

ALTERATION OF CENTRAL SEROTONIN MODIFIES ONSET AND SEVERITY OF ADJUVANT-INDUCED ARTHRITIS IN THE RAT

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SUMMARY

Objective. Previous studies have determined that depletion of serotonin reduces the severity of hind-paw inflammation in adjuvant-induced arthritis (AA) in the rat. We wished to (i) test the hypothesis that this effect may be mediated, at least in part, through a central mechanism and (ii) to investigate further the pro-inflammatory role of serotonin we determined whether increasing serotonin using a selective serotonin reuptake inhibitor (SSRI), to increase serotonin availability at the active site of release, would increase inflammation.

Methods. (i) Serotonin was depleted in the brain of rats with the selective neurotoxin 5,7'-dihydroxytryptamine. (ii) Rats were treated with an SSRI on days 10, 11 and 12 following adjuvant injection. Hind-paw inflammation was determined with plethysmometry as an index of severity of inflammation, and brain, pituitaries and blood were collected for assessment of changes in the hypothalamo–pituitary–adrenal (HPA) axis.

Results. (i) Serotonin depletion significantly reduced hind-paw inflammation. (ii) SSRI-treated animals developed hind-paw inflammation sooner, and the severity was increased compared to vehicle-treated AA rats. The changes in the HPA axis associated with inflammation were partly reversed by this treatment.

Conclusion. These data suggest a pro-inflammatory role for central serotonin in this disease model and indicate that treatment with SSRIs may exacerbate the development of inflammation.

KEY WORDS: Adjuvant-induced arthritis, Inflammation, Serotonin, SSRI, Corticosterone, Corticotrophin-releasing factor, Pro-opiomelanocortin.

A PRO-INFLAMMATORY role for serotonin (5HT) has been established in response to acute stimulation. In the rat, direct intraplantar injection of serotonin produces paw oedema and a nociceptive response which can be blocked by serotonin antagonists [1–4]. The effects of serotonin in chronic inflammatory conditions and its mechanism of action are less well established. We have used the T-cell-mediated chronic inflammatory stress model of adjuvant-induced arthritis (AA) which has been used for studies of arthritis, pain, inflammation and Reiter's syndrome. An upregulation in serotonergic activity has been reported in this model with increased serotonin in plasma, spinal cord and brain [5–11]. We have previously noted that whole-body depletion of serotonin using *p*-chlorophenylalanine (PCPA; a reversible inhibitor of tryptophan hydroxylase) at the time of onset of inflammation, but not at the time of adjuvant injection, reduces the severity of hind-paw inflammation [12], confirming a pro-inflammatory role for serotonin in this model. These studies offer little insight as to the site of action of serotonin, which could be directly at the level of the joint, through modulation of the immune system, or centrally.

Profound neuroendocrine changes are associated with the development of immune-mediated disease in humans and in animal disease models. It has been suggested that a component determining susceptibility in autoimmune disease models such as experimental arthritis [13] and experimental allergic encephalomyel-

itis (EAE; the model of choice for multiple sclerosis research [14]), resides in a defect in the hypothalamo–pituitary–adrenal (HPA) axis and more specifically in a lack of corticotrophin releasing factor (CRF) responsiveness [13]. In the Piebald–Viral–Glaxo (PVG), Lewis and Wistar strains of rat, there is a paradoxical decrease in CRF mRNA [15, 16] and decrease in CRF release into the hypophyseal portal blood [15], despite activation of the pituitary–adrenal components of the axis associated with inflammation in AA. Evidence suggests that arginine vasopressin takes over as the major stimulator of the axis in this model [15, 17]. The adrenal response is crucial to survival since adrenalectomized rats exhibit an earlier onset and greater severity of disease in AA [18] and in EAE [19]. Left untreated, the outcome is rapidly fatal, although this can be prevented with steroid replacement.

