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Effect of dexfenfluramine on the transcriptional activation of CRF and its type 1 receptor within the paraventricular nucleus of the rat hypothalamus

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1 The present study investigated the effect of intraperitoneal (i.p.) administration of the indirect 5-hydroxytryptamine (5-HT) receptor agonist, dexfenfluramine, on the transcriptional activity of corticotropin-releasing factor (CRF) and its type 1 receptor in the brains of conscious male Sprague-Dawley rats via *in situ* hybridization histochemistry (ISHH) using both intronic and exonic probe technology.

2 The immediate *early* gene (IEG) c-*fos* mRNA was also used as index of cellular activity, whereas localization between CRF-immunoreactive (ir) perikarya and the IEG was accomplished to determine the site of CRF neuronal activation in the brain of dexfenfluramine-treated rats.

3 Thirty minutes, 1, 3, and 6 h after a single injection of either dexfenfluramine (10 mg kg^{-1}) or the vehicle solution, adult male rats (230-260 g) were deeply anaesthetized and rapidly perfused with a 4% paraformaldehyde-borax solution (PF). The brains were removed from the skull, postfixed, and placed in a solution of 4% PF-10% sucrose overnight at 4°C. Frozen brains were mounted on a microtome and cut from the olfactory bulb to the medulla in 30-μm coronal sections.

4 Dexfenfluramine induced a general neuronal activation as indicated by the strong signal of c-*fos* mRNA in several structures of the brain, including the parietal cortex, caudate putamen, circumventricular organs, medial preoptic area, bed nucleus of the stria terminalis, choroid plexus, choroidal fissure, supraoptic nucleus, paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus, central nucleus of the amygdala, dorsomedial nucleus of the hypothalamus, laterodorsal tegmental nucleus, locus coeruleus, and several subdivisions of the dorsal vagal complex. In most of these structures, the signal was maximal at 30 min, still strong and positive at 60 min, largely decreased at 3 h, and had completely disappeared 6 h after injection.

5 In the parvocellular division of the PVN, the large majority of CRF-ir perikarya displayed a positive signal for the mRNA encoding c-*fos*, indicating a profound CRFergic activation within this neuroendocrine nucleus after dexfenfluramine administration.

6 Colocalization between CRF-ir neurones and c-*fos* positive cells was not detected in any other regions. This selective activation of PVN CRF neurones was also confirmed by the presence of CRF primary transcript; 30 min after i.p. injection of the indirect 5-HT agonist, a positive signal for CRF hnRNA was observed, specifically in the parvocellular PVN.

7 Transcription of the gene encoding the type 1 receptor for CRF was highly stimulated in the PVN following 5-HT activation. Although this hypothalamic nucleus exhibited a barely detectable signal under basal conditions, dexfenfluramine induced a strong signal of CRF_1 receptor mRNA in the parvocellular PVN. Interestingly, CRF-ir neurones displayed a positive signal for the mRNA encoding the CRF_1 receptor, 3 and 6 h after systemic treatment with dexfenfluramine.

8 These results indicate that although dexfenfluramine can generate a wide neuronal activation throughout the brain, this 5-HT agonist triggers the activity of CRF neurones selectively in the parvocellular division of the PVN, a mechanism possibly related to the activity of hypothalamic-pituitary-adrenal axis. Induction of CRF_1 receptor mRNA in CRF cells of the PVN indicates that neuroendocrine CRF neurones can be targeted by CNS CRF under 5-HT stimulation.

Keywords: Corticotropin-releasing factor; c-*fos*; dexfenfluramine; hypothalamic-pituitary-adrenal axis; immediate *early* genes; *in situ* hybridization histochemistry; immunocytochemistry; intronic probe technology; 5-HT

Introduction

Immunocytochemistry, (Swanson *et al.*, 1983; Swanson, 1986; Sakanaka *et al.*, 1987; Sawchenko & Swanson, 1990) as well as *in situ* hybridization studies (Imaki *et al.*, 1991; 1992b; Rivest & Rivier, 1995) have shown that corticotropin-releasing factor (CRF) can be produced in numerous regions of the central nervous system (CNS) including the olfactory bulb, bed nucleus of the stria terminalis (BnST), parvocellular division of the hypothalamic paraventricular nucleus (PVN), substantia innominata (SI), medial preoptic area (MPOA), central nucleus of the amygdala (CeA), lateral hypothalamic area (LHA), perioculomotor nucleus (POR), dorsal premammillary nucleus (PMd), medial part of the medial geniculate complex (MGCm), laterodorsal tegmental nucleus (LDT), Barrington's nucleus (B), parabrachial nucleus (PB), dorsal vagal complex (DVC), medial vestibular nucleus (MVN), nucleus prepositus (PRP), and A_5 and A_1 catecholamine cell groups. However, the exact neuroanatomical organization of most of these CRF-containing cells and the role of these pathways during various

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types of challenges are unknown. In contrast, the importance of the PVN in controlling the synthesis and release of ACTH/β-endorphin (β-End) from the pituitary gland is now established. It is generally accepted that CRF released from parvocellular PVN neurones into the hypothalamic-pituitary portal blood is the primary stimulus to anterior pituitary adrenocorticotrophic hormone (ACTH) secretion. Indeed, the parvocellular division of the hypothalamic PVN contains most of the CRF cell bodies projecting to the infundibular system and participates directly in the control of the hypothalamic-pituitary-adrenal (HPA) axis during stress (Sawchenko & Swanson, 1990). Stressors as diverse as prolonged exercise, immobilization, exposure to mild footshocks, or immune challenge markedly increase both c-*fos* expression in CRF-immunoreactive neurones and CRF mRNA in the PVN of rats, providing evidence of stress-induced alteration of neuroendocrine CRF cells (Imaki *et al.*, 1991; 1992a; Rivest & Rivier, 1994; 1995; Rivest & Laflamme, 1995).

Among the large variety of neurotransmitters able to modulate the activity of CRF neurones within the parvocellular PVN, 5-hydroxtryptamine (5-HT) has been suggested as a candidate for regulating neuroendocrine CRF cells and the activity of the HPA axis during various circumstances (Bagdy *et al.*, 1989; Calogero *et al.*, 1989; Owens *et al.*, 1990; 1991). Indeed, CRF perikarya of the parvocellular PVN receive innervation from 5-HT neurones originating from raphé nuclei (Sawchenko *et al.*, 1983; Assenmacher *et al.*, 1987; Liposits *et al.*, 1987; Halliday *et al.*, 1995) whereas 5-HT and 5-HT agonists can stimulate CRF release *in vivo* (Gibbs & Vale, 1983; Alper, 1990; Pan & Gilbert, 1992) and *in vitro* (Holmes *et al.*, 1982; Nakagami *et al.*, 1986; Calogero *et al.*, 1989; 1992). Fenfluramine, a 5-HT agonist that increases 5-HT synaptic availability in stimulating its release and blocking its reuptake, activates the secretion of CRF from the hypothalamus (Holmes *et al.*, 1982) and into the hypophyseal portal blood (Olivier *et al.*, 1990) and raises plasma corticosterone (McElroy *et al.*, 1984; Appel *et al.*, 1991).

The interaction between CRF and 5-HT is not, however, limited to the control of the HPA axis; involvement of CRF in the anorectic and thermogenic influence of fenfluramine has been reported (Lefeuvre *et al.*, 1990). On the other hand, central CRF injection is able to raise the HPA axis (Ono *et al.*, 1985), activate sympathetic outflow from the CNS and associated metabolic and cardiovascular changes (Fisher *et al.*, 1982; Brown *et al.*, 1982; 1985; 1989), induce anorectic behaviour (Levine *et al.*, 1983; Rivest *et al.*, 1989), as well as increase arousal and responsiveness to external stimuli (Dunn & Berridge, 1990). Stimulation of 5-HT pathways with fenfluramine can cause similar endocrine, behavioural and autonomic responses, suggesting possible participation of the CRF system in these phenomena. However, the influence of the indirect 5-HT agonist on the activity of CRF neurones throughout the rat brain still remains unknown as is the site of action of CRF within the brain under 5-HT stimulation. The purposes of the present study were therefore to outline the sites of activation throughout the rat brain after systemic administration of the indirect 5-HT agonist, dexfenfluramine, via detection of the immediate *early* gene (IEG) c-*fos* mRNA; to determine the sites of colocalization between CRF and c-*fos* by combining *in situ* hybridization and immunocytochemistry techniques on the same brain sections; to investigate the transcriptional activity of CRF by means of *in situ* hybridization histochemistry using intronic and exonic probe technology throughout the rat brain; and to analyze the exact sites of action as well as the type of cells targeted by CRF in the brain of dexfenfluramine-treated via expression of the mRNA encoding the CRF type 1 receptor. We have recently reported that CRF type 1 receptor was finely regulated in the brains of stressed rats suggesting that this particular receptor could be a serious candidate to mediate some of the actions of the neuropeptide within the CNS during specific challenges involving 5-HT circuitry.

Methods

Animals

Adult male Sprague-Dawley rats (230–260 g) were acclimatized to laboratory conditions (14 h light, 10 h dark cycle; lights on at 06 h 00 min and off at 20 h 00 min) and given free access to rat chow and water. The rats were conscious and freely moving at all times throughout the experimental procedure. Each rat was used for experimentation once only, and protocols were approved by Laval University's Animal Welfare Committee.

