

Interaction Between the Serotonergic and Dopaminergic Systems in *d*-Fenfluramine-Induced Activation of *c-fos* and *jun B* Genes in Rat Striatal Neurons

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Abstract: To test for the relative contributions of the dopaminergic and serotonergic systems in the striatum to the effects of *d*-fenfluramine, an indirect serotonin receptor agonist, we assessed the expression of Fos/Jun proteins induced by *d*-fenfluramine given alone or in the presence of dopaminergic or serotonergic agents. To determine the neuronal targets of *d*-fenfluramine in the striatum, we identified the phenotypes of striatal neurons in which *d*-fenfluramine induced Fos expression. Our results demonstrated that *d*-fenfluramine evokes nuclear expression of Fos/Jun B proteins in the striatum, and that the Fos expression was dose-dependent and accompanied by transient induction of *c-fos* mRNA. Fos expression was blocked by *p*-chloroamphetamine, a serotonergic neurotoxin. Pretreatment with SCH 23390, a D₁-dopamine receptor antagonist, led to a marked decrease in Fos/Jun B expression in the caudoputamen, but not in the cortex, whereas pretreatment with methiothepin, a nonselective serotonin 5-HT₁ receptor antagonist, blocked Fos expression completely in the cortex and only partially in the caudoputamen. The expression of Fos/Jun B in the striatum occurred mainly in dynorphin-containing neurons and in a subpopulation of striatal interneurons that exhibited NADPH-diaphorase activity. Most of the enkephalin-containing neurons of the striatum did not show Fos/Jun B staining. These results suggest that the mechanism by which *d*-fenfluramine induces *c-fos* and *jun B* expression in the rat caudoputamen depends at least in part on activation of the dopaminergic system by serotonin. **Key Words:** Dexfenfluramine—Striatum—*c*-Fos—Jun B—Dynorphin—*p*-Chloroamphetamine. *J. Neurochem.* **74**, 1363–1373 (2000).

The innovative method for mapping neuronal activity by monitoring the nuclear expression of immediate-early genes (IEGs) has proven to be particularly successful in identifying selective neuroanatomical loci targeted by drugs (Hughes and Dragunow, 1995). We have used this method to approach the question of what neurotransmit-

ter receptors are responsible for the activation of IEGs in the striatum following an acute exposure to *d*-fenfluramine. *d*-Fenfluramine acts both by releasing serotonin [5-hydroxytryptamine (5-HT)] from nerve terminals and by blocking 5-HT reuptake (Samanin and Garattini, 1990; Appel et al., 1991). Evidence suggests that most of the behavioral effects of this drug, including stimulation of the hypothalamo–pituitary–adrenal axis, decrease in food intake by enhancing satiety, and increase in thermogenesis, are mediated through the activation of brain serotonergic systems. *d*-Fenfluramine has been shown in vivo microdialysis studies to increase extracellular 5-HT levels in the caudoputamen and nucleus accumbens, as well as in the frontal cortex and hypothalamus (Laferrere and Wurtman, 1989; Balcioglu and Wurtman, 1998). All of these effects of *d*-fenfluramine have also been observed with *d,l*-fenfluramine, the racemic mixture of the *d* and *l* isomers of fenfluramine (Auerbach et al., 1989; Sabol et al., 1992; Kirby et al., 1995).

Immunohistochemical studies have shown that Fos-like proteins are expressed rapidly and transiently in specific regions of the rat brain following a single administration of *d*-fenfluramine to rodents (Li and Rowland, 1993; Li et al., 1994). Consistent with the serotonergic-related behavioral effects of *d*-fenfluramine, the brain regions showing the most intense Fos immunostaining include regions receiving a dense serotonin-

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Abbreviations used: 5-HIAA, 5-hydroxyindole-3-acetic acid; 5-HT, 5-hydroxytryptamine or serotonin; IEG, immediate-early gene; PBS, phosphate-buffered saline; PCA, *p*-chloroamphetamine.

ergic innervation from raphe nuclei, such as several nuclei of the hypothalamus and the lateral parabrachial nucleus. The striatum, however, may differ from other brain regions, because the close parallels between the pharmacological effects of *d*-fenfluramine and *d,l*-fenfluramine have not been found for the striatal induction of Fos-like proteins. A single systemic administration of the racemic mixture *d,l*-fenfluramine at a dose lower than 20–25 mg/kg fails to induce the nuclear expression of Fos-like proteins in the rat striatum (Richard et al., 1992; Torres and Rivier, 1993, 1994; Torres, 1995; Rouillard et al., 1996; Cook and Wirtshafter, 1998). By contrast, the *d* isomer, *d*-fenfluramine, appears more efficient in activating striatal neurons to express Fos-like proteins, because it induces Fos in the striatum even at very low doses (2–3 mg/kg) in rats (Li and Rowland, 1993). This paradoxical result is surprising given that the *d* isomer, *d*-fenfluramine, is thought to act mainly on serotonergic neurons, whereas the racemic mixture *d,l*-fenfluramine contains 50% of the *l* isomer, which can directly release dopamine (Jori and Bernardi, 1972; Garattini et al., 1975; Crunelli et al., 1980; Invernizzi et al., 1986; Bettini et al., 1987). Given that the striatum receives a massive dopaminergic innervation from the substantia nigra pars compacta along with its dense serotonergic input from the dorsal raphe nucleus (Steinbusch, 1984), a robust induction of Fos would have been predicted following the direct release of dopamine by *d,l*-fenfluramine.

Here we examined the possibility that *d*-fenfluramine triggers transcription factor gene expression in the striatum as an indirect consequence of interactions between striatal 5-HT and dopamine circuitries. Such an action of *d*-fenfluramine could reflect the major known pharmacological actions of *d*-fenfluramine, i.e., the enhanced release and decreased reuptake of endogenous 5-HT. The 5-HT in turn could stimulate the release of dopamine in the striatum, leading to the expression of Fos-like proteins in striatal neurons. Serotonergic neurons of the midbrain raphe nuclei have been shown to modulate dopaminergic nigrostriatal pathways (Soubrié et al., 1984; Yadid et al., 1994), and an in vivo microdialysis study has demonstrated an indirect dopamine-releasing capacity of *d*-fenfluramine, secondary to its ability to release 5-HT in the striatum (Balcioglu and Wurtman, 1998).

To test for the relative contributions of the dopaminergic and serotonergic systems in the striatum to the effects of *d*-fenfluramine, we assayed *d*-fenfluramine-induced expression of Fos/Jun proteins in the striatum across a range of doses of the drugs (5, 10, 20, or 40 mg/kg). We then tested for this expression of Fos/Jun proteins when *d*-fenfluramine was given in the presence of agents that interfere with dopaminergic or serotonergic neurotransmission. Finally, we studied the neuronal targets of *d*-fenfluramine in the striatum by identifying the phenotypes of striatal neurons in which *d*-fenfluramine induced Fos expression.

