Serotonin depletion does not alter lipopolysaccharide-induced activation of the rat paraventricular nucleus

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Abstract

We have investigated the effects of serotonin depletion on immune-mediated activation of the hypothalamo-pituitary-adrenal (HPA) axis. Corticotrophin-releasing factor (CRF) mRNA, *c-fos* mRNA and Fos peptide responses in the paraventricular nucleus (PVN) together with circulating levels of corticosterone were assessed in response to i.p. injections of three doses of lipopolysaccharide (LPS) both in control animals and animals pretreated with *p*-chlorophenylalanine (PCPA).

Conscious animals received either an i.p. injection of 0.5 ml saline or 200 mg/kg PCPA in 0.5 ml saline on 2 consecutive days. This treatment resulted in a 93% depletion of serotonin on the fourth day. On day 4, animals received i.p. injections of LPS (2.5 mg/0.5 ml saline, 250 µg/0.5 ml or 50 µg/0.5 ml; *E. coli* 055:B5), or saline injections as controls.

Pretreatment with PCPA had no effect on the basal levels of corticosterone, or on the elevated levels induced by the three doses of LPS. Fos peptide and *c-fos* mRNA were undetectable in control animals, and Fos-like immunoreactivity increased in a dose-

dependent manner following i.p. LPS in both control and PCPA-pretreated animals. *C-fos* mRNA expression induced by LPS was unaffected by serotonin depletion. Following the lowest dose of LPS, CRF mRNA did not change above control levels, however, the medium and high doses of LPS produced a significant ($P < 0.05$) increase in CRF mRNA levels in both depleted and intact animals.

To confirm the temporal effects of serotonin depletion on activation of the HPA axis we collected plasma at 30 min, 1, 2, 3, 4, 5 and 6 h after LPS in both intact and serotonin-depleted animals. No significant differences in plasma corticosterone levels were found at any of the time points between intact and depleted animals.

It appears that, at least under these experimental conditions, serotonergic inputs do not seem to play a major role in mediating the effects of LPS on changes in mRNA levels in the PVN or on the subsequent activation of the HPA axis.

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Introduction

The parvocellular cells of the paraventricular nucleus (PVN) produce corticotrophin-releasing factor (CRF), which is released into the hypophyseal portal blood and is generally considered to be the primary mediator of the stress response. Acute stress is known to activate the hypothalamo-pituitary-adrenal (HPA) axis and results in an increase in CRF mRNA in the PVN, increased plasma adrenocorticotrophin (ACTH) concentrations and hence increased amounts of corticosterone are released from the adrenal glands (Harbuz & Lightman 1992). Lipopolysaccharide (LPS, a component of the cell wall of gram-negative bacteria) and interleukin-1 β (IL-1 β) can induce a wide range of effects, including the activation of the HPA axis (Fontana *et al.* 1984, Besedovsky *et al.* 1986, Berkenbosch *et al.* 1987, Sapolsky *et al.* 1987, Uehara *et al.* 1987, Schobitz *et al.* 1994).

The role played by serotonin in regulating the stress response remains controversial. Serotonergic innervation of the hypothalamus originates in the brainstem raphe nuclei (Moore *et al.* 1978). It has been suggested that serotonergic innervation of the PVN is very low, compared with surrounding areas (Van de Kar & Lorens 1979, Sawchenko *et al.* 1983, Larsen *et al.* 1996). However, immunocytochemical studies have indicated that contact may exist between CRF immunoreactive cells and serotonin axons, with both axosomatic and axodendritic synapses occurring (Liposits *et al.* 1987). Acute stress paradigms such as foot-shock result in an increase in serotonin turnover (Dunn 1988). Other studies have suggested that any involvement of serotonin may be stressor dependent, as lesions of the raphe nucleus or direct neurochemical lesions of the PVN have little or no effect on basal or stress-induced changes in plasma corticosterone (Feldman *et al.* 1987, 1991). In contrast to these acute stressors, little is

known about the effects of serotonin on acute immune stimulation of the HPA axis by immune modulators such as IL-1 β or LPS.

