

p-Chlorophenylalanine Changes Serotonin Transporter mRNA Levels and Expression of the Gene Product

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Abstract: After a single intraperitoneal injection of the irreversible tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (PCPA; 300 mg/kg), there was a rapid down-regulation of serotonin (5-HT) transporter mRNA levels in cell bodies. This change was significant at 1 and 2 days after PCPA administration within the ventromedial but not the dorsomedial portion of the dorsal raphe nucleus. Seven days after PCPA treatment, 5-HT transporter mRNA levels were significantly elevated compared with controls in both regions of the dorsal raphe nucleus. PCPA administration produced no change in the [³H]-citalopram binding and synaptosomal [³H]5-HT uptake in terminal regions at 2 and 7 days after treatment but significantly reduced both these parameters by ~20% in the hippocampus and in cerebral cortex 14 days after PCPA administration. The striatum showed a lower sensitivity to this effect. No significant changes were observed in the levels of [³H]citalopram binding to 5-HT cell bodies in the dorsal raphe nucleus. In the same animals used for 5-HT transporter mRNA level measurements, levels of tryptophan hydroxylase mRNA in neurons of the ventromedial and dorsomedial portions of the dorsal raphe nucleus were increased 2 days after PCPA administration and fell to control levels 7 days after injection in the ventromedial region but not in the dorsomedial portion of the dorsal raphe nucleus, where they remained significantly higher than controls. Altogether, these results show that changes in 5-HT transporter mRNA are not temporally related to changes in 5-HT transporter protein levels. In addition, our results suggest that the 5-HT transporter and tryptophan hydroxylase genes are regulated by different mechanisms. We also provide further evidence that dorsal raphe 5-HT neurons are differentially regulated by drugs, depending on their location. **Key Words:** Serotonin uptake/serotonin uptake sites—Serotonin transporter—Tryptophan hydroxylase—*p*-Chlorophenylalanine—Gene expression—In situ hybridization—Autoradiography—Image analysis.
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The activity of 5-hydroxytryptamine (5-HT; serotonin) neurons can be altered by physiological or pharmacological interventions. The processes that control

the responses of 5-HT neurons to alterations in their environment, however, are not fully understood. Molecular biological techniques have allowed isolation of several genes that regulate presynaptic function of 5-HT neurons, and manipulations of the 5-HT system by drugs would be predicted to regulate the expression of these genes at different levels, including transcriptional regulation, mRNA turnover, and regulation of protein levels and activity.

A protein that plays an important role in the function of 5-HT neurons is the presynaptic sodium-dependent 5-HT transporter, a target for many antidepressant drugs and drugs of abuse (Åberg-Wigstedt, 1989; Blakely et al., 1991; Hoffman et al., 1991; Rudnick and Wall, 1992). Regulation of the 5-HT transporter may be an important component of the response of 5-HT system to drugs, because modulation of 5-HT transporter levels could influence the availability of 5-HT in the synaptic cleft.

Two drugs that affect 5-HT neurons are the amphetamine derivatives *d*-fenfluramine and 3,4-methylenedioxymethamphetamine (MDMA). Both drugs increase the release of 5-HT from nerve terminals and inhibit its reuptake (Garattini et al., 1986; Gobbi et al., 1992; Rudnick and Wall, 1992). Subchronic administration of high doses of *d*-fenfluramine and MDMA produced a long-lasting depletion of 5-HT in the rat CNS (Battaglia et al., 1987; Appel et al., 1989) and a long-lasting decrease in content of the 5-HT

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Abbreviations used: DRd, dorsomedial area of the dorsal raphe nucleus; DRv, ventromedial area of the dorsal raphe nucleus; 5-HT, 5-hydroxytryptamine (serotonin); MDMA, 3,4-methylenedioxy-methamphetamine; PCPA, *p*-chlorophenylalanine; SSC, saline-sodium citrate.

transporter as determined by synaptosomal [^3H]5-HT uptake (Schuster et al., 1986) or [^3H]paroxetine binding (Appel et al., 1990; Zaczeck et al., 1990; Battaglia et al., 1991). We have recently shown that these drugs also produce a down-regulation of 5-HT transporter mRNA in 5-HT neurons of the dorsal raphe nucleus (Ratray et al., 1993, 1994). Reductions of 5-HT transporter mRNA level have also been reported after chronic treatment with different antidepressant drugs that bind to the 5-HT uptake protein (Lesch et al., 1993; Kuroda et al., 1994; Lopez et al., 1994). From these studies it is not clear the extent to which changes in 5-HT availability in the synaptic cleft and/or a direct action of drugs on 5-HT transporter protein influence the expression of 5-HT transporter gene.

To investigate the influence of changes in 5-HT concentration on the regulation of 5-HT transporter gene expression, we have analyzed the effect of a treatment with *p*-chlorophenylalanine (PCPA). PCPA is an irreversible inhibitor of tryptophan hydroxylase (Jéquier et al., 1967; Koe and Weissman, 1968). This leads to rapid depletion of 5-HT levels in the brain and loss of immunoreactive 5-HT in nerve terminals (Miller et al., 1970; Chen et al., 1994; Park et al., 1994). One study has shown that the level of the 5-HT transporter protein, as determined by [^3H]paroxetine binding, is unaffected by PCPA treatment (Dewar et al., 1992), suggesting that the 5-HT uptake system may be unaffected by PCPA administration. In this study we have addressed this question further by determining the effect of PCPA on 5-HT transporter mRNA levels, [^3H]citalopram binding, and [^3H]5-HT uptake. Because recent studies have shown that PCPA induced a rapid increase in the levels of tryptophan hydroxylase mRNA (Cortés et al., 1993; Park et al., 1994), we have also examined the regulation of this gene. In addition, we have looked for differences in response in 5-HT neurons that lie in the dorsomedial (DRd) and ventromedial (DRv) areas of the dorsal raphe nucleus, as we have found previously that cells in these subdivisions can differ in their response to drugs (Bendotti et al., 1990, 1993; Gobbi et al., 1994; Ratray et al., 1994).

MATERIALS AND METHODS

Animal treatments

Male Sprague-Dawley rats (weighing 250 g; Charles-River, Italy) were injected with PCPA ethyl ester hydrochloride (Aldrich Chemical Co.; 300 mg/kg, i.p.) or saline. Animals were killed by decapitation 24 h, 48 h, or 7 days after the injection for the *in situ* hybridization analysis and 2, 7, or 14 days after treatment for the analysis of [^3H]citalopram binding and [^3H]5-HT uptake. The brains were immediately removed after decapitation. In one experiment, the hippocampus and striatum were dissected out for the determination of 5-HT levels, and brainstem was rapidly frozen in dry ice/isopentane for the *in situ* hybridization experiments or in liquid nitrogen for citalopram binding autoradiography. In a second experiment, cerebral cortices, hippocampi, and striata

were rapidly dissected and processed for crude synaptosomal preparations to be used for [^3H]5-HT uptake and [^3H]citalopram binding studies. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national laws and guidelines (EEC Council directive 86/609, OJ L358, 1, December 12, 1987; NIH *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23, 1985).

In situ hybridization

Section preparation. Coronal rat brain sections (14 μm) were cut on a cryostat and mounted onto gelatin/chrome alum-treated microscope slides. Sections were fixed in 4% paraformaldehyde for 15 min followed by two 10-min incubations in phosphate-buffered saline. Sections were then acetylated (0.1 M triethanolamine and 0.25% acetic anhydride in 0.9% NaCl), delipidated for 5 min in chloroform, dehydrated through a graded series of ethanol, air-dried, and stored frozen at -70°C . Before use, sections were dehydrated through a graded series of alcohol.