TABLE 1. Drug treatments for immunocytochemistry

Treatment	Dose (mg/kg i.p.)	No. of animals per treatment
<i>d</i> -Fenfluramine (D)		
only	10	5
D	20	12
D	40	15
SCH 23390 + D	0.3 + 20	4
SCH 23390 + D	0.3 (or 0.2) + 40	6 (2) ^a
Methiothepin + D	10 + 20	3
Methiothepin + D	10 + 40	6
SCH 23390 + methiothepin + D	0.3 + 10 + 40	2
PCA + D	(7.5 × 2 days + 10 × 3 days) + 40	7

Doses (in mg/kg of body weight, i.p.) and schedule of treatments are given. The standard series were *d*-fenfluramine with 2-h survival time (D). For combined-drug treatments, times before final *d*-fenfluramine were 30 min each for the selective D₁ receptor antagonist, SCH 23390, and for the nonselective 5-HT₁ receptor antagonist, methiothepin, and once a day for 5 days (7.5 mg/kg daily for 2 days and then 10 mg/kg daily for 3 days) for the neurotoxin PCA. In control experiments, SCH 23390 and methiothepin were also given individually at the doses indicated for the combined-drug experiments.

^a Number in parentheses indicates animals given alternative dose shown in parentheses in the middle column.

EXPERIMENTAL PROCEDURES

Animals and experimental design

Adult male Sprague–Dawley rats (180–200 g) were used in this study. Animals were housed with a 12-h light/dark cycle with free access to food and water. Drugs were dissolved in 0.9% NaCl except for methiothepin, which was dissolved in 4% polyethylene glycol in 0.9% NaCl and were given intraperitoneally in a volume of 2 ml/kg. On the day of the experiment, the rats were brought into a quiet experimental room and the drugs were administered at the following doses (Table 1): *d*-fenfluramine hydrochloride (Servier Laboratories, Neuilly-sur-Seine, France), 5, 10, 20, and 40 mg/kg; cocaine hydrochloride (Sigma), 25 mg/kg as previously described (Moratalla et al., 1993); *R*(+)-SCH 23390 hydrochloride (Research Biochemicals International, Natick, MA, U.S.A.), 0.2 or 0.3 mg/kg, given 30 min before *d*-fenfluramine (20 or 40 mg/kg); methiothepin maleate (Biomol Research Laboratories, Plymouth Meeting, PA, U.S.A.), 10 mg/kg, given 30 min before *d*-fenfluramine (20 or 40 mg/kg). Doses of drugs are expressed as their respective salts.

Immunohistochemistry

Two hours following drug treatment, rats were deeply anesthetized with pentobarbital (1 ml/kg; Sanofi Santé Nutrition Animale, Libourne, France) and were then perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer containing 0.9% NaCl (PBS), pH 7.4. Brains were removed and cut coronally at 30 μm on a sliding microtome. Single antigen immunostaining was performed on free-floating sections (Graybiel et al., 1990), with a standard avidin-biotin peroxidase complex (ABC protocol, Vectastain, Vector Laboratories, Burlingame, CA, U.S.A.). The sections were pretreated with 3% H₂O₂ and 0.3% Triton X-100 in PBS for 10 min and with 5% normal goat serum for 30 min. Incubations in primary antisera were carried out overnight at 4°C. The sections were then incubated successively in biotin-

ylated goat anti-rabbit IgG diluted 1:500 (Vector) for 1 h and in avidin-biotin peroxidase complex (Vector) for 1 h, and then were treated with 0.0001% H₂O₂ and 0.08% nickel ammonium sulfate (Farmitalia Carlo Erba Analyticals) and 0.02% 3,3'-diaminobenzidine (Sigma) as chromogen. Each step was preceded by washes in 0.01 M PBS. Sections were mounted on gelatin-coated slides, air-dried, dehydrated, coverslipped with Permount, and analyzed by light microscopy.

Some sections were also processed by a double-labeling technique to study the distribution of Fos-like immunoreactivity in NADPH-diaphorase interneurons. In the rat striatum, NADPH-diaphorase activity had been shown largely to coincide with the type I isoform of the enzyme nitric oxide synthase, which is the isoform constitutively expressed in neurons (i.e., neuronal nitric oxide synthase) (Dawson et al., 1991). Thus, for nitric oxide synthase-containing interneurons, sections were first immunostained by the ABC protocol and then were reacted for NADPH-diaphorase activity (Moratalla et al., 1996a).

Dual antigen immunohistochemistry was carried out to study the distribution of Fos/*Jun B* in dynorphin- or enkephalin-positive projection neurons in the striatum. Sections were first stained for Fos or *Jun B* as indicated above, but using 3,3'-diaminobenzidine alone. Then a second staining with the second antibody (anti-dynorphin or anti-enkephalin) was carried out with 3,3'-diaminobenzidine and nickel ammonium sulfate as chromophore (Moratalla et al., 1996a).

Antisera

To detect Fos-*Jun* family proteins, we used polyclonal rabbit antisera against *c-Fos* (1:200; Oncogene Science PC05, Cambridge, MA, U.S.A.) or *Jun B* (1:4,000; provided by Dr. R. Bravo, Bristol-Myers, Princeton, NJ, U.S.A.). To identify striatal projection neurons, we used antisera against dynorphin B 1-29 (1:10,000; provided by Dr. S. Watson, University of Michigan, Ann Arbor, MI, U.S.A.) or Met-enkephalin (1:2,000; Incstar, Stillwater, MN, U.S.A.).

Counts of Fos- and Jun B-immunopositive nuclei

The numbers of immunopositive nuclei (nuclei per mm²) were estimated for striatal sections from control and *d*-fenfluramine-treated rats, using a standard transverse level, ~10 mm rostral to the interaural line. The analysis was carried out single-blind with a 10× objective using an image analysis system (Biocom, Les Ulis, France). A horizontal strip through the middle of the caudoputamen was chosen as the sample area for all sections (Moratalla et al., 1996a). Before counting, threshold levels were set empirically by independent observers to allow detection of nuclei stained with moderate to high intensity, with suppression of lightly stained nuclei.

