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A comparison of the effects on central 5-HT function of sibutramine hydrochloride and other weight-modifying agents

[1,3]D.J. Heal, [1]S.C. Cheetham, [1]M.R. Prow, [1,2]K.F. Martin & [1]W.R. Buckett

[1]Knoll Pharmaceuticals Research & Development, Nottingham NG1 1GF

1 Effects on 5-HT function of sibutramine and its active metabolites, BTS 54 354 and BTS 54 505, were compared with fluoxetine, (+)-fenfluramine and (+)-amphetamine.

2 *In vitro* sibutramine weakly inhibited [^{3}H]-5-HT uptake into brain synaptosomes. BTS 54 354, BTS 54 505 and fluoxetine were powerful [^{3}H]-5-HT uptake inhibitors, whereas (+)-fenfluramine and (+)-amphetamine were very much weaker. Conversely, whilst sibutramine, its metabolites and fluoxetine did not release [^{3}H]-5-HT from brain slices at $\leqslant 10^{-5}$M, (+)-fenfluramine and (+)-amphetamine concentration-dependently increased [^{3}H]-5-HT release.

3 Sibutramine and fluoxetine had no effect on 5-hydroxytryptophan (5-HTP) accumulation in either frontal cortex or hypothalamus at doses <10 mg kg^{-1}. In contrast, (+)-amphetamine ($\geqslant 3$ mg kg^{-1}) reduced 5-HTP in hypothalamus, whilst (+)-fenfluramine ($\geqslant 1$ mg kg^{-1}) decreased 5-HTP in both regions.

4 Sibutramine (10 mg kg^{-1} i.p.) and fluoxetine (10 mg kg^{-1} i.p.) produced slow, prolonged increases of extracellular 5-HT in the anterior hypothalamus. In contrast, (+)-fenfluramine (3 mg kg^{-1} i.p.) and (+)-amphetamine (4 mg kg^{-1} i.p.) induced rapid, short-lasting increases in extracellular 5-HT.

5 Only (+)-fenfluramine (10 mg kg^{-1}) altered 5-HT$_{2A}$ receptors in rat frontal cortex when given for 14 days, producing a 61% reduction in receptor number and a 18% decrease in radioligand affinity.

6 These results show that sibutramine powerfully enhances central 5-HT function via its secondary and primary amine metabolites; this effect, like that of fluoxetine, is almost certainly mediated through 5-HT uptake inhibition. By contrast, (+)-fenfluramine enhances 5-HT function predominantly by increasing 5-HT release. (+)-Amphetamine, though weaker than (+)-fenfluramine, also enhances 5-HT function by release.

Keywords: Sibutramine; BTS 54 354; BTS 54 505; fluoxetine; (+)-fenfluramine; (+)-amphetamine; central 5-HT function; 5-HT uptake; 5-HT release

Introduction

Sibutramine (BTS 54 524; *N*-[1-{(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-*N*,*N*-dimethylamine HCl monohydrate) is a novel drug for the treatment of obesity which has recently been approved for prescription use in the U.S.A., Mexico and Brazil. Pharmacologically, sibutramine acts as an inhibitor of 5-hydroxytryptamine (5-HT, serotonin) and noradrenaline uptake *in vivo* (Heal & Cheetham, 1997) and consistent with this mechanism, sibutramine was initially developed as an antidepressant (Buckett *et al*., 1988). However, in Phase II clinical trials for the treatment of depression, efficacy was not proven but significant weight loss was observed in these depressed subjects (Kelly *et al*., 1995). Following the strategic switch of sibutramine to obesity from a drug for the treatment of depression, animal studies have demonstrated that sibutramine reduces body weight of rats by a dual action which involves both a decrease in food intake (Fantino *et al*., 1995; Stricker-Krongrad *et al*., 1995; Jackson *et al*., 1997a,b) by the enhancement of satiety (Halford *et al*., 1995) and also an increase in energy expenditure by the enhancement of thermogenesis (Stock, 1997). There is evidence both from experiments using selective uptake inhibitors of 5-HT and noradrenaline given alone and in combination and using monoaminergic receptor antagonists that sibutramine's effects on food intake (Stricker-Krongrad *et al*., 1996; Jackson *et al*., 1997a,b) and energy expenditure (Stock, 1997; Heal & Cheetham, 1997) are mediated via combined inhibition of 5-HT and noradrenaline uptake.

In the present study, the 5-HT mechanisms by which sibutramine exerts its effects have been further investigated using [^{3}H]-5-HT uptake into synaptosomes, release of [^{3}H]-5-HT from preloaded brain slices, accumulation of the 5-HT precursor, 5-HTP and levels of extracellular 5-HT in the anterior hypothalamus using *in vivo* microdialysis. The effects of repeated administration of sibutramine on 5-HT$_{2A}$ receptor number and affinity in rat brain have also been determined by radioligand binding. These effects were compared with those of fluoxetine and (+)-fenfluramine, which modify weight via the 5-hydroxytryptaminergic system (Samanin & Garattini, 1982; Fuller & Wong, 1989; Garattini *et al*., 1989) and (+)-amphetamine, which acts predominantly via the catecholaminergic system (Carlsson, 1970; Silverstone & Kyriakides, 1982).

Methods

Animals, drugs and reagents

Experiments were conducted using adult male CD rats (150–250 g) with the exception of the microdialysis studies in which adult male Wistar rats (265–310 g) were used. Animals were kept at 21°C and 55% humidity on a 12 h light/dark cycle (commencing 07.00 h). Rats were housed in groups of two or

[2]Present address: BTG plc, London EC4M 7SB.
[3]Author for correspondence.

three and were allowed free access to pelleted food and tap water.

