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Studies on the role of serotonin receptor subtypes in the effect of sibutramine in various feeding paradigms in rats

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1 The effect of the 5-hydroxytryptamine (5-HT) and noradrenaline (NA) reuptake inhibitor sibutramine was studied in food deprived, neuropeptide Y (NPY)- or muscimol-injected rats.

2 Sibutramine dose-dependently reduced feeding caused by food-deprivation ($ED_{50} = 5.1 \pm 0.8$ mg kg^{-1}) or by NPY injection into the paraventricular nucleus of the hypothalamus ($ED_{50} = 6.0 \pm 0.5$ mg kg^{-1}). The increase in food intake caused by muscimol injected into the dorsal raphe was not modified by sibutramine (1–10 mg kg^{-1}).

3 The hypophagic effect of 5.1 mg kg^{-1} sibutramine in food-deprived rats was studied in rats pretreated with different serotonin receptor antagonists. Metergoline (non-selective, 0.3 and 1.0 mg kg^{-1}), ritanserin (5-HT$_{2A/2C}$, 0.5 and 1.0 mg kg^{-1}) and GR127935 (5-HT$_{1B/1D}$, 0.5 and 1.0 mg kg^{-1}) did not modify the hypophagic effect of sibutramine, while SB206553 (5-HT$_{2B/2C}$, 5 and 10 mg kg^{-1}) slightly but significantly reduced it ($F_{int}(2.53) = 3.4$; $P < 0.05$).

4 The reduction in food intake caused by 6.0 mg kg^{-1} sibutramine in NPY-injected rats was not modified by GR127935 (1.0 mg kg^{-1}).

5 The results suggest that, with the possible exception of a partial involvement of 5-HT$_{2B/2C}$ receptors in sibutramine's hypophagia in food-deprived rats, 5-HT$_1$ and 5-HT$_2$ receptor subtypes do not play an important role in the hypophagic effect of sibutramine, at least in the first 2 h after injection.

Keywords: Sibutramine; food intake; 5-hydroxytryptamine; 5-HT receptors; neuropeptide-Y; muscimol

Abbreviations: DA, dopamine; 5-HT, 5-hydroxytryptamine; GR127935, N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide HCl; SB206553, 5-methyl-1-(3-pyridyl-carbamoyl)-1,2,3,5-tetrahydropyrrolo [2,3-f]indole; NPY, neuropeptide-Y; NA, noradrenaline; DR, nucleus raphe dorsalis; PVN, paraventricular nucleus of the hypothalamus

Introduction

Sibutramine (BTS 54524) and its secondary (BTS 54354) and primary (BTS 54505) metabolites inhibit the neuronal reuptake of monoamines to different degrees *in vitro* and *in vivo*. In cortical slices, used for studying [^{14}C]-noradrenaline (NA) and [^{14}C]-serotonin (5-HT) uptake, and striatal synaptosomal preparations, for [^{14}C]-dopamine (DA) uptake, sibutramine and its metabolites inhibited NA and DA uptake at concentrations considerably lower than those inhibiting 5-HT uptake. *In vivo* in models based on the depletion of brain NA and DA by alpha-methyl-m-tyrosine and of 5-HT by p-chloro-amphetamine, sibutramine and its metabolites showed similar potency in inhibiting NA and 5-HT uptake and were less potent on DA uptake (Luscombe *et al*., 1989). More recently it has been demonstrated that the monoamine uptake inhibiting properties of sibutramine are predominantly mediated by its primary and secondary metabolites (Cheetham *et al*., 1993; 1996; Luscombe *et al*., 1990).

Animal studies have shown that sibutramine reduces food intake in obese and lean rats (Stricker-Krongrad *et al*., 1995; Fantino & Souquet, 1995; Jackson *et al*., 1997a, b) probably by fastening the natural development of satiety (Halford *et al*., 1995) and this effect may be related to its ability to inhibit the reuptake of both 5-HT and NA, with the subsequent activation of a variety of NA and 5-HT receptors (Jackson *et al*., 1997a, b).