Semiquantitative RT-PCR analysis of *c-fos* mRNA expression

The expression of mRNA for *c-fos* was assessed by reverse transcription and amplified cDNA by semiquantitative PCR performed according to Kerdine et al. (1996). Striatal homogenates from three rats were pooled and total cellular RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987). Levels of *c-fos* transcripts were normalized to those of a rat β -actin probe. cDNA was synthesized in 20- μ l reactions containing 1 μ g of cellular RNA and 25 μ M oligo(dT)₁₅ primer (Bioprobe Systems, Montreuil, France), 50 mM dNTP, 40 mM sodium pyrophosphate, 62.5 mM Tris-HCl, pH 8.3, 8 U of spermidine (Promega), 25 U of RNase inhibitor (Promega), and 5 U of

AMV-reverse transcriptase (Promega). Following a 90-min incubation at 42°C, fractions of the reverse transcription products were obtained following reverse transcription of 2 μ g of the total cellular mRNA. The DNA amplification was carried out in 1× PCR buffer supplemented with 800 μ M dNTP, 0.50 μ M each of 5'- and 3'-specific primers (custom-synthesized; Bioprobe), and 2 U of *Taq* polymerase (HighTaq, Bioprobe) in a final volume of 100 μ l. The mixture was overlaid with mineral oil and amplified in 30 cycles in a thermal cycler (Hybaid Thermal Reactor TR 1L). The rat primer sequences from Bioprobe were as follows: β -actin sense, 5'-TTCCTGGGTAAGTTGTAGTC-3'; β -actin antisense, 5'-AGCACTGTGTTGGCATAGAG-3'; *c-fos* sense, 5'-GGAGCTGACAGATACGCTCCA-3'; *c-fos* antisense, 5'-AATGTTCTTGACGGCTCCA-3'. The lengths of cellular amplicon for β -actin and *c-fos* were 190 and 298 bp, respectively. The cycling conditions were 2 min at 95°C for the denaturation, 2 min at 57°C for annealing, and 2 min at 72°C for the elongation. The PCR amplification was terminated by 10 min at 72°C for the final elongation. Then a 1:20 dilution of the PCR products was used for separation of cellular amplicons by 1% agarose gel electrophoresis in the presence of ethidium bromide. The intensity of specific bands on Polaroid negatives was quantified with a densitometry system (Computing Densitometer, Molecular Dynamics, Sunnyvale, CA, U.S.A.) and the corresponding software (Image Quant software). To correct for any variation in RNA content and cDNA synthesis in the different striatal homogenates, and to perform a relative quantification of specific mRNA levels, we used the relatively invariant β -actin mRNA as control. PCR amplifications of both cellular templates were run in parallel (Bouaboula et al., 1992). Each level of *c-fos* mRNA was expressed as a percentage of β -actin mRNA expression calculated for each rat.

In the first RT-PCR experiment, rats were injected with a single dose of *d*-fenfluramine (10 mg/kg, i.p.) or saline (2 ml/kg, i.p.). Then total RNA was extracted from the caudoputamen 0.5, 1, 2, and 6 h after *d*-fenfluramine injection, and the extracted mRNA was converted to cDNA and amplified by RT-PCR. In the second RT-PCR experiment, rats received a range of *d*-fenfluramine doses (5, 10, 20, and 40 mg/kg, i.p.) or saline (2 ml/kg, i.p.), and RT-PCR analysis was performed on RNA isolated from the caudoputamen 30 min after the single drug injection. The *c-fos* and β -actin mRNA expression was then quantified with a densitometry system.

Measurement of striatal 5-HT, 5-hydroxyindole-3-acetic acid (5-HIAA), and dopamine levels in *p*-chloroamphetamine (PCA)-pretreated rats

To produce lesions of serotonergic neurons, rats were injected intraperitoneally with PCA (7.5 mg/kg for 2 days and then 10 mg/kg for 3 days) and then were allowed to recover for 1 week (D'Amato et al., 1987; Bhat and Baraban, 1993) before the administration of either saline (2 ml/kg) or *d*-fenfluramine (40 mg/kg). Rats were perfused 2 h after the last drug administration for immunocytochemical examination of brain tissue or quickly decapitated for subsequent RNA or analytical studies. Dopamine uptake sites were also measured as described by Graybiel and Moratalla (1989) after the PCA treatment. Treatments for the seven groups of rats (four to six animals per group) are summarized in Table 1.

Monoamine levels in the striatum of PCA-treated rats were measured and compared with controls following methods previously described (Gardier et al., 1994). After decapitation, brains were quickly removed, and the caudoputamen was removed (Glowinski and Iversen, 1966) and kept at -70°C for

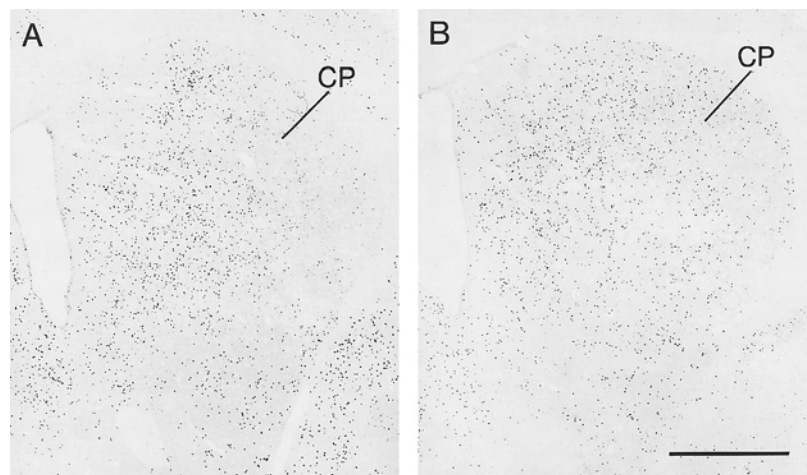


FIG. 1. Comparison of the effects of *d*-fenfluramine and cocaine on the induction of Fos-like protein expression in the rat striatum. Distributions of c-Fos-immunopositive nuclei induced in the rat caudoputamen (CP) by a single dose of either (A) cocaine (25 mg/kg, i.p.) or (B) *d*-fenfluramine (20 mg/kg, i.p.) are shown. Animals were perfused 2 h after the administration of the drug. Note the similarities in distribution of Fos-positive nuclei induced in the rat caudoputamen by *d*-fenfluramine and by cocaine. Photographs were printed from high-contrast negatives. Scale bar = 1 mm.

subsequent assays of 5-HT, its major metabolite 5-HIAA, and dopamine. On the day of analysis, tissue samples were thawed, weighed, and sonicated in a perchloric acid medium (0.2 M HClO₄) containing 0.1% EDTA, 0.1% Na₂S₂O₅, and 0.1% L-cysteine as antioxidant. The tissue homogenates (standardized to 100 mg of wet tissue/ml) were then centrifuged at 17,000 *g* for 15 min. 5-HT and 5-HIAA were measured using a liquid chromatography system coupled to an electrochemical detector.