Drugs were obtained from the following sources: sibutramine hydrochloride monohydrate, BTS 54 354 (*N*-1-{[1-(4-chlorophenyl)cyclobutyl]-3-methylbutyl}-*N*-methylamine hydrochloride), BTS 54 505 (1-[1(4-chlorophenyl)cyclobutyl]-3-methylbutylamine hydrochloride) were synthesised by the Medicinal Chemistry Department of Knoll Pharmaceuticals Research & Development Department, Nottingham, U.K.; fluoxetine hydrochloride (Eli Lilly, Indianapolis, U.S.A.); zimeldine, GBR 12909 (1-{2-[bis(4-fluorophenyl) methoxy]-ethyl}-4-(3-phenylpropyl)piperazine dihydrochloride), (+)-fenfluramine (Research Biochemicals, Natick, U.S.A.); (+)-amphetamine sulphate (SmithKline-Beecham, Welwyn Garden City, U.K.), desipramine hydrochloride, NSD-1015 (*m*-hydroxybenzylhydrazine), pargyline hydrochloride (Sigma, Poole, U.K.) and methysergide (Sandoz, Basle, Switzerland). Drugs for administration were given orally (p.o.) dissolved in distilled water (5 ml kg^{-1}) or intraperitoneally (i.p.) dissolved in 0.9% (w/v) saline (2 ml kg^{-1}).

Reagents were obtained from Sigma (Poole, U.K.), Aldrich (Gillingham, U.K.), BDH (Poole, U.K.), Kodak (Liverpool, U.K.), Rathburn Chemicals (Walkerburn, U.K.), Romil (Cambridge, U.K.) and FSA (Loughborough, U.K.). [^{3}H]-5-HT 26.7 Ci $mmol^{-1}$ and [^{3}H]-noradrenaline 11.4–24.4 Ci $mmol^{-1}$, [^{3}H]-dopamine 23.9–30.1 Ci $mmol^{-1}$ and [^{3}H]-ketanserin 60 Ci $mmol^{-1}$ were all obtained from New England Nuclear (Boston, U.S.A.). Reagents for HPLC methods were of the highest purity grade available and water was distilled and deionized prior to use. Reagents for other experiments were of analytical grade purity.

Measurement of [^{3}H]-monoamine uptake into synaptosomes

Rats were killed by stunning and cervical dislocation, brains were removed and frontal cortex (for [^{3}H]-noradrenaline and [^{3}H]-5-HT uptake) and striatum (for [^{3}H]-dopamine uptake) were immediately dissected. Measurement of [^{3}H]-monoamine uptake into synaptosomes was then carried out according to the methods described in Cheetham *et al.* (1996; [^{3}H]-noradrenaline uptake), Cheetham *et al.* (1993; [^{3}H]-5-HT uptake) and Heal *et al.* (1992a; [^{3}H]-dopamine uptake).

Measurement of [^{3}H]-5-HT release in vitro

Rats were killed by stunning and cervical dislocation and frontal cortices were rapidly removed. Tissues were prepared by slicing at 250 μm × 250 μm intervals in two directions at 90° using a McIlwain tissue chopper. The slices were incubated for 20 min at 37°C in 2 ml Krebs-Henseleit buffer (constituents, mM: NaCl 118, KCl 4.7, Na_2CO_3 25, (+)-glucose 11, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 1.3) gassed with 95% O_2–5% CO_2 to pH 7.4 containing 0.13 mM pargyline and 95 nM [^{3}H]-5-HT. Aliquots of slices (~5 mg) were placed in individual chambers of the Shearline superfusion apparatus and were superfused with Krebs-Henseleit buffer at 37°C using a Desaga STA peristaltic pump. The flow-rate was 1 ml min^{-1} and an initial superfusion of the slices was performed for 30 min to remove extraneuronal [^{3}H]-5-HT. Fractions of superfusion medium were collected at 2 min intervals using an LKB Ultrorac 2070 fraction collector. Frontal cortical slices were initially superfused for 8 min to determine basal release. The tissues were then superfused with Krebs-Henseleit buffer containing 25 mM KCl (2 min), 10^{-7}–10^{-4} M test drug solutions (8 min) or Krebs-Henseleit buffer alone (2 min or 8 min as appropriate). Finally, all slices were superfused with Krebs-Henseleit buffer for the remainder of the 26 min test. The radioactivity present in the fractions was measured using a Packard Tri-Carb 2200 CA liquid scintillation counter at an efficiency of ~45–50%. The accumulated efflux of tritium present in fractions 5–13 was then calculated as a fraction of the total radioactivity initially present in the slices.

Measurement of 5-HTP accumulation in rat brain ex vivo

Rats were administered the test drug (0.3–10 mg kg^{-1} p.o.) or distilled water (5 ml kg^{-1} p.o.) and 30 min later, all animals were injected with the *L*-aromatic amino acid decarboxylase inhibitor, NSD-1015 (*m*-hydroxybenzylhydrazine; 100 mg kg^{-1} i.p.). After a further 30 min, rats were killed and frontal cortex and hypothalamus were removed and frozen in liquid nitrogen. Tissues were homogenized in 5 (frontal cortex) or 15 (hypothalamus) volumes (w/v) of ice-cold 0.1 M perchloric acid containing 400 μM sodium metabisulphite (antioxidant). The homogenates were centrifuged twice at 15,000 × *g* for 15 min at 4°C. Fifty μl aliquots of the resulting supernatant layers were injected onto the h.p.l.c. system which consisted of a Spectra-Physics 8810 pump connected via a Waters WISP 710B autoinjector to a 250 × 4.6 mm 5 μm Spherisorb ODS-1 reversed-phase analytical column protected by a Brownlee 30 × 4.6 mm 7 μm Aquapore RP300 precolumn. The analytical column was maintained at 45°C using a Jones Chromatography column heater. The mobile phase was 0.1 M sodium acetate/citric acid, 3% (v/v) methanol, 0.1% (v/v) dibutylamine, pH 3.3 pumped at 1.0 ml min^{-1}. 5-HTP was measured using an ESA Coulochem 5100A detector with electrode 1 set at +0.15 V and electrode 2 (used for detection) at +0.35 V; this was linked to a Perkin Elmer Turbochrom data acquisition system.