In the present study, we have addressed three questions: (i) is central serotonin involved in modulating the degree of inflammation in this model; (ii) on the basis of the pro-inflammatory actions of serotonin, would using a selective serotonin reuptake inhibitor (SSRI) result in an earlier onset and/or increased severity of inflammation; (iii) does manipulation of serotonergic systems alter the HPA axis?

METHODS

Nine-week-old male PVG rats (Harlan-Olac, UK) were housed four to a cage under standard environmental conditions with free access to food and water. On day 0, the rats were given a single intradermal injection (0.1 ml containing 10 mg/ml) of ground,

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heat-killed *Mycobacterium butyricum* in paraffin oil into the base of the tail. Inflammation is usually first apparent at around day 13/14 and, following consultation with the Home Office Animal Inspectorate, experiments are terminated within 24 h of evidence of hind-paw inflammation, usually day 14. At this time, animals were decapitated, and the brains and pituitaries rapidly removed and frozen on dry-ice. The brains were taken for determination of serotonin concentrations in specific nuclei and/or for CRF mRNA in the paraventricular nucleus (PVN), and the pituitaries were taken for pro-opiomelanocortin (POMC) mRNA determination by *in situ* hybridization histochemistry. Forty-eight-base ³⁵S-labelled oligonucleotide probes complementary to part of the exonic mRNA sequences coding for CRF or POMC mRNAs were used as described previously [20, 21]. Specificities of the probes have been determined and representative photomicrographs published previously [20, 22, 23]. All control and experimental sections for each experiment were hybridized in the same hybridization reaction. The autoradiographic images of probe bound together with ¹⁴C standards (Amersham, Bucks.) 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 Wayne Rasband, NIH, Bethesda, MD, USA) and run on an Apple MacIICi. Results are presented as the mean percentage change from control \pm S.E.M.

Trunk blood was taken for plasma corticosterone measurements by radioimmunoassay using antiserum supplied by Dr G. Makara (Institute of Experimental Medicine, Budapest, Hungary). The tracer was [¹²⁵I]corticosterone (ICN Biomedicals, CA, USA) with a specific activity of 2–3 mCi/ μ g. The sensitivity of the assay was 25 ng/ml. The intra-assay coefficient of variation was <12%.

Hind paws were collected into 4% formaldehyde and the degree of inflammation measured by plethysmometry (Ugo-Basile, Milan, Italy) to provide an index of severity. Hind paws were submerged to the level of the lateral malleolus and the mean of three recordings taken. Measurements were accurate to 0.01 ml.

5'7'-Dihydroxytryptamine neurotoxin lesioning

Six days after injection of adjuvant, the animals were anaesthetized using sodium pentobarbitone and injected i.p. with desipramine hydrochloride (Sigma, Poole) at a dose of 25 mg/kg to prevent damage to catecholaminergic neurons. Thirty minutes later, 10 μ l of vehicle (0.9% saline containing 1 mg/ml ascorbic acid) or 5'7'-dihydroxytryptamine creatinine sulphate (150 μ g free base, Sigma, Poole) were infused into the right lateral ventricle. On day 14, the animals were decapitated, trunk blood collected and plasma stored at -20°C . Brains and pituitaries were collected, quickly frozen on dry-ice and stored at -80°C . Sections (3 \times 300 μ m) were taken through the hippocampus, central amygdala (CeA) and ventromedial hypothalamic nucleus (VMN). Micropunches of each of these areas were taken according to the atlas of

Palkovits and Brownstein [24]. These were processed for determination of serotonin and the major serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously [25]. Briefly, tissues were placed in cold 0.2 N acetic acid and immediately frozen on dry-ice. After thawing, they were centrifuged at 19 000 g for 3 min at 4°C . Twenty microlitres of the supernatant were assayed for serotonin and 5-HIAA content. The pellets were resuspended in 0.2 N NaOH and assayed for protein content using Pierce protein reagent (Pierce & Warrinder, Chester) with bovine serum albumin as standard. Hind paws were collected into 4% formaldehyde and paw volume determined as described above. Sections of 12 μ m through the PVN and anterior pituitary were taken for *in situ* hybridization to CRF and POMC mRNA, respectively.

