

EFFECT OF CHRONIC D-FENFLURAMINE ADMINISTRATION ON RAT HYPOTHALAMIC SEROTONIN LEVELS AND RELEASE

Judith D. Schaechter and Richard J. Wurtman

Department of Brain and Cognitive Sciences
Massachusetts Institute of Technology, Cambridge, MA

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Summary

D-fenfluramine, an anorectic agent in rats and man, was administered daily at doses 1.25, 2.5, 5 or 10 mg/kg/day for 10 days, and sacrificed 6 days later. Hypothalamic serotonin (5-HT) levels were unchanged in rats receiving 1.25-5 mg/kg/day of d-fenfluramine but reduced by 22% ($p < 0.01$) at the highest drug dose (10 mg/kg/day); hypothalamic 5-hydroxyindole acetic acid (5-HIAA) levels were reduced by 15% ($p < 0.05$) or 28% ($p < 0.01$) in rats receiving 5 or 10 mg/kg/day of the drug, respectively. Hypothalamic slices prepared from rats which were previously treated with any of the drug doses spontaneously released endogenous 5-HT at rates that did not differ from those of vehicle-treated rats. 5-HT released with electrical field-stimulation was unaffected by prior d-fenfluramine treatment at doses of 1.25-5 mg/kg/day, and was reduced by 20% ($p < 0.05$) from slices prepared from rats which received 10 mg/kg/day. 5-HIAA efflux was also attenuated by the highest drug dose. These data indicate that chronic administration to rats of customary anorectic doses of d-fenfluramine (i.e. 0.06-1.25 mg/kg) fail to cause long-lasting reductions in brain 5-HT release.

Racemic dl-fenfluramine (1, 2) and, more recently, d-fenfluramine (3, 4) have been shown to be clinically effective in reducing appetite and facilitating weight loss. The anorexigenic effect of d-fenfluramine, which accounts for the action of the racemic mixture (3, 5), probably results from an enhancement in serotonin-mediated brain neurotransmission since the drug both potentiates 5-HT release from nerve terminals and inhibits its inactivation by reuptake (6).

Numerous studies have described long-lasting depletions in brain 5-HT levels when a high dose of dl-fenfluramine (15-25 mg/kg) (7-10) or d-fenfluramine (6 mg/kg/day) (11) was administered to rats. However, these doses produce elevations in plasma and brain fenfluramine concentrations (12, 13) which far exceed those needed to reduce food intake in deprived rats (ED_{50} for d-fenfluramine = 1.3 mg/kg; for l-fenfluramine = 3.0 mg/kg) (13). Lower dose(s) of d-fenfluramine administered to rats either acutely (1.25-2.5 mg/kg) (14) or chronically (3 mg/kg/day for 6 days) (11) have not been reported to deplete brain 5-HT levels. Apparently, no data are available on possible effects of chronic d-fenfluramine treatment on the ability of serotonergic neurons to release their neurotransmitter. The study that follows has addressed this relationship using a superfused, electrically-stimulated, rat hypothalamic slice preparation.

Methods

Male Sprague-Dawley rats (130-170 g) were paired according to body weight. Each pair was housed together (lights on 0700-1900) and provided with rat chow (Charles River Rat, Mouse and Hamster Formula, 26.3% protein) and tap water ad libitum. Each rat received a single daily intraperitoneal injection (between 1200-1300), for 10 days, of either d-fenfluramine hydrochloride (provided by Servier Laboratories, Neuilly-Sur-Seine, France) dissolved in 0.9% NaCl (1.25, 2.5, 5 or 10 mg/kg) or its vehicle, in a volume of 2.5 ml/kg. The rats continued to be housed in pairs until day 16 at which time they were sacrificed by decapitation (between 1000-1100). Each brain was rapidly removed from the skull and immersed in ice-cold Krebs-bicarbonate buffer which had been previously gassed with 95% O₂/5% CO₂.