Dexfenfluramine administration

On the day of the experiment (~09 h 00 min), 16 rats received a single intraperitoneal (i.p.) injection of dexfenfluramine (10 mg kg^{-1}) dissolved in 0.5 ml of sterile 0.9% saline; control rats were injected with 0.5 ml of 0.9% sterile saline only. Dexfenfluramine is the dextrorotatory enantiomer ((+)-isomer) of fenfluramine, an amphetamine derivative described as an indirect 5-HT agonist as it increases 5-HT availability at the synaptic cleft (Garattini, 1987); although this action of the drug might well be indirect. The influence of fenfluramine on the release of 5-HT from the nerve terminals and the blockade of its reuptake is well known; however at high dose, this drug has been shown to trigger the release not only of 5-HT, but also other monoamines (Samanin & Garattini, 1993). On the other hand, dexfenfluramine is specifically activating the central and peripheral 5-HT system and, therefore, explains our choice of this particular drug. It is however important to keep in mind that the dose used in the present study was quite high in order to induce a profound activation of 5-HT pathways and involvement of other monoaminergic systems remains possible. Thirty minutes, 1, 3, and 6 h after the i.p. injection of dexfenfluramine or the vehicle solution, the animals were deeply anaesthetized via an i.p. injection of 0.3 ml of a mixture of ketamine hydrochloride (80 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5 at 4°C). The brains were removed from the skulls, postfixed for 2 to 8 days, and then placed in 10% sucrose in a solution of 4% paraformaldehyde-borax buffer overnight at 4°C. The frozen brains were mounted on a microtome (Reichert-Jung, Cambridge Instruments Company, Deerfield, IL) and cut into 30-μm coronal sections. The slices were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at −20°C.

In situ *hybridization histochemsitry*

Hybridization histochemical localization of each transcript was carried out in 1 in 6 series (every sixth section) of slices throughout the brain (from the olfactory bulb to the end of the medulla) using ^{35}S-labelled cRNA probes. Protocols for riboprobe synthesis, hybridization and autoradiographic localization of mRNA signal were adapted from Simmons *et al.* (1989). All solutions and part of the material were treated with diethylpyrocarbonate (Depc) and sterilized to prevent RNA degradation. Tissue sections mounted onto poly-L-lysine-coated slides were desiccated under vacuum overnight, fixed in 4% paraformaldehyde for 30 min, and digested by proteinase K (10 μg ml^{-1} in 100 mM Tris HCl, pH 8.0, and 50 mM EDTA, at 37°C for 25 min). Thereafter, the brain sections were rinsed in sterile Depc water followed by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated through graded concentrations of alcohol (50, 70, 95, and 100%). After vacuum-drying for a minimum of 2 h, 90 μl of hybridization mixture (10^7 cpm ml^{-1}) was spotted on each slide, sealed under a coverslip, and incubated at 60°C overnight (~15–20 h) in a slide warmer. Coverslips were then removed and the slides

were rinsed in 4 × standard saline citrate (SSC) at room temperature. Sections were digested by RNase A (20 μg ml^{-1}, 37°C, 30 min), rinsed in descending concentrations of SSC (2 ×, 1 ×, 0.5 × SSC), washed in 0.1 × SSC for 30 min at 60°C (1 × SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0), and dehydrated through graded concentrations of alcohol. After being dried for 2 h under vacuum, the sections were exposed at 4°C to X-ray film (Kodak) for 15 to 36 h (depending on the probe), defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1 : 1 with distilled water). Slides were exposed for 6 to 14 days (CRF and c-*fos* mRNA, 6 days; CRF_1 receptor mRNA, 9 to 14 days; CRF hnRNA, 14 days) developed in D19 developer (Kodak) for 3.5 min at 14–15°C, and fixed in rapid fixer (Kodak) for 5 min. Thereafter, tissues were rinsed in running distilled water for 1 to 2 h, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with DPX.

cDNA probe synthesis and preparation

The c-*fos* and CRF cRNA probes were generated from the *Eco*R I fragment of rat c-*fos* cDNA (Dr I. Verma, The Salk Institute, La Jolla, CA, USA) and rat CRF cDNA (Dr K. Mayo, Northwestern University, Evanston, IL, USA) subcloned into pBluescript SK-1 (Stratagene, La Jolla, CA, USA), and linearized with *Sma* I and *Hind* III (Pharmacia), respectively. The pGem3 plasmid containing a pure CRF intronic piece was linearized with *Hind* III (530 bp) to detect specifically CRF heteronuclear (hn) RNA {Dr S. Watson, The Universtiy of Michigan, Ann Arbor (Herman *et al.*, 1992)}. On the other hand, the 1.3 kb *Pst*I-*Pst*I fragment of rat type 1 CRF receptor cDNA (Dr W. Vale, Peptide Biology Laboratory, The Salk Institute) was subcloned into pBluescript II SK (Stratagene, La Jolla, CA, USA) and linearized with *Bam*H I and *Hind* III (Pharmacia) for antisense and sense probes, respectively (Rivest *et al.*, 1995).

Radioactive antisense cRNA copies were synthesized by incubating 250 ng of linearized plasmid in 6 mM $MgCl_2$, 30–40 mM Tris (pH 7.5), 2 mM spermidine, 10 mM dithiothreitol, 0.2 mM ATP/GTP/CTP, [α-^{35}S]-UTP, 40 u RNAsin (Promega, Madison, WI, USA), and 20 u of SP6 (CRF mRNA), or T7 (c-*fos* mRNA, CRF hnRNA, and CRF_1 receptor mRNA) RNA polymerase for 60 min at 37°C. Unincorporated nucleotides were removed using the ammonium-acetate precipitation method; 100 μl of DNAse solution (1 μl of DNAse, 5 μl of 5 mg ml^{-1} tRNA, 94 μl of 10 mM Tris/10 mM $MgCl_2$) was added, and 10 min later an extraction was accomplished with a phenol-chloroform solution. The cRNA was precipitated with 80 μl of 5 M ammonium acetate and 500 μl of 100% ethanol for 20 min on dry ice. The pellet was washed with 500 μl ethanol, dried, and resuspended in 100 μl of 10 mM Tris/1 mM EDTA (pH 8.0). A concentration of 10^7 c.p.m. probe was mixed into 1 ml of hybridization solution (500 μl formamide; 60 μl 5 M NaCl, 10 μl 1 M Tris [pH 8.0], 2 μl 0.5 M EDTA [pH 8.0], 20 μl 50 × Denhart's solution, 200 μl 50% dextran sulphate, 50 μl 10 mg ml^{-1} tRNA, 10 μl 1 M DTT, [118 μl Depc water – volume of probe used]). This solution was mixed and heated for 5 min at 65°C before being spotted on slides. Radioactive sense (control) cRNA copies were also prepared to verify the specificity of each probe. Hybridization with these probes did not reveal any positive signal in the brain of vehicle- and dexfenfluramine-treated rats.

Combination of immunocytochemistry with in situ *hybridization*

Immunocytochemistry {CRF-immunoreactive (ir) nerones} was combined with the *in situ* hybridization histochemistry protocol (c-*fos* mRNA or CRF_1 receptor mRNA) to determine whether CRF perikarya were able to express the IEG c-*fos* or the gene encoding the type 1 CRF receptor after stimulation of 5-HT pathways. Every sixth tissue slice for each double labelling (CRF-ir + c-*fos* mRNA, CRF-ir + CRF_1 receptor mRNA) was processed by using the avidin-biotin amplification bridge method with peroxidase as a substrate. Briefly, slices were washed in KPBS buffer (0.05 M potassium- phosphate) and incubated overnight with primary rabbit antihuman/rat CRF serum antibody (Code PBL rc 70, 8/9/83 bleed, Dr Wylie Vale, Peptide Biology Laboratory, The Salk Institute). The CRF antisera was diluted 1/10 000 in a solution of KPBS, 0.4% triton-X, 0.25% heparin, and 1% bovine serum albumin

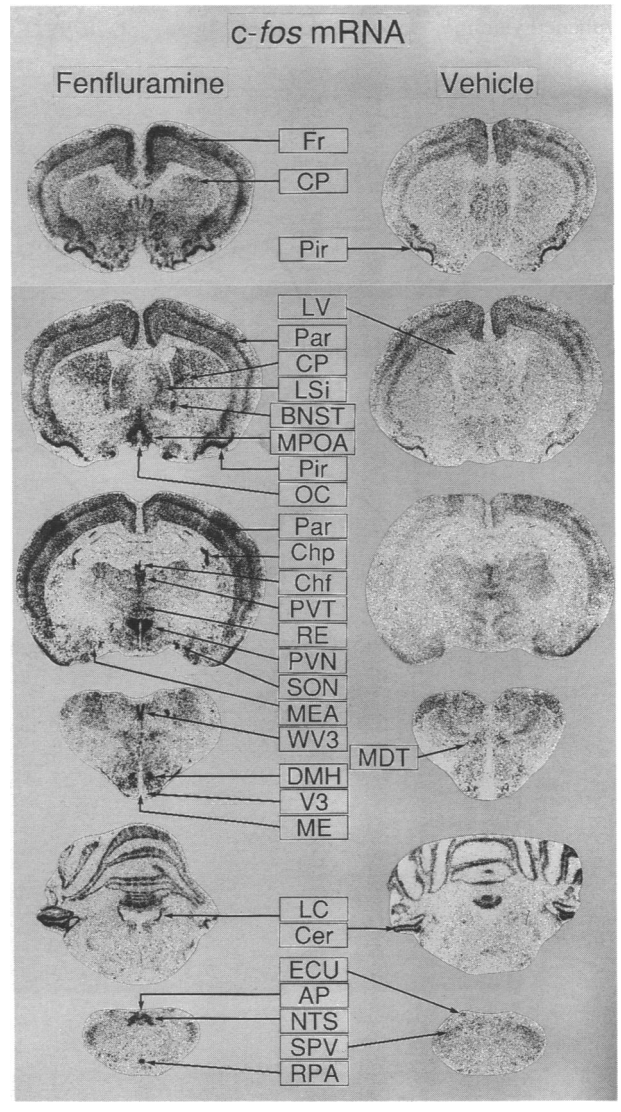

Figure 1 Representative example of the distribution of the mRNA encoding the immediate *early* gene c-*fos* in the rat brain after intraperitoneal (i.p.) administration of dexfenfluramine (Fenfluramine) or vehicle. Animals were deeply anaesthetized and rapidly perfused with 4% paraformaldehyde 30 min after treatment with dexfenfluramine (10 mg kg^{-1}) or the vehicle solution. These rostrocaudal coronal sections (30 μm) of dexfenfluramine-treated rat exhibited a positive signal on X-ray film (Kodak XAR-5) for c-*fos* transcript in multiple structures throughout the brain. AP, area postrema; BnST, bed nucleus of the stria terminalis; Cer, cerebellum; chf, choroidal fissure; Chp, choroid plexus; CP, caudate putamen; DMH, dorsomedial nucleus of the hypothalamus; ECU, external cuneate nucleus; Fr, frontal cortex; LC, locus coeruleus; LSi, intermediate part of the lateral septal nucleus; ME, median eminence; MeA, medial nucleus of the amygdala; MPOA, medial preoptic area; NTS, nucleus of the solitary tract; OC, optic chiasm; Par, parietal cortex; Pir, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; RPA, nucleus raphé pallidus; RE, nucleus reuniens; SON, supraoptic nucleus; SPV, spinal nucleus of the trigeminal; V3, third ventricle; WV3, wall of the third ventricle. Identification of brain structures are based essentially on Swanson's Atlas (1992).

