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Fenfluramine Effects on Serotonergic Measures in Vervet Monkeys

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BRAMMER, G. L., M. J. RALEIGH, E. R. RITVO, E. GELLER, M. T. McGUIRE AND A. YUWILER. *Fenfluramine effects on serotonergic measures in vervet monkeys.* PHARMACOL BIOCHEM BEHAV **40**(2) 267–271, 1991.—Chronic fenfluramine treatment reduced whole blood serotonin and CSF 5-hydroxyindoleacetic acid, but increased aggressive and locomotor behavior, in adult male vervet monkeys (*Cercopithecus aethiops sabaeus*). Following a drug-free washout period to monitor the drug recovery course, we initiated a second period of fenfluramine treatment in the same animals. When whole blood serotonin concentrations were reduced by about 40% from predrug baseline levels, we examined 11 cortical and subcortical brain regions for their content of 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, norepinephrine, and dopamine. We observed correspondence between the reduction in whole blood serotonin and the reduction in brain 5-hydroxytryptamine. Similarly, there was a correspondence between the reduced 5-hydroxyindoleacetic acid levels observed in CSF and brain. No alterations were noted in the concentrations of norepinephrine or dopamine. These observations suggest that the behavioral effects observed in monkeys after chronic fenfluramine treatment result from reduced central serotonin.

Fenfluramine Whole blood serotonin 5-HT 5-HIAA Vervet monkey

THE substance fenfluramine has been used in humans as an appetite suppressant and more recently for the alleviation of the symptoms of childhood autism (9, 11, 21, 26). In the autism trials, fenfluramine treatment regimens which resulted in clinical improvement also resulted in marked reductions in whole blood serotonin (WBS) concentrations, and the reduced WBS level has been used to monitor drug treatment (11). Changes observed in responding patients included reduced abnormal motor behavior, improved intelligence test scores, and increased socially appropriate responsiveness (21,22).

Fenfluramine has long been known to have a major influence on central serotonergic systems in experimental animals (6). In detail, however, there is some uncertainty in the characterization of fenfluramine action. Acutely, fenfluramine exhibits a modest inhibition of 5-hydroxytryptamine (5-HT) uptake (3,16) and a marked stimulation of 5-HT release (13), and these acute effects have led to the characterization of fenfluramine as enhancing 5-HT activity (20) and to its use as a puted serotonin provoking agent in neuroendocrine studies (7,15). Appearing within a few hours after acute treatment and observed uniformly with chronic fenfluramine treatment are marked reductions in 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) concentrations, tryptophan hydroxylase activity, and serotonin turnover (4, 6, 8, 16). These reductions in 5-HT-related measures might be characterized as indicating reduced 5-HT activity. In contrast, however, chronic fenfluramine treatment results in reduced [^{3}H]-5-HT binding in brain membranes (23,24), and such reduced binding in other situations is often interpreted as a "down-regulatory" consequence of hyperstimulation (17). In addition, uncertainty exists about the recovery phase. Some investigators report rapid recovery of 5-HT stores following cessation of chronic treatment courses (5), whereas others report enduring deficits from single exposures to drug (4,25). Further, neuronal cell body aberrations have been reported by one group (12), but have not been found by another group of investigators (27).

In order to clarify the relationships between drug treatment, behavioral alterations, and changes in WBS, CSF metabolites, and brain measures, we treated adult male vervet monkeys with fenfluramine. Using a within animal OFF-ON-OFF-ON design, vervet monkeys were treated with fenfluramine, and a previous report (19) has described the initial OFF-ON-OFF phases of the study. During the first drug treatment period, WBS concentrations and the CSF concentrations of 5-HIAA were reduced, CSF homovanillic acid (HVA) concentrations were unchanged, and aggression and locomotor activity were increased (19). All measures returned to predrug baseline levels during the drug-free

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TABLE 1

DRUG TREATMENT RECORD

Day Numbers	Days at Dose	Dose*
1–10	10	4
11–49	39	2
50–70	21	1
71–105	35	0
106–112	7	2
113–115	3	4

*Dose in mg/kg dl-fenfluramine hydrochloride, IM.