In the present study we examined the effect of sibutramine in feeding paradigms which are sensitive to the hypophagic effect of agents increasing central 5-HT transmission such as d-fenfluramine (Borsini *et al*., 1983; Bendotti & Samanin, 1987). First we studied the effect of sibutramine on food intake in food-deprived rats, a condition in which fenfluramine markedly reduces food intake by a mechanism involving stimulation of 5-HT receptors (Curzon *et al*., 1997), particularly the 5-HT$_{1B}$ (Grignaschi *et al*., 1992; 1995) and 5-HT$_{2C}$ subtypes (Hartley *et al*., 1995). We studied the effect in food-deprived rats in the presence of different 5-HT receptor antagonists: metergoline(non-selective), ritanserin (5-HT$_{2A/2C}$), SB 206553 (5-HT$_{2B/2C}$) and GR 127935 (5-HT$_{1B/1D}$) (Hoyer, 1988; Kennett *et al*., 1996; Skingle *et al*., 1993).

There is evidence that neuropeptide Y (NPY)-induced hyperphagia models a pathological condition involving excessive food consumption. In fact, intracerebral injection of NPY has a potent and long-lasting orexigenic effect which overrides physiological satiety signals (Morley *et al*., 1987; Paez & Myers, 1990). d-Fenfluramine potently blocked the hyperphagia caused by NYP injected into the paraventricular nucleus of the hypothalamus (PVN) by a mechanism that seemed to involve 5-HT$_{1B}$ receptors (Grignaschi *et al*., 1995). It was therefore interesting to examine whether sibutramine reduced NPY-induced eating by a mechanism similar to d-fenfluramine's. Finally, we studied the effect of sibutramine on overeating caused by injection of muscinol, a γ-aminobutyric acid (GABA) receptor agonist (Naik *et al*., 1976), in the nucleus raphe dorsalis (DR) of sated rats. Muscimol-induced

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eating selectively identifies 5-HT-dependent hypophagia since d-fenfluramine potently reduced it whereas d-amphetamine was completely ineffective (Borsini *et al*., 1983).

Some of the results of this study have already been published in an abstract (Grignaschi *et al*., 1997a, b).

Methods

Animals

Male Sprague-Dawley rats (CD-COBS, Charles River, Italy), weighing 220–250 g were housed in groups of two in a room at $21 \pm 1^{\circ}$C and 60% relative humidity, with a 12 h light 12 h dark cycle (light off at 18.00 h) and water and food (Altromin food pellets for rats) *ad libitum*. Procedures involving animals and their care are conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). In all the experiments the animals were used once.

Cannulae implantation

Rats were anaesthetized with 3.0 ml kg^{-1} equithesin injected intraperitoneally (i.p.) (sodium pentobarbitone 1.2 g; chloral hydrate 5.3 g; $MgSO_4$ 2.7 g; propylene glycol 49.5 ml; ethanol 12.5 ml and distilled water 58 ml) and placed on a stereotaxic apparatus (mod. 900, David Kopf, Tujunga, CA, U.S.A.). The skin was incised and the skull was cleaned. Guide cannulae made of 23-gauge stainless-steel tubing were implanted 2 mm above the site to be injected. The guide tubes were secured by acrylic dental cement anchored to three stainless-steel screws fixed to the skull. To prevent clogging, 30-gauge stainless-steel stylets were placed in the guide cannulae until the animals were given intracerebral injections. On the day of the test, the stylets were withdrawn and replaced by injection units (30-gauge stainless-steel tubing) terminating 2 mm below the tip of the guides. The drugs were infused in a volume of 0.5 μl over a period of 1 min, with an additional 30 s allowed for diffusion of the solution away from the needle tip. Stereotaxic coordinates for the DR and the PVN were as follows: AP = +1.2, V = +3.2, L = 0 and AP = +7.2; V = +2.0; L = +0.5 mm according to the Paxinos & Watson atlas (1982). Cannulae were implanted at a 20° angle to the sagittal plane to avoid damage to the sinus.