(BSA, fraction V). Approximately 16 h after incubation at 4°C with the primary antibody, the brain slices were rinsed in sterile KPBS and incubated with a mixture of KPBS + triton-X + heparin + biotinylated goat antirabbit IgG (1 : 1500 dilution; Vector Laboratories, CA) for 90 min. The sections were then rinsed with KPBS and incubated at room temperature for 60 min with an avidin-biotin-peroxidase complex (Vectastain ABC elite kit, Vector Laboratories, CA, USA). The peroxidase complex was amplified by means of 10-min incubation with a 7 μM solution of biotin (sulphosuccinimydyl 6-(biotinamido) hexanoate, Pierce No. 21335)-tyramine HCl (4-hydroxyphenethylamine hydrochloride, Sigma T-2879)-H_2O_2 (0.01%), followed by a second incubation of 30 min with the ABC elite solution. After several rinses in sterile KPBS, the brain slices were reacted in a mixture containing sterile KPBS, the chromagen 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.05%), and 1% hydrogen peroxide (H_2O_2).

Thereafter, the tissues were rinsed in sterile KPBS, mounted onto poly-L-lysine-coated slides, dessicated under vacuum overnight, fixed in 4% paraformaldehyde for 30 min, and digested by proteinase K (10 μg ml^{-1} in 100 mM Tris HCl, pH 8.0, and 50 mM EDTA (pH 8.0), at 37°C for 25 min). Prehybridization, hybridization with either c-*fos* or CRF_1 receptor cRNA probe, and posthybridization steps were per-

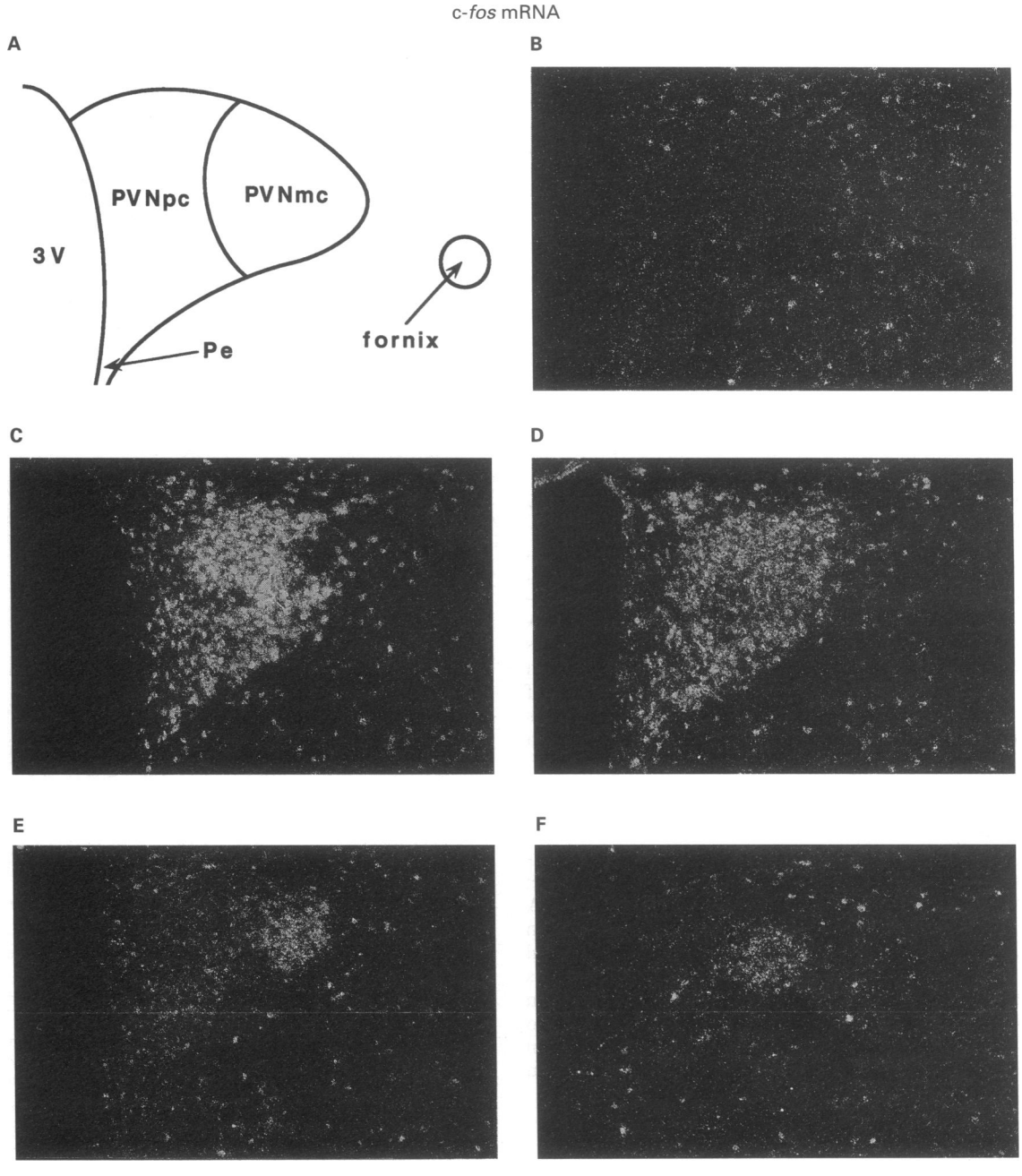

Figure 2 Time-related influence of intraperitoneal (i.p.) administration of dexfenfluramine or vehicle on the expression of c-*fos* mRNA in the paraventricular nucleus (PVN) of the rat hypothalamus. These photos depict darkfield photomicrographs of dipped autoradiographs of hybridized 30-μm sections with rat c-*fos* riboprobe through identical areas of the right medial PVN. Animals were deeply anaesthetized and rapidly perfused with 4% paraformaldehyde 0.5 (C), 1 (D), 3 (E), and 6 (F) h after treatment with dexfenfluramine (10 mg kg^{-1}) or the vehicle solution (B). Note the intense positive signal in the parvocellular PVN as early as 30 min after dexfenfluramine injection (C), while both parvocellular and magnocellular divisions exhibited strong hybridization signal for this IEG at 1 h post-injection (D). The PVN of vehicle-treated rats did not display detectable c-*fos* transcript (B). Abbreviations for (A) PVNmc, magnocellular division of the paraventricular nucleus of the hypothalamus; PVNpc, parvocellular division of the paraventricular nucleus of the hypothalamus; Pe, periventricular nucleus of the hypothalamus; 3V, inferior third ventricle.

formed as described above except for the dehydration (alcohol 50, 70, 95, 100%), which was shortened to avoid decolouration of CRF cells (brown staining). After being dried for 2 h under the vacuum, the sections were exposed at 4°C to X-ray film (Kodak) overnight, defatted in xylene, and dipped in NTB2 nuclear emulsion (Kodak; diluted 1:1 with distilled water). Slides were exposed 7 (c-*fos* mRNA) to 10 days (CRF_1 receptor mRNA), developed, rapidly dehydrated in graded concentrations of alcohol, clear in xylene, and coverslipped with DPX mountant. The presence of c-*fos* or CRF_1 receptor transcripts was evident as silver grains in perikarya, and CRF immunoreactivity within the cell cytoplasm and fibres was stained in brown.

Quantitative analysis

The c-*fos* mRNA, CRF mRNA, CRF hnRNA, and CRF_1 receptor mRNA signals revealed on dip NTB2 nuclear emulsion slides were analyzed and quantified using an Olympus Optical System (BX-50, β-MAX) coupled to a Macintosh computer (Power PC 7100/66) and Image software (version 1.55 non-FPU, W. Rasband, NIH). The optical density (OD) of the hybridization signal was measured under darkfield illumination at a magnification of × 10. Sections for the experimental and control animals were matched for rostro-caudal level. Because of the lack of endogenous expression of c-*fos* mRNA, CRF hnRNA, and CRF_1 receptor mRNA in the hypothalamic PVN, the whole medial PVN was digitized under brightfield illumination and then subjected to densitometric analysis under darkfield, yielding measurements of integrated OD (area of PVN × average OD). On the other hand, the hybridization signal for CRF mRNA in the parvocellular PVN was digitized under darkfield and expressed as intensity of signal within this hypothalamic structure. The OD of each specific transcript was then corrected for the average background signal as determined by sampling cells immediately outside the cell group of interest (McCabe & Pfaff, 1989).

