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## Effect of serotonin depletion on the neuronal, endocrine and behavioural responses to corticotropin-releasing factor in the rat

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### Abstract

Interactions between serotonin and corticotropin-releasing factor (CRF) have been demonstrated by various studies in different parts of the brain. Both are activated by stressful stimuli. Additionally, serotonergic fibres directly synapse with the parvocellular division of the paraventricular nucleus (PVN) that mainly synthesizes CRF. The functional impact of this serotonin-CRF interaction on CRF-induced responses remains unclear. CRF infusion into the brain evokes a specific pattern of behavioural, endocrine and neuronal changes that resemble those following various forms of stress. The aim of the present study was to investigate the effects of serotonin depletion on acute CRF-induced *c-fos* expression, corticosterone levels and behavioural responses. Lateral ventricular (i.c.v.) infusion of CRF (250 pmol) resulted in a significant increase in grooming, corticosterone and *c-fos* mRNA in the PVN. Adequate and specific depletion of serotonin using 5,7-DHT did not alter these CRF-induced changes. These data suggest that acute responses induced by i.c.v. CRF are independent of basal levels of serotonin.

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**Keywords:** Corticotropin-releasing factor; Serotonin; 5,7-dihydroxytryptamine; Hypothalamic-pituitary adrenal axis; Paraventricular nucleus; *c-fos*; Behaviour; Corticosterone

Corticotropin-releasing factor (CRF) is a neuropeptide particularly synthesized in the parvocellular division of the paraventricular nucleus (PVN). CRF, a 41-residue peptide, is a principal activator of adrenocorticotrophic hormone (ACTH) secretion by the anterior pituitary [8]. Activation of this axis, also called the 'stress axis' or hypothalamic-pituitary adrenal axis (HPA), results in glucocorticoid release into the systemic circulation. Glucocorticoids are in turn considered to be key players in the organism's response to stress [11]. However, the role of CRF in stress is not limited to endocrine responses. CRF-containing neurons are found throughout the limbic and other parts of the brain, and central CRF has behavioural and autonomic effects that suggest it plays an important role in the coordinated physiological and behavioural responses to stressful stimuli [1,3,15].

Serotonergic (5HT) systems are also activated by stressful stimuli. Their role is less well-defined, but, in

general, reducing serotonin levels or activity increases the responsiveness to stress, though the literature is not unanimous; the HPA axis becomes hyperactive, behavioural responses are accentuated, and animals may overreact to demanding situations (sometimes referred to as 'impulsiveness'). Are there neurochemical interactions between peptides (such as CRF) and monoamines (such as serotonin) that might underlie these phenotypic findings? In other words, does the level of serotonin regulate the animal's response to CRF?

CRF producing neurons of the PVN receive a direct (though modest) serotonergic innervation from the B7–B9 cell groups [14,17,18]. Electrical stimulation of the dorsal raphe nucleus resulted in excitation of PVN neurons that was blocked by parachlorophenylalanine, a serotonin depleting drug [16]. Feldman et al. [9] showed that serotonin depletion inhibited photic stimulation-induced increase in ACTH and corticosterone. However, in other studies using acute stress and lipopolysaccharide (LPS) to activate the HPA axis, serotonin depletion had no effect on ACTH, corticosterone and levels of *c-fos*, an immediate-

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early gene that is held to reflect neuronal activation [6,10]. In addition to behavioural and physiological effects, lateral ventricular (i.c.v.) infusions of CRF evoke a specific pattern of *c-fos* activation in the brain, which closely resembles that described following psychological stress [2,4,12]. In particular, PVN neurons show intense *c-fos* expression after CRF treatment. The present study investigated the effects of serotonin depletion using the serotonergic neurotoxin 5,7-dihydroxytryptamine (5,7-DHT) on CRF-induced behavioural, endocrine and *c-fos* responses to determine whether low serotonin accentuated the activity of this peptide.