Food intake in food-deprived rats

One day before testing, at 14.00 h, the rats were placed in individual plexiglass cages, with a wire mesh floor and a wire top and kept without food for 24 h. Normal laboratory food was then placed on the floor of the cage in a Perspex Petri dish and a paper towel was placed below the cage floor to collect any spillage. Metergoline (0.3 and 1.0 mg kg^{-1}) and ritanserin (0.5 and 1.0 mg kg^{-1}) were administered i.p. respectively 3 h and 90 min before the test. SB206553 (5 and 10 mg kg^{-1} p.o.) and GR127935 (0.5 and 1.0 mg kg^{-1} s.c.) were administered 90 min before the test and sibutramine (1–10 mg kg^{-1}) was administered p.o. 60 min before the test. Food intake in 1 h was measured by weighing all portions of food before and after the feeding test and expressed as g 100 g^{-1} of body weight.

Food intake in NPY-injected rats

The animals were accustomed to handling for 7 days before testing and on the day of the experiment sibutramine (3–10 mg kg^{-1}) was administered orally at 14.00 h, 60 min before the test. The pre-treatment time and the route of administration for GR127935 (1.0 mg kg^{-1}) was as previously described. After sibutramine injection the animals were allowed to become habituated to the test cages for 60 min (Perspex cages with a grid and blotting paper on the floor), with normal laboratory food freely available on the cage floor. NPY (235 pmol 0.5 μl^{-1}) was injected into the PVN immediately before testing.

After the NPY injection, the rats were placed in the test cages with a weighed amount of food pellets on the floor. Food intake corrected for spillage was measured in 1 h as for food deprived rats.

Food intake in muscimol-injected rats

The animals were accustomed to handling for 7 days before testing and on the day of the experiment sibutramine (1, 3 and 10 mg kg^{-1}) was administered orally at 14.00 h, 60 min before the test. After this the animals were allowed to become habituated to the test cages (Perspex cages with a grid and blotting paper on the floor) for 40 min, with normal laboratory food freely available on the cage floor. Twenty min before the test, the food was removed from the cages and the animals were injected into the DR with muscimol (100 ng 0.5 μl^{-1}). A weighed amount of food was presented 20 min later, at the beginning of the 1 h test. Food intake corrected for spillage was measured as for food deprived rats.

Histology

On completion of each experiment the animals were anaesthetized with equithesin and killed by decapitation and the locations of the cannulae were determined histologically. Only data from rats in which the cannulae were exactly located in the appropriate structures were included in the results.

Drugs

The vehicles for the various drugs were: 1% ascorbic acid for metergoline base (Pharmacia-Upjohn, Milan, Italy), distilled water containing lactic acid (2–5 drops) and 20% propylene glycol and brought to pH 5 with 10 N NaOH for ritanserin base (Janssen, Beerse, Belgium), distilled water for sibutramine HCl (Knoll Pharmaceuticals, Nottingham, U.K.), muscimol base (Biosearch, San Raphael, CA, U.S.A.), porcine neuropeptide-Y (Novabiochem, Laufelfingen, Switzerland) and GR127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′methyl-4′(5-methyl-1,2,4-oxadiazol-3-yl)[1,1-biphenyl]-4-carboxamide HCl) (Glaxo Wellcome, Hertfordshire, U.K.), 0.5% carboxymethylcellulose (CMC) for SB206553 (5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo [2,3-f]indole) (SmithKline Beecham, Harlow, U.K.). Drugs were injected systematically in a volume of 2 ml kg^{-1}, except muscimol and porcine NPY, which were given intracerebrally in a volume of 0.5 μl. Injection times before testing were: 3 h for metergoline, 90 min for ritanserin, GR127935 and SB206553, 60 min for sibutramine, 20 min for muscimol. Porcine NPY was administered immediately before the test. The doses of the antagonists were selected on the basis of previous experiments in which they reduced the hypophagic effect of d-fenfluramine (Samanin *et al*., 1989; Neill & Cooper, 1989), or of direct

agonists at 5-HT_{2C} or 5-HT_{1B} receptors (Grignaschi *et al.*, 1998; Hartley *et al.*, 1995).