5-HT transporter mRNA. For the 5-HT transporter *in situ* hybridization, an oligonucleotide S2 (5' GAA GAT GAC GAA GCC AGA GAC GAA GCT 3') complementary to the rat gene was used, as described previously (Ratray et al., 1994). The oligonucleotide was labeled with ^{35}S -dATP to a specific activity of 4,800 Ci/mmol using terminal deoxynucleotidyl transferase. The reaction mixture (50 μl) contained 4 pmol of S2, 1 \times cacodylate reaction buffer (Promega), 100 $\mu\text{g}/\mu\text{l}$ of bovine serum albumin, 60 U of terminal deoxynucleotidyl transferase (Promega), and 800 nM ^{35}S -dATP (1,200 Ci/mmol; NEN). After a 1-h incubation at 37°C , the reaction products were purified through a Sephadex G-50 column (NICK; Pharmacia), and the probe was freeze-dried. The probe was resuspended to a final concentration of 2 nM in hybridization buffer [0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 10% dextran sulfate, 50% formamide, 20 mM dithiothreitol, 50 $\mu\text{g}/\text{ml}$ of polyadenylic acid (Sigma), 100 $\mu\text{g}/\text{ml}$ of herring sperm DNA, 600 mM NaCl, and 60 mM sodium citrate, pH 7.4]. The probe was denatured at 65°C for 15 min and quenched on ice. The probe (60 μl) was applied to each slide, which was then covered with a glass coverslip and sealed in a humidified container. After incubation at 37°C overnight the coverslips were removed, and the slides were washed in 1 \times saline-sodium citrate (SSC; 150 mM NaCl and 15 mM sodium citrate, pH 7.4) at room temperature for 10 min followed by two 30-min washes in 1 \times SSC at 55°C . Slides were then washed for 30 min in 1 \times SSC at room temperature and dried through a graded series of alcohol.

Tryptophan hydroxylase mRNA. On sections cut from the same animals, *in situ* hybridization was carried out using a riboprobe directed against tryptophan hydroxylase mRNA using the protocol described previously (Bendotti et al., 1990). A full-length rat tryptophan hydroxylase cDNA (TPH-1) subcloned into pSP65 (Darmon et al., 1988) was linearised with *Sma*I and purified. In a volume of 25 μl , ^{35}S -labeled antisense RNA was transcribed from 1 μg of purified template using 15 units of T7 RNA polymerase (BRL). The reaction mixture contained 1 \times transcription buffer (BRL), 10 mM dithiothreitol, 15 U of human placental RNase inhibitor (Promega), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 10 μM UTP, and 2.5 μM ^{35}S -UTP (1,000 Ci/mmol; Amersham). After a 1-h incubation at 40°C , the template was digested with 1 U of DNase I -RNase free (Promega) for 15 min at 37°C . The 1.4-kb template was hydrolyzed in mild

alkali (80 mM NaHCO₃ and 120 mM Na₂HCO₃) at 65°C for 40 min. The mixture was neutralized, and the ³⁵S-labeled probe was purified by phenol/chloroform extraction followed by ethanol precipitation. Probe with a specific activity of 10⁸ cpm/μg of RNA was dissolved in hybridization buffer at a concentration of 3 × 10⁵ cpm/μl. Sections were incubated overnight at 55°C in sealed humidified chambers using Parafilm coverslips. After hybridization, coverslips were removed, and sections were incubated in 1× SSC for 10 min at room temperature. Sections were then washed for 30 min at 37°C in 20 μg/μl of RNase A in RNase buffer (0.5 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 8.0) followed by 30 min at 37°C in RNase buffer alone. Sections were then washed in 1× SSC for 30 min at room temperature, 0.5× SSC for 30 min at 65°C, and 0.5× SSC for 10 min at room temperature before dehydrating through a graded series of ethanol.

Image analysis. From both experiments, slides were dipped in photographic emulsion (Ilford K5; 1:1 with water) and exposed for 30 (5-HT transporter mRNA) or 15 (tryptophan hydroxylase mRNA) days. Sections were developed and counterstained with toluidine blue or cresyl violet. Sections that contained the dorsal raphe nucleus at -7.8 mm from bregma of the rat brain atlas (Paxinos and Watson, 1982) were used for the analysis (Fig. 1, diagram). At this level the dorsal raphe nucleus can clearly be divided into DRv and DRd and lateral wings. Using image analysis techniques on a Kontron IBAS system (Masseroli et al., 1993), silver grain densities over neurons lying in the DRd and DRv were obtained for four or five animals in each of the four treatment groups. The neurons of the lateral wings were not considered for the quantitative analysis as they were more dispersed and less numerous than in the other regions. For the same reason we did not consider the neurons in the raphe medialis. Silver grain densities were obtained from individual labeled cells present in the regions delimited by hatched boxes as represented in the diagram of Fig. 1. Nonspecific background was determined from the grain density of selected cells without a specific hybridization signal, and this value was automatically subtracted to give a measure of the specific signal for the positive cells. The data were expressed as number of grains for square micrometer area of the individual neuron.

Determination of 5-HT levels

5-HT levels in striatum were measured by HPLC with electrochemical detection. In brief, the tissue samples were homogenized by sonication in 300 μl of 0.1 M HClO₄ and centrifuged at 15,000 g for 10 min. Samples (20 μl) of supernatant were directly injected into a reverse-phase column, and 5-HT was quantified according to the procedure of Invernizzi et al. (1989).

Autoradiographic determination of [³H]citalopram binding

Methods used were as described by Gobbi et al. (1994). Coronal sections were cut at 14 μm on a cryostat and thaw-mounted on gelatin-coated slides. Sections were kept at -20°C until assay. For [³H]citalopram binding the sections were preincubated for 15 min at room temperature in 50 mM Tris-HCl (pH 7.4) containing 120 mM NaCl and 5 mM KCl, transferred to fresh buffer containing 1 nM [³H]citalopram (NEN; 80 Ci/mmol), and incubated for 60 min at room temperature. Nonspecific binding was determined by adding 1 μM indalpine (Pharmuka, France) to the incubation

medium. The washing procedure consisted of a rapid dip in buffer, followed by two 5-min washes in buffer and a final dip in distilled water (all these solutions were kept at 4°C). The slides were dried overnight at 4°C and apposed, together with Amersham-³H plastic standard scales, to tritium-sensitive film (Amersham) and left for 3 weeks at -50°C. The films were developed using Kodak D19 developer (5 min at 20°C), fixed (Unifix; Kodak), and washed in water. Computer-assisted autoradiographic analysis was done, using the RAS-3000 image analyzer (Loats System, Wemminster, MD, U.S.A.), on the brain structures described in a rat brain atlas (Paxinos and Watson, 1982).

[³H]5-HT uptake and [³H]citalopram binding in crude synaptosomal preparations

[³H]5-HT uptake and [³H]citalopram binding were carried out on the same crude synaptosomal preparation obtained after pooling the brain areas of five rats per group. Cerebral cortices, hippocampi, and striata were rapidly dissected, transferred into 20 volumes of ice-chilled 0.32 M sucrose (pH 7.4), and homogenized by a glass homogenizer with a Teflon pestle. Synaptosomes were obtained as described by Gray and Whittaker (1962). In brief, the homogenate was centrifuged for 10 min at 1,000 g, and the supernatant was centrifuged at 12,000 g for 60 min to yield the crude synaptosomal pellet (P2).

