Psychopharmacology (1999) 143:309–314

ORIGINAL INVESTIGATION

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Reduced satiating effect of *d*-fenfluramine in serotonin 5-HT$_{2C}$ receptor mutant mice

Received: 28 September 1998 / Final version: 17 November 1998

Abstract *Rationale: d*-Fenfluramine stimulates the release of serotonin (5-HT) and is a potent inhibitor of the re-uptake of 5-HT into nerve terminals. Administration of *d*-fenfluramine suppresses food intake in both animals and humans. *Objective:* We have investigated the role of the 5-HT$_{2C}$ receptor in mediating the effect of *d*-fenfluramine on mouse food intake and the behavioural satiety sequence. *Methods:* Mutant mice lacking serotonin 5-HT$_{2C}$ receptors and wild-type animals were habituated to a daily presentation of wet mash. Animals were non-deprived and received *d*-fenfluramine (3–30 mg/kg) 30 min prior to being assessed for the presence of stereotypy and presented with wet mash. The behaviour of animals was observed for the subsequent 40 min and food intake was recorded. *Results: d*-Fenfluramine dose-dependently inhibited the consumption of a palatable wet mash by the mice. *d*-Fenfluramine (3 mg/kg) significantly reduced the amount of wet mash consumed by wild-type mice and induced a temporal advance in the behavioural satiety sequence consistent with an enhancement of satiety. Mutant mice were less sensitive to the satiating effects of 3 mg/kg *d*-fenfluramine. Hence, this dose of *d*-fenfluramine had a reduced effect on both food consumption and the behavioural satiety sequence in the 5-HT$_{2C}$ mutant mice. In contrast, mutant mice showed an increased sensitivity to the stereotypy induced by high doses of *d*-fenfluramine (10, 30 mg/kg) compared to that of wild-type littermates. *Conclusion:* These data demonstrate a role for the 5-HT$_{2C}$ receptor in mediating *d*-fenfluramine-induced satiety.

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Key words *d*-Fenfluramine · 5-HT$_{2C}$ receptor · Serotonin · Mouse · Feeding · Satiety

Introduction

The serotonergic system has been extensively implicated in the control of feeding behaviour (for reviews see Blundell 1977; Dourish 1995); indeed, the recently withdrawn appetite suppressant *d*-fenfluramine stimulates the release of 5-HT and is a potent inhibitor of the re-uptake of 5-HT into nerve terminals (Garattini et al. 1986). At least 14 serotonin receptor subtypes have been described (Boess and Martin 1994; Martin and Humphrey 1994) and studies using non-selective antagonists at these receptors have suggested that the 5-HT$_{1B}$ and 5-HT$_{2C}$ receptor subtypes are of particular importance in mediating *d*-fenfluramine-induced hypophagia (Neill and Cooper 1989; Grignaschi and Samanin 1992; Vickers et al. 1996). However, due to the absence of selective ligands, the precise role of serotonin receptor subtypes in mediating *d*-fenfluramine-induced hypophagia has remained uncertain.

Recently, the development of mice lacking functional 5-HT$_{2C}$ receptors has provided a complementary approach (Tecott et al. 1995). It has been reported previously that 5-HT$_{2C}$ mutant mice are overweight compared to wild-type animals and that this increase in body weight is likely to be due to enhanced food intake (Tecott et al. 1995). In addition, despite there being no obvious evidence of anatomical or functional abnormalities in the CNS of these animals, the mutants display an enhanced seizure susceptibility (Tecott et al. 1995; Brennan et al. 1997). We have now used the 5-HT$_{2C}$ knockout mouse to examine the importance of the 5-HT$_{2C}$ receptor in mediating *d*-fenfluramine-induced hypophagia.

When allowed to feed to satiety, rats exhibit a characteristic sequence of behaviours. Thus, the cessation of feeding is superseded by a brief period of active behaviours including locomotion, rearing and grooming which is in turn followed by a longer period of rest and/or sleep

(Antin et al. 1975). This behavioural sequence is sensitive to manipulations that affect ingestive behaviour and has been used to discriminate between satiety-like factors and non-specific reductions in feeding. For example, pre-feeding or administration of compounds such as *d*-fenfluramine lead to a temporal advance in the progression of the sequence whilst leaving it behaviourally intact (Kitchener and Dourish 1994; Vickers et al. 1996). The present study investigates the specific role of the 5-HT_{2C} receptor in mediating *d*-fenfluramine-induced hypophagia by examining the effect of the compound on food intake and postprandial feeding behaviour in mice lacking functional 5-HT_{2C} receptors.

Preliminary accounts of these data have been presented to the Society for the Study of Ingestive Behaviour (Vickers et al. 1997) and the Society for Neuroscience (Dourish et al. 1997).

