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IS RECEPTOR ACTIVATION INVOLVED IN THE MECHANISM BY WHICH (+)-FENFLURAMINE AND (+)-NORFENFLURAMINE DEPLETE 5-HYDROXYTRYPTAMINE IN THE RAT BRAIN?

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1 The effects of (+)-fenfluramine, (+)-norfenfluramine and reserpine on the concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in brainstem and telencephalon were studied in rats treated with methergoline, a 5-HT antagonist.

2 Methergoline significantly reduced the effect of (+)-norfenfluramine (5 mg/kg) on 5-HT levels in telencephalon and brainstem but did not modify the effect of (+)-norfenfluramine (2.5 mg/kg).

3 Neither the effect of (+)-fenfluramine on 5-HT levels nor the decrease of 5-HT metabolism caused by (+)-fenfluramine and (+)-norfenfluramine was significantly modified by methergoline treatment.

4 Methergoline potentiated the effects of reserpine on brain indoles. The effect was particularly evident on 5-HIAA levels in the brainstem, although significant effects were found on 5-HT in the brainstem and 5-HIAA in the telencephalon depending on the dose of reserpine used.

5 The results show that postsynaptic receptor activation may partially contribute to the depletion of brain 5-HT caused by (+)-norfenfluramine in the rat. This mechanism does not seem to play a significant role in the effect of (+)-fenfluramine.

Introduction

Fenfluramine is an anorectic agent which causes long-lasting depletion of 5-hydroxytryptamine (5-HT) in the rat brain (Duhault & Verdavainne, 1967; Costa, Groppetti & Revuelta, 1971; Clineschmidt, Zacchei, Totaro, Pflueger, McGuffin & Wishousky, 1978; Steranka & Sanders-Bush, 1979). The mechanism of this effect is not clear. Fenfluramine releases 5-HT from nerve endings and inhibits its reuptake into the neurone (Garattini & Samanin, 1976; Garattini, Borroni, Mennini & Samanin, 1978) but neither of these mechanisms appears sufficient to explain the marked depletion of brain 5-HT induced by this drug (Ghezzi, Samanin, Bernasconi, Tognoni, Gerna & Garattini, 1973; Corrodi, Farnebo, Fuxe & Hamberger, 1975). Considerable amounts of norfenfluramine are found in the brains of fenfluramine-treated rats even several days after a single drug injection (Clineschmidt *et al.*, 1978). Norfenfluramine, besides increasing 5-HT release and inhibiting its reuptake, possesses some affinity for postsynaptic receptor sites (Garattini, Caccia, Mennini, Samanin, Consolo & Ladinsky, 1979). Although shortly after administration fenfluramine may increase 5-HT synthesis (Costa *et al.*, 1971), at later times it markedly inhibits tryptophan hydroxylase, which could contribute to 5-HT depletion (Knapp & Mandell, 1976; Fuller, Snoddy & Hemrick, 1978; Steranka & Sanders-Bush, 1979). Since neither fenfluramine nor norfenfluramine appear directly to inhibit tryptophan hydroxylase in *in vitro* experiments (Morgan, Löfstrandh & Costa, 1972) the possibility exists that prolonged receptor activation caused by fenfluramine and norfenfluramine determines tryptophan hydroxylase inhibition through a feedback mechanism and, consequently, 5-HT depletion.

Another mechanism by which fenfluramine may deplete brain 5-HT is by interference with amine storage within the granules. It has been recently found that reserpine prevents the effect of (+)-fenfluramine on 5-HT release from synaptosomal preparations of rat brains (Mennini, Borroni, Samanin & Garattini, 1981). In the same study it was found that (+)-norfenfluramine was less active than (+)-fenfluramine on the reserpine-sensitive storage pool (Mennini *et al.*, 1981). However, fenfluramine and reserpine appear to release 5-HT differently, as the former releases 5-HT directly into the extraneuronal space (Garattini & Samanin, 1976)

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whereas reserpine has been shown to act mainly on intraneuronal storage of the amine (Carlsson, 1966). This is also indicated by the fact that fenfluramine and reserpine, although causing depletion of 5-HT, decrease and increase 5-HT metabolism respectively in the rat brain (Tozer, Neff & Brodie, 1966; Garattini, Buczko, Jori & Samanin, 1975; Garattini, Bizzi, de Gaetano, Jori, Samanin 1975). In the present study the effects of (+)-fenfluramine, (+)-norfenfluramine and reserpine on brain 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) levels were studied in rats pretreated with methergoline, a central 5-HT antagonist (Mawson & Whittington, 1970). The aim was to establish the extent to which blockade of postsynaptic 5-HT receptors modifies the effects of these drugs on brain indoles. The (+)-isomers of fenfluramine and norfenfluramine were used as it has been shown recently that they are more selective than (−)-isomers in their effect on 5-HT mechanisms (Garattini *et al.*, 1979; Crunelli, Bernasconi & Samanin, 1980).