[³H]5-HT uptake. The method described by Mennini et al. (1985) was used. The P2 pellets were diluted (50–60 volumes of initial weight) with Krebs–Henseleit buffer having the following composition: 116 mM NaCl, 25 mM NaHCO₃, 1 mM NaH₂PO₄, 6 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 10 mM glucose, 10 mM pargyline, 0.07 mM EDTA, and 0.3 mM ascorbic acid, pH 7.2–7.4. Aliquots (0.5 ml) were preincubated for 5 min at 30°C in the absence or presence of 0.3 μM citalopram. To each aliquot, 100 μl of [³H]5-HT (NEN; 27.9 Ci/mmol) was added to give final concentrations ranging from 15 to 140 nM. The reactions were stopped 5 min later by adding 2 ml of ice-chilled Krebs–Henseleit buffer. Samples were then filtered through cellulose nitrate filters (pore size, 0.6 μm; Perdomini, Verona, Italy) and washed twice with 2 ml of Krebs–Henseleit buffer. The radioactivity trapped on the filters was counted in 4 ml of Filter Count (Packard, Groningen, The Netherlands) in a liquid scintillation counter.

[³H]Citalopram binding. The method described by D'Aмато et al. (1987) was used with slight modifications. The P2 pellets were resuspended (50–60 volumes of initial weight) with Tris HCl (50 mM, pH 7.4) containing 120 mM NaCl, 5 mM KCl, and 10 μM pargyline. Aliquots of the homogenate (0.5 ml) were added to 100 μl of buffer containing different concentrations of [³H]citalopram (NEN; 80 Ci/mmol) to give a final concentration ranging from 0.3 to 8 nM. Samples incubated in the presence of 100 μM 5-HT were used to define the nonspecific binding. After 60 min at 25°C the samples were rapidly filtered (model M-48RP; Brandel) under vacuum through Whatman GF/C glass fiber filters presoaked in 0.5% polyethylenimine and washed three times. The radioactivity on the filters was measured by liquid scintillation.

Calculations and statistics. The saturation curves were analyzed by nonlinear least squares fitting, according to the Michaelis–Menten equation, using the LIGAND program (Munson and Rodbard, 1984). This analysis estimates the kinetic parameters of the saturation curves, the Michaelis constant (K_m) and the maximal rate of [³H]5-HT uptake

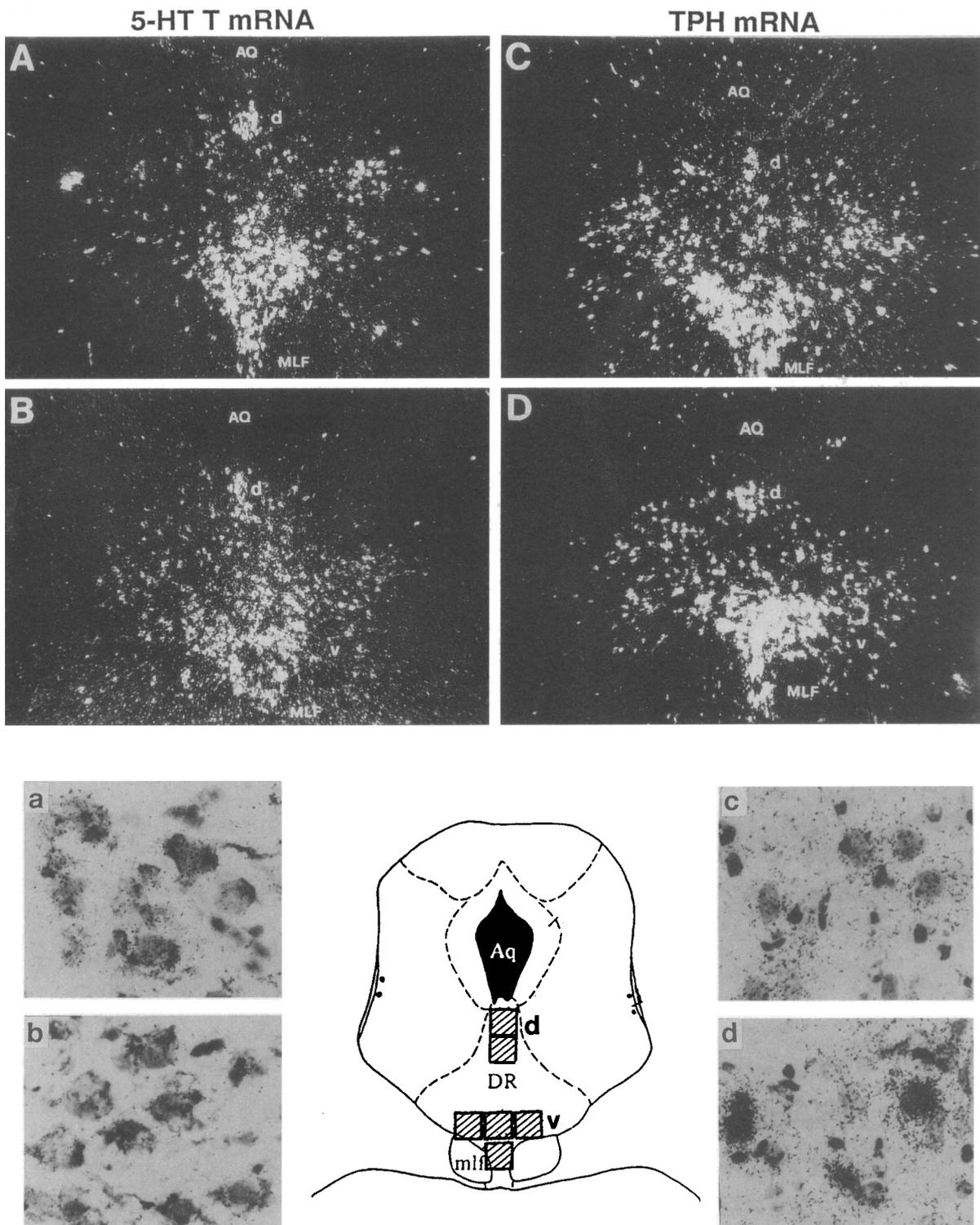


FIG. 1. Dark-field photomicrographs of hybridization signal for the 5-HT transporter (5-HT T) mRNA and tryptophan hydroxylase (TPH) mRNA in the dorsal raphe nucleus. 5-HT T mRNA is shown in controls (**A**) and 1 day after PCPA treatment (**B**). TPH mRNA is shown in controls (**C**) and 2 days after PCPA treatment (**D**). **a-d**: At high power in bright-field, the grain density over individual neurons of the DRv representative of the hybridization signal shown in the ventromedial region in A-D, respectively. The diagram shows a representation of the rat brain at the level of analysis (-7.8 mm from bregma) based on the rat brain atlas of Paxinos and Watson (1982). The DRd (d) and the DRv (v) are indicated. The hatched boxes, two in the dorsomedial and four in the ventromedial subdivision, represent the measuring frames (0.065 mm² each) for the analysis of the neuronal grain density and the number of positive neurons. DR, dorsal raphe; mlf/MLF, medial longitudinal fasciculus; Aq, aqueduct. A-D, $\times 60$; a-d, $\times 224$.