Measurement of extracellular 5-HT in vivo

Rats were anaesthetised with either Equithesin (1 ml, i.p.) or with a mixture of isoflurane, oxygen and nitrous oxide and then held in a stereotaxic frame (David Kopf Instruments). A microdialysis probe (Carnegie Medicin AB) was implanted into the anterior hypothalamus (co-ordinates for the probe tip relative to bregma: anterior-posterior −1.8 mm, medio-lateral +0.8 mm, dorso-ventral −9.3 mm according to Paxinos & Watson, 1986). The probe was continuously perfused at a flow rate of 1.2 μl min^{-1} with an aqueous salt solution containing (mM): NaCl 147, KCl 4 and $CaCl_2$ 1.5. During surgery, anaesthesia was maintained with supplementary doses of Equithesin (0.2 ml, i.p.) when required (in experiments involving sibutramine, fluoxetine and (+)-fenfluramine). Rats were allowed to recover overnight in specially adapted holding chambers. The dialysis probes were connected to a fluid swivel which allowed free movement. Experiments were performed 16 to 20 h after surgery when dialysates were collected every 20 min and analysed for their 5-HT content by h.p.l.c. with electrochemical detection as described below. Four 20 min baseline samples were taken before any drug intervention was made. Drug or saline was administered (i.p.) and 20 min samples were collected for a further 3 h.

The h.p.l.c. system consisted of a Gynkotec 300 pump connected via a Spark Holland Triathlon refrigerated autosampler (4°C) to a Hypersil 3 μm ODS 2 mm × 15 cm reversed phase analytical column (HPLC Technology; for sibutramine, fluoxetine and (+)-fenfluramine) or to a Spherisorb 3 μm ODS2 2.1 mm × 7.5 cm column (Presearch;

for (+)-amphetamine). The h.p.l.c. mobile phase (sibutramine, fluoxetine and (+)-fenfluramine) consisted of 10 mM sodium dihydrogen phosphate, 0.46 mM sodium 1-octanesulphonic acid (ion pair reagent), 0.67 mM EDTA, 20% v/v methanol and distilled deionised water to 100%. The pH was adjusted to 3.0 with phosphoric acid. For the (+)-amphetamine study, the h.p.l.c. mobile phase was 150 mM sodium dihydrogen phosphate, 0.46 mM sodium 1-octanesulphonic acid, 1 mM EDTA, 4% v/v isopropanol and distilled deionized water to 100%. The pH was adjusted to 5.0 with sodium hydroxide. The mobile phases were filtered and degassed before use and pumped at a flow rate of 0.3 or 0.2 ml min^{-1}. 5-HT was detected using an Antec electrochemical detector set at a potential of +0.7 V versus Ag/AgCl reference electrode, linked to a Spectra Physics Chromjet integrator. Dialysate 5-HT concentrations were determined using peak height by reference to the ratio with an external 200 fmol 5-HT standard.

Measurement of 5-HT$_{2A}$ receptors in rat frontal cortex

Rats were administered 10 mg kg^{-1} p.o. of sibutramine, fluoxetine, (+)-fenfluramine, or (+)-amphetamine once daily for 14 days. Control rats received distilled water (5 ml kg^{-1} p.o.) over the same dosing regime. Twenty-four hours after the final treatment, rats were killed and frontal cortices were rapidly removed and frozen at −80°C until required. Membranes were prepared as described by Cheetham *et al.* (1988). Aliquots (400 μl; equivalent to 5 mg wet weight tissue/tube) were incubated with 50 μl [^{3}H]-ketanserin at eight concentrations ranging from 0.5–4.8 nM, 50 μl 10% ethanol (total binding) or 50 μl methysergide in 10% ethanol (5×10^{-6} M; non-specific binding) for 15 min at 37°C. Membrane-bound radioactivity was recovered by filtration under vacuum through Skatron 11734 filters using a Skatron cell harvester. Filters were rapidly washed with 50 mM Tris-HC1, pH 7.7 (wash setting 9,9,0) and punched into vials. Ultima Gold™ MV (1 ml; Packard, U.K.) was then added to each vial and radioactivity (d.p.m.) determined by liquid scintillation counting using a 1900 CA Tri-Carb Liquid Scintillation counter at ~45–50% efficiency. Aliquots of membrane were stored at −20°C for subsequent protein determination (Lowry *et al.*, 1951) using bovine serum albumin as the reference standard. Equilibrium dissociation constants (K_d) and the maximal number of binding sites (B_{max}) were determined by non-linear regression analysis fitting to a one-site binding model (LIGAND; Munson & Rodbard, 1980).

Statistics

Although control data have been pooled in some figures and tables for the sake of clarity, statistical comparisons were always made against the appropriate subgroup of controls.

The following statistical analyses were used: (a) For studies involving [^{3}H]-5-HT release *in vitro* and 5-HT$_{2A}$ receptor number and affinity, a 1-way ANOVA followed by Bonferroni's test was used; (b) 5-HTP accumulation was statistically analysed using a 2-way ANOVA and Williams' test; (c) Microdialysis (extracellular 5-HT levels) data used either the least significant difference test ((+)-amphetamine) or Williams' test (sibutramine, fluoxetine and (+)-fenfluramine).

Microdialysis data were considered invalid and, therefore, not included in the analysis if the pre-intervention baseline values varied by >40%. The maximum value for each treatment was compared to other treatments using Newman-Keuls' test and the times to maximum compared using the Wilcoxon rank-sum test with significance determined by exact critical values (allowing for ties).