Hypothalamic slices (0.3 x 3 x 3 mm) were prepared using a McIlwain tissue slicer. Some hypothalamic tissue (approximately every fourth slice in the rostro-caudal direction) was retained, promptly frozen over dry ice and stored at -70°C until subsequent biochemical assay. The slices (20-30 from each hypothalamus; 3-4 mg protein) from the fenfluramine- and the vehicle-treated rats were transferred, within 10 minutes of decapitation, into parallel superfusion chambers (0.75 ml) and equilibrated for 50 minutes at 37°C. The physiologic medium superfusing the slices (0.6 ml/min) was of the following composition (in mM): NaCl 130; KCl 3.5; CaCl₂ 1.3; MgSO₄ 1.5; NaH₂PO₄ 1; NaHCO₃ 25; d-glucose 10; and 0.002 fluoxetine hydrochloride (provided by Eli Lilly Laboratories, Indianapolis, IN) to block the reuptake of synaptic 5-HT (15). The medium was continuously gassed with 95% O₂/5% CO₂ to maintain pH 7.3-7.4. From 50 to 130 minutes, five minute fractions were collected in 100 µl of 7 mM ascorbate containing two internal standards: 5-hydroxy-N-methyltryptamine oxalate (5-HT-CH₃) and 5-hydroxy-2-indolecarboxylic acid (5-HICA) (both purchased from Aldrich Chemical Co., Milwaukee, WI). Once collected, each superfusate fraction was vortexed and stored in the dark and on ice until further processing. The slices were electrically field-stimulated (5 Hz, 2 ms, 100 mA/cm², 1400 bipolar pulses) for three periods during the experiment, at 60, 85 and 110 minutes. At the end of the experiment, at 130 minutes, the slices were removed from the chamber, rinsed with distilled water and frozen at -70°C.

Each fraction of superfused medium was passed through a preparative column to concentrate its contents of 5-HT and 5-HIAA. These columns were prepared by loading 100 mg of dry C₁₈-reverse phase sorbent (40 µM; Analytichem International, Harbor City, CA) into glass wool-plugged pasteur pipettes (9"). The columns were conditioned with 1.5 ml of methanol followed by 0.75 ml of 0.1 M NaH₂PO₄ (pH 3.0). Each sample fraction (32 x 3.1 ml) and standard fractions prepared with known amounts of 5-HT and 5-HIAA were brought to pH 2.8-3.0 before passage through its column. The aqueous phase in the column was displaced by 125 µl of 70% methanol/30% acetic acid, and the 5-hydroxyindoles were then eluted with 300 µl of this solution. The solvent was evaporated under nitrogen. The dried eluents were reconstituted with 50 µl of 0.15 N HCl containing 0.25 mM ascorbate. Recoveries of 5-HT and 5-HIAA were generally 85-95% based on calculations using the internal standards 5-HT-CH₃ and 5-HICA, respectively.

The release of 5-HT, spontaneous and electrically-evoked, and of 5-HIAA were assayed by high performance liquid chromatography with electrochemical detection. Samples (45 µl of 50 µl) were automatically injected (Waters Intelligent Systems Program; Milford, MA) over a reverse-phase C₁₈ column (5 µm, 25 cm; Beckman Instruments, San Ramon, CA). The mobile phase was of the following composition: 0.2 M NaH₂PO₄; 0.1 mM Na₂EDTA; 0.17 mM octyl sodium sulfate; 13% methanol; pH 4.3. The substances were detected at 2 nA/V when the potential of the glassy carbon electrode was set at 0.55 V against the Ag/AgCl reference electrode.

Tissue levels of tryptophan, 5-HT and 5-HIAA were determined using this chromatographic system, though the applied potential was set at 0.85 V and the sensitivity at 5 nA/V. Frozen tissue samples were sonicated in 0.2 N HClO₄, containing 0.5 mM ascorbate and internal standards (approximately 0.4 ml/mg protein), and centrifuged (17,000 rpm, 10 min). An aliquot of this supernatant was injected over the reverse-phase column. The amounts of indole measured in each sample, superfused medium and tissue supernatant, were normalized by the amount of protein in the tissue pellets, as determined by the Lowry method (16).

The amounts of 5-HT released from the slices, under basal conditions and with electrical field-stimulation, were calculated as the average rate (fmol/mg protein/min) during the four rest periods and the three periods of electrical stimulation, respectively. The rate of 5-HIAA efflux was taken as the average across the 80 minute (16 fraction) collection period. Results were evaluated by the Student's paired t-test. Values are reported here as means \pm the standard error of the mean (s.e.m.).