TABLE 1. Effect of PCPA administration on mRNA levels in raphe nuclei

	DRv		DRd	
	5-HT transporter mRNA	Tryptophan hydroxylase mRNA	5-HT transporter mRNA	Tryptophan hydroxylase mRNA
Saline	0.17 ± 0.03 (61)	0.20 ± 0.01 (52)	0.13 ± 0.01 (20)	0.13 ± 0.01 (15)
PCPA				
1 day	0.10 ± 0.01 (55) ^a	0.28 ± 0.01 (50)	0.10 ± 0.01 (17)	0.13 ± 0.01 (15)
2 days	0.11 ± 0.01 (60) ^a	0.50 ± 0.05 (55) ^a	0.12 ± 0.01 (22)	0.20 ± 0.02 (17) ^a
7 days	0.25 ± 0.01 (69) ^a	0.23 ± 0.01 (53)	0.24 ± 0.02 (20) ^b	0.17 ± 0.01 (16) ^b

Levels of mRNA are expressed as mean ± SEM grain density for square micrometer area of individual neurons (mean no. of positive neurons included in the fields analyzed at the level of the DRd and the DRv for each brain section according to the scheme in Fig. 1). In each case, one or two sections were analyzed from four or five rats.

Data were analyzed by one-way ANOVA followed by Dunnett's test: ^a $p < 0.01$, ^b $p < 0.05$, compared with saline group.

(V_{max}), or the dissociation constant of [³H]citalopram binding (K_D) and the maximal number of binding sites (B_{max}). For the statistical comparison between the kinetic parameters of the curve of PCPA-treated rats and those of the curve of controls rats, we used the "extra sum of squares" F -test criterion on the residual variances, included in the LIGAND program.

RESULTS

Effect of PCPA on 5-HT transporter and tryptophan hydroxylase mRNA levels in dorsal raphe nucleus

Using a ³⁵S-labeled oligonucleotide probe for the 5-HT transporter and a ³⁵S-labeled riboprobe for tryptophan hydroxylase, specific hybridization was found over neurons in the DRd, DRv, and lateral subdivisions of the dorsal raphe nucleus, as indicated in Fig. 1. No hybridization signal was observed when probes were used on RNase-pretreated sections (data not shown).

Image analysis techniques were used to determine the grain densities above 5-HT neurons in the dorsal raphe nucleus as a measure of the cellular level of expression of 5-HT transporter and tryptophan hydroxylase mRNA (Table 1). In control animals, the average levels of tryptophan hydroxylase and 5-HT transporter mRNA were highest in cells that lay in the DRv.

In cells that lie in the DRv, PCPA produced a significant decrease in 5-HT transporter mRNA levels 1 and 2 days after injection ($p < 0.01$). The peak reduction was to ~60% of control levels, 1 day after PCPA administration. Seven days after the injection, levels of 5-HT transporter mRNA were significantly increased to 147% of controls. A greater increase of the levels of this transcript, to 187% of control levels, was found in the DRd 7 days after PCPA. In DRd, however, there were no significant changes in 5-HT transporter mRNA levels 1 or 2 days after drug treatment (Table 1).

In contrast to the regulation of 5-HT transporter mRNA by PCPA, there was a different pattern of

change for tryptophan hydroxylase mRNA. In DRv, PCPA induced an increase in tryptophan hydroxylase mRNA levels to 250% of control levels, 2 days after drug administration. In DRd, there was a less pronounced but significant increase in tryptophan hydroxylase mRNA levels 2 and 7 days after PCPA administration (Table 1).

Histograms showing the distribution of grain densities in positive neurons of the DRd (Fig. 2) and DRv (Fig. 3) are shown for both 5-HT transporter and tryptophan hydroxylase mRNAs. In the DRv there was a clear shift to the left of the expression of 5-HT transporter mRNA 1 and 2 days after PCPA, whereas at 7 days there was an increase in the number of cells expressing a high level of this transcript. An increase in the number of cells expressing higher levels of tryptophan hydroxylase mRNA was observed 2 days after PCPA administration in both the DRd and the DRv.

The number of positively hybridizing cells did not change over the course of drug treatment (Table 1). There were similar numbers of cells per section that expressed tryptophan hydroxylase mRNA and 5-HT transporter mRNA.

The different time courses of the effects on 5-HT transporter and tryptophan hydroxylase mRNA levels in DRv and DRd are shown in Fig. 4. In the same animals, the levels of 5-HT in the striatum were measured. At all time points after PCPA administration, there were significant reductions in the levels of 5-HT to 24 ± 4 , 19 ± 4 , and $51 \pm 2\%$ of controls at 1, 2, and 7 days, respectively.

Effect of PCPA on 5-HT transporter protein in the dorsal raphe nucleus

Because PCPA treatment did not affect the K_D value of [³H]citalopram in forebrain regions (see below), for the autoradiographic experiments we used a single ligand concentration (1 nM) to give comparative measures of binding levels in the dorsal raphe nucleus. Table 2 shows that, in control animals, binding levels in the DRv were higher than in the DRd. PCPA did not

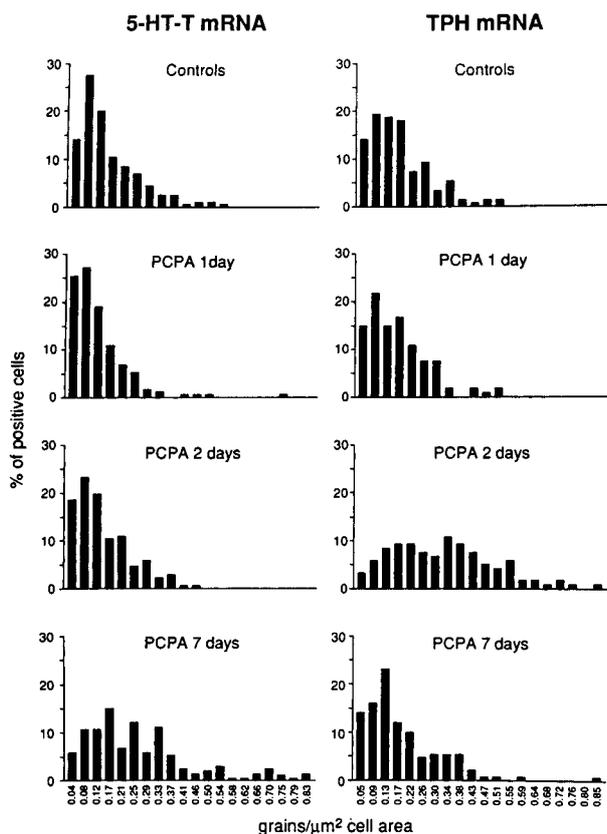


FIG. 2. Frequency histograms of the neuronal grain densities in the DRd represent the levels of 5-HT transporter (5-HT-T) mRNA and tryptophan hydroxylase (TPH) mRNA.

