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NORFENFLURAMINE AND SEROTONIN TURNOVER RATE IN THE RAT BRAIN

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Abstract—Norfenfluramine (40 μmoles/kg, i.v.) causes generalized tremors, rigidity, backwards walking, salivation and hyperthermia lasting about 40 min. This dose also markedly lowers serotonin concentrations in tel-diencephalon lasting longer than 50 hr. In contrast, the serotonin concentrations of brain stem and hypothalamus are slightly reduced at 7 hr after norfenfluramine and return to normal at 18 hr. This dose of norfenfluramine does not affect brain norepinephrine and dopamine content. Atropine (72 μmoles/kg, i.p. 30 min before) reduces the pharmacological effects, but fails to affect the persistent decrease of tel-diencephalic serotonin concentration elicited by norfenfluramine. The lowering of tel-diencephalic and brain stem serotonin is associated neither with a decrease of the turnover rate of this brain amine nor with a decrease of 5-hydroxyindoleacetic acid concentrations in brain tissue. Actually, serotonin turnover rate is increased, suggesting that norfenfluramine, unlike its chemical congener *p*-chloroamphetamine, lowers brain serotonin but does not inhibit the synthesis of this amine.

FENFLURAMINE (*N*-ethyl-α-methyl-3-trifluoromethylphenylethylamine)[1–3] and norfenfluramine (α-methyl-3-trifluoromethylphenylethylamine)[4] persistently decrease the serotonin (5-HT) concentrations of various areas of the rat brain; this action resembles that of its chemical congener, *p*-chloro-amphetamine and that of other *p*-halo derivatives of amphetamine.[4–6] Since *p*-chloro-amphetamine reduces the turnover rate of brain 5-HT,[7,8] we tested whether norfenfluramine decreases brain 5-HT content by virtue of a similar action. It has been reported that fenfluramine fails to inhibit the conversion of tryptophan to 5-HT *in vitro*,[9] and actually increases the turnover rate of brain 5-HT measured *in vivo*.[3] Indeed, norfenfluramine has been shown to be present in high concentrations in rat brain 4 hr after a dose of fenfluramine,[10] and one cannot exclude the possibility that the long-lasting reduction of brain 5-HT elicited by fenfluramine is mediated by its conversion to norfenfluramine. The present results show that norfenfluramine increases the turnover rate of 5-HT in tel-diencephalon, although it persistently reduces the concentration of this amine.

METHODS

Male Sprague–Dawley rats (Zivic Miler, Allison Park, Pa.) weighing 180–200 g were housed for 7 days preceding the experiment in our facilities, ten per cage at 20° with free access to food and water. The animals were injected intraperitoneally with 40 μmoles/kg of *dl*-norfenfluramine and 4 hr later received intravenously 1·7 μmoles/kg of ^{14}C-L-tryptophan (specific activity, 45 mc/m-mole) obtained from Amersham Searle (Des Plaines, Ill.). Groups of three rats each were decapitated 10, 40, 80 and 120 min after the injection of the amino acid. A similar experiment was run in parallel, with rats injected with saline 4 hr prior to the injection of the labeled amino acid.

The brain was removed and dissected into parts (tel-diencephalon, brain stem and hypothalamus) according to the method of Meyerson.[11] These parts of brain tissue were stored at −15° until the specific radioactivity of 5-HT and tryptophan was assayed by existing ion-exchange chromatography methods.[12] The significance of differences in amine or amino acid concentrations and/or their specific radioactivity between treated and control animals was assessed using Student's *t*-test. The fractional rate constant (k) for the efflux of 5-HT from the storage sites of various brain areas was calculated according to Neff *et al.*[12] from equation (1).

$$k \approx \frac{\dfrac{\text{SA 5-HT}_{t_2} - \text{SA 5-HT}_{t_1}}{t_2 - t_1}}{\dfrac{(\text{SA TP} - \text{SA 5-HT})_{t_2} + (\text{SA TP} - \text{SA 5-HT})_{t_1}}{2}} \tag{1}$$