Statistics

Statistical analyses were performed with StatView 4.02 (Abacus Concepts, Inc, Berkeley, CA, U.S.A.). Statistical analyses of group differences were carried out for the quantification of immunopositive nuclei as well as for concentrations of biogenic amines in rat brain homogenates expressed in nanograms per gram of wet weight tissue. Values are means \pm SEM for five to eight determinations. A two-way ANOVA was used to establish a significant interaction between pretreatment (PCA, SCH 23390, or methiothepin) and treatment (saline or *d*-fenfluramine). These analyses were performed by using one- or two-way ANOVA followed by Scheffé's *F* tests for comparison of pairs of treatments when appropriate. Statistical significance was considered when $p < 0.05$.

RESULTS

Expression of Fos-like and Jun B-like proteins in the striatum following *d*-fenfluramine treatment: comparison with cocaine

Fos-like and Jun B-like proteins were distributed in a characteristic pattern in the caudoputamen following *d*-fenfluramine treatment. As shown for Fos expression in Fig. 1B, the most intense expression was in the central and centromedial parts of the caudoputamen. Only low levels of induction occurred laterally. This general distribution pattern closely resembled that evoked by acute exposure to cocaine (Fig. 1A). At more caudal levels, Fos-positive and Jun B-positive nuclei in the caudoputamen appeared mainly in localized dorsal and ventral parts of this area, again paralleling distributions for cocaine induction (data not shown; Graybiel et al., 1990; Moratalla et al., 1993).

Counts of immunopositive nuclei in rats prepared for the dose-response study showed that the induction of Fos in the striatum was dose-dependent, whereas that of Jun B was not (Fig. 2). Fos expression increased steadily for *d*-fenfluramine doses from 5 to 40 mg/kg, whereas Jun B expression reached a maximum at the lowest dose tested (10 mg/kg). Higher doses of *d*-fenfluramine did not increase Jun B expression further.

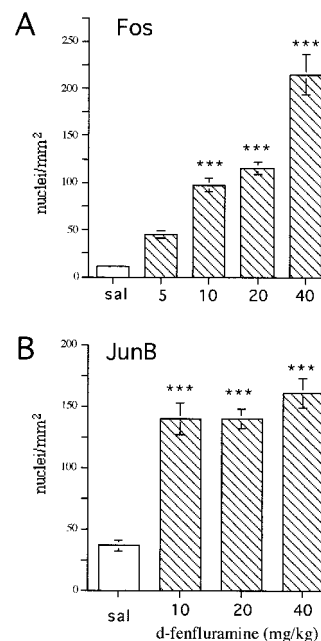
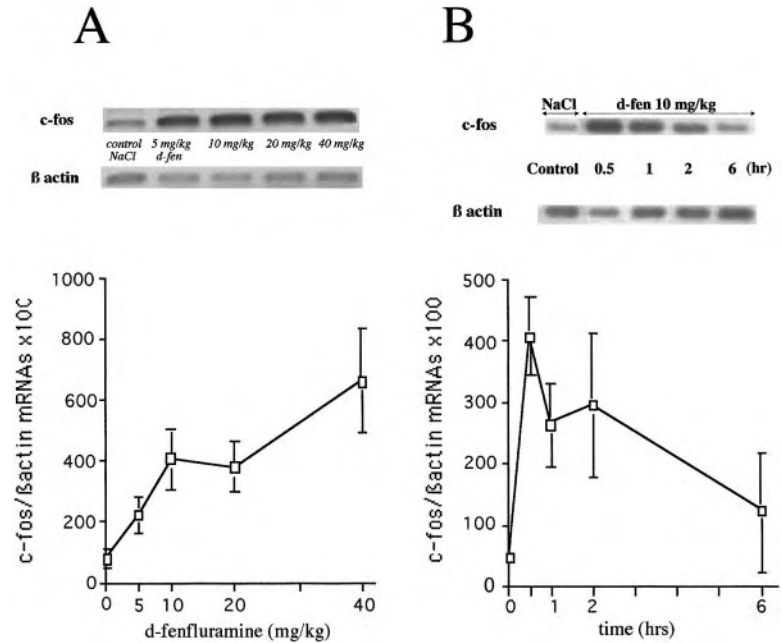


FIG. 2. Numbers of nuclei immunopositive for (A) c-Fos and (B) Jun B counted in sections through the dorsomedial part of the caudoputamen from rats receiving a single intraperitoneal injection of either saline (sal; 2 ml/kg) or *d*-fenfluramine at various doses (5, 10, 20, or 40 mg/kg of body weight) and perfused 2 h later. Data are from single sections with counts in both hemispheres and are expressed as the number of cells per mm² (means \pm SEM for $n = 4-5$ determinations per group). *** $p < 0.001$, significantly different from controls.

FIG. 3. Semiquantitative RT-PCR analysis of *c-fos* mRNA expression in the striatum of *d-fenfluramine*-treated rats. **A:** Dose-response relationship of systemic *d-fenfluramine* administration on striatal *c-fos* mRNA expression. Rats received a range of *d-fenfluramine* doses (*d-fen*; 0, 5, 10, 20, and 40 mg/kg, i.p.), and RT-PCR analysis was performed on RNA isolated from the striatum 1 h after the single-drug injection. Results are expressed as percentage of β -actin mRNA expression. Data show results in a typical experiment representative of three. See the text for details. **B:** Time-course study of *c-fos* mRNA induction in the striatum by *d-fenfluramine* in the rat striatum. Rats were injected with a single dose of *d-fenfluramine* (10 mg/kg, i.p.) or saline (2 ml/kg). Total striatal mRNA was extracted from the striatum 0.5, 1, 2, and 6 h after the drug injection, and then converted to cDNA by reverse transcription and amplified cDNA by polymerase chain reaction (RT-PCR; 1/20 dilution) for 30 cycles. Results are expressed as percentage of β -actin mRNA expression. Data detail a typical experiment representative of three. See the text for details.



Semiquantitative RT-PCR analysis of *c-fos* mRNA expression in the striatum of *d-fenfluramine*-treated rats

To analyze the induction of *c-fos*, rats were injected with a single dose of *d-fenfluramine* (5, 10, 20, or 40 mg/kg, i.p.) or with saline (2 ml/kg), and total RNA was extracted from the striatum 1 h after treatment. The results of RT-PCR (Fig. 3A) demonstrated that *c-fos* mRNA expression increased in a dose-dependent manner in the range of *d-fenfluramine* doses studied. To determine the time course of this induction, a second set of rats was injected intraperitoneally with a single dose (2 ml/kg) of 10 mg/kg *d-fenfluramine* or saline, and total RNA was extracted from the caudoputamen 0.5, 1, 2, and 6 h after the injections. RT-PCR analysis (Fig. 3B) showed that the *d-fenfluramine*-induced *c-fos* mRNA expression was transiently increased, reaching its maximum at 0.5 h after *d-fenfluramine* injection and then returning to control levels by 6 h.