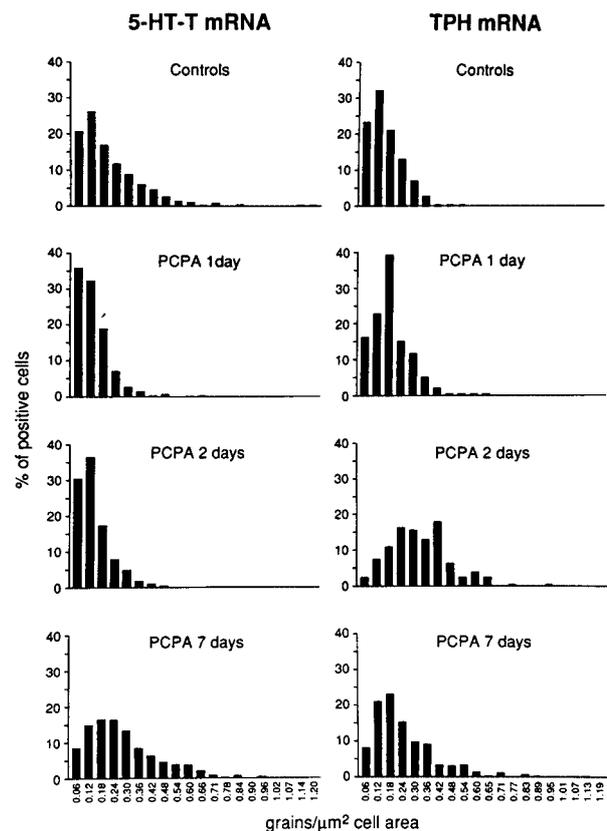


FIG. 3. Frequency histograms of the neuronal grain densities in the DRv represent the levels of 5-HT transporter (5-HT-T) mRNA and tryptophan hydroxylase (TPH) mRNA.

alter [^3H]citalopram binding to the 5-HT transporter protein at 2, 7, or 14 days after injection (Table 2).

Effect of PCPA on 5-HT uptake in synaptosomes of forebrain regions

[^3H]Citalopram binding and [^3H]5-HT uptake were measured on crude synaptosomal preparations obtained from cerebral cortex, hippocampus, and striatum of controls and PCPA-treated rats at 2, 7, or 14 days after injection. Table 3 shows that PCPA did not produce any significant alteration in the affinity (K_D) or in the number (B_{max}) of [^3H]citalopram binding sites 2 or 7 days after the treatment. After 14 days, the maximal number of [^3H]citalopram binding sites was decreased by 20% in the hippocampus and cortex and, to a lower but significant extent (10%), in the striatum. In the striatum 14 days after PCPA injection, the K_D value was also significantly decreased. Consistent with these data, there was no effect of PCPA on the K_m or the maximal velocity (V_{max}) of [^3H]5-HT uptake into synaptosomes at 2 and 7 days after PCPA administration, whereas at 14 days after the treatment there was a significant decrease of [^3H]5-HT uptake by 20–28% in cerebral cortex and hippocampus but not in striatum (Table 4).

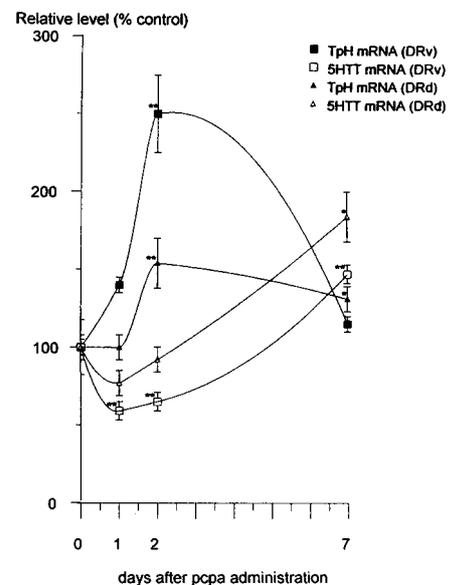


FIG. 4. Contrasting effects of PCPA on 5-HT transporter (5-HTT) and tryptophan hydroxylase (TpH) mRNA levels in the DRd and the DRv. Changes in mRNA levels are shown after a single administration of PCPA, 300 mg/kg i.p., at day 0. * $p < 0.05$, ** $p < 0.01$.

TABLE 2. Effect of PCPA (300 mg/kg *i.p.*) on [³H]citalopram binding in raphe nuclei

	DRv	DRd
Control	377 ± 12 (7)	265 ± 8 (7)
PCPA		
2 days	358 ± 18 (4)	275 ± 18 (4)
7 days	391 ± 16 (4)	280 ± 12 (4)
14 days	395 ± 16 (4)	303 ± 23 (4)

Binding of [³H]citalopram was measured on rat brain coronal sections by quantitative autoradiography. Data are mean ± SEM values (no. of rats), picomoles per gram of tissue. There were no statistically significant changes in [³H]citalopram binding to either the DRv or the DRd by Student's *t* test.

DISCUSSION

The distribution of 5-HT transporter mRNA and tryptophan hydroxylase mRNA was consistent with their expression in 5-HT neurons, as observed previously by various authors (Bendotti et al., 1990; Kim et al., 1991; Fujita et al., 1993; Rattray et al., 1994). We observed a similar number of cells expressing the 5-HT transporter mRNA and the tryptophan hydroxylase mRNA in the same anatomical location of the dorsal raphe, suggesting that all 5-HT neurons synthesize both mRNAs. However, the patterns of regulation of these transcripts clearly differ following treatment with PCPA.

Consistent with recent observations by Cortés et al. (1993) and Park et al. (1994), a single administration of 300 mg/kg of PCPA caused a rapid increase in the levels of tryptophan hydroxylase mRNA in the dorsal raphe. The level of tryptophan hydroxylase mRNA returned to control levels 7 days after PCPA administration in the DRv. The level of expression of the tryptophan hydroxylase gene appears to be related to the availability of the transmitter, as drug treatments that decrease 5-HT levels by different mechanisms all lead to increases in levels of tryptophan hydroxylase mRNA (Bendotti et al., 1990, 1993; Cortés et al., 1993; Park

et al., 1994). A possible exception is the tryptophan hydroxylase inhibitor, 6-fluorotryptophan, which blocks 5-HT synthesis but does not change the levels of tryptophan hydroxylase protein (Richard et al., 1990). The effect of 6-fluorotryptophan on tryptophan hydroxylase mRNA levels, however, remains to be determined. As regards the mechanism involved, as for the related tyrosine hydroxylase gene, tryptophan hydroxylase mRNA expression may be induced by cyclic AMP. This has been directly demonstrated in the pineal gland (Ehret et al., 1991), and analysis of the human tryptophan hydroxylase promoter region shows that there are AP-2 binding sites corresponding to a cyclic AMP response element (Boularand et al., 1995). Although the role of cyclic AMP in the PCPA-induced up-regulation of TPH mRNA remains to be determined, it is possible that the reduction in extracellular 5-HT concentrations induced by PCPA decreases the response of 5-HT receptor subtypes negatively coupled to the adenylyl cyclase.

Tryptophan hydroxylase may not only be subject to transcriptional control. The tryptophan hydroxylase gene has multiple transcripts (Delort et al., 1989; Dumas et al., 1989). These transcripts differ in the number of a consensus degradation site (AUUUA) in the 3' untranslated region that is typical of mRNAs that are targeted for rapid turnover (Malter, 1989). These observations suggest that tryptophan hydroxylase may be subject to several complex control mechanisms. Changes in the steady-state levels of tryptophan hydroxylase mRNA may be due to a reduction in the turnover of the mRNA rather than an elevation of gene transcription. Further experiments are necessary to determine which mechanism(s) applies.