Treatment with SSRI

The SSRI (Lu10-134) was kindly supplied by Dr Jens Mikkelsen, Lundbeck A/S, Copenhagen, Denmark. This SSRI is a potent inhibitor of 5HT *in vitro* and has low noradrenergic (NA) and dopaminergic (DA) uptake inhibitory potencies with no significant affinities for serotonergic, noradrenergic, dopaminergic, histaminergic or cholinergic receptors. The *in vivo* 5HT uptake inhibitory potency occurs at lower doses compared to NA and DA uptake inhibition, demonstrating potent and selective 5HT reuptake inhibition [26]. The SSRI was chosen precisely because it is a very selective SSRI with little effect on catecholaminergic systems, which does not appear to be the case with other SSRIs, e.g. Prozac [27]. Ten days after injection of the adjuvant, and prior to the development of inflammation, control and adjuvant-injected animals received an injection (i.p.) of either vehicle or Lu10-134 (10 mg/kg). This was repeated on days 11 and 12. Brains, pituitaries, plasma and paws were collected on day 13 after adjuvant injection (due to the earlier onset and severity of inflammation seen at this time), and processed as described above.

Statistics

Statistical comparisons were made between groups using the Fisher PLSD test following one-way analysis of variance. A value of $P < 0.05$ was considered significant.

RESULTS

5'7'-Dihydroxytryptamine neurotoxin lesioning

The development of inflammation did not alter concentrations of either serotonin or 5-HIAA in the CeA, hippocampus or VMN compared with levels in sham-lesioned controls (Table I). Following 5'7'-dihydroxytryptamine lesions, there were highly significant (>90%) reductions in both serotonin and 5-HIAA levels in the areas investigated, with no difference between the adjuvant-injected and control rats.

Fourteen days after adjuvant injection, vehicle-infused rats had developed hind-paw inflammation, as

TABLE I

Serotonin and 5-HIAA concentrations in the hippocampus, ventromedial nucleus (VMN) and central amygdala (CeA) (pg/ μ g protein) as determined by HPLC with electrochemical detection. The study comprised control and adjuvant-injected (AA) animals receiving intracerebroventricular (icv) injections of either vehicle or 5/7'-dihydroxytryptamine (5/7'-DHT). The values represent the mean \pm S.E.M. for $n = 7-9$ per group

	Treatment	Hippocampus	VMN	CeA
Control	Serotonin	1.03 \pm 0.10	3.06 \pm 0.32	3.36 \pm 0.43
	5-HIAA	5.95 \pm 0.47	9.11 \pm 0.35	8.10 \pm 0.58
5/7'-DHT	Serotonin	0.10 \pm 0.05**	0.08 \pm 0.08**	0.04 \pm 0.04**
	5-HIAA	0.56 \pm 0.17**	0.72 \pm 0.09**	1.07 \pm 0.18**
AA	Serotonin	1.23 \pm 0.24	3.36 \pm 0.25	3.13 \pm 0.28
	5-HIAA	6.87 \pm 0.57	8.51 \pm 0.73	9.92 \pm 0.76
AA \pm 5/7'-DHT	Serotonin	0.18 \pm 0.06**	0.08 \pm 0.08**	0.17 \pm 0.07**
	5-HIAA	0.84 \pm 0.10**	0.64 \pm 0.03**	0.83 \pm 0.11**