TABLE 2

WHOLE BLOOD SEROTONIN AND CSF METABOLITE CONCENTRATIONS AT DIFFERENT PERIODS OF THE STUDY*

Period	WBS	CSF 5-HIAA	CSF HVA
Baseline	1070 ± 89	89 ± 13	238 ± 19
ON Drug (2 mg/kg)	360 ± 55†	40 ± 8‡	248 ± 9
OFF Drug (17–33 days)	1254 ± 92	71 ± 14	256 ± 16
ON Drug Terminal	605 ± 199	30 ± 10	187 ± 10

*Values are mean ± SEM (in ng/ml) of each animal's characteristic (mean) value determined by 3 to 7 repeated samples for the first three periods and are the mean ± SEM of single values for the terminal period.

†WBS value on drug different from baseline, $F(3,12) = 11.82$, $p < 0.01$.

‡CSF 5-HIAA value on drug different from baseline, $F(3,12) = 5.71$, $p < 0.01$.

washout period. These results left unanswered what changes fenfluramine might induce in brain tissue and what correspondence there might be between brain tissue level changes and the more peripheral measures of CSF and WBS. Consequently, a second drug treatment period was carried out. While ON drug for the second time, the animals were sacrificed, and samples of brain tissue, in addition to CSF and blood, were collected. Several brain areas were assayed for their concentrations of 5-HT, 5-HIAA, dopamine (DA) and norepinephrine (NE). This report presents measurements of WBS, CSF metabolite concentrations, and brain neurotransmitter and metabolite concentrations.

METHOD

Subjects

Drug-treated animals. Four adult male vervet monkeys were used as subjects. The monkeys were feral raised animals from St. Kitts or Nevis. They were air-shipped to California and quarantined prior to the beginning of the study. The animals were individually housed in stainless steel cages ($1 \times 1 \times 1$ m) outdoors in a shelter protecting against wind, rain, and sun. Food (monkey chow) and water were continuously available, and fresh citrus and other fruits and vegetables were provided twice weekly. Forced warm air heating was provided during cold weather.

Reference animals. Adult male vervet monkeys captured and maintained on St. Kitts were used as reference animals. These animals were housed in mixed sex, multimale groups in outdoor enclosures protected from wind, rain, and sun. Water was continuously available, and fresh fruits and vegetables were provided twice daily. Previous experience has shown comparability of measures from socially living animals between the locations of St. Kitts and Los Angeles.

Drug Treatment Record

The overall design of this study was OFF-ON-OFF-ON. An initial two-month baseline period was followed by a 70-day drug treatment period. Then a 35-day drug-free period intervened before the final, 10-day drug treatment period. From the initiation of the first fenfluramine treatment period, the number of days each dose was administered is recorded in Table 1. Fenfluramine (dl-, hydrochloride, Pondimin) was dissolved in saline and administered IM once daily. Each animal received its last drug treatment 24 h before sacrifice.

Sample Collection

Blood and CSF samples were collected as described previously (1). Animals were immobilized with ketamine (10–15 mg/kg, IM) prior to each sample collection procedure. Blood was collected by femoral venipuncture using a plastic syringe, stabilized with EDTA, and held on ice if assayed the same day or frozen at −70°C for later assay. CSF samples were obtained at the C1 level. Ascorbic acid was added at a rate of 1 mg/ml, and the sample was held on ice. CSF samples were freed of any debris by centrifugation and then frozen and stored at −70°C.

Terminal Procedures

The animals were sacrificed and brain samples were collected as described previously (2). Ketamine (10–15 mg/kg) and sodium pentobarbital (6 mg/kg, IV) provided deep anesthesia. Skeletal musculature was relaxed, the corneal eye-blink reflex was absent, and respiration was shallow, but regular, and was maintained unaided. After induction of deep anesthesia, no animal was responsive to any remaining procedures. Brain samples were freehand dissected from whole brain maintained on a bed of ice, and individual samples were frozen and stored at −70°C until assay.

Assays

The concentration of 5-HT in whole blood samples was determined by the fluorometric assay of Yuwiler (28) as described previously (18).