Results

Effects on [^{3}H]-monoamine uptake

The [^{3}H]-monoamine uptake inhibition profiles of sibutramine and its primary and secondary amine metabolites are shown in comparison to those of fluoxetine, (+)-amphetamine and (+)-fenfluramine (Table 1). The results clearly demonstrated that sibutramine was a relatively weak inhibitor of [^{3}H]-noradrenaline uptake with no effect on that of [^{3}H]-5-HT or [^{3}H]-dopamine. By contrast, BTS 54 354 and BTS 54 505 were very powerful inhibitors of [^{3}H]-noradrenaline uptake *in vitro* with slightly less activity against [^{3}H]-5-HT and [^{3}H]-dopamine. Fluoxetine, a potent and selective 5-HT uptake inhibitor, yielded a K_i value against [^{3}H]-5-HT uptake which was very similar to those of BTS 54 354 and BTS 54 505. (+)-Fenfluramine weakly inhibited [^{3}H]-5-HT and [^{3}H]-noradrenaline uptake, but had no effect on the uptake of [^{3}H]-dopamine. Finally, (+)-amphetamine was a moderately potent inhibitor of [^{3}H]-noradrenaline uptake *in vitro*, but was weaker against [^{3}H]-dopamine and was essentially inactive against [^{3}H]-5-HT.

Effects on [^{3}H]-5-HT release

Sibutramine, BTS 54 354 and BTS 54 505 had no significant effect on [^{3}H]-5-HT overflow from rat cortical slices at concentrations of 10^{-7}–10^{-5} M. However at 10^{-4} M, the highest concentration tested, these three compounds increased [^{3}H]-5-HT release by 100–150% (Figure 1). An identical pattern of results was observed with fluoxetine (Figure 1). (+)-Fenfluramine, on the other hand, concentration-dependently enhanced [^{3}H]-5-HT release, producing a significant increase in overflow at the much lower concentration of 10^{-6} M (Figure 1). (+)-Amphetamine also potentiated the release of [^{3}H]-5-HT, but its effects were less pronounced than those of (+)-fenfluramine (Figure 1).

Effects on 5-HT synthesis

Sibutramine (0.3–10 mg kg^{-1} p.o.) was without effect on the accumulation of 5-HTP (a measure of 5-HT synthesis) in rat hypothalamus following NSD-1015 pretreatment (Figure 2a). A similar pattern was observed for the lower doses of sibutramine in the frontal cortex, the exception being the

Table 1 Comparison of the monoamine uptake inhibition profiles of sibutramine and its metabolites with those of other weight-modifying agents

	K_i (nM)		
Drug	*[^{3}H]-5-HT*	*[^{3}H]-Noradrenaline*	*[^{3}H]-Dopamine*
Sibutramine	1811±193	283±25	2309±104
BTS 54 354	17±2	2.7±0.3	24±1
BTS 54 505	25±1	4.9±0.3	31±2
Fluoxetine	11±1	320±33	2025±85
(+)-Fenfluramine	272±14	260±21	6227±499
(+)-Amphetamine	1441±72	45±2	132±13

The inhibition of uptake of [^{3}H]-monoamines into rat brain synaptosomes was performed as described in Methods. Results are the mean±s.e.mean for three to four observations. The Hill slopes ranged from 0.9–1.4.

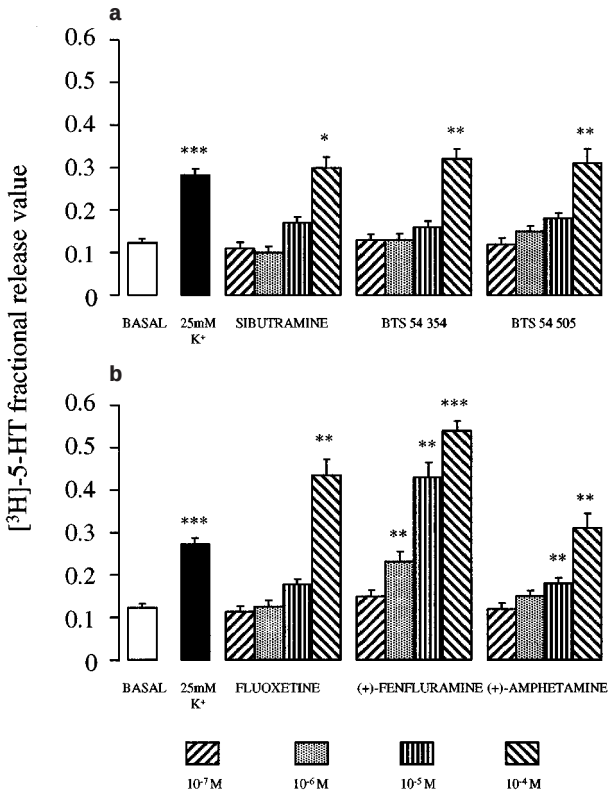

Figure 1 The effects of sibutramine, its metabolites and other weight-modifying agents on [^{3}H]-5-HT release *in vitro*. [^{3}H]-5-HT release from slices of rat frontal cortex was measured by superfusion as described in Methods. The effects of $10^{-7}-10^{-4}$ M sibutramine, BTS 54 354, BTS 54 505, fluoxetine, (+)-fenfluramine and (+)-amphetamine were determined. Results are the mean ± s.e.mean for six observations at each drug concentration. Although controls have been combined for the sake of clarity, significance values were always calculated using the appropriate subgroup of basal controls. Significantly different from appropriate basal control. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