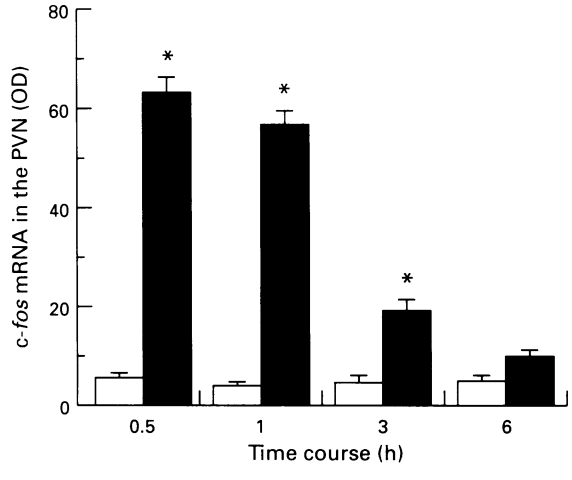

Figure 3 Influence of i.p. dexfenfluramine (10 mg kg^{-1}, solid columns) or vehicle (open columns) administration on the average optical density (OD) for c-*fos* mRNA signal in the paraventricular nucleus (PVN) of the rat hypothalamus. The OD was quantified in both sides of the medial PVN with an Olympus Optical System (BX-50, β-MAX) coupled to a Macintosh computer (Power PC 7100/66) and IMAGE Software (version 1.55 non-FPU, W. Rasband, NIH). Results represent means ± s.e. mean of 3–4 rats. The scale for OD is relative and in arbitrary units. Results were analyzed by a 2 × 4 analysis of variance (ANOVA), followed by a Bonferroni/Dunn test procedure as *post hoc* comparisons (Statview 4.01). For more information on image analysis and statistical analysis, see Methods. *$P<0.05$ from vehicle-treated rats at their respective times post-injection.

Statistical analysis

Data from Figures 3, 6 and 9 are expressed as OD for c-*fos* mRNA, CRF hnRNA (top panel) and CRF mRNA (bottom panel), and CRF_1 receptor mRNA, respectively. Results were analyzed by a 2 × 4 analysis of variance (ANOVA), followed by a Bonferroni/Dunn test procedure as post hoc comparisons (Statview 4.01). Factors were identified as follows: systemic treatment, which was composed of two levels (i.p. vehicle or i.p. dexfenfluramine), and time post-injection, which combined four levels (0.5, 1, 3, and 6 h post-injection).

Results

*Distribution of c-*fos *mRNA in the brain of rat injected with dexfenfluramine*

Figure 1 illustrates a representative example of the rostro-caudal distribution of c-*fos* mRNA expression in the rat brains

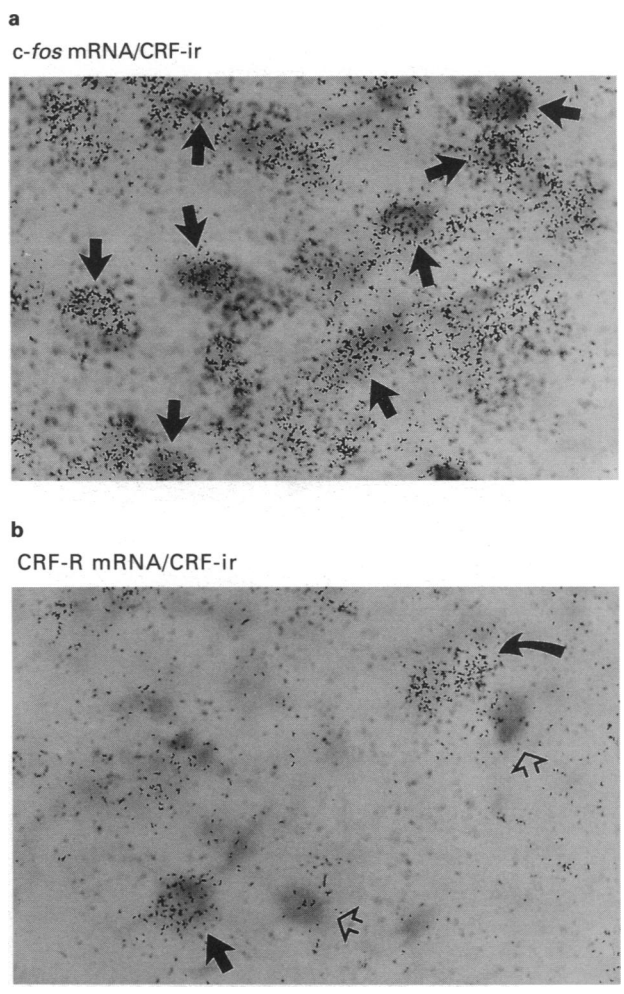

Figure 4 Colocalization of CRF-immunoreactive (ir) neurones with cells expressing c-*fos* mRNA (a) or CRF type 1 receptor transcript (CRF-R, b) in the parvocellular division of the paraventricular nucleus of dexfenfluramine-treated rat hypothalamus. The animals were killed 30 min (a) or 3 h (b) after intraperitoneal (i.p.) administration of dexfenfluramine. Immunocytochemistry (CRF protein, stained cytoplasm and fibres) was performed on same brain sections (30 μm) before *in situ* hybridization histochemistry (silver grains). For more details on the combination of both immunocytochemistry and *in situ* hybridization techniques, see Methods. (a) Filled arrowheads, CRF-ir neurones expressing the mRNA encoding Fos. Note that the large majority of CRF-ir perikarya were positive for c-*fos* mRNA. (b) Filled arrowhead, CRF-ir neurones expressing the mRNA encoding the CRF_1 receptor. Open arrowheads, CRF-ir cell bodies alone; curved arrowhead, CRF-R positive neurone alone. Magnification × 250.

30 min after an i.p. injection of dexfenfluramine (left column) or vehicle solution (right column). Hybridization with the sense probe did not detect any of the positive signal observed with the antisense probe (data not shown). A weak to moderate basal signal (vehicle-treated rats, right column) was observed in several layers of the frontal (Fr) and parietal cortex (Par), piriform (Pir), caudate putamen (CP), suprachiasmatic nucleus (SCh), arcuate nucleus (ARC), dorsomedial nucleus of the hypothalamus (DMH), mediodorsal nucleus of the thalamus (MDT), pontine gray (PG), parabrachial nucleus (PB), laterodorsal tegmental nucleus (LDT), several layers of the cerebellum, external cuneate nucleus (ECU), and spinal nucleus of the trigeminal (SPV). Thirty min after dexfenfluramine injection, a strong hybridization signal was detected in several areas of the brain, including various layers of the Fr and Par, CP, organum vasculosum of the lamina terminalis (OVLT), medial preoptic area (MPOA), bed nucleus of the stria terminalis (BnST), choroid plexus (Chp), choroidal fissure (Chf), subfornical organ (SFO), supraoptic nucleus (SON), paraventricular nucleus of the hypothalamus (PVN), paraventricular nucleus of the thalamus (PVT), central nucleus of the amygdala (CeA), wall of the third ventricle, superior and