The time course of changes in levels of tryptophan hydroxylase mRNA differed from changes in the levels of 5-HT transporter mRNA after PCPA treatment. The levels of 5-HT transporter mRNA first decreased in the DRv and then increased in both the DRv and the DRd. The time course of the decrease was similar to the time course and extent of the decrease in content of

TABLE 3. Effect of PCPA (300 mg/kg *i.p.*) on [³H]citalopram binding to synaptosomes from terminal regions

	Cortex		Hippocampus		Striatum	
	<i>K_D</i>	<i>B_{max}</i>	<i>K_D</i>	<i>B_{max}</i>	<i>K_D</i>	<i>B_{max}</i>
Control	1.02 ± 0.11	239 ± 12	0.7 ± 0.04	180 ± 4	0.76 ± 0.13	201 ± 13
PCPA (2 days)	1.14 ± 0.08	263 ± 3	0.97 ± 0.17	213 ± 18	0.78 ± 0.09	222 ± 12
Control	1.01 ± 0.17	218 ± 9	0.71 ± 0.08	194 ± 9	0.71 ± 0.08	228 ± 9
PCPA (7 days)	1.08 ± 0.11	219 ± 13	0.72 ± 0.07	189 ± 7	0.72 ± 0.07	204 ± 9
Control	0.94 ± 0.14	292 ± 23	0.79 ± 0.06	261 ± 11	0.82 ± 0.03	233 ± 5
PCPA (14 days)	0.66 ± 0.03	230 ± 5 ^a	0.69 ± 0.04	209 ± 6 ^a	0.64 ± 0.03 ^a	209 ± 7 ^a

Saturation curves of [³H]citalopram binding (four concentrations in triplicate) were measured on crude synaptosomal membranes obtained after pooling the brain areas of five rats per group. The *K_D* (in nM) and *B_{max}* (in fmol/mg of protein) data are means ± SE values obtained by analyzing the saturation curves with the LIGAND program.

^a *p* < 0.05, as assessed by the *F* test (see Materials and Methods).

TABLE 4. Effect of PCPA (300 mg/kg i.p.) on [³H]5-HT uptake into synaptosomes from terminal regions

	Cortex		Hippocampus		Striatum	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Control	27.3 ± 2.2	995 ± 40	20.7 ± 6.8	884 ± 115	10.2 ± 0.8	749 ± 15
PCPA (2 days)	18.5 ± 5.9	883 ± 106	19.3 ± 2.1	950 ± 88	13.0 ± 2.5	860 ± 52
Control	24.1 ± 0.5	626 ± 6	13.0 ± 1.3	515 ± 16	12.6 ± 0.3	502 ± 5
PCPA (7 days)	18.8 ± 0.7	647 ± 7	15.5 ± 2.5	576 ± 35	10.0 ± 0.1	547 ± 5
Control	22.6 ± 1.6	1063 ± 32	19.5 ± 2.9	1040 ± 62	19.5 ± 4.1	802 ± 64
PCPA (14 days)	16.7 ± 2.5	851 ± 42 ^a	15.4 ± 0.9	749 ± 15 ^a	20.4 ± 4.1	978 ± 78

Saturation curves of [³H]5-HT uptake (four concentrations in triplicate) were measured on synaptosomes obtained after pooling the brain areas of five rats per group. The K_m (in nM) and the V_{max} (in fmol/min/g of protein) data are mean ± SE values obtained by analyzing the saturation curves with the LIGAND program.

^a $p < 0.05$, as assessed by the *F*-test (see Materials and Methods).

5-HT in the striatum, in that there was a rapid decrease within 2 days after PCPA administration followed by a recovery at 7 days posttreatment. However, whereas 5-HT levels recovered partially 7 days after PCPA administration, the levels of 5-HT transporter mRNA in cell bodies were higher than control levels. Our results agree with a recent report by Linnet et al. (1995), who showed, using northern blotting, that PCPA decreased 5-HT transporter mRNA levels. The observation that tryptophan hydroxylase and 5-HT transporter mRNAs are differentially regulated confirms our previous findings using a subchronic treatment with *d*-fenfluramine that produced a marked depletion of 5-HT levels in forebrain regions. In that case, there was a down-regulation of 5-HT transporter mRNA in the DRv, but not the DRd, and an up-regulation in tryptophan hydroxylase mRNA in all subdivisions of the dorsal raphe nucleus (Bendotti et al., 1993; Rattray et al., 1994).

The changes in expression of 5-HT transporter mRNA observed in the present study are difficult to reconcile with other studies. Previous studies of changes in 5-HT transporter mRNA levels have used drugs that bind to the 5-HT transporter. Most of these studies, with some exceptions (Burnet et al., 1994; Spurlock et al., 1994), strongly suggest that drugs that inhibit or enhance 5-HT uptake lead to a down-regulation of 5-HT transporter mRNA levels (Lesch et al., 1993; Rattray et al., 1993, 1994; Kuroda et al., 1994; Lopez et al., 1994). Because PCPA does not directly interact with the 5-HT transporter, our studies suggest that reduction of 5-HT levels produced by PCPA or *d*-fenfluramine activates a mechanism that reduces 5-HT transporter mRNA levels. As the neurons recover their 5-HT and tryptophan hydroxylase contents, other factors may intervene to produce a net elevation of 5-HT transporter mRNA content. The nature of these factors is unknown but may be related to intracellular 5-HT content or rate of turnover of the 5-HT transporter protein. Whether changes in 5-HT transporter protein result from changes in the rate of transcription

or the rate of degradation of the 5-HT transporter mRNA is not yet known.

Whatever the mechanisms involved, the reduction of 5-HT transporter mRNA content does not seem temporally related to functional changes of the protein. This differs from tryptophan hydroxylase, where up-regulation in mRNA levels precedes similar changes in the levels and activity of active tryptophan hydroxylase protein (Richard et al., 1990; Weissmann et al., 1990; Cortés et al., 1993; Park et al., 1994). At times when PCPA significantly reduced or increased 5-HT transporter mRNA levels, no changes in either [³H]-citalopram binding and [³H]5-HT uptake occurred in forebrain regions, whereas 14 days after drug injection both parameters were lowered by 20% in the cortex and hippocampus. The striatum appeared to be less sensitive to the late (14 days) modifications induced by PCPA treatment. We cannot exclude that larger changes may occur in the striatum at time points not measured in this study. Changes in the level of striatal 5-HT transporter are especially relevant in the present study because it receives a large projection from the dorsal raphe. The observation that a decrease in 5-HT transporter mRNA content at 1 and 2 days after PCPA administration is followed by a decrease in amount of functional protein in forebrain regions after 14 days suggests that the 5-HT transporter may have a low turnover rate and may be slowly transported from the cell bodies to the terminals. It is more difficult to relate the lack of changes of [³H]citalopram binding in the raphe nuclei at any times after PCPA administration.

In general, our results with the [³H]citalopram binding and 5-HT uptake confirm and extend a previous study showing that PCPA administration produced no changes in [³H]paroxetine binding in different brain regions (Dewar et al., 1992) 24 h after the injection of PCPA. Several studies have demonstrated that the levels of 5-HT transporter protein, as determined by citalopram or paroxetine binding, can be altered in the brain by various treatments (Battaglia et al., 1991; Brunswick et al., 1992; Hrdina and Vu, 1993; Kovach-

ich et al., 1992; Kuroda et al., 1994); however, 5-HT transporter mRNA was not examined in these studies. Our study is the first to evaluate in the same experimental conditions the levels of the 5-HT transporter mRNA in 5-HT neurons and the functional expression of the protein in terminal areas.