Methods

Female CD-COBS (Charles River, Italy) rats, body weight 175–200 g were used. The animals were housed at a constant room temperature (22 ± 1°C) and relative humidity (60%) with food and water freely available.

The animals were injected intraperitoneally with 5 or 10 mg/kg (+)-fenfluramine hydrochloride, 2.5 or 5 mg/kg (+)-norfenfluramine hydrochloride (Servier Laboratory-Neully-Sur-Seine, France), 1 mg/kg methergoline base (Farmitalia-Carlo Erba, Milan, Italy) dissolved in 1% ascorbic acid or 2 or 5 mg/kg reserpine (Serpasil, Ciba-Geigy) as free base dissolved in a vehicle consisting of polyethyleneglycol 300, benzyl alcohol, citric acid and water. Control animals received an equal volume of the vehicle.

Four hours after treatment the animals were killed by decapitation, the brains were rapidly removed, the brain stem and remaining telencephalon were dissected, frozen on dry ice and stored at −20°C until biochemical assay.

Brain 5-HT was determined by high performance liquid chromatography with electrochemical detection, according to the method of Ponzio & Jonsson (1979). A slight modification of the Wightman, Plotsky, Strope, Delcore & Adams (1977) technique for the determination of amine metabolites in cerebrospinal fluid was used for measuring 5-HIAA in brain tissues. 5-HT and 5-HIAA were determined in the same sample. After extraction of 5-HT from tissue homogenates according to the method of Ponzio & Jonsson (1979) the organic phase containing the metabolite was transferred to a centrifuge tube containing 100 µl 0.3 M sodium acetate (pH 8.1). After shaking on a Vortex-Rotamixer for 3 min the tubes were centrifuged at 3090 *g* for 5 min and the organic phase was removed by aspiration. Ten µl of this solution was injected into the HPLC with a 25 cm glass column packed with strong anion exchange resin ZIPAX-SAX (Dupont). The values were corrected for recovery calculated on separate samples with the addition of known amounts of authentic 5-HIAA. Biochemical data were analyzed statistically by analysis of variance ANOVA (2 × 2); experimental groups were compared by Tukey's test.

Table 1 Effect of combined treatment with methergoline and (+)-fenfluramine on concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindole-acetic acid (5-HIAA) in the rat brain

Treatment (mg/kg i.p.)	*5-HT*		*5-HIAA*	
	Telencephalon	*Brainstem*	*Telencephalon*	*Brainstem*
Controls	285 ± 14 (6)	756 ± 18 (6)	176 ± 8 (6)	565 ± 12 (6)
(+)-Fenfluramine 5.0	139 ± 6** (6)	588 ± 12** (6)	116 ± 7** (6)	368 ± 16** (6)
Methergoline 1.0	256 ± 8 (6)	780 ± 43 (6)	196 ± 5 (6)	644 ± 22 (6)
Methergoline 1.0 plus (+)-fenfluramine 5.0	149 ± 5 (6)	574 ± 3 (6)	134 ± 3 (6)	396 ± 5 (6)
Controls	229 ± 15 (6)	700 ± 10 (6)	182 ± 11 (6)	534 ± 16 (6)
(+)-Fenfluramine 10.0	72 ± 4** (6)	371 ± 36** (6)	98 ± 3** (6)	352 ± 8** (6)
Methergoline 1.0	215 ± 13 (6)	785 ± 44 (6)	212 ± 10 (6)	595 ± 45 (6)
Methergoline 1.0 plus (+)-fenfluramine 10.0	87 ± 9 (6)	465 ± 37 (6)	188 ± 4 (6)	366 ± 11 (6)

The animals were killed 4 h after injection.
Values are expressed as ng/g tissue and represent the means ± s.e. Number of animals for each group are given in parentheses.
**$P < 0.01$, Tukey's test (vs control).