Serotonin release within the brain is known to be affected by IL-1 β . Intracerebroventricular administration of IL-1 β or i.p. LPS both lead to an increase in serotonin and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) within the hippocampus (Mohankumar *et al.* 1993, Shintani *et al.* 1993, Linthorst *et al.* 1994, 1995, 1996). In addition to elevating serotonin levels, intrahippocampal IL-1 β also increases HPA axis activity as evidenced by increases in plasma ACTH and corticosterone levels (Linthorst *et al.* 1994). Immobilization stress elevates plasma ACTH and monoamines including serotonin within the hypothalamus, an effect which can be blocked by the use of an IL-1 receptor antagonist (Shintani *et al.* 1995). Hence evidence exists suggesting that serotonin could play a role in mediating the effects of IL-1 β , or LPS on the HPA axis.

The immediate early gene *c-fos* provides a useful means of identifying neuronal activation. Expression is usually very low within the central nervous system (CNS). In response to a stimulus, *c-fos* mRNA appears transiently and rapidly, with peak levels usually being seen at around 30 min to 1 h (Morgan & Curran 1989), however, following stimulus with an immune mediator such as LPS, *c-fos* mRNA is high 3 h following i.p. LPS, declines by 6–9 h post-injection, and is totally absent 12 h following i.p. LPS (Rivest & Laflamme 1995).

In the present study we have depleted serotonin using the reversible tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA), to determine if this treatment has any effect on the LPS-induced increase in CRF and *c-fos* mRNAs and Fos-like immunoreactivity in the PVN and on plasma concentrations of corticosterone.

Materials and Methods

Adult male Sprague–Dawley rats weighing 210–250 g were housed four to a cage with free access to food and water. They were maintained on a ratio of 12 h light:12 h darkness (lights on at 0700 h). All studies were begun between 0930 h and 1030 h. On day 1 rats received a single i.p. injection of PCPA (Sigma Chemical Co., Poole, Dorset, UK) at a dose of 200 mg/kg body weight in a final volume of 0.5 ml. Control animals received a similar injection of water. This procedure was repeated on day 2, and on day 4 animals received an i.p. injection of LPS (*E. coli* 055:B5) at a dilution of 2.5 mg/0.5 ml saline (high dose), 250 μ g/0.5 ml saline (medium dose) or 50 μ g/0.5 ml saline (low dose). Control animals received an i.p. injection of saline. This depletion protocol has previously been demonstrated to result in a greater than 90% depletion in hypothalamic serotonin (McElroy *et al.* 1984, Harbuz *et al.* 1993).

Groups of animals were killed by decapitation either 30 min ($n=8$ per treatment group) or 3 h ($n=8$ per treatment group) following injection. Brains were quickly removed, frozen on dry ice and stored at -70°C . Trunk blood was collected into ice-cold heparinized tubes, centrifuged and the plasma stored at -20°C prior to radioimmunoassay.

A second group of animals received an indwelling jugular cannula on day 1. On days 1 and 2, the animals received either PCPA treatment as described above (depleted, $n=8$) or saline (controls, $n=8$). On day 4, blood samples were taken from all animals, which then received an i.p. injection of LPS (medium dose, 250 μ g/0.5 ml saline). Blood samples were taken 30 min, 1, 2, 3, 4, and 5 h following the LPS injection, centrifuged and the plasma stored at -20°C prior to radioimmunoassay. Animals were killed by decapitation 6 h following LPS, and trunk blood was collected and treated as described above prior to radioimmunoassay.

In situ hybridization histochemistry (ISHH)

ISHH was performed as described previously (Harbuz & Lightman 1989, Harbuz *et al.* 1993). The probes used were 48-mer oligonucleotides complementary to part of the exonic mRNA sequences coding for CRF or *c-fos* (bases 138–185) (Perkin Elmer, Norwalk, CT, USA). The specificity of the CRF probe has been previously determined (Young *et al.* 1986) and representative photomicrographs have been previously published (Lightman & Young 1988, Harbuz *et al.* 1991). The specific activities of the CRF and *c-fos* probes (d.p.m./mol) were 8.65×10^{18} and 6.46×10^{18} respectively. All control and experimental sections were hybridized in the same hybridization reaction. The autoradiographic images of probe bound to parvocellular CRF mRNA or *c-fos* mRNA, together with ^{14}C -labelled standards (to compensate for the non-linear response of the film to radioactivity) were measured using a computer assisted image analysis system (Image 1.22 developed by W Rasband, NIH, Bethesda, MD, USA) and run on an Apple Mac IICx. The CRF results are presented as the mean percentage change from control with a standard error about the mean. The *c-fos* data are presented as actual values with a standard error about the mean.