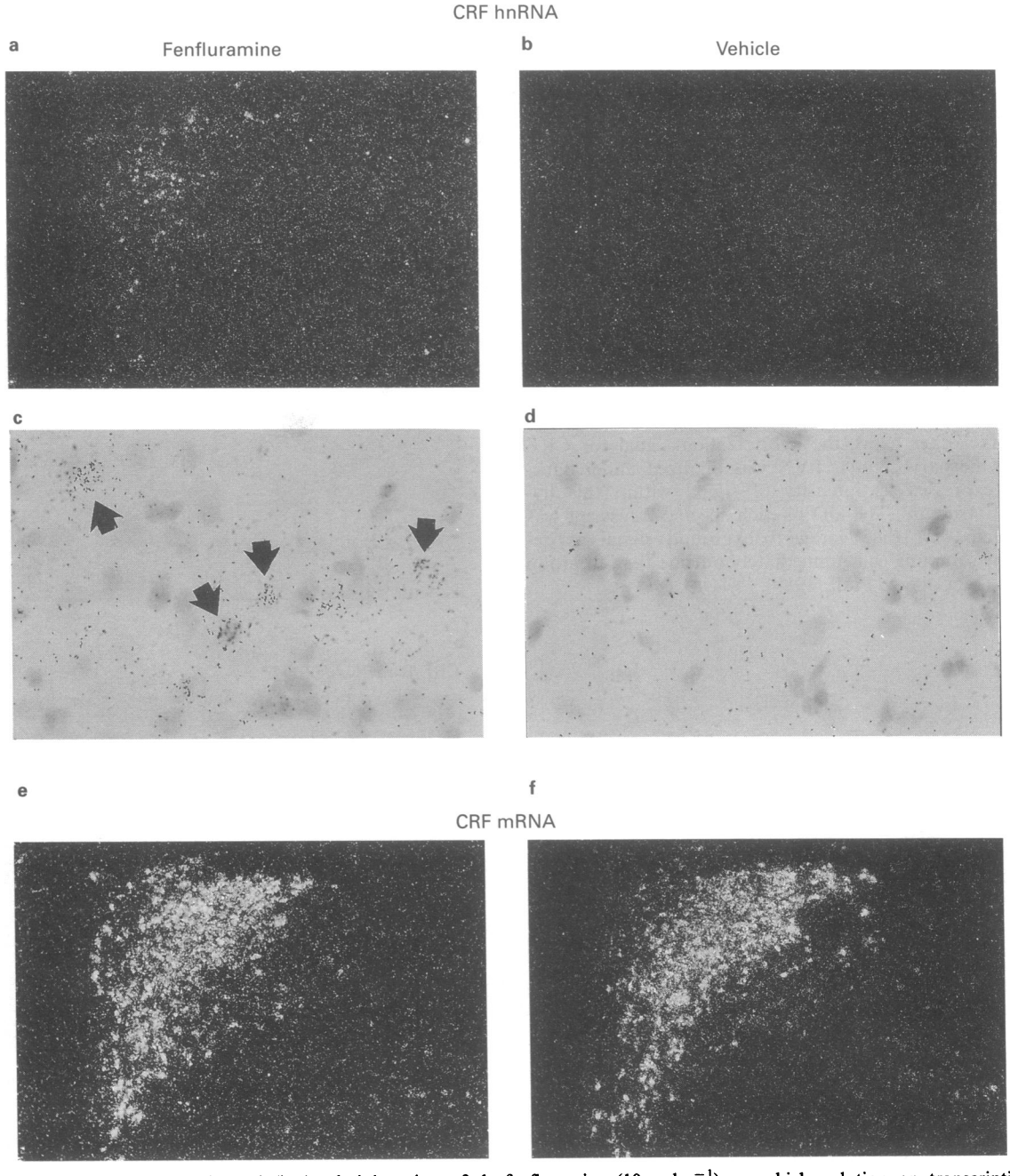

Figure 5 Effect of intraperitoneal (i.p.) administration of dexfenfluramine ($10\,mg\,kg^{-1}$) or vehicle solution on transcriptional activity of CRF in the paraventricular nucleus of the rat hypothalamus. These photos depict dark- and brightfield photomicrographs of dipped autoradiographs of hybridized 30-μm sections with the CRF intronic (top and middle panels) or exonic (bottom panels) riboprobes through identical areas of the right medial PVN. Top and middle panels represent CRF primary transcript of rat killed 30 min after the intraperitoneal (i.p.) injection of dexfenfluramine (Fenfluramine, left column) or vehicle solution (right column), whereas bottom panels show examples of CRF mRNA levels in the PVN of animals killed 3 h after treatment. Note the rapid but small induction of CRF heteronuclear (hn)RNA in the PVN 30 min after injection with the indirect 5-HT agonist, whereas the PVN of vehicle-treated rats did not exhibit positive signal for CRF hnRNA during experimentation. On the other hand, control animals exhibited strong basal levels of CRF mRNA (f) and no notable increase in the intensity of CRF signal was observed after the i.p. administration with the indirect 5-HT agonist (e) in the rat hypothalamic PVN. Magnification $\times 25$ (a,b,e,f); $\times 250$ (c,d).

inferior (WV3), DMH, laterodorsal tegmental nucleus (LDT), locus coeruleus (LC), area postrema (AP), several subdivisions of the nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus nerve (X), and the nucleus raphé pallidus (RPA). Systemic dexfenfluramine administration also caused a moderate to low positive signal in numerous other regions, such as the intermediate part of the lateral septal nucleus (LSi), nucleus reuniens (RE), medial nucleus of the amygdala (MEA), ARC/median eminence (ME), ventromedial hypothalamic nucleus (VMH), PB, nucleus incertus (NI), dorsal tegmental nucleus (DTN), pontine central gray (PCG), nucleus raphé magnus (RM), nucleus raphé obscurus (RO), and hypoglossal nucleus (XII).

Time course of c-fos *induction by dexfenfluramine*

In most of the areas and nuclei expressing the gene encoding c-*fos* after treatment with dexfenfluramine, the intensity of the hybridization signal was maximum at 30 min, still strong and positive at 60 min, largely decreased at 3 h, and had totally disappeared 6 h following the i.p. injection. Exceptions were, however, observed in some subdivisions of specific nuclei; an example of this phenomenon is depicted in Figure 2. Schematic drawing of the PVN subdivisions is presented by Figure 2a. The PVN of vehicle-treated rats did not exhibit a positive signal for c-*fos* mRNA (Figure 2b), whereas i.p. dexfenfluramine induced a rapid and robust transcription of that gene in this hypothalamic nucleus. Thirty minutes after dexfenfluramine injection, the parvocellular division of the PVN displayed a very strong signal, although a lower but positive c-*fos* expression was detected in the magnocellular PVN (Figure 2c). At 60 min post-dexenfluramine treatment, c-*fos* was highly expressed in both parvo- and magnocellular subdivisions of the PVN (Figure 2d). Whereas c-*fos* signal in the parvocellular PVN totally vanished 3 and 6 h after dexfenfluramine administration, this IEG remained low but positive in the magnocellular division of this hypothalamic nucleus (Figure 2e and f, respectively). The optical density (OD) of the hybridization signal for c-*fos* transcript in the whole PVN is representative of this phenomenon (Figure 3).

Dexfenfluramine and CRF neuronal activity and gene expression

To determine whether CRF-immunoreactive (CRF-ir) neurones express the gene encoding c-*fos* after dexfenfluramine injection, immunocytochemistry (CRF-ir) was performed before *in situ* hybridization histochemistry (c-*fos* mRNA) on the same brain sections. Figure 4a shows examples of CRF-ir neurones expressing c-*fos* mRNA in the dorsomedial parvocellular PVN 30 min after a single i.p. injection of dexfenfluramine. Although none of the CRF-ir neurones exhibited a positive signal for c-*fos* transcript in the PVN of vehicle-administered rats (results not shown), dexfenfluramine caused the expression of mRNA encoding this IEG in most of the CRF-ir perikarya located in this hypothalamic nucleus (Figure 4a, filled arrowheads). However, some c-*fos*-expressing perikarya not colocalized in CRF-ir neurones were also noticed in the PVN of dexfenfluramine-injected rats, particularly in the magnocellular division. Among all the structures evaluated throughout the rat brain, PVN was the unique nucleus that exhibited clear colocalization between CRF-ir perikarya and cells positive for c-*fos* mRNA.

Dexfenfluramine induced rapid but transient expression of CRF primary transcript specifically in the parvocellular PVN. Figure 5 depicts representative dark- (a, b) and brightfield (c, d) photomicrographs of dipped autoradiographs of hybridized 30-μm sections with a selective probe complementary to the rat CRF intron and provides an indication of the early transcription of CRF through identical areas of the right PVN after injection of dexfenfluramine (Figure 5, left column) or a vehicle solution (Figure 5, right column). Although CRF heteronuclear (hn)RNA was not expressed in the PVN of vehicle-treated rats, a low but positive signal for this primary transcript was detected 30 min after dexfenfluramine treatment. Indeed, several positive neurones containing nuclear agglomerations of silver grains were detected in the parvocellular PVN 30 min following injection with the 5-HT agonist (Figure 5c, filled arrowheads).

Dexfenfluramine did not cause a strong and prolonged activation of CRF transcription because the signal for CRF hnRNA in the hypothalamic PVN rapidly vanished after the systemic treatment. This might explain the lack of significant increase in the levels of CRF mRNA in the PVN of dexfenfluramine-treated rats, a phenomonen illustrated by the bottom panels of Figures 5 and 6. The parvocellular PVN displayed strong basal levels of CRF mRNA (Figure 5f), and dexfenfluramine did not provoke a notable increase in the expression of the mRNA encoding CRF in this hypothalamic subdivision (Figure 5e). Indeed, the average OD for the CRF-hybridized signal in the PVN did not differ significantly throughout the time course of the experiment between dexfenfluramine- and vehicle-treated animals, but a small ten-

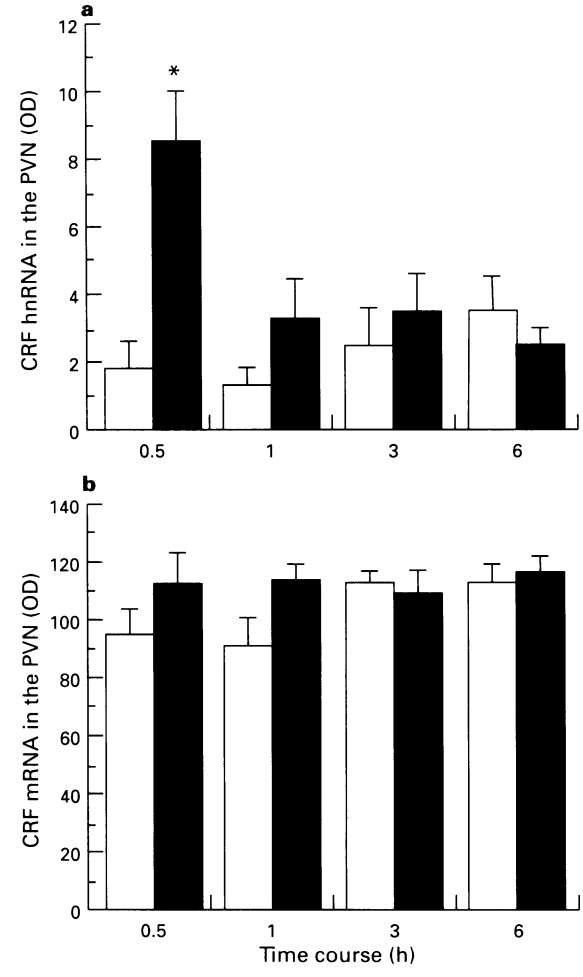

Figure 6 Influence of systemic (i.p.) dexfenfluramine (solid columns) or vehicle (open columns) administration on the average optical density (OD) for CRF heteronuclear (hn)RNA (a) and CRF mRNA (b) signal in the paraventricular nucleus (PVN) of the rat hypothalamus. The OD was quantified in both sides of the medial PVN with an Olympus Optical System (BX-50, β-Max) coupled to a Macintosh computer (PowerPC 7100/66) and Image software (version 1.55 non-FPU, W Rasband, NIH). The scale for OD is relative and in arbitrary units. Results represent means $\pm$ s.e. mean of 3–4 animals. Results were analysed by a 2 $\times$ 4 analysis of variance (ANOVA), followed by a Bonferroni/Dunn test procedure as *post hoc* comparisons (Statview 4.01). $^*P<0.05$ from vehicle-treated rats of the same time post-injection. For more information on image analysis, see Methods.

dency to increase was observed 0.5 and 1 h after a single i.p. administration with the indirect 5-HT agonist (Figure 6b). In contrast, a significant increase in the average OD was obtained at 30 min post-dexfenfluramine treatment for CRF hnRNA in the hypothalamic PVN (Figure 6a). Because of the very strong basal expression of CRF mRNA in the PVN, it is difficult to evaluate small changes in levels of mRNA, whereas intronic probe technology was able to detect induction of primary transcript for this stress-related neuropeptide specifically in the parvocellular PVN following stimulation of 5-HT pathways.