Effects of PCA on Fos-like proteins in the striatum of *d-fenfluramine*-treated rats (40 mg/kg for 2 h)

To assess the role of the 5-HT system in mediating the effects of *d-fenfluramine* on the expression of Fos-like proteins in the striatum, we administered PCA to induce degeneration of 5-HT-containing fibers (Fig. 4). To ensure that the PCA treatment targeted principally 5-HT-containing nerve terminals relative to dopamine-containing nerve terminals, we assessed the effects of PCA treatment on 5-HT and dopamine metabolism by measuring striatal 5-HT, 5-HIAA, and dopamine content in a set of rats treated with a single 40 mg/kg dose of *d-fenfluramine* or with saline 1 week after PCA treatment (Table 2). PCA pretreatment decreased 5-HT and 5-HIAA levels in the striatum to 18 and 10% of the

respective control values measured in saline-treated rats. Similar results were obtained in rats treated with the combination PCA + *d-fenfluramine*. This combined treatment decreased 5-HT and 5-HIAA levels in the striatum to 12 and 21% of the respective control values measured in saline-treated rats, while decreasing dopamine levels to 72%. These results demonstrate that our

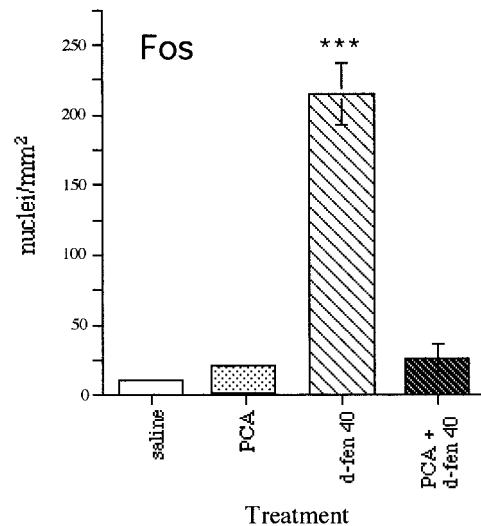


FIG. 4. Numbers of nuclei expressing Fos-like protein in the dorsomedial part of the caudoputamen counted bilaterally in single sections, from rats pretreated for 5 days with PCA (7.5 mg/kg/day for 3 days and 10 mg/kg/day the next 2 days), given a 7-day washout period, and then given either saline (2 ml/kg) or *d-fenfluramine* (*d-fen*; 40 mg/kg, i.p.). Animals were perfused 2 h after the last injection. Data are expressed as the number of cells per mm² (means \pm SEM for $n = 4-5$ determinations per group). *** $p < 0.001$, significantly different from controls.

TABLE 2. Effects of 5-HT depletion on *d*-fenfluramine-induced changes in striatal 5-HT, 5-HIAA, and dopamine contents

Treatment	5-HT	5-HIAA	DA
Saline	282 ± 12(5)	346 ± 18(5)	6,477 ± 495(5)
PCA	52 ± 9 (6) ^a	35 ± 4 (6) ^a	ND
PCA + <i>d</i> -fenfluramine	34 ± 7 (6) ^a	74 ± 10(6) ^a	4,658 ± 797(6)

A single dose of *d*-fenfluramine (40 mg/kg, i.p.) or saline (2 ml/kg) was administered to rats. These animals were pretreated with either five doses (24-h interval) of saline or PCA (7.5 mg/kg for 2 days and then 10 mg/kg for the next 3 days) 1 week before *d*-fenfluramine administration. Animals were killed 2 h after the last drug injection as described in the text. Concentrations in brain homogenates are expressed in ng/g of wet tissue. Values are means ± SEM. Numbers of determinations are in parentheses. ND, not determined.

^a*p* < 0.001 when compared with saline by using a one-way ANOVA followed by Student's *t* test.

PCA treatment targeted mainly the serotonergic rather than the dopaminergic terminals. Thus, pretreatment with PCA markedly decreased the expression of Fos-like protein following *d*-fenfluramine treatment compared with the expression in saline-pretreated controls.

Effect of methiothepin on the inducibility of Fos-like and Jun B-like protein expression in the striatum following *d*-fenfluramine treatment

Pretreatment with the nonselective 5-HT₁ receptor antagonist, methiothepin (10 mg/kg), produced a small reduction in the number of cell nuclei that express Fos and Jun B proteins in the striatum following *d*-fenfluramine (20 mg/kg) administration. This reduction was ~18% for Fos (Fig. 5A) and was statistically significant and ~12% for Jun B (Fig. 5B). Similar effects were obtained with the highest dose of *d*-fenfluramine tested (40 mg/kg). Methiothepin pretreatment, however, completely blocked the *d*-fenfluramine-induced expression of Fos-like and JunB-like proteins in the neocortex (data not shown). Rats treated with methiothepin alone did not exhibit significant differences in the expression of Fos-like and JunB-like proteins in the brain regions studied when compared with control animals treated with vehicle.

Effect of SCH 23390 on the inducibility of Fos-like and Jun B-like protein expression in the striatum following *d*-fenfluramine treatment

Pretreatment with low doses (0.3 mg/kg, i.p.) of the D₁-class selective dopamine receptor antagonist, SCH 23390, partially blocked the expression of Fos-like (Fig. 5A) and Jun B-like (Fig. 5B) proteins by *d*-fenfluramine (20 mg/kg) in the striatum. SCH 23390 produced a significant reduction in the number of cell nuclei that express Fos-like (58%; Fig. 5A) and Jun B-like proteins (40%; Fig. 5B) in the striatum following *d*-fenfluramine. This partial inhibition was still present at the 40 mg/kg dose of *d*-fenfluramine (data not shown). Control rats treated with saline vehicle only showed very small Fos-like and Jun B-like pro-