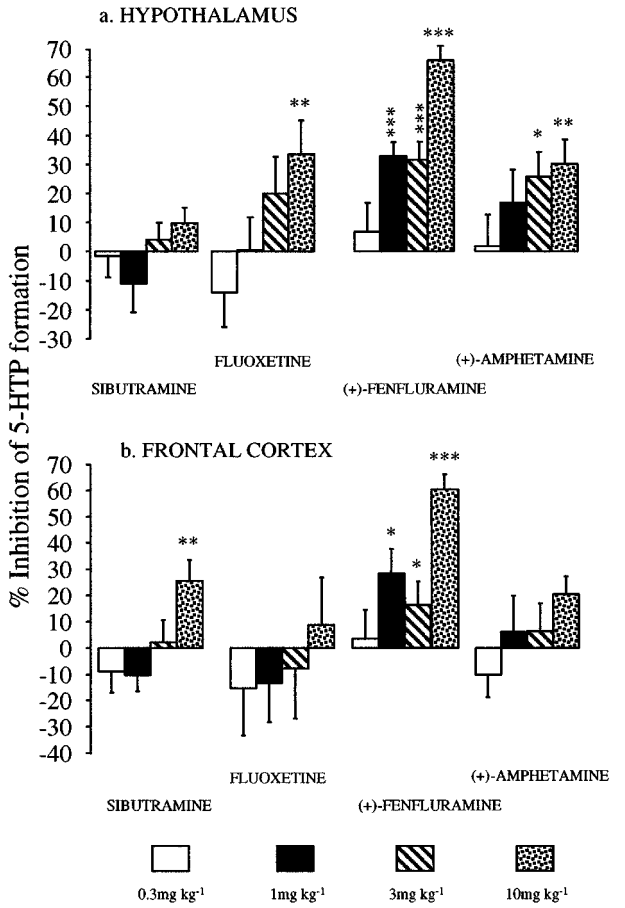

Figure 2 The effects of sibutramine and other weight-modifying drugs on 5-HTP formation in rat (a) hypothalamus and (b) frontal cortex. Groups of rats were orally administered sibutramine, fluoxetine, (+)-fenfluramine, (+)-amphetamine or distilled water vehicle 30 min prior to NSD-1015 (100 mg kg^{-1}). Animals were killed after a further 30 min and 5-HTP concentrations were measured as described in Methods. Results are the mean ± s.e.mean, $n=6-8$ for each drug dose; controls, $n=8-15$. Significantly different from appropriate vehicle control. $^{*}P<0.05$, $^{**}P<0.01$.

highest dose used (10 mg kg^{-1} p.o.), which produced a significant 25% ($P<0.01$) reduction in 5-HTP formation (Figure 2b). Fluoxetine (0.3–10 mg kg^{-1} p.o.) did not alter 5-HTP accumulation in the frontal cortex and only significantly decreased (−34%; $P<0.01$) the level of this 5-HT precursor in the hypothalamus at the high dose of 10 mg kg^{-1} p.o. (Figure 2a and b). (+)-Fenfluramine at doses of 1, 3 and 10 mg kg^{-1} p.o., evoked marked reductions of 5-HTP formation in both the hypothalamus and frontal cortex (maximum decreases −66% and −60% respectively, see Figure 2a and b). (+)-Amphetamine (3 and 10 mg kg^{-1} p.o.) caused significant decreases in 5-HTP levels in the hypothalamus but was without effect on 5-HTP accumulation in the frontal cortex (Figure 2a and b).

Effects on extracellular 5-HT in the anterior hypothalamus

Drugs were administered at doses which are effective in reducing food intake in rats (~3× oral ED_{50} to inhibit feeding at time of maximum effect, except fluoxetine). It was found that sibutramine (10 mg kg^{-1} i.p.; Jackson *et al.*, 1997b) increased dialysate 5-HT content slowly (maximum increase 214% [$P<0.05$] of pre-intervention level, 100 min after injection, see Figure 3 and Table 2). Fluoxetine (10 mg kg^{-1} i.p.; Wong *et al.*, 1988) increased 5-HT levels maximally after 100 min (406%; $P<0.001$) and this elevation remained significantly greater than saline-treated controls for the remainder of the experiment (up to 180 min, see Figure 3 and Table 2). (+)-Fenfluramine (3 mg kg^{-1} i.p.; Jackson *et al.*, 1997b) caused a rapid increase of extracellular 5-HT which was maximal (528%; $P<0.001$) 40 min after drug administration, and remained significantly higher than control levels for up to 180 min (Figure 3 and Table 2). (+)-Amphetamine (4 mg kg^{-1} i.p.; Jackson, unpublished data) also evoked a rapid elevation of extracellular 5-HT (544%; $P<0.001$) 20 min after administration. Following this peak, dialysate 5-HT levels declined slowly, but they remained significantly above basal values for ⩽160 min post-injection (Figure 3 and Table 2).

The effects of the four drugs on extracellular 5-HT levels in the rat anterior hypothalamus were compared in terms of maximum 5-HT levels attained and times of such maxima (Table 2). It was found that (+)-fenfluramine (3 mg kg^{-1}) and (+)-amphetamine (4 mg kg^{-1}) both increased dialysate 5-HT significantly more ($P<0.05$ or $P<0.01$) than either sibutramine (10 mg kg^{-1}) or fluoxetine (10 mg kg^{-1}) and also that fluoxetine caused a significantly ($P<0.01$) higher increase in 5-HT than sibutramine. Thus, the overall rank order of

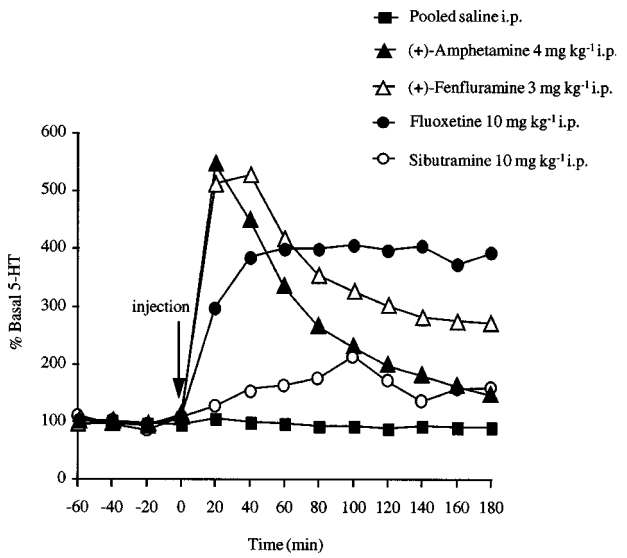

Figure 3 The effects of sibutramine and other weight-modifying drugs on extracellular 5-HT levels in rat anterior hypothalamus. Each point represents the mean % of pre-intervention value (s.e.mean $\leqslant \pm 23\%$ of mean, $n=4-5$ animals (for each drug). Saline-treated controls ($n=17$) were pooled for the sake of clarity. Statistical comparisons were made as in Table 2. Significantly different from saline-treated controls: (+)-amphetamine $0.05>P<0.001$ (20–160 min after injection), (+)-fenfluramine or fluoxetine $P<0.001$ (20–180 min), sibutramine $P<0.05$ (80–180 min).