In equation (1), t_1 and t_2 are minutes after the injection of ^{14}C-L-tryptophan; the subscript indicates minutes between successive time intervals, the higher number being the longer interval; SA TP and SA 5-HT indicate specific radioactivity of tryptophan and 5-HT respectively. The k was calculated at various times after labeling, using the specific radioactivity of the precursor and that of the product at successive 10-min intervals beginning at 10 min after the amino acid injection. The specific radioactivity values were obtained from a semilogarithmic plot of the changes with time of precursor and product specific radioactivities measured at the time specified above. For each brain area and each experiment, 11 values of k were estimated; these values were averaged to obtain the mean k reported in Table 3. The derivation of equation 1 from equation (2) was described in a previous paper.[12]

$$\frac{d\ \text{SA 5-HT}}{dt} = k(\text{SA 5-HT} - \text{SA TP}). \tag{2}$$

The turnover rate (TR) of 5-HT was calculated from equation (3).

$$\text{TR} = k\ [\text{5-HT}]. \tag{3}$$

In other experiments rats were injected with 40 μmoles/kg, i.v. of *dl*-norfenfluramine. Several brain areas were analyzed (striatum, hypothalamus, brain stem, tel-diencephalon) for 5-HT, norepinephrine (NE) and dopamine (DM) content at various times after the injection. The three amines were assayed spectrofluorometrically after they were separated by ion-exchange chromatography and the catecholamines were further purified by Al_2O_3 adsorption.[12] In some rats the heart NE concentrations were also measured spectrophotofluorometrically after purification with ion-exchange chromatography and Al_2O_3 adsorption.[12] We assayed spectrofluorometrically 5-hydroxyindole acetic acid (5-HIAA) as proposed by Ashcroft and Sharman[13] in brain extracts purified according to Lindqvist.[14] Body temperature before and after drug injections was recorded rectally (Telethermometer, Yellow Springs, Ohio) in rats kept one in each cage at 20°.

TABLE 1. EFFECT OF NORFENFLURAMINE (NF) WITH OR WITHOUT ATROPINE ON THE BODY TEMPERATURE (°F ± S.E.M.) OF RATS*

Pretreatment (μmoles/kg, i.p.)	Time before NF (min)		Treatment (μmoles/kg, i.v.)	Time after NF (min)			
	60	30		30	60	90	240
Saline	98·3 ± 0·31	97·8 ± 0·41	Saline	98·8 ± 0·87	98·9 ± 0·42	99·1 ± 0·52	98·7 ± 0·42
Saline	98·9 ± 0·92	97·9 ± 0·42	NF (40)	100·8 ± 0·85	101·9 ± 0·32†	102·2 ± 0·52†	97·9 ± 0·38
Atropine (72)	98·5 ± 0·60	97·5 ± 0·61	Saline	97·3 ± 0·60	97·1 ± 0·51	97·4 ± 0·61	97·6 ± 0·39
Atropine (72)	97·9 ± 0·82	97·8 ± 0·58	NF (40)	98·3 ± 0·42	99·6 ± 0·72	100·1 ± 0·52†	97·8 ± 0·52

* Each value is the mean of six rats. The dose injected into a 200 g rat was dissolved in 1 ml.
† $P < 0.01$ when compared to means before NF.

RESULTS

Symptomatology elicited by norfenfluramine

Rats receiving 40 μmoles/kg, i.v., of norfenfluramine exhibit generalized tremor, muscular rigidity, backwards walking and salivation. Table 1 reports data on the rectal temperature of rats receiving either 5 ml/kg, i.v., of saline or 40 μmoles/kg, i.v., of norfenfluramine given alone or 20 min after atropine, 72 μmoles/kg, i.p. Norfenfluramine causes hyperthermia; pretreatment with atropine not only delays the onset of hyperthermia elicited by norfenfluramine but also reduces the intensity of this response. The pretreatment with atropine reduces the intensity of or abolishes the tremors, salivation, backwards walking, and rigidity elicited by norfenfluramine. Rats receiving 40 μmoles/kg, i.p. of norfenfluramine exhibit neither tremor nor rigidity; when these symptoms are present they are attenuated as compared with those of rats receiving a similar dose of the drug intravenously.