Immunohistochemistry (IHC)

IHC was performed as described previously (Conde *et al.* 1995) but on unfixed frozen 12 μ m cryostat sections. Sections through the PVN were fixed in 4% paraformaldehyde for 10 min prior to immunostaining. The antibody used was a polyclonal rabbit anti-Fos (Oncogene Science, Cambridge, MA, USA, 1:1000) raised against the N-terminal (4–17) region of the rat Fos peptide. Sections

from all groups of animals were processed simultaneously, and immunoreactive nuclei were counted using a Leitz D M R B microscope.

Radioimmunoassay

Total plasma corticosterone was measured directly in plasma (1 μ l diluted in 100 μ l buffer) using antiserum kindly supplied by G Makara (Institute of Experimental Medicine, Budapest, Hungary). The tracer was [125 I] corticosterone (ICN Biomedicals, Irvine, CA, USA) with a specific activity of 2–3 mCi/ μ g. The sensitivity of the assay was 25 ng/ml.

HPLC

In order to confirm the extent of the serotonin depletion, 700 μ m tissue punches were taken from the cerebellum. Serotonin and its metabolite were measured as described previously (Kilpatrick *et al.* 1986), and protein content was assessed using Pierce protein reagent (Pierce and Warrinder, Chester, UK).

Statistics

Statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Fisher's PLSD (parametric least significant difference).

Results

PCPA resulted in a highly significant ($P < 0.05$) depletion in serotonin concentrations in the brain compared with saline-injected control rats. Control levels were 71.9 ± 16.1 ng/g wet weight. Levels in PCPA-treated rats were reduced to 5.1 ± 3.1 ng/g. This level of depletion (93%) is in accord with previous studies using similar protocols (McElroy *et al.* 1984, Harbuz *et al.* 1993).

Basal plasma corticosterone levels were unaffected by PCPA treatment (Fig. 1). Intraperitoneal injection of all three doses of LPS resulted in a significant ($P < 0.05$) increase in plasma corticosterone 3 h following the injection. This increase was unaffected by serotonin depletion with PCPA.

CRF mRNA in the PVN (Figs 2 and 3) was not significantly altered by PCPA treatment, compared with vehicle-injected animals. Injection of the medium and high doses of LPS caused a significant ($P < 0.05$) and similar increase in CRF mRNA, in both vehicle-treated controls and serotonin-depleted animals. Injection of the lowest dose of LPS did not evoke an increase in CRF mRNA levels from controls.

Levels of *c-fos* mRNA were undetectable in all control animals and PCPA treatment itself did not induce *c-fos* mRNA (Figs 4 and 5). Injection of LPS did not induce any

Figure 1 Plasma corticosterone levels 3 h following i.p. injection of either saline (S) or LPS (L) in intact (S+S, $n=8$; S+L, $n=8$) or serotonin-depleted (P+S, $n=8$; P+L, $n=8$) animals (means $\pm$ S.E.M.). With all doses of LPS there was a significant increase in plasma corticosterone levels (* or +, $P < 0.05$; Fisher's PLSD). Serotonin depletion had no significant effect on the LPS-induced increase in plasma corticosterone, compared with non-depleted animals.

change in *c-fos* mRNA at the 30 min time point (data not shown), however, 3 h after the highest dose of LPS, there was a significant increase in *c-fos* message in the PVN. This increase was similar following serotonin depletion. The low and medium doses of LPS did not result in measurable levels of *c-fos* mRNA in any of the groups of animals studied.