Results

Hypothalamic contents of tryptophan, 5-HT and 5-HIAA in vehicle-treated rats were 194 \pm 9, 81 \pm 3 and 51 \pm 2 pmol/mg protein, respectively. The levels of 5-HT in the hypothalamus of rats treated with d-fenfluramine for 10 days at a dose of 1.25, 2.5 or 5 mg/kg/day were not different, 6 days after the last injection, from those of vehicle-treated rats (Table I). Only those rats which received the highest dose of d-fenfluramine, 10 mg/kg/day, showed a significant decrease in

TABLE I

Effect of chronic d-fenfluramine administration on hypothalamic indole levels

	d-fenfluramine dose (mg/kg/day)			
	1.25	2.5	5.0	10.0
TRYPTOPHAN				
vehicle	182.1 \pm 19.0	196.9 \pm 29.6	185.8 \pm 17.1	207.7 \pm 12.4
drug	207.7 \pm 19.9*	205.4 \pm 24.2	184.8 \pm 14.3	226.7 \pm 19.2
%	115.4 \pm 4.4	107.6 \pm 7.2	101.7 \pm 8.0	108.8 \pm 5.3
5-HT				
vehicle	78.9 \pm 4.7	75.7 \pm 7.8	85.2 \pm 6.8	83.7 \pm 5.1
drug	78.1 \pm 6.7	72.3 \pm 5.9	72.2 \pm 5.3	65.8 \pm 5.5**
%	98.3 \pm 5.5	97.2 \pm 6.9	86.0 \pm 6.4	78.4 \pm 3.8
5-HIAA				
vehicle	45.8 \pm 3.6	46.6 \pm 4.2	51.2 \pm 5.1	57.9 \pm 3.6
drug	47.7 \pm 4.0	49.7 \pm 4.8	42.8 \pm 3.1*	41.3 \pm 3.3**
%	103.9 \pm 3.6	107.1 \pm 6.3	85.1 \pm 5.0	71.6 \pm 4.8

Rats received d-fenfluramine dissolved in 0.9% NaCl (drug) or 0.9% NaCl (vehicle) for 10 days; tissues were taken 16 days from the onset of treatment. Values are given as group means \pm s.e.m. for N = 5-8 pairs; levels are in pmol/mg protein. * p<0.05, ** p<0.01 differs from vehicle-treated group by Student's paired t-test.

hypothalamic content of 5-HT, to $78.4 \pm 3.8\%$ of control. Hypothalamic levels of the major 5-HT metabolite, 5-HIAA, were unchanged at the two lower doses, 1.25 and 2.5 mg/kg/day; higher doses of d-fenfluramine, 5 or 10 mg/kg/day, lowered 5-HIAA levels to $85.1 \pm 5.0\%$ and $71.6 \pm 4.8\%$ of those in vehicle-treated rats, respectively. Tryptophan levels in the hypothalamus were elevated by administration of 1.25 mg/kg/day of d-fenfluramine, but this effect was not detected at the higher doses.

Hypothalamic slices prepared from vehicle-treated rats spontaneously released 5-HT at a rate of 54 ± 4 fmol/mg protein/min (Figure 1A). Exposing the slices to electrical field-stimulation increased 5-HT release to 281 ± 13 fmol/mg protein/min for a period of 10 minutes, after which there was a return to the basal rate. The efflux of 5-HIAA was 634 ± 23 fmol/mg protein/min over the 80 minute collection period (Figure 1B).

The amount of 5-HT released spontaneously from the hypothalamic slices was unaltered by d-fenfluramine pretreatment at any dose tested (Table II). D-fenfluramine pretreatment at a dose of 1.25-5 mg/kg/day produced no change in 5-HT release elicited by electrical field-stimulation. Slices prepared from rats which received 10 mg/kg/day reduced their rate of electrically-evoked 5-HT release to $80 \pm 5\%$ of control; their efflux of 5-HIAA was decreased to $63 \pm 4\%$. Thus, there were parallel long-lasting effects, or lack thereof, of chronic d-fenfluramine administration on hypothalamic 5-HT content, evoked 5-HT release, and 5-HIAA efflux, with significant (and proportionate) reductions occurring only at the highest dose (Figure 2). Basal 5-HT release did not exhibit this relationship to hypothalamic 5-HT levels.