Lister hooded male rats ( $n = 48$ ; Harlan), average body weight 300 g, were kept under reversed light-dark cycles (12 h each phase: lights off 11:00 h) in a temperature-controlled room. The rats were fed ad libitum and handled daily for 7 days before experiments. Depletion procedure was adopted from Chung et al. [5]. Twenty to 30 min prior to surgery all animals received i.p. injections of 15 mg/kg nomifensine maleate (RBI, UK) and 20 mg/kg desipramine (Sigma, UK) to prevent any effect of 5,7-DHT on neuronal levels of dopamine (DA) and noradrenaline (NA). Under halothane anaesthesia, animals of the lesion group were treated bilaterally with intracerebroventricular (i.c.v.) injections of 150  $\mu\text{g}$  5,7-DHT free base (Sigma) in a volume of 10  $\mu\text{l}$  using a 10  $\mu\text{l}$ -Hamilton syringe (5  $\mu\text{l}$  per ventricle; coordinates: AP  $-1.0$  mm, ML 1.5 mm from bregma and V  $-3.5$  mm from skull). Animals of the sham group received vehicle (0.1 M ascorbate dissolved in 0.9% saline). In the same experimental conditions each animal a unilateral stainless steel cannula was implanted (23-gauge stainless steel) directed at the lateral ventricle (coordinates: AP  $-1.0$ , ML 1.5 mm from bregma and V  $-2.5$  mm from skull). Cannulae were fixed in place by dental cement attached to three stainless steel screws inserted into the skull. Flush-fitting stylets (30-gauge stainless steel) were inserted into the cannulae. After these procedures the rats were left to recover for 10 days.

CRF was obtained from Cambridge Biochemicals U.K., and dissolved in artificial cerebrospinal fluid (CSF) to a final concentration of 250  $\mu\text{mol}/\mu\text{l}$ . Sham operated animals received similar volume of artificial CSF (1  $\mu\text{l}$ ). All infusions were made on hand-held conscious rats. The stylet was removed from the guide cannula, and a 10.5 mm steel cannula (31-gauge) was inserted. This was connected via tubing (PE 10, Portex Ltd.) to a 5  $\mu\text{l}$  Hamilton syringe driven by a Harvard infusion pump. Rats were either infused with CRF (250 pmols) or CSF (1  $\mu\text{l}$ ). These procedures resulted in four experimental groups: (1) 5,7-DHT/CRF, (2) 5,7-DHT/CSF, (3) SHAM/CRF, (4) SHAM/CSF. After infusion all animals were monitored for grooming, burrowing, exploring and drinking behaviour for 25 min. The total time of each behaviour was measured.

Blood samples of the rats were collected by cardiac puncture when the animals were decapitated 30 min after infusion. All samples were collected around the same time

phase in the dark-light cycle (between 12:00 and 14:00 h) into heparinized syringes and centrifuged. Plasma was stored at  $-20$  °C until assay. Corticosterone was measured by radioimmunoassay (intra-assay variation 4.3%) using a validated procedure [16]. After decapitation the brains were rapidly removed. Samples of the prefrontal cortex were taken on an ice-cooled aluminium block for high pressure liquid chromatography (HPLC) determination of serotonin and other amines. Together with the brains, these samples were snap frozen in dry ice and stored at  $-70$  °C. For assay, the frontal samples were thawed and weighed, ten volumes of homogenization solution (0.4 M perchloric acid, 0.1% sodium metabisulphate, 0.02% cysteine and 0.01% EDTA) was added, homogenized and centrifuged. Supernatants were collected and the concentrations of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), NA, DA and 3,4-dihydroxyphenylacetic acid (DOPAC) were measured using HPLC.

The remainder of each brain was sectioned at 10  $\mu\text{m}$  at  $-20$  °C and mounted onto poly-lysine coated slides (Sigma, Dorset, UK). These were dried at room temperature and were then fixed with 4% paraformaldehyde for 5 min, washed in PBS, dehydrated and finally stored in fresh 95% ethanol at 4 °C. In-situ hybridization was carried out using previously published methods [16]. An oligonucleotide complementary to mRNA coding for *c-fos* was designed using special programmes (Oligo-NBI, Plymouth, USA). Specificity of the sequences was checked by basic BLAST search on NCBI. After labelling the probe with  $^{35}\text{S}$ , hybridization and incubation overnight, the slides were washed and air-dried at room temperature before exposure to X-ray film (Amersham, UK) for 1 week prior to development; which was carried out automatically. Subsequently, slides containing the PVN were dipped twice in 43 °C LM-1 (Amersham, UK) emulsion in a dark room. The slides stored at 4 °C in the light proof box. They were developed in 50% D19 Kodak developer for 5 min and counter stained with cresyl violet (Sigma, UK), dehydrated and cover-slipped with DPX mountant (BDH, UK). Analysis was carried out on Macintosh-based image analysis system (NIH image). Mean optical densities were obtained for each rat. This mean was used to calculate the group means. The total PVN was analysed.