Fos peptide was found to be present in all groups of animals examined, although control groups showed very few Fos-immunoreactive nuclei per section. Fos-positive cells in the PVN increased significantly ($P < 0.05$) with increasing doses of LPS, with the highest number of nuclear profiles being seen in the animals receiving the highest dose of LPS (Fig. 6). This effect was also apparent in the PCPA-treated animals, and serotonin depletion did not significantly alter the numbers of positive cells, except in the group of animals which received the medium dose of LPS. Here, serotonin depletion resulted in a significant ($P < 0.05$) decrease in the numbers of Fos-immunoreactive nuclear profiles, compared with non-depleted animals which had received the same dose of LPS.

In the second study the medium dose of LPS caused a significant increase ($P < 0.01$, Fisher PLSD) in plasma corticosterone levels within 30 min of injection, in both intact and serotonin-depleted animals (Fig. 7). Corticosterone

Figure 2 CRF mRNA levels in the PVN, expressed as % of controls (S+S) (means $\pm$ S.E.M.). With the low dose of LPS there was no change in CRF mRNA levels. However following the medium and high doses, CRF mRNA increased significantly (* or +, $P < 0.05$; Fisher's PLSD), compared with the saline-treated controls. Serotonin depletion did not significantly affect this increase.

levels continued to rise reaching a maximum 2 h following LPS injection, and remaining significantly ($P < 0.01$; Fisher's PLSD) elevated up to 6 h following the LPS injection. Depletion of serotonin did not have any significant effect on this increase at any of the time points measured.

Discussion

We have confirmed that in control animals, *c-fos* mRNA levels are undetectable in the PVN, indicating very low basal levels of expression (Harbuz *et al.* 1993). In addition, minor procedures, such as i.p. injection of vehicle had no effect on *c-fos* mRNA levels. In response to i.p. LPS, activation of *c-fos* mRNA was not apparent 30 min after injection, as would be expected for acute stress, such as restraint stress which produces a peak in *c-fos* mRNA after 30 min (McElroy *et al.* 1984, Harbuz *et al.* 1993) and which has all but disappeared by 1 h. *C-fos* mRNA was present 3 h following the i.p. injection of LPS within both the magno- and parvocellular portions of the PVN. This expression was unaffected by PCPA-induced depletion of serotonin. Serotonin depletion did not alter the numbers of neurons within the PVN which were immunoreactive for Fos-like peptide 3 h following i.p. saline, and these control

Figure 3 Representative autoradiographs showing CRF mRNA in the PVN in controls (S+S), depleted animals (P+S), and controls and depleted animals which had received an i.p. injection of the highest dose of LPS (S+L and P+L respectively), 3 h prior to killing. 3 V, third ventricle; Bar=200 μ m.

Figure 4 *C-fos* mRNA in the PVN (means $\pm$ S.E.M.) 3 h following i.p. LPS (high dose). In control animals which had not received LPS, *c-fos* mRNA was below the level of detection. However, following LPS, there is a significant increase (* or +, $P < 0.01$; Fisher's PLSD) in *c-fos* mRNA compared with controls, with serotonin depletion having no effect on this LPS-induced expression.

animals exhibited few Fos-positive nuclear profiles. Treatment with LPS induced a dose-dependent increase in Fos-positive cells within the PVN. This response was

Figure 5 Representative autoradiographs showing *c-fos* mRNA in the PVN in controls (S+S), serotonin-depleted animals (P+S) and animals following LPS treatment (S+L; P+L). 3V=third ventricle; Bar=200 μ m.

unaffected by PCPA pretreatment, although the response to the medium dose was reduced. This suggests that serotonin does not play a major role in mediating this *c-fos* response.

PCPA pretreatment did not affect either basal or LPS-activated CRH mRNA 3 h after LPS injection. LPS-induced plasma corticosterone secretion, as evidence of end-point activation of the HPA axis, was not altered over a period of 6 h. This suggests that serotonin depletion does not alter the response or the time course of the response of the LPS-induced activation of the HPA axis. We have previously demonstrated that changes seen following acute restraint stress are unaffected by PCPA pretreatment (Harbuz *et al.* 1993) and other workers have shown that lesions of the raphe nucleus do not alter basal or stress-induced changes in plasma corticosterone (Feldman *et al.* 1987, 1991). This suggests that HPA axis responses to a variety of stimuli are not altered following serotonin depletion.