Table 2 Comparison of maximum extracellular 5-HT levels and time of maximum effect evoked by four weight-modifying drugs in microdialysis studies

Treatment	*Maximum effect (%) ± s.e.mean*	*Time to maximum (min) ± s.e.mean*
Sibutramine ($10\,mg\,kg^{-1}$ i.p.)	214 ± 36	100 ± 23
Fluoxetine ($10\,mg\,kg^{-1}$ i.p.)	406 ± 56^{a}	75 ± 24
(+)-Fenfluramine ($3\,mg\,kg^{-1}$ i.p.)	$528\pm67^{a,b}$	$24\pm4^{c,d}$
(+)-Amphetamine ($4\,mg\,kg^{-1}$ i.p.)	$544\pm33^{a,b}$	25 ± 5^{c}

For maximum effect on dialysate 5-HT levels, comparisons between treatments used Newman-Keuls' test. $^{a}P<0.01$ versus sibutramine, $^{b}0.05>P<0.01$ versus fluoxetine. For time of maximum effect on dialysate 5-HT levels, comparisons between treatments used Wilcoxon's rank-sum test. $^{c}P<0.05$ versus sibutramine, $^{d}P<0.05$ versus fluoxetine.

potency was (+)-fenfluramine = (+)-amphetamine > fluoxetine > sibutramine. It was also observed that the effects of (+)-fenfluramine on dialysate 5-HT were significantly ($P<0.05$) more rapid in onset than those of either sibutramine or fluoxetine and also that (+)-amphetamine evoked a more rapid increase ($P<0.05$) in extracellular 5-HT than sibutramine (Table 2).

Effects of repeated drug administration on 5-HT$_{2A}$ receptors

Sibutramine, fluoxetine, (+)-fenfluramine and (+)-amphetamine were each given to groups of rats at a dose of 10 mg kg^{-1} p.o. once daily for 14 days. Controls received distilled water vehicle (5 ml $kg^{-1} \times 14$). The effects of these drugs on 5-HT$_{2A}$ receptors in frontal cortex were then determined 24 h after the final treatment. (+)-Fenfluramine produced a striking 61% decrease in 5-HT$_{2A}$ receptor number accompanied by a small, but significant, decrease in K_d. The other three drugs were without effect (Table 3).

Table 3 Effects of repeated administration of sibutramine and other weight-modifying agents on rat cortical 5-HT$_{2A}$ receptors

Drug	B_{max} (fmol mg protein^{-1})	K_d (nM)
Vehicle	433 ± 16	0.51 ± 0.02
Sibutramine	442 ± 13	0.47 ± 0.01
Fluoxetine	461 ± 12	0.53 ± 0.02
(+)-Fenfluramine	175 ± 5***	0.60 ± 0.01**
(+)-Amphetamine	444 ± 15	0.50 ± 0.01

Groups of rats were administered drugs at a dose of $10\,mg\,kg^{-1}$ p.o. or vehicle (distilled water) once daily for 14 days. 5-HT$_{2A}$ receptor binding was measured in rat frontal cortex 24 h after the final treatment as described in Methods. Results are given as mean ± s.e.mean ($n=10$ drug-treated groups; $n=20$ vehicle-treated group). Significantly different from vehicle-treated control. $^{**}P<0.01$, $^{***}P<0.001$.

Discussion

Sibutramine is a noradrenaline and 5-HT uptake inhibitor *in vivo* which has been shown to reduce body weight in animal studies by a dual action that decreases food intake (Fantino *et al*., 1995; Stricker-Krongrad *et al*., 1995; Halford *et al*., 1995; Jackson *et al*., 1997a,b) and increases resting energy expenditure (Stock, 1997). In both instances, its anti-obesity actions are mediated *via* a synergistic interaction between noradrenaline and 5-HT in the central nervous system (Jackson *et al*., 1997a,b; Heal & Cheetham, 1997). Sibutramine's pharmacological mode of action as an anti-obesity agent differs from those of all other anti-obesity drugs. The selective 5-HT uptake inhibitor, fluoxetine, has been reported to decrease the food intake of rats by enhancing satiety (Wong *et al*., 1988) but its potency is relatively weak compared with that of the marketed anti-obesity drugs, sibutramine, (+)-fenfluramine and (+)-amphetamine (Jackson *et al*., 1997b; Jackson, unpublished observations, Wong *et al*., 1988). In addition, fluoxetine has no effect on thermogenesis (Heal & Cheetham, 1997). These findings are consistent with the results of clinical trials which have clearly shown that while fluoxetine produces moderate short-term weight-loss in obese subjects, this effect is not maintained with longer-term treatment (Goldstein *et al*., 1994). (+)-Fenfluramine, like sibutramine, decreases food intake by enhancing satiety (Halford *et al*., 1995). It produces this behavioural effect by selectively enhancing central 5-hydroxytryptaminergic function (Fibiger *et al*., 1973; Heal & Cheetham, 1997) and this enhancement is again mediated almost exclusively by enhanced monoamine release (Gundlah *et al*., 1997; this study). (+)-Fenfluramine has been reported to increase energy expenditure (Stallone & Levitsky, 1994). Once again, however, the mechanism differs from that of sibutramine because (+)-fenfluramine increases meal-induced thermogenesis, but it has no effect on resting metabolic rate (Stallone & Levitsky, 1994). In the case of (+)-amphetamine, this drug reduces food intake. However, this reduction is not mediated through enhanced satiety. Rather, it occurs secondarily to the