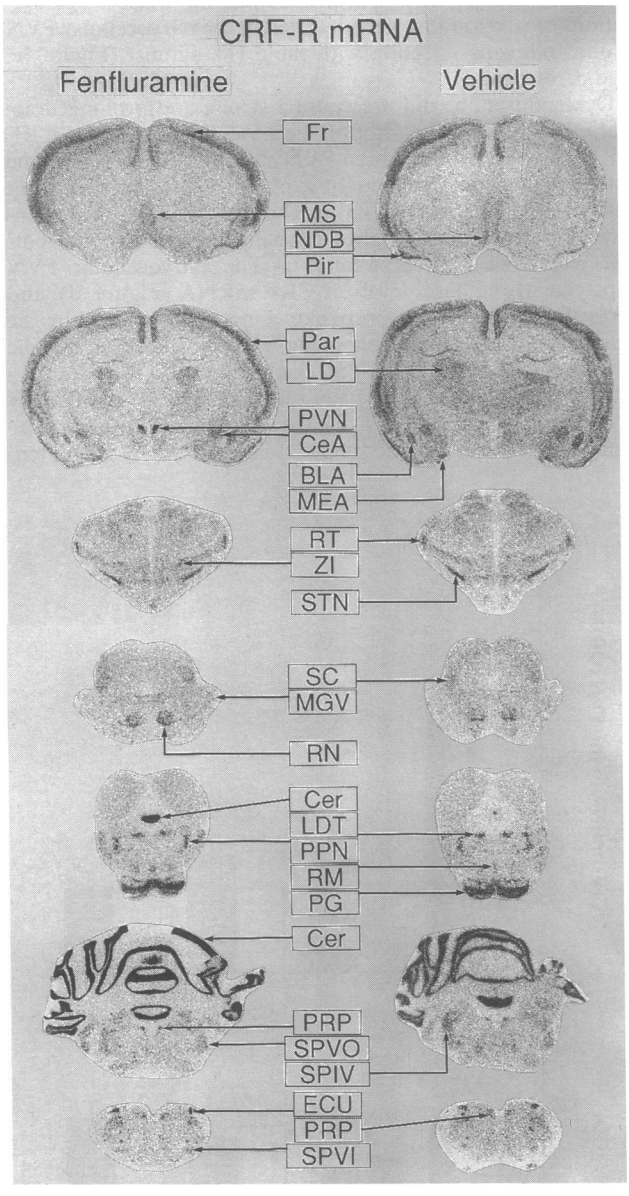

Figure 7 Representative example of the distribution of the mRNA encoding the type 1 receptor for corticotropin-releasing factor (CRF-R) in the rat brain after the intraperitoneal administration of the indirect 5-HT agonist, dexfenfluramine (Fenfluramine) or vehicle. Animals were deeply anaesthetized and rapidly perfused with 4% paraformaldehyde 6 h after treatment with dexfenfluramine (10 mg kg^{-1} of body weight) or the vehicle solution. These rostro-caudal coronal sections (30 μm) of both dexfenfluramine- and vehicle-treated rat brains exhibited positive signal on X-ray film (Kodak XAR-5) for the CRF_1 receptor transcript in various structures. However, note the selective induction of the CRF-R mRNA in the hypothalamic paraventricular nucleus (PVN) of dexfenfluramine-injected rat (left column). BLA, basolateral nucleus of the amygdala; Cer, cerebellum; DMH, dorsomedial nucleus of the hypothalamus; ECU, external cuneate nucleus; Fr, frontal cortex; IPNc, interpeduncular nucleus, central subnucleus; LDT, laterodorsal tegmental nucleus; MEA, medial nucleus of the amygdala; MGV, medial part of the geniculate complex; MS, medial septal nucleus; NI, nucleus incertus; NDB, nucleus of the diagonal band; Par, parietal cortex; LD, lateral dorsal nucleus of the thalamus; Pir, piriform cortex; PG, pontine gray; PPN, pedonculopontine nucleus; PRP, nucleus prepositus; RM, raphé magnus; RN, red nucleus; RT, reticular nucleus of the thalamus; SC, various layers of the superior colliculus; SPIV, spinal vestibular nucleus; SPVI, spinal nucleus of the trigeminal nerve, interpolar part; SPVO, spinal nucleus of the trigeminal nerve, oral part; STN, subthalamic nucleus; ZI, caudal division of the zona incerta. Identification of brain structures are based essentially on Swanson's Atlas (1992).

Selective induction of CRF_1 receptor in the PVN of dexfenfluramine-administered rats

As already reported (Potter *et al.*, 1994; Rivest *et al.*, 1995), the gene encoding the type 1 CRF receptor is widely distributed in the rat brain and this phenomonen is depicted by Figure 7. Hybridized tissues with sense probe did not exhibit detectable signals in any of the regions that showed a positive signal with an antisense probe (results not shown). Basal levels of CRF_1 receptor mRNA can be observed in numerous regions, including various layers of the Fr and Par, Pir, medial septal nucleus (MS), nucleus of the diagonal band (NDB), lateral dorsal nucleus of the thalamus (LD), MEA, basolateral nucleus of the amygdala (BLA), CeA reticular nucleus of the thalamus (RT), zona incerta (ZI), subthalamic nucleus (STN), compact part of the substantia nigra (SNc), various layers of the superior colliculus (SC), red nucleus (RN), Purkinje and granule cell layers of the cerebellum (Cer), LDT, pedonculopontine nucleus (PPN), RM, PG, spinal vestibular nucleus (SPIV), nucleus prepositus (RRP), oral part of the spinal nucleus of the trigeminal (SPVO), interpolar part of the spinal nucleus of the trigeminal (SPVI), and ECU.

Detailed qualitative analysis of the hybridization signal for CRF_1 receptor transcript X-ray film did not detect any clear differences in most of the endogenously expressing structures of the dexfenfluramine- and vehicle-treated rats. In contrast, as shown in Figure 7, dexfenfluramine injected systemically caused a profound induction of the mRNA encoding the CRF type 1 receptor selectively in the PVN. Indeed, although the hypothalamic PVN barely displayed a positive signal for CRF_1 receptor transcript in vehicle-treated rats (Figure 8, right column), a strong expression of that gene was detected 3 and 6 h after dexfenfluramine treatment (Figure 8, left column). The CRF_1 receptor signal in the PVN (OD) was not positive at 30 min and 1 h, but was significantly increased with respect to vehicle-treated animals at the 3 and 6 h time points after the dexfenfluramine injection (Figure 9).

Although a low hybridization signal was detected in the magnocellular PVN, the strong induction of CRF_1 receptor transcription after the i.p. treatment with the 5-HT agonist, dexfenfluramine, was quite selective for the parvocellular division of this hypothalamic nucleus. In this region, CRF-ir neurones expressed the gene encoding the receptor for CRF (Figure 4b, filled arrowhead), although not all the CRF-ir neurones of the parvocellular PVN were positive for CRF_1 receptor transcript (Figure 4b, open arrowheads). Figure 4b) also shows an example of CRF_1 receptor-expressing perikarya not colocalized in CRF-ir neurones in the PVN of the dexfenfluramine-injected rat (curved arrowhead). In vehicle-treated rats, none of the CRF-ir neurones exhibited a positive signal for the gene encoding CRF_1 receptor protein (results not shown).

Discussion

The present study provides evidence that i.p. injection of dexfenfluramine induces strong to moderate expression of c-*fos* transcript in a large number of structures of the brain, such as Fr, Par, CP, LSI, OVLT, MPOA, BnST, Chp, Chf, SFO, SON, PVN, PVT, RE, CeA, WV3, DMH, VMH, ARC/ME, LDT, LC, PB, NI, AP, NTS, X, XII, DTN, RO, RM and RPA (see Figure 1). In most of these stuctures, the hybridization signal was maximal 30 min after treatment with the 5-HT-