Statistical analysis

The effects of muscimol and NPY on food intake were analysed by Student's *t*-test. The reduction in food intake caused by sibutramine was analysed by one-way analysis of variance followed by Dunnett's test. The effect of pretreatment with 5-HT receptor antagonists on the hypophagic effect of sibutramine was analysed by factorial ANOVA for balanced or unbalanced designs (GLM procedure; SAS, SAS, Institute, NC, U.S.A.) followed by Tukey's test. ED_{50} were calculated using the ALLFIT program (De Lean *et al.*, 1978).

Results

Effect of sibutramine on food intake

As shown in Figure 1, sibutramine (1–10 mg kg^{-1}) significantly and dose-dependently reduced food intake in food deprived rats with an ED_{50} of 5.1±0.8 mg kg^{-1}.

An intra-PVN injection of 235 pmol 0.5 μl^{-1} NPY significantly increased the amount of food eaten in 1 h by free-feeding rats (saline = 0.08±0.03 and NPY = 1.14±0.09 g 100 g^{-1} b.w.; $P<0.01$ Student's *t*-test). Feeding caused by NPY injected into the PVN was inhibited by sibutramine (3–10 mg kg^{-1} p.o.) with an ED_{50} of 6.0±0.5 mg kg^{-1} (Figure 2).

An intra-DR injection of muscimol (100 ng 0.5 μl^{-1}) caused a significant increase in food intake of free-feeding rats (saline = 0.18±0.08 and muscimol = 2.47±0.30 g 100 g^{-1} b.w.; $P<0.01$ Student's *t*-test) and this effect was not modified by 1, 3 and 10 mg kg^{-1} p.o. sibutramine (muscimol + vehicle = 2.47±0.30; muscimol + 1 mg kg^{-1} sibutramine = 2.73±0.21; muscimol + 3 mg kg^{-1} sibutramine = 2.21±0.49; muscimol + 10 mg kg^{-1} sibutramine = 2.60±0.33 g 100 g^{-1} b.w.).

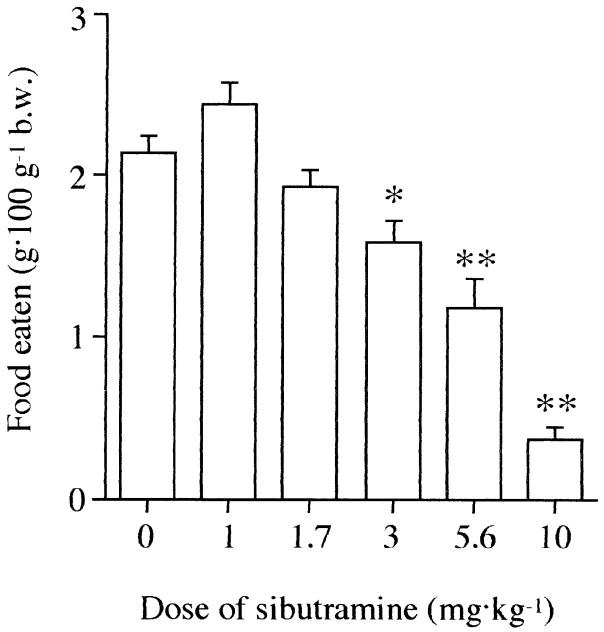

Figure 1 Effect of various doses of sibutramine on food intake of 24 h food-deprived rats. Sibutramine (1–10 mg kg^{-1}) was dissolved in distilled water and given p.o. 60 min before the test. Food intake corrected for spillage was measured for 1 h and expressed as g 100 g^{-1} of body weight. Values are the mean±s.e.mean of ten animals per group. * = $P<0.05$ ** = $P<0.001$ vs controls. Dunnett's test.