Table 2 Effect of a combined treatment with methergoline and (+)-norfenfluramine on concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) in the rat brain

Treatment (mg/kg i.p.)	*5-HT*		*5-HIAA*	
	Telencephalon	*Brainstem*	*Telencephalon*	*Brainstem*
Controls	226±8(12)	700±9(12)	167±9(12)	547±14(12)
(+)-Norfenfluramine 2.5	142±9** (12)	578±23** (12)	122±3** (12)	431±11** (12)
Methergoline 1.0	215±6(12)	769±28(12)	192±9(12)	615±25(12)
Methergoline 1.0 plus (+)-norfenfluramine 5.0	142±6(12)	572±28(12)	139±4(12)	504±14(12)
Controls	223±5(6)	706±12(12)	152±11(6)	591±21(12)
(+)-Norfenfluramine 5.0	76±3** (6)	395±22** (12)	82±3** (6)	376±15** (12)
Methergoline 1.0	215±6(6)	723±25(12)	172±11(6)	615±19(12)
Methergoline 1.0 plus (+)-norfenfluramine 5.0	105±9†† (6)	517±20† (12)	107±6(6)	444±9(12)

The animals were killed 4 h after injection.
Values are expressed as ng/g tissue and represent the means±s.e. Number of animals for each group given in parentheses.
**$P<0.01$, Tukey's test (vs control); †$P<0.05$; ††$P<0.01$ ANOVA.

Results

At the doses used, (+)-fenfluramine and (+)-norfenfluramine caused a dose-dependent decrease of 5-HT and 5-HIAA levels in telencephalon and brainstem of rats 4 h after treatment (Tables 1 and 2).

Reserpine, 2 and 5 mg/kg, caused a reduction of 5-HT levels comparable to that observed with the two doses of (+)-fenfluramine and (+)-norfenfluramine but, unlike these compounds, markedly raised 5-HIAA levels in the two brain areas examined (Table 3). As shown in the various tables, no changes in 5-HT and 5-HIAA levels were observed in the telencephalon and brainstem of animals treated with methergoline.

Methergoline significantly reduced the effect of (+)-norfenfluramine (5 mg/kg) on 5-HT levels in telencephalon and brainstem but did not modify the effect of 2.5 mg/kg (Table 2). Neither the decrease of 5-HT levels caused by (+)-fenfluramine nor the reduction of 5-HIAA concentrations induced by (+)-fenfluramine and (+)-norfenfluramine were sig-

Table 3 Effect of combined treatment with methergoline and reserpine on concentrations of 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid 5-HIAA in the rat brain

Treatment (mg/kg i.p.)	*5-HT*		*5-HIAA*	
	Telencephalon	*Brainstem*	*Telencephalon*	*Brainstem*
Controls	223±5(6)	699±17(6)	154±5(14)	580±11(8)
Reserpine 2.0	122±12** (6)	357±43** (6)	300±17** (14)	1345±40** (8)
Methergoline 1.0	215±6(6)	752±37(6)	177±6(14)	583±19(8)
Methergoline 1.0 plus reserpine 2.0	116±10(6)	280±11(6)	409±12° (14)	1663±89†† (8)
Controls	215±8(6)	670±35(6)	172±8(6)	594±15(6)
Reserpine 5.0	77±8** (6)	236±28** (6)	363±17** (6)	1032±71** (6)
Methergoline 1.0	229±15(6)	671±21(6)	181±5(6)	596±15(6)
Methergoline 1.0 plus reserpine 5.0	82±8(6)	160±4† (6)	406±27(6)	1273±41† (6)

The animals were killed 4 h after injection.
Values are expressed as ng/g tissue and represent the means±s.e. Number of animals of each group is given in parentheses.
**$P<0.01$, Tukey's test (vs control); †$P<0.05$; ††$P<0.01$ ANOVA.

nificantly modified by methergoline pretreatment (Tables 1 and 2). Methergoline significantly potentiated the decrease of 5-HT levels caused by reserpine (5 mg/kg) in the brainstem ($P<0.05$, ANOVA) whereas no significant effect was found with reserpine (5 mg/kg) in the telencephalon or with reserpine (2 mg/kg) in the two brain areas examined.

A marked potentiation of the effect of reserpine (2 mg/kg) on 5-HIAA levels was observed in the telencephalon and brainstem of methergoline-treated rats (Table 3). With the highest dose of reserpine only the effect on brainstem 5-HIAA was significantly enhanced by methergoline treatment.

Discussion

A non significant tendency to an increase of 5-HIAA was found in the telencephalon and brainstem of rats treated with methergoline. At 1 mg/kg, methergoline was previously reported to increase significantly 5-HT metabolism in various brain areas of rats (Invernizzi & Samanin, 1981). On the other hand, at doses of 5 and 10 mg/kg, methergoline has been found to decrease 5-HT metabolism and synthesis in brainstem and forebrain of rats (Bourgoin, Artaud, Bockaert, Hèry, Glowinsky & Hamon, 1978). One likely explanation for these apparently contradictory data is that methergoline possesses some agonist action on 5-HT receptors located in presynaptic nerve cell membranes (Haigler & Aghajanian, 1977). This effect, which is known to cause a decrease in impulse flow in 5-HT containing neurones (Haigler & Aghajanian, 1977) may be better revealed at the highest doses of methergoline. Whatever the reason for these differences in the effect of methergoline on 5-HT metabolism, there is little doubt that it is a potent 5-HT antagonist at central synapses (Mawson & Whittington, 1970; Fuxe, Agnati & Everitt, 1975; Sastry & Phillis, 1977) and at doses ranging from 0.5 to 1 mg/kg it has been found to block completely various responses to agents which increase central 5-HT transmission (Fuxe, Ögren, Agnati & Jonsson, 1978; Samanin, Mennini, Ferraris, Bendotti, Borsini & Garattini, 1979).