When IL-1 β is infused into the medio-basal hypothalamus using a push-pull cannula, serotonin and levels of its metabolite 5-HIAA rise, indicating that IL-1 β can affect the serotonin system and suggests that serotonin might mediate some of the central actions of IL-1 β (Shintani *et al.* 1993). More recent evidence also indicates that

Figure 6 Number of Fos-immunoreactive profiles per section through the PVN (means $\pm$ s.e.m.). Fos peptide was seen in all groups of animals, and increased significantly with increasing doses of LPS ($P < 0.05$), with the highest number being seen in the animals which received the highest dose of LPS. Serotonin depletion did not prevent this increase, although it did attenuate significantly (comparing S+L with P+L) the increase seen following the medium dose of LPS (* or +, $P < 0.05$; Fisher's PLSD).

when recombinant human IL-1 β is infused into the hippocampus, extracellular serotonin levels are elevated (Linthorst *et al.* 1994, 1995, 1996). This appears to be in parallel to an increase in the activity of the HPA axis, as plasma ACTH is also elevated. Similar results are obtained using immobilization stress in conjunction with an intra-hypothalamic infusion of IL-1 β (Shintani *et al.* 1995). The inference from these studies is of a link between the observed changes in serotonin in response to either LPS or IL-1 β , and the subsequent activation of the HPA axis. In the present study, our data suggest that these events may occur independently and that under these conditions serotonin does not appear to be involved in mediating the activation of the HPA axis in response to LPS, although serotonin may be involved in mediating the behavioural changes noted following intrahippocampal IL-1 β (Linthorst *et al.* 1996). In contrast to the lack of effects of serotonin on the acute stimulation of the HPA axis by LPS, there is evidence for the involvement of serotonin following chronic immune activation. Adjuvant induced arthritis (AA) is an immune-mediated disease model resulting in hind paw inflammation in susceptible strains of rat, appearing 12–14 days after injection of adjuvant. Associated with the development of inflammation there is an activation of the HPA axis (Harbuz *et al.* 1992). Depletion

Figure 7 Time course showing plasma corticosterone levels in both serotonin-depleted animals and intact animals 30 min, 1, 2, 3, 4, 5, and 6 h following i.p. injection of LPS (250 µl/0.5 ml saline). LPS causes a significant ($P < 0.01$; Fisher's PLSD) increase in plasma corticosterone in both depleted and non-depleted animals. There are no differences in plasma corticosterone levels between depleted and non-depleted groups of animals throughout the sampling period.

of serotonin using PCPA at the time of injection of the adjuvant was without effects on the progress of the disease. However, serotonin depletion immediately prior to the onset of inflammation resulted in a significant reduction in the severity of the inflammation (Harbuz *et al.* 1996). Alterations in the HPA axis associated with inflammation were also reversed in these rats. It appears therefore that serotonin has an important role in modulating the severity of disease and the attendant changes in the HPA axis in this chronic inflammatory stress model.

The tryptophan hydroxylase inhibitor used in the present study, has also been demonstrated to inhibit phenylethanolamine N-methyltransferase, the enzyme responsible for the conversion of noradrenaline to adrenaline (Coen *et al.* 1993). It is possible therefore that the results obtained here represent the combination of both serotonin and adrenaline depletion. As the role played by adrenaline in controlling the HPA axis is poorly understood, the use of specific lesions and selective antagonists may resolve this issue.

In summary, PCPA pretreatment resulted in a significant decrease in hypothalamic serotonin concentrations. This decrease had no effect on basal levels of corticosterone, nor on levels stimulated by LPS, as ascertained over a considerable period of sampling. Similarly, activation of *c-fos* mRNA and peptide, and of CRF mRNA were unaffected by serotonin depletion. Although serotonin appears to play a relatively important role in mediating

chronic inflammatory stress, it does not appear to be involved in modulating the HPA axis response to acute immune activation.

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