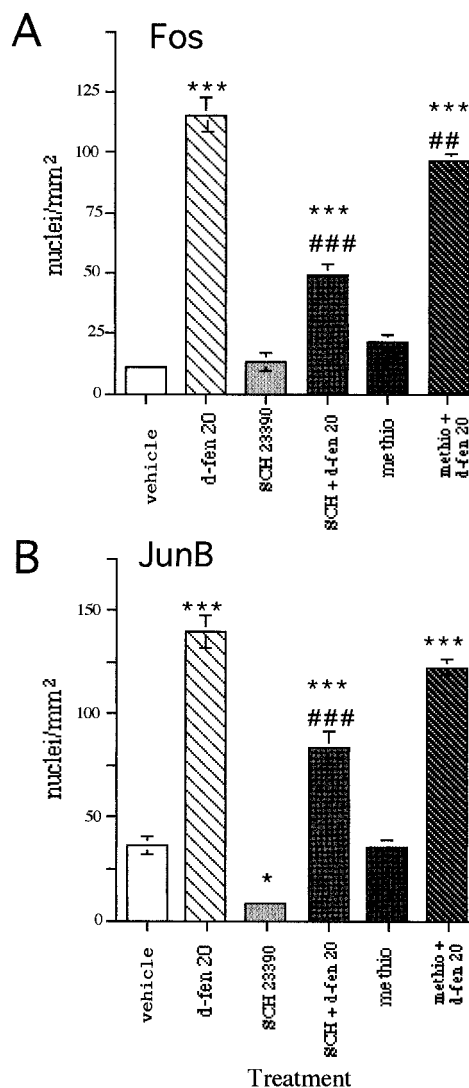
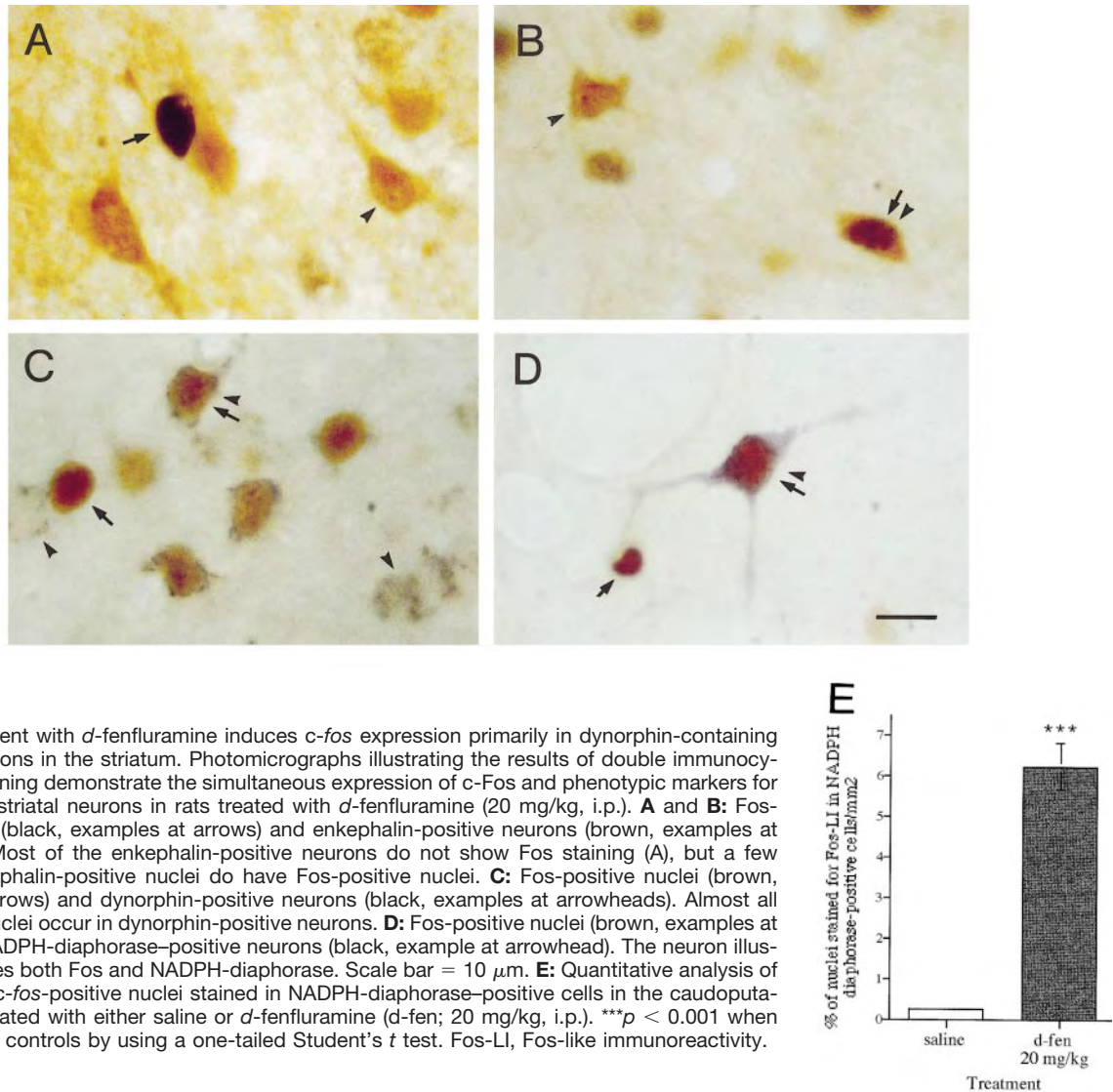


FIG. 5. Numbers of nuclei that express (A) Fos-like protein and (B) Jun B-like protein in the dorsomedial caudoputamen counted in both hemispheres in single sections from rats that received either vehicle (polyethylene glycol or saline, 2 ml/kg) or an acute dose of *d*-fenfluramine (*d*-fen; 20 mg/kg, i.p.) given alone or with the selective D₁-class dopamine receptor antagonist, SCH 23390 (SCH; 0.2 mg/kg, i.p.), or the nonselective 5-HT₁ receptor antagonist, methiothepin (methio; 10 mg/kg, i.p.). Animals were perfused 2 h after the last injection. Data are expressed as the number of cells per mm² (means ± SEM for n = 4–5 determinations per group). **p* < 0.01, ****p* < 0.001, significantly different from controls. ##*p* < 0.01, ###*p* < 0.001, significantly different from *d*-fenfluramine alone.

tein expression in the striatum and other brain regions. Administration of SCH 23390 alone did not affect Fos expression in the striatum, but slightly reduced Jun B expression.