The fact that methergoline significantly reduced the effect of (+)-norfenfluramine (5 mg/kg) on 5-HT levels suggests that activation of 5-HT receptors contributes to the ability of (+)-norfenfluramine to deplete brain 5-HT. It has been found recently that (+)-norfenfluramine shows some affinity for [^{3}H]-5-HT receptor binding in brain membranes (Garattini *et al.*, 1979), a finding which suggests that (+)-norfenfluramine may mimic the action of 5-HT on postsynaptic receptors. Inhibition of tryptophan hydroxylase following receptor activation may therefore be one mechanism by which (+)-norfenfluramine reduces 5-HT levels in the brain. However, the effect of methergoline on (+)-norfenfluramine was only partial and was not seen with a lower dose of (+)-norfenfluramine, suggesting that mechanisms other than receptor activation contribute to its effect on brain 5-HT. The ability of (+)-norfenfluramine to release 5-HT directly from nerve endings (Mennini *et al.*, 1981) may contribute to 5-HT depletion in the brain.

This mechanism may be more important for (+)-fenfluramine, as methergoline did not modify the effect of any dose of this compound. Only 5-HT in the brainstem of animals treated with (+)-fenfluramine 10 mg/kg, tended to be reduced by methergoline, probably because substantial amounts of (+)-norfenfluramine are formed in these animals.

(+)-Fenfluramine has been shown recently to possess much less affinity than (+)-norfenfluramine for [^{3}H]-5-HT binding to rat brain membranes (Garattini *et al.*, 1979). As regards the mechanism by which (+)-fenfluramine and (+)-norfenfluramine release 5-HT from nerve terminals, reserpine prevents the effect of (+)-fenfluramine but not that of (+)-norfenfluramine on 5-HT release from synaptosomes of rat brain (Mennini *et al.*, 1981). These findings suggest that (+)-fenfluramine but not (+)-norfenfluramine releases 5-HT from a granular reserpine-sensitive pool. However, the mechanism by which (+)-fenfluramine and reserpine act on 5-HT may be different since the former increases the availability of 5-HT at postsynaptic receptors by enhancing 5-HT release (Garattini & Samanin, 1976) whereas the latter interferes mainly with intraneuronal storage of the amine (Carlsson, 1966).

This is confirmed in the present study by the fact that (+)-fenfluramine and reserpine had opposite effects on 5-HT metabolism and by recent findings that (+)-fenfluramine, unlike reserpine, is apparently not stored in vesicles of 5-HT-containing nerve terminals (Mennini, Pataccini, Crunelli, Caccia, Ballabio, Samanin & Garattini, 1980). Moreover, methergoline, which did not modify the effect of (+)-fenfluramine on 5-HIAA levels, significantly potentiated the increase of 5-HIAA caused by reserpine. This is the effect one would expect from a combined treatment with an intraneuronal releaser and an agent which tends to increase impulse flow in presynaptic neurones. That methergoline increases impulse flow of 5-HT containing neurones through a feedback mechanism triggered by blockade of postsynaptic receptors has been suggested by some authors (Fuxe *et al.*, 1975; Invernizzi & Samanin, 1981) although others (Bourgoin *et al.*, 1978), using doses of methergoline higher than those used in the present study, found a reduction in 5-HT synthesis and metabolism in the rat brain. Methergoline did not significantly modify the reduction of 5-HIAA caused

by (+)-norfenfluramine. Should changes in 5-HIAA levels simply reflect changes in 5-HT synthesis, it would be surprising if methergoline partially prevented the effect of (+)-norfenfluramine on 5-HT but not that on 5-HIAA. However, additional mechanisms by which (+)-norfenfluramine reduces 5-HT metabolism may involve enhanced release of 5-HT combined with inhibition of 5-HT reuptake into the nerve endings. (+)-Norfenfluramine (and (+)-fenfluramine) has been found to block effectively the accumulation of 5-HT into synaptosomal preparations of rat brain (Garattini *et al.*, 1979).

In conclusion, although receptor sites sensitive to methergoline may partially contribute to the ability of (+)-norfenfluramine to reduce 5-HT levels in the rat brain, it is clear that other, as yet unknown mechanisms are responsible for 5-HT depletion caused by (+)-fenfluramine and (+)-norfenfluramine.

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