Statistical comparisons were made by one- or two-way analyses of variance (ANOVA). Post-hoc comparisons between groups, after significant one-way ANOVA, were evaluated using Bonferroni test. Values given in the graphs are means and standard errors.

Neurochemical analysis using HPLC of the dissected brain samples showed that 5,7-DHT infusions had been successful in selectively reducing serotonin. Levels of 5-HT in the depleted animals ranged from 7 to 17% ( $F = 17.0$ ,  $P < 0.001$ ) of those in the sham operated groups. There were corresponding reductions in levels of its main metabolite, 5-HIAA. No significant changes were observed in NA, DA or DOPAC as a result of 5,7-DHT treatment.

Following infusion of CRF or CSF behaviour was

observed for the period of 25 minutes. Statistical analysis of the variance of the behavioural data, followed by Bonferoni's test, demonstrated that the CRF infusion increased grooming behaviour (Fig. 1). This was found to be significant in the 5,7/CRF and S/CRF group ( $F = 16$ ,  $P < 0.001$ ). 5-HT depletion did not alter this CRF-induced behaviour ( $F = 2.6$ , not significant). Other behavioural patterns showed no significant reactions to CRF or depletion.

CRF infusion induced a significant increase in the corticosterone levels in depleted and non-depleted animals ( $F = 22$ ,  $P < 0.0001$ ). However, corticosterone level in the 5,7/CRF group were not significantly different from the S/CRF group ( $F = 0.16$ , not significant). In addition, corticosterone levels in the 5,7-DHT/CSF group were also not different from the S/CSF group (Fig. 2).

Quantification of the *c-fos* mRNA in the PVN showed that i.c.v. CRF infusion increased *c-fos* expression significantly ( $F = 10.6$ ,  $P = 0.012$ ). However, post-hoc tests showed that although *c-fos* expression in the S/CRF group is significantly increased compared to the S/CSF it is not significantly different from the 5,7-DHT/CRF group (Fig. 3).

The objective of the present study was to investigate whether CRF infusion would induce modified neuronal, endocrine and behavioural responses in serotonin-depleted rats. The depletion achieved in this study with the neurotoxin 5,7-DHT was profound and specific. Serotonin levels and levels of 5-HIAA, its main metabolite, were significantly reduced without affecting tissue levels of other monoamines.

Early immediate genes, such as *c-fos*, are widely accepted to be markers for neuronal activation. It is well known that i.c.v. CRF treatment induces increased expression of *c-fos* in the PVN, as well as in other brain areas, and increases plasma corticosterone concentration [4, 6]. In our study we confirmed these responses and showed

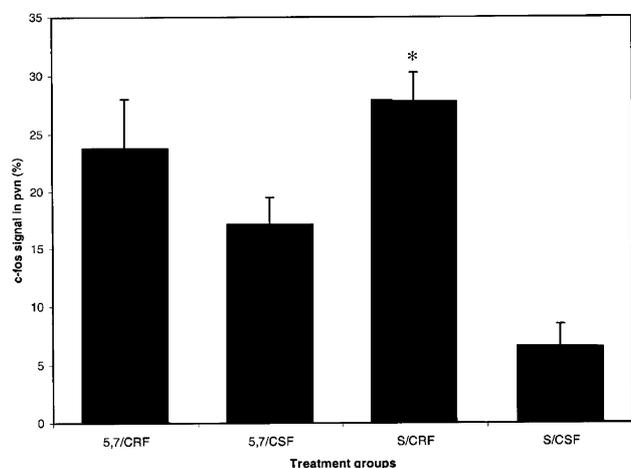


Fig. 1. CRF infusion induced a significant increase in grooming behaviour in the 5,7/CRF and S/CRF groups. 5,7/CRF group was not significantly different from the S/CRF group.  $*P < 0.05$  (ANOVA).

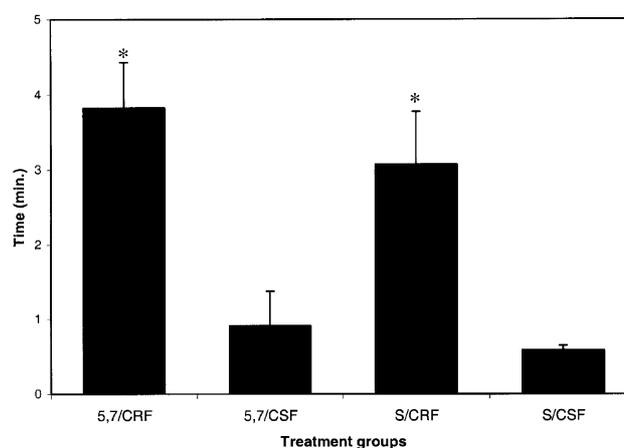


Fig. 2. Infusion of CRF into the brain resulted in increased corticosterone release into the systemic circulation in the 5,7/CRF and S/CRF groups. This increase was not affected by the depletion since 5,7/CRF is not significantly different from the S/CRF.  $*P < 0.05$  (ANOVA)

that CRF-induced increase in *c-fos* expression in the PVN and corticosterone levels are not dependent on serotonin, since depleting this monoamine did not influence these changes.