TABLE II

Effect of chronic d-fenfluramine administration on *ex vivo* release of 5-hydroxyindoles from the rat hypothalamus

	d-fenfluramine dose (mg/kg/day)			
	<u>1.25</u>	<u>2.5</u>	<u>5.0</u>	<u>10.0</u>
Basal 5-HT				
vehicle	55±6	50±8	57±4	55±6
drug	49±8	51±5	53±3	54±8
%	87±6	108±10	93±4	100±15
Evoked 5-HT				
vehicle	285±30	239±25	290±30	301±28
drug	274±30	261±30	267±34	241±26 *
%	96±4	110±6	92±8	80±5
5-HIAA				
vehicle	609±56	535±57	670±101	781±60
drug	586±52	650±84	588±83	486±35 **
%	97±4	123±11	90±11	63±4

Rats received d-fenfluramine dissolved in 0.9% NaCl (drug) or 0.9% NaCl (vehicle) for 10 days; hypothalamic slices were prepared 16 days from the onset of treatment. Values are given as group means \pm s.e.m. for N = 6-7 pairs; amounts are expressed as fmol/mg protein/min. * $p < 0.05$, ** $p < 0.01$ differs from vehicle-treated group by Student's paired t-test.

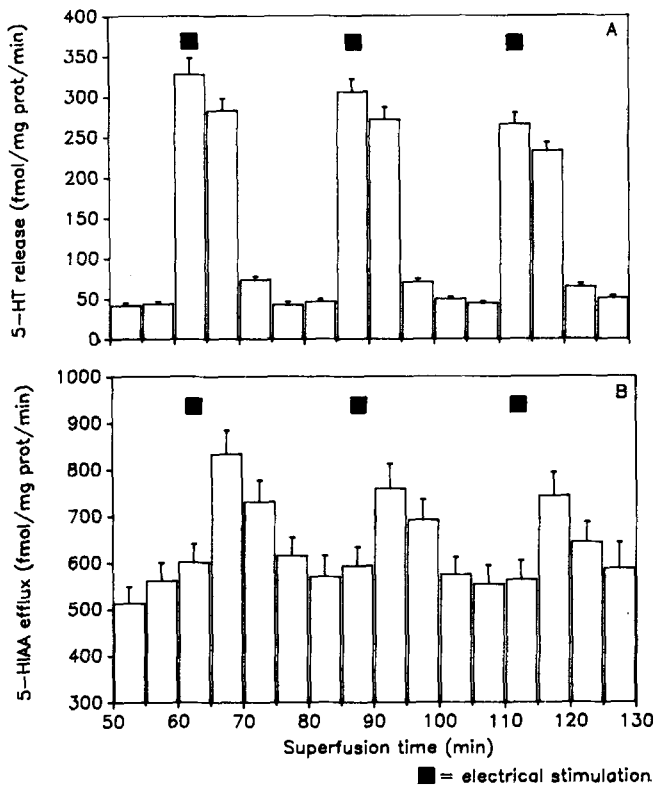


FIG. 1

Time-course of 5-HT release (A) and 5-HIAA efflux (B) from hypothalamic slices prepared from control rats. Rats were given daily intraperitoneal injections of saline for 10 days. On day 16 following the onset of treatment, slices were prepared and equilibrated in physiologic medium for 50 minutes; 5 minute fractions were collected during the subsequent 80 minutes. The slices were electrically field-stimulated for 3 periods. The amounts of 5-HT and 5-HIAA (fmol/mg protein/min) released into the medium were monitored. N = 24.

Discussion

These results indicate that chronic administration of d-fenfluramine to rats, even in doses of 5 mg/kg/day, which are considerably higher than those needed to produce anorexia (17), does not cause long-lasting decreases in hypothalamic 5-HT levels (that is an effect which persists beyond the period of drug administration for at least 6 days). This treatment also has no effect on the ability of hypothalamic nerve terminals to release endogenous 5-HT, basally or during membrane depolarization. Repeated injections of even higher doses (10 mg/kg/day) can reduce hypothalamic 5-HT content (by $21.6 \pm 3.8\%$), the evoked release of 5-HT (by $20 \pm 5\%$) and the efflux of 5-HIAA (by $37 \pm 4\%$). However, basal 5-HT release remains unaltered even after such doses, a finding in accord with the demonstration by Mennini that basal ^3H -5-HT release from cortical synaptosomes prepared from rats chronically treated (for 14 or 28 days) with d-fenfluramine was unaffected, but the release evoked by *in vitro* challenge with d-fenfluramine was reduced (18).