** $P < 0.01$ compared with control levels.

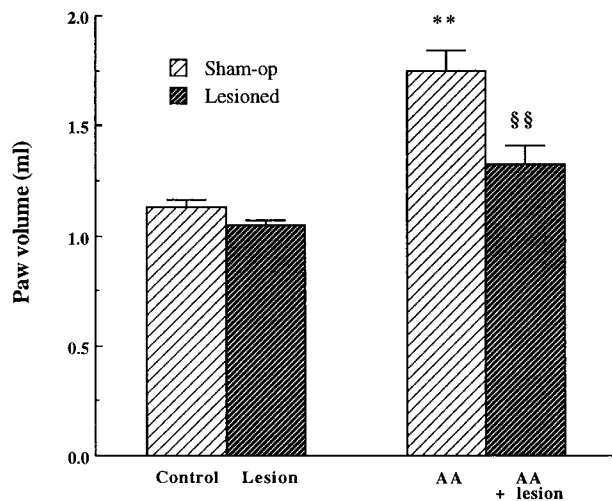


FIG. 1.—Paw volume following 5/7'-dihydroxytryptamine neurotoxin lesioning. Six days after injection of adjuvant (AA) or vehicle (control), animals were anaesthetized and injected i.p. with desipramine hydrochloride at a dose of 25 mg/kg (Sigma, Poole, Dorset) to prevent damage to catecholaminergic neurons. Thirty minutes later, 10 μ l of vehicle (0.9% saline containing 1 mg/ml ascorbic acid) or 5/7'-dihydroxytryptamine creatinine sulphate (Lesion; 150 μ g free base, Sigma, Poole, Dorset) were infused into the right lateral ventricle. Fourteen days after adjuvant injection, the hind paws were collected and measured. Serotonin depletion had no effect on paw volume in the non-adjuvant-injected animals. AA rats had significantly increased paw volume which was reduced by serotonin depletion. ** $P < 0.01$ against control; §§ $P < 0.01$ against AA + lesion. Values represent means with S.E.M. for $n = 7-9$ per group.

expected in this model (Fig. 1). The serotonin-depleted animals had significantly reduced inflammation at this time. Indeed, only two of the nine animals in this group exhibited a significant degree of swelling in their hind paws.

The changes in the HPA axis are summarized in Table II. Depletion of serotonin had no effect on CRF mRNA levels in the PVN. Associated with inflammation, there was a significant decrease in CRF mRNA. In the lesioned, adjuvant-injected animals, CRF mRNA levels were not significantly different from control levels and significantly increased from those in sham-operated AA rats. There was a significant ($P < 0.001$) negative correlation ($r = -0.681$) between

TABLE II

Changes in components of the hypothalamo-pituitary-adrenal axis following central (intracerebroventricular) serotonin depletion with the neurotoxin 5/7' dihydroxytryptamine (5/7'-DHT) or injection of vehicle in control and adjuvant-injected (AA) animals. Values represent means \pm S.E.M. for $n = 7-9$ per group

Treatment	CRF mRNA (% change)	POMC mRNA (% change)	Corticosterone (ng/ml)
Control	100 \pm 4.3	100 \pm 11.2	28 \pm 7
5/7'-DHT	111.7 \pm 10.2	89.3 \pm 12.5	149 \pm 48**
AA	53.3 \pm 11.9**	344.7 \pm 69.9**	154 \pm 70**
AA \pm 5/7'-DHT	96.7 \pm 9.9	222.9 \pm 54.3*	189 \pm 40**

** $P < 0.01$ and * $P < 0.05$ compared with control levels.

decreased levels of CRF mRNA in the PVN and the increased hind paw inflammation. POMC mRNA in the anterior pituitary was not altered by serotonin depletion. Associated with inflammation, there was a significant increase ($P < 0.01$) in POMC mRNA, which was also significantly ($P < 0.05$) increased in the serotonin-depleted AA rats. Depletion of central serotonin elevated plasma corticosterone concentrations in the non-AA animals. Plasma corticosterone concentrations were also elevated to a similar extent in both the sham-operated and lesioned AA rats.