Table 4 A summary of the anti-obesity actions of sibutramine, fluoxetine, (+)fenfluramine and (+)-amphetamine

	Anti-obesity actions			
Drug	*Reduces food intake*	*Increases thermogenesis*	*Mediating neurotransmitters*	*Mechanism*
Sibutramine	✓	✓[a]	NA + 5-HT	Uptake inhibition
Fluoxetine	✓	x	5-HT	Uptake inhibition
(+)-Fenfluramine	✓	✓[b]	5-HT	Release
(+)-Amphetamine	✓	✓[c]	DA + NA	Release

For detailed explanation see Discussion. [a]Increases resting metabolic rate; [b]Meal-induced thermogenesis; [c]Behavioural activation.

behavioural activation induced by this powerful psychostimulant (Halford *et al*., 1995). Since (+)-amphetamine increases locomotor activity at doses which reduce food intake (Halford *et al*., 1995), its anti-obesity actions must inevitably result in part from increased energy expenditure. This effect on energy expenditure differs markedly from that of sibutramine, which increases resting metabolic rate by selectively activating central sympathetic drive to brown adipose tissue (Stock, 1997). Evidence from lesioning and antagonist studies indicate that (+)-amphetamine reduces food intake via enhanced central dopaminergic and noradrenergic function (Fibiger *et al*., 1973; Samanin *et al*., 1975, 1977; Jackson, unpublished observations) and this effect predominantly derives from neurotransmitter release (Westerink *et al*., 1987; Martin *et al*., 1995; Wortley *et al*., 1997, 1998). The data on the respective anti-obesity actions of the drugs used in this study are summarized in Table 4.

In the present study, the actions of sibutramine and its active metabolites, BTS 54 354 and BTS 54 505, have been compared with those of the other weight-reducing drugs, i.e. (+)-fenfluramine and (+)-amphetamine, and the selective 5-HT uptake inhibitor, fluoxetine.

In vitro, sibutramine has no actions to inhibit [^{3}H]-5-HT or [^{3}H]-dopamine uptake, but it is a weak inhibitor of [^{3}H]-noradrenaline uptake. However, its metabolites, BTS 54 354 and BTS 54 505, are very potent inhibitors of [^{3}H]-noradrenaline uptake with $\sim$10 fold less potent effects on that of [^{3}H]-5-HT and [^{3}H]-dopamine. *In vivo*, however, the actions of sibutramine and its metabolites on central dopamine function are much lower than those exerted on noradrenaline and 5-HT function (Heal *et al*., 1992b).

Focusing on [^{3}H]-5-HT uptake, BTS 54 354 and BTS 54 505 are potent inhibitors of [^{3}H]-5-HT uptake with K_i values similar to that of fluoxetine; the K_i value of 11 nM for fluoxetine is in close agreement with results published by Richelson & Pfenning (1984) and Wong *et al*. (1985). Comparing these values for the inhibition of [^{3}H-5-HT uptake with the concentrations required to increase [^{3}H]-5-HT overflow from rat cortical slices revealed a separation of 400 fold for sibutramine and 4000 to 6000 fold for its metabolites; these separations compare closely with a separation of $\sim$10,000 fold for fluoxetine. However, these findings contrast markedly with those obtained for (+)-fenfluramine and (+)-amphetamine. (+)-Fenfluramine is a weak inhibitor of [^{3}H]-5-HT uptake with a K_i of 272 nM which concurs with previously reported data (Wong *et al*., 1985; Garattini *et al*., 1989). (+)-Fenfluramine significantly increased [^{3}H]-5-HT efflux from rat cortical slices at a concentration of 1000 nM yielding a separation between the K_i for the inhibition of [^{3}H]-5-HT uptake versus the concentration to significantly increase [^{3}H]-5-HT release *in vitro* of only 4 fold. Similarly, (+)-amphetamine has a K_i value for the inhibition of [^{3}H]-5-HT uptake of 1441 nM and it increased [^{3}H]-5-HT release *in vitro* at 10,000 nM, yielding a potency separation of $\sim$6 fold.

From the above, it is apparent that for drugs which enhance central 5-HT function by inhibition of uptake, e.g. fluoxetine and sibutramine, there is a very marked separation between their *in vitro* K_is for inhibition of [^{3}H]-5-HT uptake and the concentrations at which they will significantly increase [^{3}H]-5-HT release; in fact, these latter concentrations are so high that they are almost certainly not of pharmacological relevance. However, for drugs which enhance central 5-HT function by release, e.g. (+)-fenfluramine and (+)-amphetamine, there is only a very small separation between the drug concentrations which produce these two differential pharmacological effects. Since inhibition of monoamine uptake only measures competition for the uptake carrier between the releasing drug and the [^{3}H]-monoamine under investigation, the term 'uptake inhibitor' is probably inappropriate as both the releasing drug and the monoamine are substrates for the monoamine uptake transporter.

In vivo, the actions of sibutramine and fluoxetine on extracellular 5-HT concentrations measured by microdialysis in the hypothalamus of freely-moving rats differ markedly from those of (+)-fenfluramine and (+)-amphetamine. Consistent with the previous findings of Rutter & Auerbach (1993) and Gundlah *et al*. (1997), we observed that sibutramine and fluoxetine produce moderate increases in extracellular 5-HT concentrations, which are slow to plateau and are of prolonged duration. In contrast, at pharmacologically equivalent doses that reduce food intake (Jackson *et al*., 1997b, unpublished data), (+)-fenfluramine and (+)-amphetamine produce significantly greater elevations in extracellular 5-HT concentrations than sibutramine and in addition, these increases are significantly more rapid in onset (see Table 2). In the more extensive study of Gundlah *et al*. (1997), it has been shown that in addition to differences in the pharmacodynamics of extracellular 5-HT concentrations after (±)-fenfluramine as opposed to sibutramine and fluoxetine, the action of (±)-fenfluramine is also independent of neuronal firing rate, unlike the actions of sibutramine and fluoxetine. Finally, it was demonstrated that the (+)-fenfluramine-induced enhancement of extracellular 5-HT concentration is not mediated *via* uptake inhibition, but derives almost exclusively from 5-HT release following entry into the presynaptic nerve terminal *via* the high affinity uptake transporter; this action is prevented by pretreating the rats with the 5-HT uptake inhibitors, fluoxetine and sibutramine.