Effect of norfenfluramine on brain monoamine content

Figure 1 shows the per cent change in the concentrations of NE and 5-HT in tel-diencephalon of rats at various times after 40 μmoles/kg, i.v. of norfenfluramine. This dose fails to lower the NE content, but it reduces the 5-HT content for longer than 50 hr. 5-HT concentrations in tel-diencephalon are reduced maximally within 2 hr and they remain lower than controls for about 3 days. In the same rats we have also assayed 5-HT concentrations in hypothalamus and brain stem. We found them to be decreased by about 30 per cent at 7 hr, but the 5-HT decrease in these brain areas appears to be less persistent; in fact, 18 hr after the drug injection, the 5-HT content is equal to that of rats receiving only saline. The concentrations of DM in striatum and NE in hypothalamus and brain stem are unaffected by 40 μmoles/kg, i.v., of norfenfluramine.

Since during hyperthermia brain 5-HT turnover rate is accelerated,[15] we evaluated whether rats pretreated with atropine doses that prevent norfenfluramine hyper-

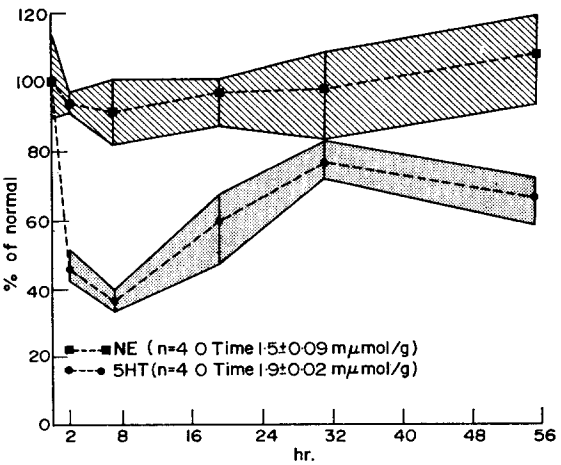

FIG. 1. Per cent change with time of NE (upper) and 5-HT (lower) concentrations in tel-diencephalon of rats receiving 40 μmoles/kg, i.v., of norfenfluramine. Shaded areas show the range of values. Each point is the average of four assays.

thermia still display a decrease of brain 5-HT concentrations. The results of the experiment are reported in Table 2 and show that the decrease of brain 5-HT concentrations elicited by norfenfluramine was not curtailed by such pretreatment with atropine. Actually, the decrease of 5-HT at 90 min after norfenfluramine was evident in hypothalamus and brain stem of rats pretreated with atropine but not in the same brain areas of rats receiving only norfenfluramine.

TABLE 2. INTERACTIONS BETWEEN NORFENFLURAMINE (NF) AND ATROPINE ON TISSUE CONCENTRATIONS OF MONOAMINES*

Tissue	Mono-amine	Saline (nmoles/g)	Atropine (nmoles/g)	NF (nmoles/g)	Atropine and NF (nmoles/g)
Heart	NE	6·8 ± 0·27	6·7 ± 0·30	6·9 ± 0·49	5·5 ± 0·63
Striatum	DM	35 ± 3·3	41 ± 4·2	40 ± 2·1	40 ± 4·5
Tel-diencephalon	NE	1·5 ± 0·085	1·4 ± 0·11	1·4 ± 0·086	1·3 ± 0·12
	5-HT	1·9 ± 0·021	1·8 ± 0·051	0·88 ± 0·055†	0·92 ± 0·11†
Hypothalamus	NE	9·4 ± 0·43	9·1 ± 0·51	9·4 ± 1·0	8·6 ± 0·27
	5-HT	9·6 ± 0·36	9·3 ± 0·51	8·5 ± 2·1	7·6 ± 0·57†
Brain stem	NE	2·2 ± 0·069	2·1 ± 0·051	1·9 ± 0·18	1·9 ± 0·28
	5-HT	3·0 ± 0·15	2·7 ± 0·12	2·8 ± 0·23	2·3 ± 0·13†

* Rats received atropine (72 μmoles/kg, i.p.) or saline (5 ml/kg, i.p.) 40 min before norfenfluramine (40 μmoles/kg, i.v.) and were killed 90 min after the latter treatment. Saline or atropine doses were injected 130 min before killing the rats. Each value is the average of three determinations ± S.E.M.
† $P < 0·01$.