Another interesting observation that extends our earlier findings using treatment with *d*-fenfluramine or MDMA (Bendotti et al., 1993; Rattray et al., 1993, 1994) is the different sensitivity of the neurons lying in the DRv in comparison with 5-HT neurons in the DRd. The response of 5-HT transporter and tryptophan hydroxylase mRNA to PCPA administration differed depending on the location of the cell bodies. For both genes, cells that were found in the DRv exhibited greater sensitivity to PCPA. This difference may be accounted for by the presence of 5-HT neurons that are relatively insensitive to PCPA and neurons that are sensitive to PCPA. Tohyama et al. (1988) in an anatomical study have noted that 5-HT nerve fibers differ in their sensitivities to PCPA administration. The basis of this phenomenon is unknown but may relate to subtle biochemical differences among 5-HT neurons.

Conclusions

We showed for the first time that a drug, PCPA, that does not directly interact with the 5-HT transporter leads to changes in the expression of its mRNA that are not temporally related with changes in levels of the expressed protein. The mechanisms by which this occurs are unknown, but are unlikely to be the same that influence the expression of the tryptophan hydroxylase gene in the same neurons. These results confirm our previous findings that 5-HT neurons in different subregions of the dorsal raphe nucleus differ in their sensitivity to drugs.

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REFERENCES

- Åberg-Wigstedt A. (1989) The antidepressant effects of 5-HT uptake inhibitors. *Br. J. Psychiatry* **155** (Suppl. 8), 32–40.
- Appel N. M., Contrera J. F., and De Souza E. B. (1989) Fenfluramine selectively and differentially decreases the density of serotonergic nerve terminals in rat brain: evidence from immunocytochemical studies. *J. Pharmacol. Exp. Ther.* **249**, 928–943.
- Appel N. M., Mitchell W. M., Contrera J. F., and De Souza E. B. (1990) Effects of high dose fenfluramine treatment on monoamine uptake sites in rat brain: assessment using quantitative autoradiography. *Synapse* **6**, 33–44.
- Battaglia G., Yeh S. Y., O'Hearn E., Molliver M. E., Kuhar M. J., and De Souza E. B. (1987) 3,4-Methylenedioxyamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin terminals in rat brain: quantification of neurodegeneration by measurement of [³H]paroxetine-labeled serotonin uptake sites. *J. Pharmacol. Exp. Ther.* **242**, 911–916.
- Battaglia G., Sharkey J., Kuhar M. J., and de Souza E. B. (1991) Neuroanatomic specificity and time course of alterations in rat brain serotonergic pathways induced by MDMA (3,4-methylenedioxyamphetamine): assessment using quantitative autoradiography. *Synapse* **8**, 249–260.
- Bendotti C., Servadio A., Forloni G., Angeretti N., and Samanin R. (1990) Increased tryptophan hydroxylase mRNA in raphe serotonergic neurons spared by 5,7-dihydroxytryptamine. *Mol. Brain Res.* **8**, 343–348.
- Bendotti C., Baldessari S., Ehret M., Tarizzo G., and Samanin R. (1993) Effect of *d*-fenfluramine and 5,7-dihydroxytryptamine on the levels of tryptophan hydroxylase and its mRNA in rat brain. *Mol. Brain Res.* **19**, 257–261.
- Blakely R. D., Berson H. E., Fremeau R. T. Jr., Caron M. G., Peck M. M., Prince H. K., and Bradley C. C. (1991) Cloning and expression of a functional serotonin transporter from rat brain. *Nature* **354**, 66–70.
- Boularand S., Darmon M. C., Ravassard P., and Mallet J. (1995) Characterization of the human tryptophan hydroxylase gene promoter. Transcriptional regulation by cAMP requires a new motif distinct from the cAMP-responsive element. *J. Biol. Chem.* **270**, 3757–3764.
- Brunswick D. J., Benmansour S., Tejani-Butt S. M., and Hauptmann M. (1992) Effects of high-dose methamphetamine on monoamine uptake sites in rat brain measured by quantitative autoradiography. *Synapse* **11**, 287–293.
- Burnet P. W. J., Michelson D., Smith M. A., Gold P. W., and Sternberg E. M. (1994) The effect of chronic imipramine administration on the densities of 5-HT_{1A} and 5-HT₂ receptors and the abundances of 5-HT receptor and transporter mRNA in the cortex, hippocampus and dorsal raphe of three strains of rat. *Brain Res.* **638**, 311–324.
- Chaput Y., Lesieur P., and de Montigny C. (1990) Effects of short-term, serotonin depletion on the efficacy of serotonin neurotransmission: electrophysiological studies in the rat central nervous system. *Synapse* **6**, 328–337.
- Chen L., Hamaguchi K., Ogawa M., Hamada S., and Okada N. (1994) PCPA reduces both monoaminergic afferents and nonmonoaminergic synapses in the cerebral cortex. *Neurosci. Res.* **19**, 111–115.
- Cortés R., Mengod G., Celada P., and Artigas G. (1993) *p*-Chlorophenylalanine increases tryptophan-5-hydroxylase mRNA levels in the rat dorsal raphe: a time course study using in situ hybridization. *J. Neurochem.* **60**, 761–764.
- D'Amato R. J., Largent B. L., Snowman A. M., and Snyder S. H. (1987) Selective labeling of serotonin uptake sites in rat brain by [³H]-citalopram contrasted to labeling of multiple sites by [³H]-imipramine. *J. Pharmacol. Exp. Ther.* **242**, 364–371.
- Darmon M. C., Guibert B., Levieil V., Ehret M., Maître M., and Mallet J. (1988) Sequence of two mRNAs encoding active rat tryptophan hydroxylase. *J. Neurochem.* **51**, 312–316.
- Delort J., Dumas J. B., Darmon M. C., and Mallet J. (1989) An efficient strategy for cloning 5' extremities of rare transcripts permits isolation of multiple 5'-untranslated regions of rat tryptophan hydroxylase mRNA. *Nucleic Acids Res.* **17**, 6439–6448.
- Dewar K. M., Grondin L., Carli M., Lima L., and Reader T. A. (1992) [³H]Paroxetine binding and serotonin content of rat cortical areas, hippocampus, neostriatum, ventral mesencephalic tegmentum, and midbrain raphe nuclei following *p*-chlorophenylalanine and *p*-chloroamphetamine treatment. *J. Neurochem.* **58**, 250–257.
- Dumas S., Darmon M. C., Delort J., and Mallet J. (1989) Differential control of tryptophan hydroxylase expression in raphe and in pineal gland: evidence for a role of translation efficiency. *J. Neurosci. Res.* **24**, 71–76.
- Ehret M., Pevet P., and Maître M. (1991) Tryptophan hydroxylase synthesis is induced by 3',5'-cyclic adenosine monophosphate during circadian rhythm in the rat pineal gland. *J. Neurochem.* **57**, 1516–1521.
- Fujita M., Shimada S., Maeno H., Nishimura T., and Tohyama M.