Role of 5-HT_1 and 5-HT_2 receptors in the hypophagic effect of sibutramine

Table 1 shows the effect of the different 5-HT receptor antagonists on the reduction in food intake caused by

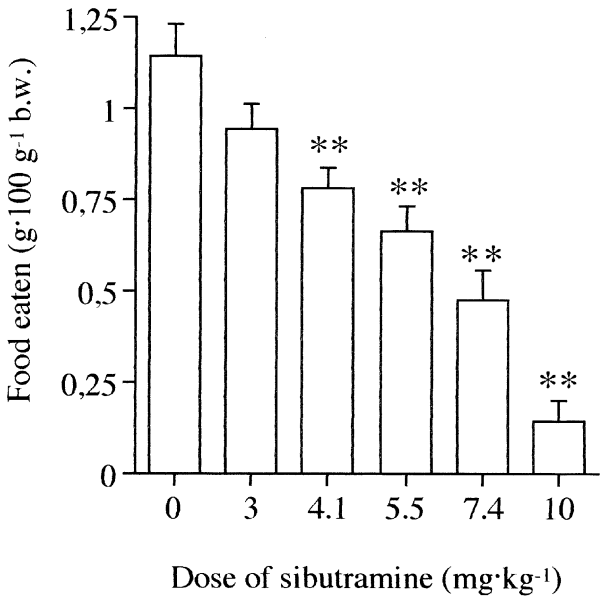

Figure 2 Effect of various doses of sibutramine on eating caused by NPY injected into the PVN. Sibutramine (3–10 mg kg^{-1}) was dissolved in distilled water and given p.o. 60 min before the test. NPY (235 pmol 0.5 μl^{-1}) was injected into the PVN immediately before the test. Food intake corrected for spillage was measured for 1 h and expressed as g 100 g^{-1} of body weight. Values are the mean±s.e.mean of ten animals per group. ** = $P<0.01$ vs controls. Dunnett's test.

Table 1 Effects of various 5-HT receptor antagonists on the reduction in food intake caused by sibutramine in food-deprived rats

		Food eaten (g 100 g^{-1} b.w.)		
Pretreatment	*Dose* (mg kg^{-1})	*Vehicle*	*Sibutramine* (5.1 mg kg^{-1})	*Fint*
Vehicle		3.41±0.14	2.10±0.24**	1.1 N.S.
Metergoline	0.3	3.28±0.07	2.30±0.20**	
Metergoline	1.0	3.28±0.02	2.50±0.17*	
Vehicle		2.02±0.16	1.01±0.10**	2.4 N.S.
Ritanserin	0.5	2.33±0.21	1.12±0.06**	
Ritanserin	1.0	1.73±0.21	1.17±0.10	
Vehicle		2.14±0.10	0.97±0.13**	3.4[a]
SB206553	5.0	2.32±0.16	1.07±0.15**	
SB206553	10.0	1.97±0.10	1.36±0.14*	
Vehicle		2.61±0.22	1.08±0.09**	1.0 N.S.
GR127935	0.5	2.37±0.18	1.08±0.15**	
GR127935	1.0	2.11±0.21	1.06±0.10**	

Values are the mean±s.e.mean of at least six animals per group. Sibutramine (5.1 mg kg^{-1}) was given p.o. 60 min before the test. Metergoline (0.3 and 1.0 mg kg^{-1} i.p.) and ritanserin (0.5 and 1.0 mg kg^{-1} i.p.) were injected 3 h and 90 min before the test. SB206553 (5 and 10 mg kg^{-1} p.o.) and GR127935 (0.5 and 1.0 mg kg^{-1} s.c.) were given 90 min before the test. ** $P<0.01$ * $P<0.05$ vs respective controls. Tukey-Kramer's test. [a] $P<0.05$, Fint (2.53); N.S. = $P>0.05$; Two-way analysis of variance.