Effect of norfenfluramine on 5-HT turnover rate in various brain areas

To understand the nature of the interaction between norfenfluramine and the mechanisms that control brain 5-HT concentrations, we studied the conversion of ^{14}C-L-tryptophan into ^{14}C-5-HT in tel-diencephalon of rats receiving intraperitoneally either saline or norfenfluramine (40 μmoles/kg) 4 hr before the pulse injection of the labeled amino acid. The changes with time of the specific radioactivity of 5-HT and tryptophan in tel-diencephalon of these animals are reported in Fig. 2. At 10 min after labeling, the specific radioactivity of 5-HT in the saline-treated rats was greater than in rats receiving norfenfluramine. However, 40 min after the amino acid injection when the specific radioactivity of tryptophan approached that of 5-HT (crossover point), the values of the 5-HT specific radioactivity were equal in the two groups of rats. After the crossover point, the specific radioactivity of 5-HT declined in both groups of rats, but this decline was faster in rats receiving norfenfluramine than in those receiving saline. In the two groups of rats, the specific radioactivity of the precursor (tryptophan) and that of the product (5-HT) were read from the graph shown in Fig. 2 for successive 10-min intervals. Using these values, we calculated k following equation (1).

Figure 1 indicates that between 4 and 6 hr after norfenfluramine injection, the 5-HT concentration in the tel-diencephalon of rats is at steady state; the efflux of labeled 5-HT gives information about the efflux of cold 5-HT which is being made and metabolized in the tel-diencephalon. Thus, the average value of k reported in Fig. 2 can be used to calculate the turnover rate of tissue 5-HT according to equation

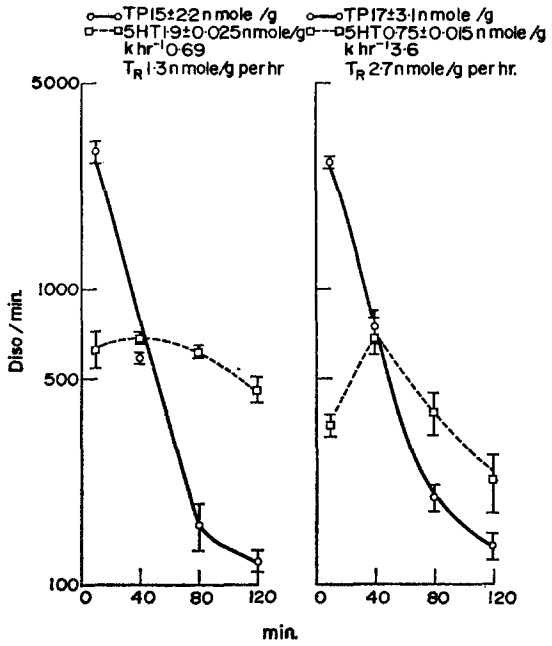

FIG. 2. Specific radioactivity of 5-HT and tryptophan (TP) in the tel-diencephalon of rats at various times after ^{14}C-L-tryptophan (1·7 μmoles/kg, i.v.). The rats received 4 hr earlier either (right) 40 μmoles/kg, i.p., of *dl*-norfenfluramine or (left) saline (5 ml kg, i.p.).

(2). This calculation shows that in saline-treated rats the tel-diencephalic 5-HT turned over at a rate of 1·3 nmoles/g/hr; in the same brain area of rats receiving norfenfluramine this turnover rate was twice as fast. In the same animals, we have assayed the specific radioactivity of 5-HT and tryptophan in hypothalamus and brain stem. In the hypothalamus, the change with time of the specific radioactivity of tryptophan and 5-HT does not crossover at the highest point of the 5-HT specific activity, as would be expected if the two substances follow the precursor product relationship proposed by Zilversmit *et al.*[16] Therefore, we cannot use these data for turnover rate measurements. In contrast, the values obtained in the brain stem display such a relationship and, therefore, allow estimation of the *k* values for 5-HT efflux. The results of these calculations are reported in Table 3. These data show that the 5-HT concentrations in tel-diencephalon and brain stem of rats receiving norfenfluramine were lower than those in controls. Despite this decrease, the turnover rate of 5-HT stored in brain stem and tel-diencephalon was greater in norfenfluramine-treated than in saline-treated rats.