The dose of d-fenfluramine that we found to cause a long-lasting reduction in hypothalamic 5-HT levels and release, 10 mg/kg/day, is much greater than doses used to inhibit food intake in a variety of test paradigms, 0.06-1.25 mg/kg (17). Caccia et. al. has shown that the concentration of d-fenfluramine reaching the brain is nonlinearly related to dose, such that an 8-fold increase in dosage (i.e. from a potent anorectic dose of 1.25 mg/kg to an excessive dose of 10 mg/kg) causes a greater than 30-fold increase in the area under the curve relating brain d-fenfluramine levels to

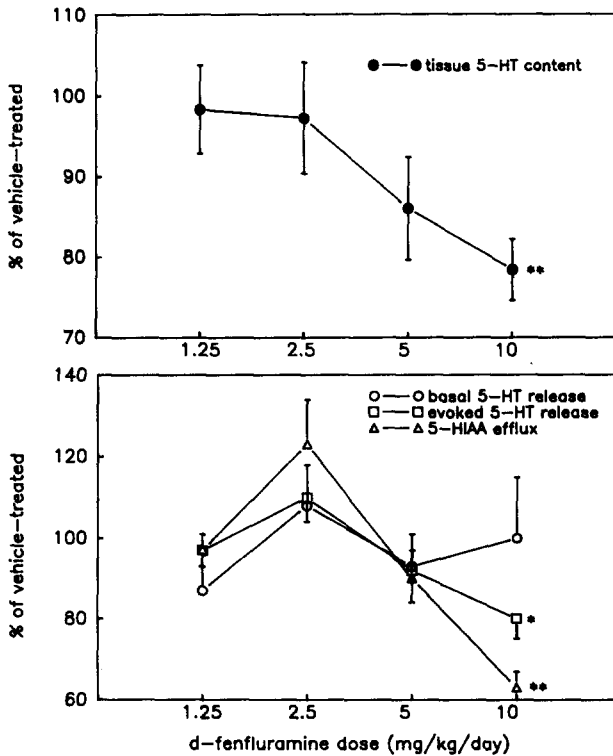


FIG. 2

Effect of chronic d-fenfluramine pretreatment on hypothalamic 5-HT levels and 5-hydroxyindole release from hypothalamic slices. Paired rats were treated for 10 days with either d-fenfluramine (1.25-10 mg/kg/day) or its vehicle. On day 16 following the onset of treatment, hypothalamic tissue was taken for 5-HT assay, and was prepared for study of 5-HT release (basal and electrically-evoked) and 5-HIAA efflux. Values are means \pm s.e.m. of percentages for drug-treated group relative to paired vehicle-treated group; N = 5-8 pairs. * $p < 0.05$, ** $p < 0.01$ differs from vehicle-treated group by Student's paired t-test.

time (19). These data, together with the present *ex vivo* findings, suggest that the brain d-fenfluramine concentration needed to produce even small (20%) long-lasting decreases in 5-HT release is greatly in excess of that required for behavioral efficacy.

The parallel effects of chronic d-fenfluramine treatment on rat hypothalamic 5-HT levels and *ex vivo* electrically-evoked 5-HT release suggest that the amount of 5-HT released from brain nerve terminals is coupled to intracellular 5-HT levels: Hypothalamic 5-HT levels and release were unchanged with 1.25-5 mg/kg/day doses of d-fenfluramine, but were proportionally reduced at the 10 mg/kg/day dose. Serotonergic neurons may be able to partially compensate for reductions in brain 5-HT content by increasing the proportion of 5-HT molecules which are available for functional activity (either by being released from nerve terminals or by being stored intracellularly for future release) relative to amounts irreversibly metabolized to 5-HIAA. This adaptive mechanism is suggested by our finding that the reductions in hypothalamic 5-HIAA levels and efflux of 5-HIAA (in the presence of fluoxetine, a 5-HT reuptake inhibitor) from slices prepared from brains of rats receiving the 10 mg/kg/day d-fenfluramine dose were proportionally greater than the reductions in 5-HT levels and release. Hence, these serotonergic neurons were able to partially restore their functional pool of 5-HT by slowing the degradation of 5-HT to 5-HIAA.

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