcnCRF RNA preceding the increase in c-fos mRNA (Kovacs & Sawchenko 1996).

However, it should be noted that ether stress does not result in an increase in CRF mRNA (Watts 1991, Kovacs & Sawchenko 1996). As a consequence it is perhaps not the best system with which to investigate these relationships. In contrast to the response to ether stress, Imaki et al. (1996) reported that in response to restraint stress (following which CRF mRNA levels in the PVN are increased), both hcnCRF RNA and c-fos mRNA were increased within 5 min, suggesting these events could be related.

There is compelling evidence for an increase in fos within CRF-containing neurons in the PVN following exposure to acute stimuli. In addition to CRF, it is firmly established that these neurons may also contain arginine vasopressin (AVP) and/or enkephalins. It is possible that these components may be under independent control. Whereas in normal rats approximately 50% of the CRF-positive secretory vesicles in the external zone of the median eminence also contain AVP (Whitnall et al. 1985, 1987), in response to stress and adrenalectomy the proportion of AVP-containing CRF vesicles increases (Whitnall 1988, 1989). This may reflect a general stimulation of all components within the CRF neurons, but could also represent a more specific effect on other components besides CRF within the CRF neuron. Evidence for a differential activation of releasing factors within CRF neurons has been reported in the AA model, where CRF mRNA and CRF peptide release are paradoxically reduced following the development of inflammation and the attendant increase in pituitary–adrenal activity, but AVP mRNA and AVP peptide release are increased (Harbuz et al. 1992, Chowdrey et al. 1995). A switch to a predominantly AVP-driven system has also been reported following repeated stress (Dimphena et al. 1991, Scacciance et al. 1991, de Goeij et al. 1992, Bartanusz et al. 1993). Examination of post-mortem tissue from patients with multiple sclerosis has reported increased AVP within CRF-containing neurons in the PVN (Erkut et al. 1995), again suggesting a differential activation of these components.

In addition to AVP, there is also evidence supporting a role for c-fos mRNA in relation to proenkephalin A
(PEA) mRNA (the enkephalin precursor), in the medial parvicellular cells of the PVN following stress. Despite the lack of CRF mRNA response to ether stress, there is an increase in PEA mRNA in the PVN (Watts 1991). In a recent study it was noted that both novelty stress and i.p. HS elevated both hnPmA and c-fos mRNA in the PVN (Yukhananov & Handa 1997). We have previously noted that in the AA rat although there is no CRF mRNA activation of immediate early genes with the response to acute stress, the PEA mRNA response to i.p. HS stress is intact (Harbuz et al. 1993b). The presence of an AP-1 recognition site on the PEA gene further supports the possibility of PEA being the target gene for c-fos (Sonnenberg et al. 1989).

If an increase in c-fos mRNA is not directly related to an increase in CRF, the question remains as to which immediate early genes might be involved in this signalling. In addition, it is also of interest to know which transcription factors are related to which releasing factors in the PVN. Despite considerable research effort these relationships remain to be elucidated. There are a number of candidates and many of the mRNAs for these transcription factors have been determined in the PVN, e.g. fos B, c-jun, jun B, a POU-domain factor (brn-2), nerve growth factor-induced gene A (NGFI-A), NGFI-B and the cAMP response element protein binding protein (CREB). Brn-2 is expressed constitutively in the PVN, although it does not appear to be responsive to stress (Kovacs & Sawchenko 1996). The remaining factors have been reported to be increased in response to acute stress (Rivest & Laflamme 1995, Kovacs & Sawchenko 1996, Umemoto et al. 1997), via a mechanism regulated by glucocorticoid steroids, with the exception of NGFI-A, which does not appear to be down-regulated by corticosterone (Umemoto et al. 1997). CREB mRNA is not closely associated with the time-course of increased CRF; however, it is the phosphorylation of CREB which provides the activity and this appears to rise in parallel to the appearance of hncRF RNA following ether stress (Kovacs & Sawchenko 1996). The protein synthesis inhibitor cycloheximide prevented the appearance of fos immunoreactivity after ether stress, but not CREB phosphorylation after ether stress. This treatment attenuated the stress-induced rise in AVP, but not hncRF RNA in the PVN (Kovacs et al. 1998), providing further evidence for distinct mechanisms governing AVP and CRF expression. However, the discrepancy between hncRF RNA and CRF mRNA in response to ether stress suggests a more complicated picture.

In summary, the present data confirm a number of previous observations suggesting that, under these conditions, c-fos mRNA is not a good marker of stimulation of the HPA axis. It is clear that independent estimation of the involvement of CRF in relation to the HPA axis should be determined. In the AA rat, CRF mRNA and peptide release is inhibited and animals with hind-paw inflammation are unable to respond to acute stress. Comparison of the activation of immediate early genes and hnRNAs in response to acute stress with the response to acute immune stimuli may provide a useful model to tease apart the molecular mechanisms underlying the control of releasing factor stimulation at the level of the PVN.

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