I.c.v. infusion of CRF produced a significant increase in grooming behaviour, corticosterone plasma levels and *c-fos* expression in the PVN confirming previously published data [2,4,5]. In our experimental conditions serotonin depletion did not alter these CRF-induced changes. It is likely that grooming behaviour is not dependant on the activation of the HPA axis but on the activation of other circuits since this behaviour is not inhibited by hypophysectomy or dexamethasone treatment [8]. In the present study CRF infusion induced grooming behaviour in non-depleted and depleted animals suggesting that serotonin probably does not actuate this action of CRF.

This data supports parallel conclusions from experiments using acute stress (i.p. injections of LPS and physical stress) rather than CRF infusions. Depletion of serotonin by pharmacological means had no effect on stress-induced *c-*

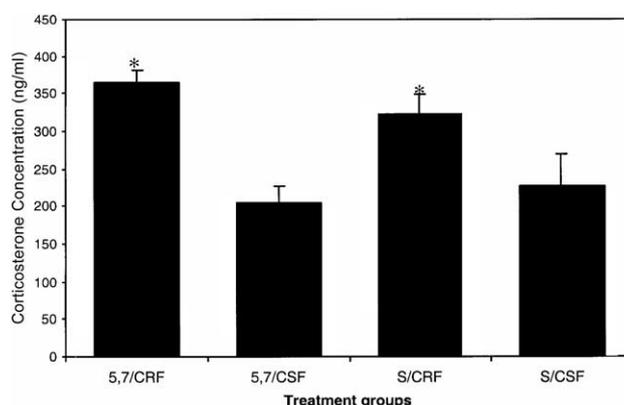


Fig. 3. *C-fos* levels were increased after CRF infusion. Although the S/CRF group had significantly more *c-fos* expression than the S/CSF group, it was not different from the 5,7/CRF group.  $*P < 0.05$  (ANOVA).

*fos* expression and corticosterone release [7,10]. Adequate depletion of serotonin had no interference with *c-fos* expression and corticosterone levels. Other authors have published varying results providing data that serotonin may have an excitatory influence on the HPA axis [9,13,16,19].

In the present study we have shown that these actions of CRF are not affected by depletion of serotonin. Although some reports show that the serotonergic system has a close interaction with the HPA axis, our data support the idea that acute CRF-induced changes are independent of reduced levels of serotonin.

The effects of the administration of CRF on behavioural, endocrine and neuronal responses are well-described in the literature. Our aim was investigate the effects of serotonin depletion on these CRF-induced changes in behaviour, corticosterone levels and *c-fos* expression in the PVN. CRF infusion generated a significant increase in grooming behaviour, corticosterone levels and *c-fos* expression in the PVN, whereas these increases were not affected by serotonin depletion. Our results support the hypothesis that altering basal levels of serotonin has a very limited role in those responses to an acute stress dependent on the central actions of CRF.