Treatment with SSRI

In the second study, adjuvant-injected rats or controls were treated with a daily injection of an SSRI or vehicle beginning 10 days after adjuvant injection and prior to any visible signs of inflammation. Treatment with the SSRI produced an earlier onset of inflammation with visible hind-paw swelling clearly apparent in all nine animals in this group at day 12, one day prior to the expected time of onset. No animals in the vehicle-injected AA group had inflammation at this time. In accordance with the Home Office guidelines, the study was terminated on day 13. At this time, the vehicle-injected AA rats had a significant ($P < 0.01$) hind-paw inflammation (Fig. 2). The SSRI-treated rats had significantly ($P < 0.01$) increased inflammation compared with vehicle-injected AA rats.

Treatment with the SSRI had no effect on CRF mRNA in the PVN, POMC mRNA in the anterior pituitary or circulating levels of corticosterone

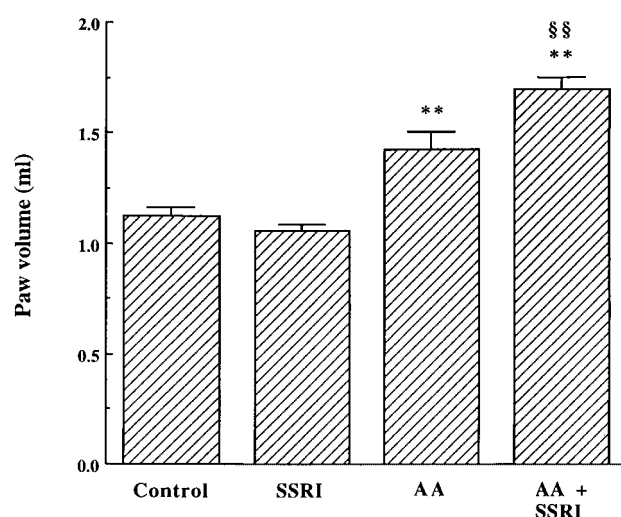


FIG. 2.—Paw volume after treatment with SSRI (Lu10–134). Ten days after injection of the adjuvant, and prior to the development of inflammation, control and adjuvant-injected (AA) animals received an injection (i.p.) of either vehicle or Lu10–134 (10 mg/kg). This was repeated on days 11 and 12. Animals were killed 13 days after injection of the adjuvant. Paws were collected as described previously. Values represent means with S.E.M. for $n = 7-9$. ** $P < 0.01$ compared with control; §§ $P < 0.01$ compared with AA.

TABLE III

Changes in components of the hypothalamo–pituitary–adrenal axis following treatment with the selective serotonin reuptake inhibitor Lu10–134 or vehicle injection in control and adjuvant-injected (AA) animals. Values represent means \pm S.E.M. for $n = 7-9$ per group

Treatment	CRF mRNA (% change)	POMC mRNA (% change)	Corticosterone (ng/ml)
Control	100 \pm 8.8	100 \pm 6.3	47 \pm 19
SSRI	96.9 \pm 9.5	96.1 \pm 10.1	46 \pm 21
AA	53.5 \pm 7.5**	166.3 \pm 12.7**	177 \pm 54**
AA \pm SSRI	46.3 \pm 3.5**	178.1 \pm 11.9**	187 \pm 23**

** $P < 0.01$ compared with control levels.

(Table III). Increased levels of POMC mRNA in the anterior pituitary and of plasma corticosterone in the AA group were not altered by SSRI treatment. Similarly, the decrease in CRF mRNA seen in rats with AA was also not altered by treatment with the SSRI.

DISCUSSION

Central depletion of serotonin resulted in a significant reduction in hind-paw inflammation compared with vehicle-injected controls. These data confirm our previous report demonstrating a reduction in severity of inflammation following total body serotonin depletion and confirm a pro-inflammatory role for serotonin in AA [12]. Furthermore, we extend our original observations to suggest that this inflammatory effect of serotonin is mediated, at least in part, through a central mechanism. Centrally injected serotonin has previously been determined to influence the severity of inflammation in an acute paw oedema model [28],

supporting our contention of a role for central serotonin in modifying peripheral inflammation.