In addition to concurring with the findings of Auerbach and his colleagues (Rutter & Auerbach, 1993; Gundlah *et al*., 1997), the magnitude of the effect of (+)-fenfluramine on extracellular 5-HT concentrations in the anterior hypothalamus is similar to those reported by Schwartz *et al*. (1989) and Raiteri *et al*. (1995). Moreover, the ability of (+)-amphetamine to evoke release of 5-HT *in vivo* confirms the finding of Kuczenski *et al*. (1995) who reported that (+)-amphetamine

(2 mg kg^{-1} s.c.) caused an increase of 600–650% over baseline levels of striatal 5-HT.

Comparing the effects of fluoxetine and the anti-obesity drugs, sibutramine, (+)-fenfluramine and (+)-amphetamine on the accumulation of 5-HTP after inhibition of *L*-aromatic amino acid decarboxylase with NSD-1015 specifically in the hypothalamus and frontal cortex, revealed differences between the actions of the 5-HT uptake inhibitors, sibutramine and fluoxetine, and the 5-HT releasing agents, (+)-fenfluramine and (+)-amphetamine. In the case of the former, sibutramine and fluoxetine had no effect on 5-HTP accumulation at doses <10 mg kg^{-1}; at 10 mg kg^{-1}, sibutramine and fluoxetine reduced 5-HTP formation in the frontal cortex and hypothalamus, respectively. On the other hand, (+)-fenfluramine dose-dependently decreased 5-HTP formation in both hypothalamus and frontal cortex with significant changes being observed with doses as low as 1 mg kg^{-1}. This reduction in 5-HT synthesis has also been reported by Invernizzi *et al.* (1986) and probably reflects a reflex inhibition of 5-HT synthesis in response to the rapid increase in extraneuronal concentrations of 5-HT evoked by (+)-fenfluramine. Similar but less pronounced effects were produced by administration of (+)-amphetamine, which significantly decreased 5-HTP accumulation in the hypothalamus at doses $\geqslant 3$ mg kg^{-1}. Once again this probably reflects reflex inhibition of 5-HT synthesis in response to marked elevations in extraneuronal concentrations of 5-HT. In this case, however, no corresponding effect was observed in the frontal cortex. As our microdialysis studies were only conducted in the anterior hypothalamus, it is not possible to specify whether (+)-fenfluramine and (+)-amphetamine have profoundly different effects on 5-HT release in rat cortex.

The longer term impact of the drugs on postsynaptic 5-HT receptor function was determined by their abilities to induce 5-HT_{2A} receptor down-regulation. Here, the difference between (+)-fenfluramine and the other drugs was most pronounced with the former reducing cortical 5-HT_{2A} receptors by a massive 61%, whilst sibutramine, fluoxetine and (+)-amphetamine were without effect. The finding with (+)-fenfluramine is consistent with the earlier report of Garattini *et al.* (1992). The observed lack of effect of chronic administration of fluoxetine on 5-HT_{2A} receptor density in our studies is also consistent with previous reports (Peroutka & Snyder, 1980; Goodnough & Baker, 1994). The effects of sibutramine and (+)-amphetamine on 5-HT_2 receptor function do not appear to have been determined previously. In this regard, it is interesting to note that although (+)-fenfluramine and (+)-amphetamine were approximately equipotent as 5-HT releasing agents *in vivo*, (+)-fenfluramine again produced the greater attenuation of central 5-HT function.

Overall, the data support the hypothesis initially presented by Fuller *et al.* (1988) and supported by Gundlah *et al.* (1997) that (+)-fenfluramine potentiates 5-HT function predominantly by neuronal 5-HT release. They do not support the view of Garattini *et al.* (1989) that 5-HT uptake inhibition forms a significant component of this drug's mechanism. In addition, the findings also demonstrate that sibutramine and its primary and secondary amine metabolites act, like fluoxetine, to enhance 5-HT function by uptake inhibition; again these results differ from the postulate that 5-HT release may be involved in the anorectic mechanism of fluoxetine (Caccia *et al.*, 1992). Although the failure of 5-HT antagonists to inhibit the anorectic actions of fluoxetine (Wong *et al.*, 1988) has prompted some authors to question its mode of action (Caccia *et al.*, 1992), Angel *et al.* (1988) have shown an excellent correlation between the potency of fluoxetine and other drugs of this type as anorectants and their ability to inhibit 5-HT uptake *in vivo*.

In the case of (+)-amphetamine, the results show that this drug potently releases 5-HT in addition to the catecholamines, dopamine and noradrenaline. Whilst receptor antagonist and lesioning studies demonstrate a role for dopaminergic and noradrenergic, but not 5-hydroxytryptaminergic neuronal systems in the hypophagic actions of (+)-amphetamine (see review by Clineschmidt & Bunting, 1980), its profound effect on hypothalamic 5-HT release suggests this neurotransmitter may well play a role in (+)-amphetamine's anorectic effects.

Finally, sibutramine has been shown to enhance central 5-HT function by a mechanism which is consistent with potent uptake inhibition. *In vitro*, sibutramine's metabolites, BTS 54 354 and BTS 54 505, are approximately as potent as fluoxetine, and *in vivo* sibutramine and fluoxetine produced very similar effects on 5-HT synthesis. Furthermore, the results provide clear evidence that, unlike (+)-fenfluramine and (+)-amphetamine, sibutramine is not a 5-HT releasing agent.

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