Concentrations of 5-HIAA *in brain of rats receiving norfenfluramine*

Rats were injected intravenously with 40 μmoles/kg of norfenfluramine and killed at various times after the injection. The data reported in Table 4 show that the brain concentrations of 5-HIAA increased above those of normal rats at 30 and 60 min after injection and returned to normal at 2 hr. Comparing the data reported in Fig. 1 to those of Table 4, it is evident that a discrepancy exists between the changes of 5-HT

TABLE 3. TURNOVER RATE OF 5-HT IN TEL-DIENCEPHALON AND BRAIN STEM OF RATS RECEIVING NORFENFLURAMINE*

Treatment	Brain part	Tryptophan (nmoles g^{-1} ± S.E.M.)	5-HT (nmoles g^{-1} ± S.E.M.)	k (hr^{-1})	Turnover rate (nmoles g^{-1} hr^{-1})
Saline	Tel-diencephalon	15 ± 2·2	1·9 ± 0·025	0·69	1·3
Norfenfluramine	Tel-diencephalon	17 ± 2·1	0·75 ± 0·015†	3·6	2·7
Saline	Brain stem	19 ± 1·2	2·3 ± 0·021	0·84	1·9
Norfenfluramine	Brain stem	21 ± 1·8	1·7 ± 0·0012†	1·7	2·9

* Norfenfluramine (40 μmoles/kg, i.p.) or saline (5 ml/kg, i.p.) was injected 4 hr before 1·7 μmoles/kg, i.v., of ^{14}C-L-tryptophan (sp. act., 45 mc/m-mole). For each treatment, four groups of three rats each were killed between 10 and 120 min after the amino acid injection, at the times indicated in the graph shown in Fig. 2. k was calculated from the specific radioactivities of 5-HT and tryptophan at each 10-min interval from 10 to 120 min after the label injection. Each mean ± S.E. refers to one assay made in each of the 12 rats.
† $P < 0·01$.

TABLE 4. CONCENTRATION OF 5-HIAA IN BRAIN OF RATS RECEIVING NORFENFLURAMINE (NF)*

Time after NF (min)	5-HIAA (nmoles/g ± S.E.M.)
0	1·2 ± 0·090
30	1·5 ± 0·10†
60	1·7 ± 0·070†
120	1·1 ± 0·082

* Norfenfluramine (40 μmoles/kg, i.v.) was injected into three groups of five rats each.
† $P < 0·05$.

and 5-HIAA concentrations: when 5-HT levels are depleted, the brain concentrations of 5-HIAA are either greater than or equal to those of normal rats.

DISCUSSION

Norfenfluramine injected in high doses elicits a syndrome reminiscent of the effects elicited by tremorine in rats.[17] This drug, like tremorine,[18] causes generalized tremor, rigidity and marked parasympathetic stimulation. The effects elicited by norfenfluramine appear almost immediately after its intravenous injection, while tremorine action becomes manifest after a time delay. Presumably, the delay is required to allow for the biotransformation of tremorine into oxotremorine which is its biologically active metabolite.[19,20] Unlike tremorine, norfenfluramine causes hyperthermia, and this response, like the tremor and salivation, is reduced by pretreatment with atropine. Conceivably, implication of the cholinergic system may explain the effects elicited by tremorine and norfenfluramine; however, there are some differences which suggest that the mode and site of action of these two drugs may be quite different. The administration of tremorigenic doses of tremorine is followed by a decrease in the