- (1993) Cellular localisation of serotonin transporter messenger RNA in the rat brain. *Neurosci. Lett.* **162**, 59–62.
- Garattini S., Mennini T., Bendotti C., Invernizzi R., and Samanin R. (1986) Neurochemical mechanism of action of drugs which modify feeding via the serotonergic system. *Appetite* **7**, 15–38.
- Gobbi M., Frittoli E., Mennini T., and Garattini S. (1992) Releasing activities of *d*-fenfluramine and fluoxetine on rat hippocampal synaptosomes preloaded with [³H]serotonin. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **345**, 1–6.
- Gobbi M., Regondi M. C., Pompeiano M., Palacios J. M., and Mennini T. (1994) Differential effects of 5,7-dihydroxytryptamine-induced serotonergic degeneration on 5-HT_{1A} receptors and 5-HT uptake sites in the rat brain. *J. Chem. Neuroanat.* **7**, 65–73.
- Gray E. G. and Whittaker V. P. (1962) The isolation of nerve endings from brain: an electron-microscope study of cell fragments derived by homogenization and centrifugation. *J. Anat.* **96**, 79–87.
- Hoffman B. J., Mezey E., and Brownstein M. J. (1991) Cloning of a serotonin transporter affected by antidepressants. *Science* **254**, 579–580.
- Hrdina P. D. and Vu T. B. (1993) Chronic fluoxetine treatment upregulates 5-HT uptake sites and 5-HT receptors in rat brain: an autoradiographic study. *Synapse* **14**, 324–331.
- Invernizzi R., Fracasso C., Caccia S., Di Clemente A., Garattini S., and Samanin R. (1989) Effect of L-cysteine on the long term depletion of brain indoles caused by *p*-chloroamphetamine and *d*-fenfluramine in rats. Relation to drug concentrations. *Eur. J. Pharmacol.* **163**, 77–83.
- Jéquier E., Lovenberg W., and Sjoerdsma A. (1967) Tryptophan hydroxylase inhibition: the mechanism by which *p*-chlorophenylalanine depletes rat brain serotonin. *Mol. Pharmacol.* **3**, 274–278.
- Kim K. S., Wessel T. C., Stone D. M., Carver C. H., Joh T. H., and Park D. H. (1991) Molecular cloning and characterisation of cDNA encoding tryptophan hydroxylase from rat central serotonergic system. *Mol. Brain Res.* **9**, 277–283.
- Koe B. K. and Weissman A. (1968) The pharmacology of *para*-chlorophenylalanine, a selective depletor of serotonin stores, in *Advances in Pharmacology, Vol. 6, Part B* (Garattini S. and Shore P. A., eds), pp. 29–48. Academic Press, San Diego.
- Kovachich G. B., Aronson C. E., and Brunswick D. J. (1992) Effect of repeated administration of antidepressants on serotonin uptake sites in limbic and neocortical structures of rat brain determined by quantitative autoradiography. *Neuropsychopharmacology* **7**, 317–324.
- Kuroda Y., Watanabe Y., and McEwen B. S. (1994) Tianeptine decreases both serotonin transporter messenger RNA and binding sites in rat brain. *Eur. J. Pharmacol. Mol. Pharmacol.* **268**, R3–R5.
- Lesch K. P., Aulakh C. S., Wolozin B. L., Tolliver T. J., Hill J. L., and Murphy D. L. (1993) Regional brain expression of serotonin transporter mRNA and its regulation by reuptake inhibiting antidepressants. *Mol. Brain Res.* **17**, 31–35.
- Linnet K., Koed K., Wiborg O., and Gregersen N. (1995) Serotonin depletion decreases serotonin transporter mRNA levels in rat brain. *Brain Res.* **697**, 251–253.
- Lopez J. F., Chalmers D. T., Vazquez D. M., Watson S. J., and Akil H. (1994) Serotonin transporter mRNA in rat brain is regulated by classical antidepressants. *Biol. Psychiatry* **35**, 287–290.
- Malter J. S. (1989) Identification of an AUUUA-specific messenger RNA binding protein. *Science* **246**, 664–666.
- Masseroli M., Bollea A., Bendotti C., and Forloni G. (1993) In situ hybridisation histochemistry quantification: automatic count on single cells in a digital image. *J. Neurosci. Methods* **47**, 93–103.
- Mennini T., Garattini S., and Caccia S. (1985) Anorectic effect of fenfluramine isomers and metabolites: relationship between brain levels and in vitro potencies on serotonergic mechanisms. *Psychopharmacology (Berlin)* **85**, 111–114.
- Miller F. P., Cox R. H. Jr., Snodgrass W. R., and Maickel R. P. (1970) Comparative effects of *p*-chlorophenylalanine, *p*-chloroamphetamine and *p*-chloro-*N*-methylamphetamine on rat brain norepinephrine, serotonin and 5-hydroxyindole-3 acetic acid. *Biochem. Pharmacol.* **19**, 435–442.
- Munson P. J. and Rodbard D. (1984) Computerized analysis of ligand binding data: basic principles and recent development. in *Computers in Endocrinology* (Rodbard D. and Forti G., eds), pp. 117–145. Raven Press, New York.
- Park D. H., Stone D. M., Baker H., Kim K. S., and Joh T. H. (1994) Early induction of rat brain tryptophan hydroxylase (TPH) mRNA following parachlorophenylalanine (PCPA) treatment. *Mol. Brain Res.* **22**, 20–28.
- Paxinos G. and Watson C. (1982) *The Rat Brain in Stereotaxic Coordinates*. Academic Press, Sydney.
- Ratray M., Wotherspoon G., Savery D., Marden C., Lopez Costa J. J., Bendotti C., and Priestley J. V. (1993) Fenfluramine and MDMA down-regulate serotonin transporter messenger RNA in subdivisions of the rat dorsal raphe complex. *Soc. Neurosci. Abstr.* **19**, 495.
- Ratray M., Wotherspoon G., Savery D., Baldessari S., Marden C., Priestley J. V., and Bendotti C. (1994) Chronic *d*-fenfluramine decreases serotonin transporter messenger RNA expression in dorsal raphe nucleus. *Eur. J. Pharmacol. Mol. Pharmacol.* **268**, 439–442.
- Richard F., Sanne J. L., Bourde O., Weissmann D., Ehret M., Cash C., Maitre M., and Pujol J. F. (1990) Variation of tryptophan-5-hydroxylase concentration in the rat raphe dorsalis nucleus after *p*-chlorophenylalanine administration. I. A model to study the turnover of the enzymatic protein. *Brain Res.* **536**, 41–45.
- Rudnick G. and Wall S. C. (1992) The molecular mechanism of "ecstasy" 3,4-methylenedioxymethamphetamine (MDMA): serotonin transporters are targets for MDMA-induced serotonin release. *Proc. Natl. Acad. Sci. USA* **89**, 1817–1821.
- Schuster C. R., Lewis M., and Seiden L. S. (1986) Fenfluramine: neurotoxicity. *Psychopharmacol. Bull.* **22**, 148–151.
- Spurlock G., Buckland P., O'Donovan M., and McGuffin P. (1994) Lack of effect of antidepressant drugs on the levels of mRNAs encoding serotonergic receptors, synthetic enzymes and 5HT transporter. *Neuropharmacology* **33**, 433–440.
- Tohyama I., Kameyama M., and Kimura H. (1988) Quantitative morphometric analysis of two types of serotonin-immunoreactive nerve fibres differentially responsive to *p*-chlorophenylalanine treatment in the rat brain. *Neuroscience* **26**, 971–991.
- Weissmann D., Chamba G., Debure L., Rousset C., Richard F., Maitre M., and Pujol J. F. (1990) Variation of tryptophan 5-hydroxylase concentrations in the rat dorsalis nucleus after *p*-chlorophenylalanine administration. II. Anatomical distribution of the tryptophan-5-hydroxylase protein and regional variation of its turnover rate. *Brain Res.* **536**, 46–55.
- Zaczek R., Battaglia G., Culp S., Appel N. M., Contrera J. F., and De Souza E. B. (1990) Effects of repeated fenfluramine administration on indices of monoamine function in rat brain: pharmacokinetic, dose response, regional specificity and time course data. *J. Pharmacol. Exp. Ther.* **253**, 104–112.