The complicated nature and the multiple receptor subtypes (at least 15 to date) which comprise the serotonergic system have confounded analysis of the role of serotonin. While some insight has been gained through the use of serotonin receptor antagonists, the relative lack of specificity of these compounds has made interpretation difficult. Thus, antagonists directed against the serotonin -1, -2 and -3 receptor subtypes have been shown to be beneficial in reducing acute inflammation in a number of animal studies [3, 10, 28, 29, 30]. In contrast to these reports, others have reported an exacerbation of inflammatory disease activity following treatment with serotonin antagonists [28, 31]. These discrepancies are likely to be due to differential specificities of the drugs used and the different receptor subtypes involved. An alternative method to ascertain the receptor subtypes involved concerns the determination of changes in receptor subtype mRNAs as a means of identifying their involvement. There is one report of a suppression in serotonin 2C-receptor subtype mRNA in the hippocampus of rats with experimental arthritis [32].

On the basis of the pro-inflammatory actions of serotonin, we hypothesized that increasing endogenous serotonin concentrations would have a detrimental effect on the time of onset and on the severity of the inflammation. However, direct application of exogenous serotonin would act on multiple receptors and produce a variety of non-specific effects which might be difficult to interpret. To avoid this possibility, we chose to use an SSRI, highly selective for serotonergic and not catecholaminergic systems, to reduce reuptake of serotonin and hence increase available serotonin at the active site of release. Not only did acute treatment with the SSRI increase the severity of the inflammation, but there was also a very rapid onset within 2 days of beginning treatment with the SSRI. Using the AA model, we have consistently observed the onset of inflammation on day 13 with a termination of the experiments on day 14 when all the animals have inflamed hind paws. In contrast, in this study, we observed an earlier onset of inflammation which was apparent by day 12 in all the SSRI-treated animals. As noted previously, there was no evidence of hind-paw inflammation in the vehicle-treated animals. Because of the earlier onset of inflammation in the SSRI-treated rats, the experiment was terminated on day 13 when the vehicle-injected rats were exhibiting a mild inflammation compared with the significantly increased inflammation of the SSRI-treated animals at this time. This demonstrates in this model that treatment with an SSRI at around the time of onset of clinical symptoms increases the severity of inflammation. In the light of these observations, the role of SSRIs in increasing inflammation in humans needs to be addressed. It is possible that these effects would be manifest before any effects of these drugs on mood would be apparent and could easily be overlooked in the clinical setting.

In order to determine a possible locus of action for serotonin, we measured levels of serotonin and the major metabolite 5-HIAA in the VMN, CeA and hippocampus in vehicle-injected and serotonin-depleted animals. AA did not alter concentrations of either of these compounds in any of the areas investigated. We have previously reported no change in serotonin levels in the PVN of the hypothalamus in rats with inflamed hind paws [12]. Together, these data suggest that these four areas, known to receive serotonergic inputs and implicated in aspects of the neural response to stress, acute inflammation and behaviour, may not be involved in mediating the pro-inflammatory effects of serotonin in this chronic inflammatory stress model. Reports in the literature suggest differences in serotonin metabolism within the brain in AA, but in these studies relatively large areas were dissected for analysis, such as 'forebrain' or 'hypothalamus plus midbrain' [5, 7], making it difficult to assess the precise location of these changes and the brain nuclei involved. Further investigation will be required to ascertain the central site(s) of action of serotonin in mediating alterations in the severity of hind-paw inflammation in this model.