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### References

- [1] D.S. Albeck, C.R. McKittrick, D.C. Blanchard, R.J. Blanchard, J. Nikulina, B.S. Mc Ewen, R.R. Sakai, Chronic social stress alters levels of corticotropin-releasing factor and arginine vasopressin mRNA in rat brain, *J. Neurosci.* 17 (1997) 4895–4903.
- [2] L.C. Andreae, J. Herbert, Expression of *c-fos* in restricted areas of the basal forebrain and brainstem following single or combined intraventricular infusions of vasopressin and corticotropin-releasing factor, *Neuroscience* 53 (1993) 735–748.
- [3] L. Arborelius, M.J. Owens, P.M. Plotsky, C.B. Nemeroff, The role of corticotropin-releasing factor in depression and anxiety disorders, *J. Endocrinol.* 160 (1999) 1–12.
- [4] F.J.L. Arnold, M. De Lucas-Bueno, H. Shiers, D.C. Hancock, G.I. Evan, J. Herbert, Expression of *c-fos* in regions of the basal limbic forebrain following intra-cerebroventricular corticotropin-releasing factor (CRF) in unstressed or stressed male rats, *Neuroscience* 51 (1992) 377–390.
- [5] K.K.K. Chung, M. Martinez, J. Herbert, Central serotonin depletion modulates the behavioural, endocrine and physiological responses to repeated social stress and subsequent *c-fos* expression the brains of male rats, *Neuroscience* 92 (1999) 613–625.
- [6] G.L. Conde, D. Renshaw, S.L. Lightman, M.S. Harbuz, Serotonin depletion does not alter lipopolysaccharide-induced activation of the rat paraventricular nucleus, *J. Endocrinol.* 156 (1998) 245–251.
- [7] B. Connor, M. Dragunow, The role of neuronal growth factors in neurodegenerative disorders of the human brain, *Brain Res. Rev.* 27 (1998) 1–39.
- [8] A.J. Dunn, C.W. Berridge, Physiological and behavioural responses to corticotropin-releasing factor administration: is CRF a mediator of anxiety or stress responses? *Brain Res. Rev.* 15 (1990) 71–100.
- [9] S. Feldman, J. Weidenfeld, Posterior hypothalamic deafferentation or 5,7-DHT inhibit corticotropin-releasing hormone, ACTH and corticosterone responses following photic stimulation, *Neurosci. Lett.* 198 (1995) 143–145.
- [10] M.S. Harbuz, J. Chalmers, L. De Souza, S.L. Lightman, Stress-induced activation of CRF and *c-fos* mRNAs in the paraventricular nucleus are not affected by serotonin depletion, *Brain Res.* 609 (1993) 167–173.
- [11] J.P. Herman, C.M.F. Prewitt, W.E. Cullinan, Neuronal circuit regulation of the hypothalamo-pituitary-adrenocortical stress axis, *Crit. Rev. Neurobiol.* 10 (1996) 371–394.
- [12] T. Imaki, T. Shibasaki, M. Hotta, H. Demura, Intracerebroventricular administration of corticotropin-releasing factor induces *c-fos* mRNA expression in brain regions related to stress responses: comparison with pattern of *c-fos* mRNA induction after stress, *Brain Res.* 616 (1993) 114–125.
- [13] S.M. Korte, S. van Duin, G.A.H. Bouws, J.M. Koolhaas, B. Bohus, Involvement of hypothalamic serotonin in activation of the sympathoadrenomedullary system and hypothalamo-pituitary-adrenocortical axis in male Wistar rats, *Eur. J. Pharmacol.* 197 (1991) 225–228.
- [14] P. Larsen, A. Hay-Schmidt, N. Vrang, J.D. Mikkelsen, Origin of projections from the midbrain raphe nuclei to the hypothalamic paraventricular nucleus in the rat: a combined retrograde and anterograde tracing study, *Neuroscience* 70 (1996) 963–988.
- [15] A.C.E. Linhorst, C. Flackskamm, S.J. Hopkins, M.E. Hoadley, M.S. Labeur, F. Holsboer, J.M.H.M. Reul, Long-term intracerebroventricular infusion of corticotropin-releasing hormone alters neuroendocrine, neurochemical, autonomic, behavioural, and cytokine responses to a systemic inflammatory challenge, *J. Neurosci.* 17 (1997) 4448–4460.
- [16] D. Saphier, S. Feldman, Paraventricular nucleus neuronal responses following electrical stimulation of the midbrain dorsal raphe: evidence for cotransmission, *Exp. Brain Res.* 78 (1989) 407–414.
- [17] D. Saphier, J.E. Welch, G.E. Farrar, N.Q. Nguyen, F. Aguado, T.R. Thaller, D.S. Knight, Interactions between serotonin, thyrotropin-releasing hormone, and substance P in the CNS regulation of adrenocortical secretion, *Psychoneuroendocrinology* 19 (1994) 779–797.
- [18] P.E. Sawchenko, L.W. Swanson, H.W.M. Steinbusch, A.A.J. Verhofstad, The distribution and cells of origin of serotonergic inputs to the paraventricular and supraoptic nuclei of the rat, *Brain Res.* 277 (1983) 355–360.
- [19] J.E. Welch, G.E. Farrar, A.J. Dunn, D. Saphier, Central 5-HT1A receptors inhibit adrenocortical secretion, *Neuroendocrinology* 57 (1993) 272–281.