The concentrations of neurotransmitters and metabolites in CSF and brain samples were determined by high pressure liquid chromatography with electrochemical detection (1). Briefly, C-18 reversed phase columns were used (4.6×150 mm, 5 μm particle size), and the electrochemical detector was maintained at 0.7 V (Ag/AgCl reference). CSF samples were mixed with a solution of 4-hydroxy-3-methoxyphenethyl alcohol (MOPET, internal standard) and 5-HIAA and HVA were separated employing a mobile phase of 100 mM sodium acetate, 20 mM citric acid, and 0.1 mM disodium EDTA, with methanol added to 7.5%. Brain samples were homogenized in 10 volumes of 0.1 N perchloric acid, and the homogenate was centrifuged. An aliquot of the supernatant was mixed with MOPET, and 5-HT, 5-HIAA, and HVA measured using the previous mobile phase. Another aliquot of the supernatant was mixed with a solution of dihydroxybenzylamine (DHBA, internal standard), adsorbed/desorbed

TABLE 3

NEUROTRANSMITTER AND METABOLITE CONCENTRATIONS IN VERVET MONKEY BRAIN*

Tissue	Group	5-HT	5-HIAA	DA	NE
Cortex					
Orb. front.	Ref	229 ± 42	125 ± 34	172 ± 52	171 ± 26
	Fen	108 ± 20	54 ± 5	180 ± 32	162 ± 17
Parietal	Ref	150 ± 23	186 ± 8	84 ± 17	256 ± 44
	Fen	104 ± 16	80 ± 9	137 ± 46	282 ± 20
Sup. temp.	Ref	380 ± 85	270 ± 32	53 ± 8	150 ± 10
	Fen	99 ± 31	60 ± 9	72 ± 11	145 ± 17
Occipit.	Ref	235 ± 26	302 ± 29	NA	76 ± 14
	Fen	100 ± 14	60 ± 1	NA	66 ± 7
Cingulate	Ref	214 ± 25	169 ± 3	104 ± 14	250 ± 26
	Fen	88 ± 11	65 ± 12	144 ± 24	286 ± 40
Hippocampus	Ref	406 ± 49	505 ± 16	38 ± 16	169 ± 35
	Fen	120 ± 14	118 ± 35	44 ± 18	117 ± 14
Amygdala	Ref	798 ± 116	360 ± 40	374 ± 64	359 ± 45
	Fen	366 ± 113	108 ± 39	325 ± 37	346 ± 69
Caud./Put.	Ref	364 ± 58	546 ± 27	17390 ± 2020	77 ± 16
	Fen	238 ± 48	138 ± 46	22580 ± 1740	80 ± 11
Hypothal.	Ref	1478 ± 38	890 ± 41	441 ± 54	5589 ± 644
	Fen	1064 ± 278	426 ± 68	566 ± 45	4368 ± 850
Subst.nigra	Ref	2002 ± 363	1294 ± 177	3121 ± 516	364 ± 30
	Fen	928 ± 383	616 ± 88	1940 ± 593	188 ± 27
Raphe	Ref	3067 ± 448	6798 ± 470	46 ± 10	638 ± 72
	Fen	2114 ± 509	3090 ± 332	70 ± 16	728 ± 73

*Data are mean ± SEM (N = 4) in ng/g. NA = not assayable. The substances reported are 5-hydroxytryptamine (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), and norepinephrine (NE), for fenfluramine-treated (Fen) and reference (Ref) animals. Treatment effects were significant for 5-HT, $F(1,66) = 18.5$, $p<0.001$, and 5-HIAA, $F(1,66) = 111.3$, $p<0.001$, but not for DA, $F(1,66) = 1.46$, $p>0.2$, nor for NE, $F(1,66) = 1.52$, $p>0.2$.

from alumina, and NE and DA measured using the mobile phase of 100 mM sodium acetate, 50 mM citric acid, 0.1 mM disodium EDTA, and 0.43 mM sodium 1-octanesulfonate, with methanol added to 6.25%. The concentrations of substances were calculated with reference to the internal standards by the peak height method.

Statistical Tests

A principal focus of this study was the postdrug recovery of measures, and this interest resulted in the within animal design. Consequently, the one-time terminal brain samples can be compared only with measures from animals not in this experiment. The significance of treatment differences were evaluated by ANOVA with repeated measures, conducted separately for each substance. The grouping factor was treated vs. reference animals, and the repeated measures were the eleven brain regions analyzed. Additionally, post hoc *t*-tests were used to identify the source of significant treatment-brain region interactions.

RESULTS

Table 2 shows WBS and CSF 5-HIAA and HVA concentrations during stable portions of each treatment phase during this study. With the exception of the single terminal value, between 3 and 7 repeat measurements of each parameter were used to establish within-subject means for a given period. The values in Table 2 are the Mean ± SEM of the individual animals' means.