Serotonin, particularly in the hippocampus, which expresses a wide variety of serotonin receptors, has been implicated as a potential neuroimmunomodulator providing a link between the nervous and immune systems. This may represent a mechanism whereby serotonin can exert these pro-inflammatory effects. Serotonin is able to stimulate expression of a number of cytokine mRNAs, such as interleukin (IL)-6, tumour necrosis factor alpha and transforming growth factor beta in rat hippocampal astrocytes [33]. These cytokines have been implicated in inflammatory processes. In addition to the effects of serotonin on cytokine mRNA expression, the converse is also true in that administration of IL-1 and IL-6 is able to increase serotonin turnover in the hippocampus and other brain areas [34–37], where this increased turnover is likely to be involved in the behavioural changes associated with cytokine administration [37]. In contrast, serotonin may not be involved in mediating the effects of acute cytokine administration on the response of the HPA axis as we found no difference in the activation of the HPA axis in response to icv IL-1 β following total body depletion of serotonin using PCPA [38]. These data suggest the possibility of different serotonin pathways mediating responses to different acute stimuli and also between acute and chronic stimuli.

One possible mechanism involved in determining the susceptibility and/or severity of inflammation is the HPA axis. There are profound neuroendocrine changes associated with the development of immune-mediated disease, such as increased circulating levels of adrenocorticotrophic hormone (ACTH) and corticosterone and increased POMC mRNA in the anterior pituitary [15, 39, 40]. This activation occurs despite the paradoxical decrease in CRFergic activity seen in this model. Previous studies have demonstrated the fundamental importance of a functioning HPA axis to an organism's

survival and, in particular, the ability to mount a glucocorticoid response to either acute injection of cytokines or immune modulators such as lipopolysaccharide (LPS), or following the development of clinical symptoms in autoimmune disease models such as AA and EAE [18, 19, 41]. In the present study, we noted a significant decrease in CRF mRNA in the vehicle-injected AA rats, which is in accord with previous reports [15, 16]. In the serotonin-depleted adjuvant-injected rats, which did not show an increase in hind-paw volume, no decrease in CRF mRNA was observed. Indeed, a good inverse correlation between the decrease in CRF mRNA and the increase in paw volume was observed in this study. Such a relationship has been noted previously [12, 42] and supports the notion of a non-glucocorticoid-mediated mechanism responsible for the downregulation of CRF mRNA influenced by the severity of inflammation [18]. Evidence suggests a role for substance P in mediating this inhibitory effect on the CRF neuron [43]. POMC mRNA was significantly increased in the AA rats, as has been noted previously [15, 39, 40]. Plasma corticosterone concentrations were increased in the serotonin-depleted rats despite the lack of effect of serotonin depletion on either CRF mRNA in the PVN or POMC mRNA in the anterior pituitary. We have previously made a similar observation in PCPA-treated rats where plasma corticosterone levels were increased in the absence of any elevation in plasma ACTH or CRF mRNA in the PVN [22], suggesting an action on corticosterone release, perhaps mediated via arginine vasopressin as noted previously [17, 18], rather than through an action on CRF neurons.

In non-arthritic animals, treatment with the SSRI on days 10, 11 and 12 had no effect on levels of CRF mRNA in the PVN, POMC mRNA in the anterior pituitary or circulating levels of corticosterone on day 13. In contrast in AA animals, levels of CRF mRNA in the PVN were reduced and levels of POMC mRNA in the anterior pituitary and corticosterone were increased as noted previously [15, 18, 40, 44]. These levels were not significantly affected by SSRI treatment despite the effects on disease. It appears likely, therefore, that the SSRI may be exerting its pro-inflammatory actions independently of the HPA axis. This is intriguing as we have previously seen good correlations between changes in the HPA axis associated with increased inflammation.

Together, these data support a pro-inflammatory role for serotonin in this chronic inflammatory stress model and suggest that these actions are mediated, at least in part, through a central mechanism. Thus, the data provide further evidence for the proposition that manipulation of central neurotransmitter systems can influence the course of peripheral inflammation [28, 42]. Furthermore, the evidence suggests that acute treatment with SSRIs may exacerbate the development of peripheral inflammation. The mechanism of action and the receptor subtypes involved in mediating these effects, particularly at the central level, remain to be determined. Overall, these data suggest that serotonin

antagonists targeted to specific receptor subtypes may be useful therapeutically as anti-inflammatory agents.

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