Phenotypes of striatal neurons expressing Fos-like proteins in response to *d*-fenfluramine

To determine the phenotypes of the striatal neurons expressing Fos/Jun B following treatment with *d*-fenflur-



amine (20 mg/kg), we carried out dual staining for Fos and neuropeptide markers for the two principal classes of striatal projection neurons (dynorphin and enkephalin), and also for a marker (NADPH-diaphorase) of one class of striatal interneurons (Fig. 6). Of the Fos-positive nuclei counted, $22 \pm 1.2\%$ (mean \pm SEM for 24 determinations) were also stained for dynorphin (Fig. 6C and D). By contrast, only $7.9 \pm 1.1\%$ (mean \pm SEM for 30 determinations) of Fos-positive nuclei expressed enkephalin-like immunoreactivity (Fig. 6A and B). Numerous NADPH-diaphorase-stained neurons with Fos-positive nuclei ($\sim 6\%$) were also found in the striatum following *d*-fenfluramine administration. Fewer than 0.25% of the NADPH-diaphorase-positive neurons contained Fos-positive nuclei in saline-treated control rats (Fig. 6E). Expression of Jun B was localized in the same types of neurons that expressed Fos (data not shown).

DISCUSSION

The results reported here suggest that *d*-fenfluramine, a drug that selectively releases 5-HT and blocks its reuptake, triggers transcription factor gene expression in the striatum as an indirect consequence of interactions between striatal 5-HT and dopamine circuitries. We show that *d*-fenfluramine evokes the expression of Fos-like proteins in a subset of striatal neurons including dynorphin-positive projection neurons and NADPH-diaphorase-positive interneurons, and we show by RT-PCR that the induction of *c-fos* mRNA by *d*-fenfluramine was transient and dose-dependently correlated with the expression of the protein. *d*-Fenfluramine also increased the expression of Jun B-like protein in the striatum, but produced an all-or-none response in the range of doses tested. Lesions of 5-HT terminals by prior PCA treatment completely blocked *d*-fenfluramine's induction of

the expression of Fos-like proteins. Furthermore, the *d*-fenfluramine-induced IEG expression in the striatum depended not only on 5-HT (including 5-HT₁ receptors), but also partially on D₁-class dopamine receptors. We suggest that the effects of a single administration of *d*-fenfluramine on the striatum result from interactions between cellular events transduced by 5-HT and dopamine receptors. An attractive possibility is that *d*-fenfluramine elicits the release of endogenous 5-HT, which in turn stimulates striatal dopamine release leading to the gene induction in the striatum.

Our work confirms and extends previous studies showing that *d*-fenfluramine induces *c-fos* expression in the striatum. In addition, we report the novel finding that this drug also induces concomitantly the expression of Jun B. This finding is important, inasmuch as it is known that different Fos and Jun proteins selectively interact to form heterodimeric complexes that activate or inhibit the expression of specific genes. Therefore, the selective activation of certain c-Fos-like and Jun B-like proteins by *d*-fenfluramine implies that proteins of these two classes can interact in regulating specific target genes induced by this drug. Similar coactivation of Fos- and Jun-family proteins has been demonstrated for treatments with typical neuroleptics (Simpson and Morris, 1994) and cocaine (Moratalla et al., 1993, 1996a).

Treatment with *d*-fenfluramine induced a dose-dependent expression of Fos-like protein in the striatum (5–40 mg/kg, i.p.). This response was more intense in the middle and dorsomedial caudoputamen and, at caudal levels, dorsal and ventral zones. *d*-Fenfluramine also induced the expression of Jun B-like proteins in the striatum, and the similarity between the patterns of distribution of Fos- and Jun B-immunoreactive neurons suggests that the regulation of the two genes occurs in the same neurons. However, we did not find a dose-response effect of *d*-fenfluramine on the expression of Jun B-like proteins in the striatum within the range of doses tested. Therefore, the response of Jun B was different from that of Fos. Our immunohistochemical findings for *c-fos* are in good agreement with our evidence that *c-fos* mRNA is induced transiently in the striatum following a single *d*-fenfluramine administration in a dose-dependent manner over doses from 5 to 40 mg/kg.

Our evidence strongly suggests that the capacity of *d*-fenfluramine to induce the expression of Fos-like and Jun B-like proteins in the striatum is partially dependent on activation of D₁-class dopamine receptors. We accumulated two different sets of data in support of this assertion. First, we observed that the *d*-fenfluramine-induced IEG expression is partially antagonized by low doses of SCH 23390. Higher doses of this drug also produced a partial blockade of striatal IEG expression (data not shown). Second, we found that the Fos-like protein is colocalized in striatal neurons with dynorphin immunoreactivity, whereas most of the enkephalin-positive neurons of the striatum did not show Fos staining. D₁-class dopamine receptors are thought to be preferentially expressed on the projection neurons coexpressing

the neuropeptides dynorphin (Xu et al., 1994; Moratalla et al., 1996a) and substance P (Gerfen et al., 1990), whereas D₂-class dopamine receptors are strongly expressed by projection neurons coexpressing the neuropeptide enkephalin (Gerfen et al., 1990). Results similar to the present findings have been obtained recently with the racemic mixture *d,l*-fenfluramine showing that it induces a specific expression of Fos-like proteins within the striatum, which was partially blocked by pretreatment with higher doses of SCH 23390 (Torres and Rivier, 1993; Rouillard et al., 1996; Guerra et al., 1998). We used two different doses for SCH 23390 and obtained similar results with each, but only the lower dose (0.2 mg/kg) is thought to be selective for D₁-class dopamine receptors and to avoid the interactions with 5-HT₂ receptors found for higher doses (Bischoff et al., 1986).

The dopamine D₁-class receptor-mediated effect of *d*-fenfluramine is not likely to reflect a direct action on dopamine or 5-HT receptors, because neither *d*-fenfluramine nor its main active metabolite, *d*-norfenfluramine, binds to any particular 5-HT or dopamine receptor subtype with high affinity (Samanin and Garattini, 1990). However, we cannot exclude the possibility that another receptor-mediated mechanism of action could be involved, especially at the high doses (20 and 40 mg/kg) used in this study.

Destruction of serotonergic projection systems by PCA, which depleted striatal tissue 5-HT levels by 88% and slightly reduced those of dopamine (by 28%), totally suppressed increases in Fos-like protein expression induced in the striatum by *d*-fenfluramine. This result suggests that the integrity of the serotonergic system is a necessary requirement for the induction of Fos expression by *d*-fenfluramine, in agreement with the results of Guerra et al. (1998). Taken together with the only partial blockade of *d*-fenfluramine induction of striatal Fos and Jun B expression by a low dose of the D₁-class dopamine receptor antagonist, SCH 23390, these data suggest that the Fos and Jun B protein expression is related to the activation of serotonergic neurons but, in addition, depends on dopamine active at D₁-preferring sites.