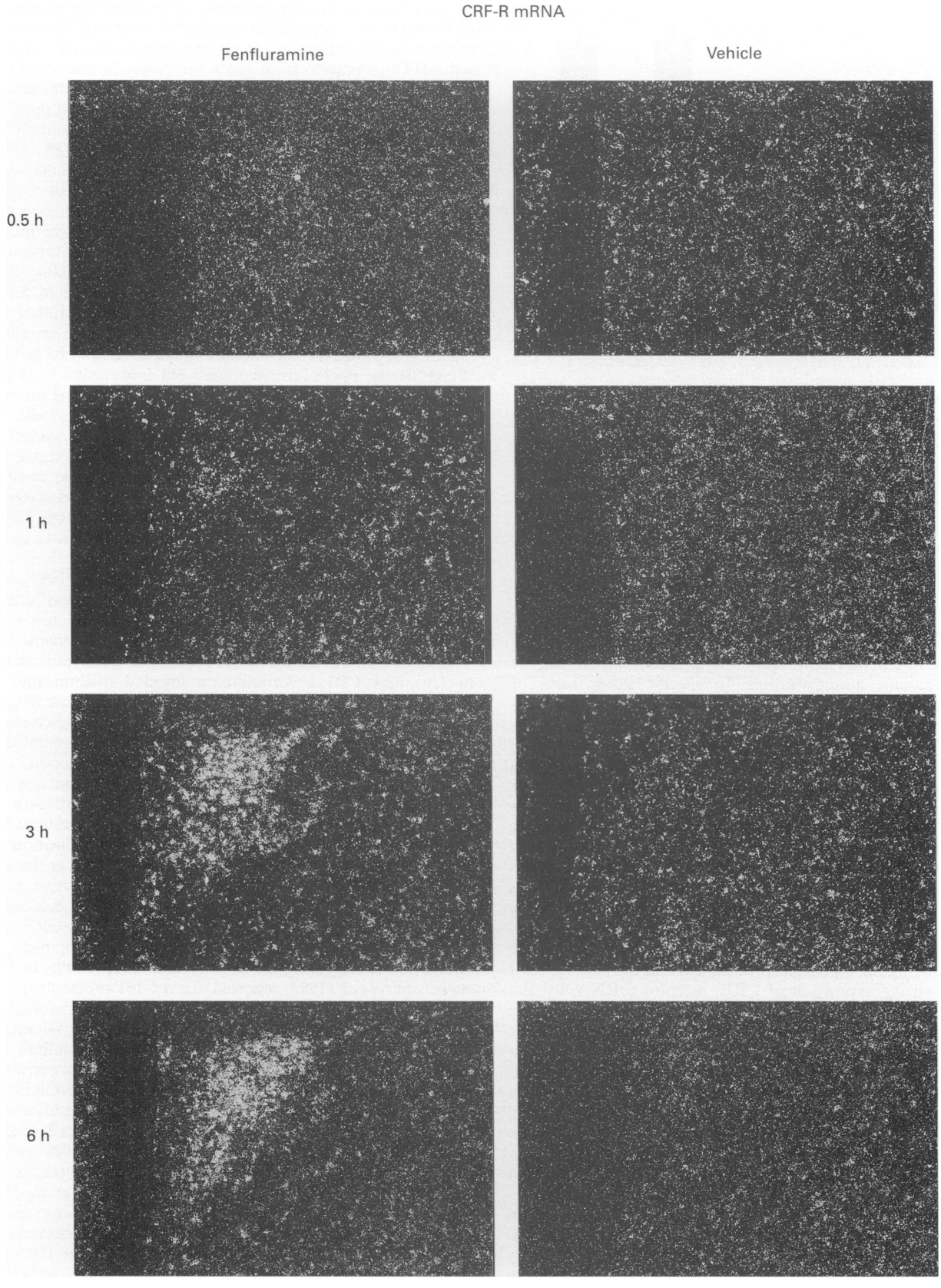

Figure 8 Influence of systemic (i.p.) administration of dexfenfluramine (Fenfluramine) or vehicle on the expression of CRF type 1 receptor (CRF-R) mRNA in the paraventricular nucleus (PVN) of the rat hypothalamus. These photos depict darkfield photomicrographs of dipped autoradiographs of hybridized 30-μm sections with the rat CRF_1 receptor riboprobe through identical areas of the right medial PVN. Animals were deeply anaesthetized and rapidly perfused with 4% paraformaldehyde 0.5, 1, 3, and 6 h after the treatment with the dexfenfluramine (10 mg kg^{-1} of body weight, left column) or the vehicle solution (right column). Note the strong signal in the parvocellular division of the PVN, 3 and 6 h following dexfenfluramine injection whereas the PVN of control rats displayed hardly detectable CRF-R transcript. Magnification $\times 25$.

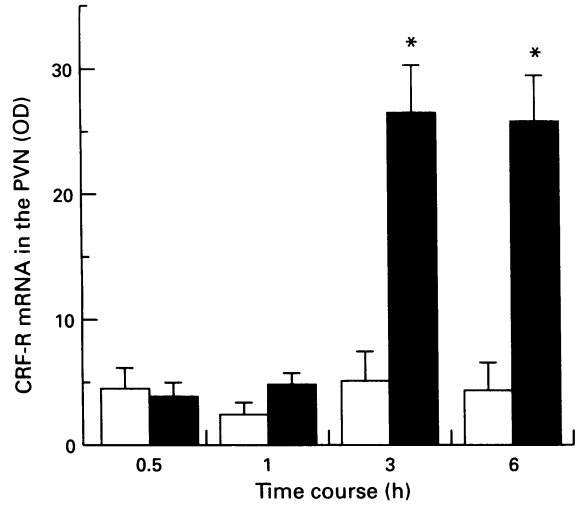

Figure 9 Effect of intraperitoneal (i.p.) dexfenfluramine (solid columns) or vehicle administration (open columns) on the average optical density (OD) for CRF type 1 receptor (CRF-R) mRNA signal in the paraventricular nucleus (PVN) of the rat hypothalamus. The OD was quantified in both sides of the medial PVN with an Olympus Optical System (BX-50, β-Max) coupled to a Macintosh computer (PowerPC 7100/66) and Image software (version 1.55 non-FPU, W. Rasband, NIH). The scale for OD is relative and in arbitrary units. Results were analyzed by a 2 × 4 analysis of variance (ANOVA), followed by a Bonferroni/Dunn test procedure as *post hoc* comparisons (Statview 4.01). For more information on image analysis and statistical analysis, see Methods. $*P<0.05$ from vehicle-treated rats at their respective times post-injection.

stimulating drug, still strong and positive at 60 min, largely decreased at 3 h, and had totally disappeared 6 h following the dexfenfluramine administration. In specific subdivisions of some nuclei, however, the time course of induction was different. In the PVN, c-*fos* was highly expressed in the parvocellular division 30 min after treatment, although the signal was positive but low in the magnocellular division. At 60 min post-dexfenfluramine injection, the signal for c-*fos* mRNA was strong in both subdivisions, totally vanished in the parvocellular PVN 2 h after, whereas at that time, it remained positive in the magnocellular division. In the parvocellular division of this hypothalamic nucleus, most of the CRF-ir perikarya expressed the IEG c-*fos* mRNA, and this colocalization was consistent with the increase in CRF transcription revealed by the presence of CRF hnRNA in the PVN of dexfenfluramine-treated animals. This drug also activated transcription of the gene encoding the type 1 receptor for CRF in the parvocellular PVN; a strong expression of CRF_1 receptor mRNA was detected 3 and 6 h after treatment with dexfenfluramine while the PVN of vehicle-injected rats exhibited a low to undetectable signal for this transcript. Interestingly, CRF-ir perikarya expressed CRF_1 receptor mRNA following the i.p. administration of the indirect 5-HT agonist. Taken together, these results suggest that although dexfenfluramine causes a wide neuronal activation throughout the rat brain, this 5-HT agonist seems to stimulate CRF neurones selectively within the PVN, the hypothalamic structure highly responsible for the control of HPA axis.

Neurones respond to a variety of extracellular stimuli, including potassium- or neurotransmitter-induced depolarization and stimulation by growth factors or hormones, by manifesting rapid and transient synthesis of IEGs, which provide a powerful tool for measuring neuronal activation (Morgan & Curran, 1989; 1991a, b; Sheng & Greenberg, 1990; Armstrong & Montminy, 1993). In this study we used the proto-oncogene encoding the transcription factor c-*fos* as an indicator of neuronal activation because of its sensitivity to be induced in neurones during particular physiological conditions, multiple stresses, and pharmacological stimuli (Richard *et al.*, 1992; Rivest *et al.*, 1992; Chan *et al.*, 1993; Parkes *et al.*, 1993; Ericsson *et al.*, 1994; Rivest & Rivier, 1994; Culliman *et al.*, 1995; Rivest & Laflamme, 1995). The wide distribution of c-*fos* mRNA following i.p. administration of the 5-HT agonist, dexfenfluramine, revealed a profound neuronal activation within numerous structures, a phenomenon in accord with the rich 5-HT innervation throughout the brain. In activating the release and blocking the reuptake of 5-HT, dexfenfluramine was capable of triggering the transcription of c-*fos* in most of the brain nuclei innervated by 5-HT pathways. For example, c-*fos* was induced in the cortex, striatum, BnST, MPOA, SFO, SON, PVN, CeA, and NTS, and these regions are innervated by ascending 5-HT fibres arising from B3, B6, B7, B8, or B9 cell groups of the raphé nucleus (for review, see Halliday *et al.*, 1995). Although dexfenfluramine did not cause transcription of c-*fos* in 5-HT containing perikarya located from B4 through B9 subdivisions, a positive signal was detected in RPA (B1), RO (B2), and RM (B3) regions, the major source of 5-HT innervation to the spinal cord (Bowker *et al.*, 1981; Millhorn *et al.*, 1987; 1989; Araneda *et al.*, 1989). The fact that these caudal groups of 5-HT cell bodies, in particular the B3 groups of cells (Beitz, 1982), receive dense 5-HT innervation from B6, B7, B8, and B9 might explain the capacity of dexfenfluramine to induce the IEG c-*fos* in B1, B2, and B3 groups of cells.

The role of the central 5-hydroxytyptaminergic system in modulating various stress responses has long been investigated and it is of interest to mention that neurogenic stress, such as immobilization (Drolet & Rivest, unpublished) and systemic stress, including immune challenge (Rivest & Laflamme, 1995), induces c-*fos* in several structures that displayed positive signal in the brains of dexfenfluramine-injected animals. Restraint (Culliman *et al.*, 1995), footshock (Rivest & Rivier, 1994), and acute exercise (Rivest & Rivier, 1995) are other stressful conditions able to activate the transcription of IEGs in a large number of nuclei activated under 5-HT agonist treatment, such as the PVN. In the parvocellular division of this hypothalamic nucleus, most CRF-ir perikarya displayed a positive signal for c-*fos* 30 and 60 min after dexfenfluramine treatment (Figure 4a), a phenomenon also observed in footshock (Rivest & Rivier, 1994), immobilization (Nappi and Rivest, unpublished data), and immune (Rivest & Laflamme, 1995) stressed animals as well as after central (Rivest *et al.*, 1992) and systemic (Brady *et al.*, 1994; Ericsson *et al.*, 1994) treatment with cytokine interleukin-1β. Among all the structures evaluated in the brains of dexfenfluramine-injected rats, colocalization between CRF-ir and c-*fos* mRNA was detected only in the parvocellular PVN, providing evidence of a selective CRF neuronal activation in this neuroendocrine nucleus. It is highly possible that dexfenfluramine specifically activates CRF neurones that project to the infundibulum, a mechanism responsible for triggering the activity of the HPA axis. In fact, Sawchenko *et al.* (1983) reported direct 5-HT projections from the rostral and caudal raphé to the parvocellular division of the PVN and synaptic interaction of 5-hydroxytryptaminergic axons and CRF synthesizing perikarya were found in this particular structure (Liposits *et al.*, 1987). Electrical excitation of the rat rostral raphé stimulates neuroendocrine CRF cells (Saphier & Feldman, 1989) whereas chemical stimulation of the caudal raphé raised plasma ACTH levels in cats (Bereiter & Gann, 1990). Moreover, 5-HT and 5-HT agonists are recognized as potent inducers of hypothalamic CRF release and HPA axis activity (Gibbs & Vale, 1983; Calogero *et al.*, 1989; Appel *et al.*, 1991; Pan & Gilbert, 1992). It is consequently tempting to speculate that some parts of the 5-HT circuitry are involved in activating neuroendocrine CRF and the HPA axis under stressful conditions; immobilization-induced ACTH secretion is potentiated by the 5-HT reuptake inhibitor fluoxetine and reversed by antagonists (Bruni *et al.*, 1982). The participation of specific 5-hydroxytyptaminergic pathways in modulating activation of neuroendocrine CRF might, however, depend on the type and duration of the stress because of the lack of 5-HT involvement in mediating activation of the HPA axis in ether (Feldman *et al.*, 1987; 1991), swim (Fuller & Snoddy, 1977), and hypoglycaemic (Fuller & Snoddy, 1977)