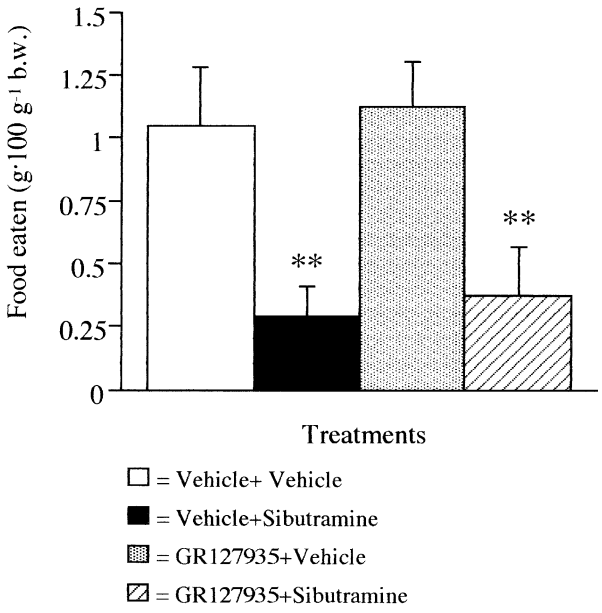

Figure 3 Effect of GR127935 on the reduction in food intake caused by sibutramine in NPY injected rats. Sibutramine (6.0 mg kg^{-1} p.o.) and GR127935 (1.0 mg kg^{-1} s.c.) were dissolved in distilled water and given 60 min and 90 min before the test. NPY (235 pmol 0.5 μl^{-1}) was dissolved in distilled water and injected into the PVN immediately before the test. Values are the mean ± s.e.mean of at least five animals per group. ** = $P<0.01$ vs vehicle treated rats. Tukey's test. Fint(1.24) = 0.0; $P>0.05$; Two-Way-ANOVA.

5.1 mg kg^{-1} sibutramine in food deprived rats. The 5-$HT_{1/2}$ receptor antagonist metergoline (0.3 and 1.0 mg kg^{-1}) did not modify feeding by itself and failed to antagonize the hypophagic effect of sibutramine (Fint(2.30) = 1.1; $P>0.05$). The 5-$HT_{2A/2C}$ receptor antagonist ritanserin (0.5 and 1.0 mg kg^{-1}) tended to reduce feeding by itself but the effect was not statistically significant and failed to modify the effect of sibutramine (Fint(2.36) = 2.4; $P>0.05$). The 5-$HT_{1B/1D}$ receptor antagonist GR127935 (0.5 and 1.0 mg kg^{-1}) did not modify the amount of food eaten or the effect of sibutramine (Fint(2.35) = 1.0; $P>0.05$). SB206553 (5 and 10 mg kg^{-1}) did not modify feeding by itself and slightly reduced the hypophagic effect of sibutramine (Fint(2.53) = 3.4; $P<0.05$).

As shown in Figure 3, in NPY-treated rats GR127935 (1.0 mg kg^{-1} s.c.) did not modify feeding or the hypophagic effect of 6.0 mg kg^{-1} p.o. sibutramine (Fint(1.24) = 0.0; $P>0.05$).

Discussion

Sibutramine dose-dependently reduced food intake of food deprived rats without causing any apparent changes in the animals' behaviour or motor activity.