At least three alternative mechanisms might account for our findings. First, a synergism between striatal 5-HT and dopamine circuitries may have occurred at the level of the striatum to promote the activation of IEG expression. Such a direct effect would be consistent with data obtained by *in vivo* microdialysis demonstrating that in anesthetized rats, *d*-fenfluramine can directly release striatal dopamine by a Ca²⁺-dependent, tetrodotoxin-sensitive mechanism independent of striatal 5-HT itself, and that local administration of 5,7-dihydroxytryptamine into the dorsal raphe nucleus did not modify this effect (De Deurwaerdère et al., 1995). This hypothesis is also consistent with the fact that some 5-HT receptor agonists can induce Fos expression through a direct action on striatal cell bodies and that stimulation of dopamine receptors positively linked to adenylate cyclase plays a permissive role in this effect (Cook and Wirtshafter, 1998).

Alternatively, the effects of *d*-fenfluramine in the striatum may have been indirect, reflecting activation of the massive glutamatergic corticostriatal system (McGeorge and Faull, 1989). Evidence suggests that L-glutamate can activate the release of dopamine in the striatum. The activation of L-glutamate receptors located on dopaminergic and serotonergic axon terminals in the striatum could also modulate the *d*-fenfluramine-induced dopamine and 5-HT release, and influence effects of *d*-fenfluramine on striatal Fos expression. It has been suggested that D₁-class dopamine receptors and glutamate NMDA postsynaptic receptors have combinatorial actions in mediating the effects of fenfluramine on striatal Fos expression (Torres and Rivier, 1993).

A third possibility, favored here, is that the involvement of the dopaminergic system in inducing striatal IEG expression in response to the *d*-fenfluramine is mostly an indirect consequence of *d*-fenfluramine's ability to activate 5-HT release from serotonergic axon terminals in the substantia nigra. 5-HT-containing axons have been shown to project from the dorsal raphe nucleus to the substantia nigra pars compacta and to modulate the dopaminergic nigrostriatal pathway (Van der Kooy and Hattori, 1980; Soubrié et al., 1984; Yadid et al., 1994). Furthermore, 5-HT released in the striatum by *d*-fenfluramine may stimulate 5-HT receptors, which in turn could increase striatal dopamine release. Both 5-HT receptor agonists and fenfluramine facilitate striatal dopamine release (Benloucif and Galloway, 1991; Benloucif et al., 1993), and an *in vivo* microdialysis study performed in freely moving rats has demonstrated directly that *d*-fenfluramine has an indirect dopamine-releasing capacity via 5-HT release in the striatum at doses higher than 2.5 mg/kg *i.p.* (Balcioglu and Wurtman, 1998). Given the converging lines of evidence obtained here with PCA pretreatment, with predominant *d*-fenfluramine-induced increases in Fos expression in the class of striatal projection neurons known to express D₁-class dopamine receptors, we favor this third alternative.

We extended these results by showing that the striatal Fos and Jun B responses to *d*-fenfluramine (20 mg/kg) not only are partially blocked by pretreatment with a low dose of SCH 23390, but also are slightly decreased by prior administration of a nonselective 5-HT₁ receptor antagonist, methiothepin (10 mg/kg). This dose of methiothepin has been shown to be effective in blocking *d*-fenfluramine's effects on brain tissue levels of 5-HT as well as on extracellular 5-HT levels in the rat frontal cortex (Gardier et al., 1992). Methiothepin is a nonselective 5-HT₁ receptor antagonist, but it displays high affinities for 5-HT_{1A} and 5-HT_{1B} receptor subtypes in rats (Engel et al., 1986). 5-HT_{1B} receptors are concentrated within the striatum (Boulenguez et al., 1996). Furthermore, the nonselective 5-HT_{1A/1B} receptor agonist, RU 24969, can induce the expression of Fos-like proteins within the rat striatum, thus reproducing the effects of *d*-fenfluramine, and the response to RU 24969 is not blocked by pretreatment with a selective 5-HT_{1A} receptor antagonist (Wirtshafter and Cook, 1998). Taken

together with the fact that 5-HT_{1A} and 5-HT_{2A/2C} receptor antagonists fail to attenuate *d*-fenfluramine-induced Fos-like protein expression in the brain (Rouillard et al., 1996; Javed et al., 1998), these data suggest that the response to methiothepin was mediated by the blockade of 5-HT_{1B} receptors. A comparison of the results we obtained with methiothepin and with SCH 23390 indicates, however, that the activation of 5-HT_{1B} receptors contributes only moderately to the *d*-fenfluramine-induced Fos expression in the striatum, whereas the activation of D₁-class dopamine receptors is more important.

It is also remarkable that RU 24969 dose-dependently induces striatal Fos expression in mice in anatomical patterns similar to those induced by cocaine (Lucas et al., 1997, 1998), as we have found for *d*-fenfluramine. The effect of RU 24969 was reduced in 5-HT_{1B} receptor knockout mice and was blocked completely in wild-type mice pretreated with the mixed 5-HT_{1B/1D} receptor antagonist GR 127935 (Lucas et al., 1997). The Fos expression induced by fenfluramine was also reduced substantially in the brains of the 5-HT_{1B} receptor knockout mice (Lucas et al., 1998). Destruction of 5-HT-containing axons by PCA pretreatment attenuates the increase in *c-fos* mRNA levels induced in the rat striatum by cocaine (Graybiel et al., 1990; Bhat and Baraban, 1993) and completely blocks Fos induction by *d*-fenfluramine. These results suggest that both *d*-fenfluramine and cocaine may induce *c-fos* expression by mechanisms depending conjointly on dopaminergic and serotonergic systems, but in different proportions. Our findings show a striking parallel between *d*-fenfluramine and cocaine in many aspects of their IEG activation effects, including the general distribution of Fos-like protein expression in the caudoputamen and the prominence of the induction in dynorphin-positive projection neurons and NADPH-diaphorase-positive interneurons (Graybiel et al., 1990; Berretta et al., 1992; Moratalla et al., 1993, 1996a,b). It has also been shown recently that both cocaine and fenfluramine induce the expression of Fos-like proteins in striatal neurons expressing the protein phosphatase inhibitor DARPP-32 (dopamine and cyclic AMP-regulated phosphoprotein), considered as a marker for neurons bearing D₁-class dopamine receptors (Berretta et al., 1992; Torres and Rivier, 1994). There is no evidence, however, that these effects lead to similar behavioral consequences in mammals. Cocaine suppresses appetite by inhibiting consumption of all the macronutrients and leads to hyperactivity and addiction; *d*-fenfluramine induces hypophagia by enhancing satiety and by inhibiting overconsumption of carbohydrates, decreases locomotion in mice and rats, does not possess abuse liability, and does not produce addiction in human. The functional and physiological significance of interactions between 5-HT and dopamine at the level of the induction of transcription factors in the striatum is unknown (Rouillard et al., 1996).

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