Daily fenfluramine treatments were accompanied by large decreases in WBS and CSF 5-HIAA concentrations. We have previously reported that the WBS and CSF 5-HIAA decrements of the first drug treatment period were statistically significant ($p<0.01$ and $p<0.02$, respectively) (19). CSF HVA concentrations were not affected by fenfluramine treatment except, perhaps, at the terminal sample. However, the terminal value was a single sample from each animal, and, in all cases, each animal's final CSF HVA concentration was within the range of all its previous values. Behavior in the subjects was not formally evaluated during the second drug treatment period, but markedly increased experimenter directed aggression occurred as previously noted (19).

Table 3 lists the mean ± SEM of neurotransmitter and metabolite concentrations (in ng/g) in regions of vervet monkey brains (2), 4 from fenfluramine-treated animals and 4 from reference animals. There were significant treatment differences for both 5-HT, $F(1,66) = 18.5$, $p<0.001$, and 5-HIAA, $F(1,66) = 111.3$, $p<0.001$. Both 5-HT and 5-HIAA concentrations were reduced 25–75% in nearly every area examined in brains from fenfluramine-treated animals compared with referent animals.

Mean 5-HT content in samples from fenfluramine animals was approximately 50% of reference values, ranging from about 25% in superior temporal cortex and hippocampus to about 70% in parietal cortex, hypothalamus, and raphe. Similarly, mean 5-HIAA content was only approximately 35% of reference values, ranging from about 20% in superior temporal and occipital cortex and hippocampus to about 45% in orbitofrontal and parietal cortex, substantia nigra, and raphe. In addition, there was a treatment by brain region interaction for 5-HIAA, $F(10,66) = 31.1$, $p<0.001$. Post hoc *t*-tests indicated that fenfluramine treatment resulted in greater than average decrement in 5-HIAA concentration in occipital and superior temporal cortex and hippocampus. In contrast, the catecholamine levels were not con-

sistently altered by fenfluramine treatment, dopamine, $F(1,66) = 1.46$, $p > 0.2$, and norepinephrine, $F(1,66) = 1.52$, $p > 0.2$.

DISCUSSION

We have observed substantial reductions in central 5-HT and 5-HIAA concentrations in vervet monkeys under a fenfluramine treatment regimen similar to that where we previously observed reduced WBS and CSF 5-HIAA but increased aggressive and locomotor behavior. At sacrifice, 5-HIAA levels were reduced by about 40% in CSF and in brain. Tissue 5-HT concentrations and WBS levels also were found to be reduced by about 50%. These results suggest that in clinical situations where fenfluramine treatment reduces WBS levels by about 50% (11,21), substantial changes have occurred in central serotonin measures.

To emphasize the parallel between central and peripheral drug effects, we noticed a gradation in 5-HT decrease between animals. At termination, the WBS measure in one animal had decreased to only 82% of its immediately predrug level, whereas the other three animals WBS was decreased to 32, 34, and 37% of their predrug levels. Overall, the fenfluramine-treated animals exhibited brain 5-HT concentrations about 50% of reference animals. But the animals whose WBS was 82% of predrug levels exhibited brain 5-HT concentrations about 90% of reference animals, and the three animals with more substantially reduced WBS exhibited brain 5-HT concentrations about 42% of reference animals.

In contrast, CSF HVA concentrations and brain sample concentrations of NE and DA are seemingly not influenced by fenfluramine treatment. Our results are similar to other investigators who have reported decreased 5-HT and 5-HIAA with no alterations of catecholamines after either acute or chronic fenfluramine treatment (5,10). We chose to examine dl-fenfluramine to mimic clinical use. When examined acutely, l-fenfluramine has been reported to increase HVA in striatum and nucleus accumbens in rats after one hour (14), but this effect is actually reversed four or eight hours later. It is unknown how prominent this effect on DA metabolism might be after chronic treatment or on treatment schedules using the mixed isomers.

The lack of consistent alterations in catecholamine levels suggests that there is no direct drug effect on catecholamine neurons. But some apparent catecholamine differences might reflect real, albeit indirect, consequences of fenfluramine treatment. Serotonin and dopamine or norepinephrine interactions have been described, and the marginally different concentrations of DA in the substantia nigra or NE in the hypothalamus might be secondary to fenfluramine alteration of serotonergic processes. However, some of the apparent differences in catecholamine levels between fenfluramine-treated and reference animals might have resulted from the different social environment of the treated and reference animals.

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