brain stem concentrations of NE in rats, mice and guinea pigs.[21] In the rat brain stem, the NE decrease elicited by tremorine is followed by a progressive increase of the 5-HT concentration.[21] In this regard, norfenfluramine differs from tremorine, for it lowers brain 5-HT selectively. This decrease of 5-HT content is prominent in the tel-diencephalon where it persists for several days (Fig. 1). Although norfenfluramine decreases the 5-HT content of hypothalamus and brain stem, this decrease of 5-HT is short-lived. The tremorigenic and hyperthermic actions of norfenfluramine can be reduced by pretreating the rats with atropine (70 μmoles/kg, i.p.), but such pretreatment does not change the decrease of 5-HT concentrations in tel-diencephalon. Our data suggest that in rats receiving both drugs the concentrations of 5-HT in the hypothalamus were reduced at a faster rate than in rats receiving only norfenfluramine. One might conclude that tremors elicited by norfenfluramine are unrelated to the change of brain 5-HT, because the rat receiving first atropine does not display tremors when injected with norfenfluramine, but the brain 5-HT decrease is unchanged. However, it might be argued that atropine blocks the response elicited by a decrease of brain 5-HT. This possibility seems to be weakened by the finding that in rats receiving 40 μmoles/kg of norfenfluramine intraperitoneally the decrease of tel-diencephalic 5-HT is comparable to that elicited by the same dose of norfenfluramine given intravenously (Fig. 1 and Table 3), but the tremor after the intraperitoneal injection of norfenfluramine is usually lessened. Reports have been published supporting a role of brain 5-HT in thermoregulation,[15] and suggesting that the turnover rate of brain 5-HT is increased when the rats are hyperthermic after exposure to 38°. To exclude that hyperthermia elicited by norfenfluramine interferes with the turnover rate measurements of brain 5-HT, we tested the effects of norfenfluramine on brain 5-HT turnover rate 4 hr after its administration when the body temperature was back to normal (Table 1), but the 5-HT concentration was still depleted. The experiments with labeled tryptophan have shown that norfenfluramine enhanced the turnover rate of 5-HT stored in tel-diencephalon and brain stem at a time when the rats were not hyperthermic. In other experiments,[4] we have tested the action of norfenfluramine on 5-HT uptake by slices of various brain parts and on tryptophan hydroxylation by tissue homogenates. We concluded that norfenfluramine added *in vitro* (10^{-5} M) or given intraperitoneally (90 μmoles/kg) does not block 5-HT uptake by striatum, brain stem and hypothalamus. In both experimental conditions, the drug inhibited the uptake of NE.[4] Norfenfluramine in concentrations of 10^{-3} M partially inhibits tryptophan hydroxylase. It is difficult, however, to relate this inhibition *in vitro* to the lowering of brain 5-HT, because 10^{-3} M concentrations of drug are not attained in brain of rats receiving 40 μmoles/kg of norfenfluramine. Since norfenfluramine binds to brain tissue,[10] we are left with these alternatives: (1) the drug inhibits re-uptake of 5-HT, and therefore the increased turnover reflects an increased efflux rate of 5-HT from storage; or (2) the drug inhibits storage, and the increased turnover reflects an increased rate of 5-HT catabolism by intraneuronal monoamine oxidase.

It is difficult to explain why inhibition of re-uptake should decrease the concentrations of brain 5-HT, and therefore this alternative explanation appears improbable. Our data on the effects of norfenfluramine on the brain concentrations of 5-HIAA help us to ascribe some value to the second alternative. We can suggest that increased intraneuronal metabolism of 5-HT is plausible because 5-HIAA is elevated when 5-HT is being depleted and brain 5-HIAA is equal to that of normal rats when 5-HT con-

centrations are decreased by 60 per cent. Our data do not allow us to suggest why the decrease of 5-HT was less severe and persistent in hypothalamus and brain stem than in tel-diencephalon. A similar difference in the extent of 5-HT depletion in these brain areas was reported by Boissier *et al.*[22] for *p*-chloro-*N*-methylamphetamine. They proposed that this difference reflects a localization of the drug in certain brain structures. We have not studied the distribution of norfenfluramine in various brain structures and therefore we cannot ascribe the less persistent and severe depletion of hypothalamic and brain stem 5-HT elicited by norfenfluramine to a drug localization in the tel-diencephalon.

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