stressed animals. In addition, Harbuz *et al.* (1993) recently reported that depletion of hypothalamic 5-HT had no effect on basal levels of ACTH or the increase in response to restraint or hypertonic saline stresses. This group also found that activation of c-*fos* mRNA in the PVN of restrained animals was unaffected by 5-HT depletion (Harbuz *et al.*, 1993).

The colocalization of c-*fos* transcript within a large proportion of CRF-ir cells in the parvocellular division of the PVN suggests that CRF neurones were selectively activated within this neuroendocrine nucleus following dexfenfluramine treatment. The use of CRF intronic probe technology as an index of activation of CRF transcription also confirmed the selectivity of dexfenfluramine in stimulating the stress-related neuropeptide in the parvocellular PVN. Indeed, CRF hnRNA was expressed in this region as soon as 30 min after 5-hydroxytryptaminergic activation, whereas the primary transcript was not induced in other structures. These results also present evidence of the rapid and transient effect of the drug on neuroendocrine CRF transcription, an effect that might explain the lack of significant influence of the indirect 5-HT agonist on CRF mRNA levels in the PVN. Because of the strong basal levels of CRF mRNA in numerous regions of the brain, such as those observed in the PVN, it is difficult to evaluate small changes in the level of mRNA and also to determine whether a higher concentration of mRNA corresponds to activation of the transcription or inhibition of the mRNA degradation. The CRF intronic probe, containing a 530 bp sequence that encodes for a part of the first intron of the genomic CRF, allows detection of the primary transcript (hnRNA) just before splicing and evaluates the activation of CRF transcription. Although the transcription of CRF was clearly activated, as revealed by the presence of CRF hnRNA in the parvocellular PVN, the hybridization signal for the primary transcript was not intense and prolonged after dexfenfluramine injection, which may explain the difficulty in detecting significant differences in the expression of PVN CRF mRNA between dexfenfluramine- and vehicle-treated rats. In contrast, an increase in CRF mRNA levels in the PVN was observed following various types of stressors, including systemic immune challenge (Kakucska *et al.*, 1993; Rivest & Laflamme, 1995). However, this particular stressful situation caused a robust and prolonged induction of CRF primary transcript in the PVN; a strong signal of CRF hnRNA can be detected 1 h after i.p. injection of LPS and remains at this intensity up to 6 h post-treatment (Rivest & Laflamme, 1995). The increased expression of PVN CRF mRNA is thus a reflection of the severe and long-lasting influence of immune stress on neuroendocrine CRF transcription, a phenomenon that appears to be more discrete in dexfenfluramine-treated rats.

Although c-*fos* mRNA was expressed in PVN CRF-ir perikarya, the role of the Fos/Jun complex in regulating neuroendocrine CRF biosynthesis remains unclear; c-*fos* is probably not involved in directly triggering the transcription of the stress-related neuropeptide. Indeed, the CRF promoter does not contain any AP-1 consensus site (Seasholtz *et al.*, 1988; Thompson *et al.*, 1990) and the time course of c-*fos* expression versus the induction of CRF hnRNA in the parvocellular PVN after the i.p. injection of dexfenfluramine does not support the evidence that the formation of AP-1 precedes the transcription of CRF. Thirty minutes after stimulation of 5-HT pathways, a positive signal for c-*fos* mRNA and CRF primary transcript was detected in the PVN, although at 1 h post-injection, the hybridization signal for CRF hnRNA declined while strong expression of the gene encoding c-*fos* was still maintained. Because of the rapid presence of CRF hnRNA in the PVN of dexfenfluramine-administered rats, it seems unlikely that the AP-1 complex can be formed before transcriptional activation of CRF. The consensus sequence for nerve growth factor-inducible gene-B (Watson & Milbrandt, 1989), POU-domain transcription factor Brain-II (He *et al.*, 1989), and cyclic AMP response element binding (CREB) protein (Seasholtz *et al.*, 1988; Spengler *et al.*, 1992) are present on the CRF promoter. Although not determined in the present experiment, these transcription factors could be other potential candidates to activate directly CRF transcription after dexfenfluramine treatment.

As recently reported (Potter *et al.*, 1994; Nappi & Rivest, 1995; Rivest *et al.*, 1995), the mRNA encoding the CRF type 1 receptor is widely distributed throughout the rat brain under basal conditions, whereas the brain distribution of the recently cloned second CRF receptor subtype is quite different (Lovenberg *et al.*, 1995; Perrin *et al.*, 1995); few structures of the limbic system display a positive hybridization signal for the gene encoding this new CRF receptor, which was not assessed in the present study. After a detailed analysis of CRF_1 receptor hybridization signal, it was not possible to detect any significant effect of dexfenfluramine on these endogenously expressing structures. It is nevertheless important to keep in mind that small changes might be very difficult to perceive. On the other hand, the positive influence of the indirect 5-HT agonist in the PVN was quite impressive; although the signal for the mRNA encoding CRF_1 receptor was barely detectable in vehicle-treated rats, dexfenfluramine caused a profound activation of CRF_1 receptor transcription specifically in this hypothalamic structure. In contrast to immune (Rivest *et al.*, 1995) and salt-loading (Luo *et al.*, 1994) stresses, which increase CRF_1 receptor transcript in both parvocellular and magnocellular divisions of the PVN as well as in the SON, a more selective expression of that gene was observed in the parvocellular PVN after dexfenfluramine administration. This selectivity of parvocellular induction of the mRNA encoding the type 1 receptor for CRF was also found in the PVN of immobilized male (Luo *et al.*, 1994; Rivest *et al.*, 1995) and female (Nappi & Rivest, 1995) rats. Whether 5-HT pathways participate in mediating the transcription of CRF_1 receptor in the parvocellular PVN during specific stressors remains an open question.

The exact role of the type 1 CRF receptor in the hypothalamic PVN is unknown, but the fact that in the parvocellular division this gene is expressed in CRF-ir perikarya indicates that CRF *per se* may play a role in controlling the activity of neuroendocrine CRF neurones under 5-HT stimulation. Indeed, CRF may be a potential modulator of its own biosynthesis selectively in the PVN; intracerebroventricular (i.c.v.) administration of CRF to conscious rats induces IEGs c-*fos* and NGFI-B in the CRF-ir neurones (Parkes *et al.*, 1993) and activates the expression of CRF hnRNA (Mansi, Drolet & Rivest, unpublished data) and CRF mRNA (Parkes *et al.*, 1993) in the parvocellular neurones of the PVN. The existence of CRF neuronal fibre projections that terminate and connect with parvocellular CRF perikarya in the PVN was reported (Silvermann *et al.*, 1989), and the PVN itself could be the origin of these fibres (Swanson *et al.*, 1987), although other hypothalamic and extrahypothalamic regions also contribute to CRFergic innervation of the rat PVN (Beaulieu & Drolet, 1994; Moga & Saper, 1994). The selectivity in the induction of CRF_1 receptor mRNA in PVN CRF neurones might indicate that neuroendocrine CRF is the only type of CNS CRF that can be regulated by its own receptor in dexfenfluramine-treated rats. The temporal changes in the expression of both CRF_1 receptor mRNA and CRF hnRNA after 5-HT stimulation do not provide eivdence that induction of the receptor is a mechanism involved in activating CRF transcription, because CRF primary transcript is present as soon as 30 min in the parvocellular PVN, whereas a positive signal for CRF_1 receptor mRNA was detected quite late, that is, 3 and 6 h after dexfenfluramine treatment. It is possible, however, that CRF participates in post-transcriptional events to restore the depletion of neuroendocrine CRF secreted into the infundibulum following treatment with the 5-HT agonist, a hypothesis that merits investigation.

In conclusion, systemic administration of the indirect 5-HT agonist dexfenfluramine induces extensive neuronal activation, revealed by the distribution of c-*fos* mRNA throughout the brain. Despite this fact, colocalization between c-*fos* mRNA and CRF-ir perikarya was observed only in the hypothalamic

PVN, suggesting a selective activation of neuroendocrine CRF. Moreover, dexfenfluramine caused a rapid induction of the CRF primary transcript and late transcription of the type 1 receptor for this stress-related neuropeptide within the parvocellular subdivision of the PVN but not in other structures. Taken together, these results provide evidence that parvocellular CRF neurones of the PVN are the only CRF cells activated in the brains of dexfenfluramine-injected male rats, a mechanism probably related to the activation of the HPA axis. Moreover, the PVN itself appears to be the main target of CNS CRF after 5-HT stimulation because of the profound and selective transcription of the gene encoding the type 1 receptor for the stress-related neuropeptide in this hypothalamic nucleus. Although CRF_1 receptor transcript was detected in CRF-ir perikarya of the PVN after dexfenfluramine administration, whether these cells are neuroendocrine CRF neurones remains to be established by tract-tracing studies.

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