None of the 5-HT receptor antagonists reduced the effect of sibutramine on rats food intake, with the possible exception of SB206553 for which a significant interaction with sibutramine was found. This compound blocks both 5-HT_{2B} and 5-HT_{2C} receptors (Kennett *et al.*, 1996). Since stimulation of 5-HT_{2B} receptors was recently found to cause hyperphagia (Kennett *et al.*, 1997), the results with SB206553 suggest an involvement of 5-HT_{2C} receptors in the effect of sibutramine. However this idea is weakened by the fact that two 5-HT_{2C} receptor antagonists, metergoline and ritanserin, had no effect on sibutramine-induced hypophagia. Moreover, Jackson *et al.* (1997a) found that another 5-$HT_{2B/2C}$ receptor antagonist, SB200646, did not modify the effect of sibutramine on 2 h food intake.

The mechanism by which SB206553 slightly reduces the effect of sibutramine remains to be elucidated, but it seems clear that 5-HT receptors have hardly any role in sibutramine's suppression of food intake in our conditions. It is interesting that Jackson *et al.* (1997a) found that blockade of α_1 or β_1 adrenoceptors significantly antagonized the effect of sibutramine on 2 h food intake whereas 5-HT_{2C} receptor antagonists reduced it only in the second part of an 8 h period. These findings led them to suggest that noradrenaline is mainly involved in sibutramine-induced hypophagia in the first few hours after drug administration whereas 5-HT seems to partially mediate the hypophagic effect at a later stage.

Sibutramine significantly reduced eating caused by NPY injected in the PVN at doses similar to those active on food intake of food deprived rats. These results are particularly interesting in view of the finding that d-fenfluramine, which is particularly effective in reducing NPY hyperphagia (Bendotti & Samanin, 1987), reduced eating in patients with bulimia nervosa (Robinson *et al.*, 1986). On the basis of a series of experiments investigating the effect of d-flenfluramine in the presence of different 5-HT receptor antagonists, we suggested that d-fenfluramine reduced NPY hyperphagia by indirectly stimulating 5-HT_{1B} receptors outside the PVN (Grignaschi *et al.*, 1995). The present results with GR127935 seem to exclude that sibutramine uses 5-HT_{1B} receptors to modify NPY-induced eating.

Subsequently, we found that stimulation of 5-HT_{2A} receptors in the PVN blocked eating caused by NPY, probably by stimulating release of the corticotropin-releasing factor (Grignaschi *et al.*, 1996). Although sibutramine does not seem to use a 5-HT mechanism to reduce food intake at early times (Jackson *et al.*, 1997a), further experiments are necessary to exclude a role of 5-HT_{2A} receptors in its effect on NPY hyperphagia. That sibutramine has little effect in enhancing 5-HT transmission at early times after administration is clearly indicated by its inability to modify eating caused by muscimol injected into the DR. Agents increasing 5-HT transmission are particularly effective in reducing muscimol-induced hyperphagia (Borsini *et al.*, 1983).

In explaining the apparent inability of sibutramine to use 5-HT mechanisms, at least at early times after drug administration, it should be recalled that the fact that selective 5-HT reuptake inhibitors activate somatodentritic 5-HT_{1A} receptors by increasing endogenous 5-HT in the raphe area reduces the firing of serotonergic cells and attenuates the effect of 5-HT reuptake inhibitors on extracellular 5-HT in terminal regions (Invernizzi *et al.*, 1992). That this also occurs for sibutramine is suggested by the fact that the drug slightly increased extracellular 5-HT in the hypothalamus (Prow *et al.*, 1996) and its hypophagic effect was potentiated by the 5-HT_{1A} receptor antagonist WAY100635 (Jackson *et al.*, 1997c).

In conclusion, sibutramine reduces food intake of food deprived rats and eating caused by NYP injected into the paraventricular hypothalamus of free-feeding rats. Except for a reduction of sibutramine's hypophagia in food deprived rats by the 5-$HT_{2B/2C}$ receptor antagonist SB206553, attempts to prove that 5-HT receptor subtypes were involved were unsuccessful, probably because a noradrenergic component of sibutramine action prevails at early times after injection. This is further suggested by sibutramine's lack of effect on muscimol-induced hyperphagia, which is particularly sensitive to agents increasing central 5-HT transmission.

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