Materials and methods

Animals

The mutants were originally generated from a 129-derived ES cell line bearing a targeted disruption of the X-linked 5-HT_{2C} receptor gene (Brennan et al. 1997). Animals were subsequently backcrossed for seven to eight generations to a C57BL/6 background. Heterozygous females were mated with wild-type males; the resulting males were hemizygous mutants hemizygous wild-types. Genotyping was performed by PCR analysis. The mutant allele was detected using primers complimentary to neomycin resistance gene (Neo) sequences: NeoD (5′-CACCTTGCTCCTGCCGAG AAA-3′) and NeoH (5′-AGAAGGCGATAGAAGGCGATG-3′). The wild-type allele was detected using primers derived from 5-HT_{2C} receptor gene sequences flanking the Neo insertion: 5N2 (5′-CAACTTGGTTGTTACACACGG-3′) and 3N2 (5′-TCTACC CTTCTCATAC TAGTT-3′). For the behavioural experiments, mice were transferred from the University of California at San Francisco to Cerebrus and subsequently to the University of Sussex at the age of 6 weeks.

Twenty-four mice were used in the study. Two mutant animals died during the course of the study and the data presented are for 11 mutant and 11 wild-type mice. Animals used in the experiments were individually housed under a 12-h light/dark cycle (lights on: 0530 hours) with ad libitum access to standard mouse diet and tap water. Ambient temperature was 21–22°C. A red light was the sole source of illumination during the dark period. Experimenters were blind to the genotype of individual animals until completion of the study. The work reported in this manuscript was performed in accordance with Home Office regulations as outlined in the Animals (Scientific Procedures) Act 1986.

Procedures

Test diet

Animals were habituated to a daily presentation of wet mash (1 part powdered diet:1 part water). Wet mash was presented on clear plastic Petri dish lids and spillage was collected at the end of each session such that consumption was recorded to the nearest 0.1 g.

Assessment of drug-induced stereotypy

Mice were individually assessed for the presence of drug-induced stereotypy 25 min after drug administration. Stereotypy was rated as 0, no stereotypy apparent; 1, equivocal stereotyped movements; 2, clear evidence of stereotyped head movements and flat body posture; 3, pronounced stereotyped head movements and flat body posture.

Behavioural satiety sequence

Immediately after assessment for the presence of stereotypy, animals were individually observed at 30-s intervals for a period of 40 min. Animals were assessed in cohorts of 12. An identical technique to that described in detail for rats (Clifton et al. 1989; Vickers et al. 1996) was used. Briefly, a microprocessor was programmed to illuminate individually a small LED adjacent to each cage every 2.5 s. As an LED lit up, the observer struck the key on the keyboard corresponding to the behaviour exhibited by the indicated animal. The key press turned the LED off and 2.5 s later the next LED was lit. This process was repeated for the 40-min test period. Accordingly, each animal was observed every 30 s and 80 times in total. Behaviour was subdivided into four mutually exclusive categories for scoring: feeding (the acquisition and eating of the food substrate and the rare occasions where animals took a drink); active (included locomotion, rearing and sniffing and was used when no other category was deemed appropriate); grooming (face and body washing and also including episodes of scratching); resting (with or without eyes closed).

The wet mash meal was presented 30 min after *d*-fenfluramine (or vehicle) administration. Each animal acted as its own control and a period of at least 72 h was left between successive drug administrations. In total, each animal received three *d*-fenfluramine administrations.

Drugs

d-Fenfluramine hydrochloride was dissolved in 0.9% saline and administered via the intraperitoneal route. All drug administrations were in the volume 10 ml/kg and the appropriate volumes of vehicle were used as controls.

Data analysis

Food intake data were analysed by two-factor ANOVA with genotype (between-subjects) and dose (within-subjects) as factors. A significant interaction was succeeded by performance of one-factor ANOVA and comparisons made using Bonferroni *t*-test. Comparisons at each level of dose were made using Tukey's test. Stereotypy data were analysed by two-factor ANOVA in an identical manner to the food consumption data.

Behavioural data were organised into 5-min bins and analysed using three-factor ANOVA (time was an additional within-subjects factor) supplemented by the performance of planned comparisons. The four behavioural categories were analysed separately. Data analysis was performed using the Genstat statistical package.

Results

Food intake

As in previous studies, mutant mice (37.5 g; n=11) were significantly heavier than their wild-type littermates (33.7 g; n=11) [$F(1,20)=5.01$, $P<0.05$]. Furthermore, two mutant animals died during the course of the experiment; one death was observed to be due to a spontaneous seizure.

Treatment with *d*-fenfluramine led to a dose-dependent decrease in food intake in both mutant and wild-type mice [main effect of dose: $F(3,60)=100.3$, $P<0.01$;

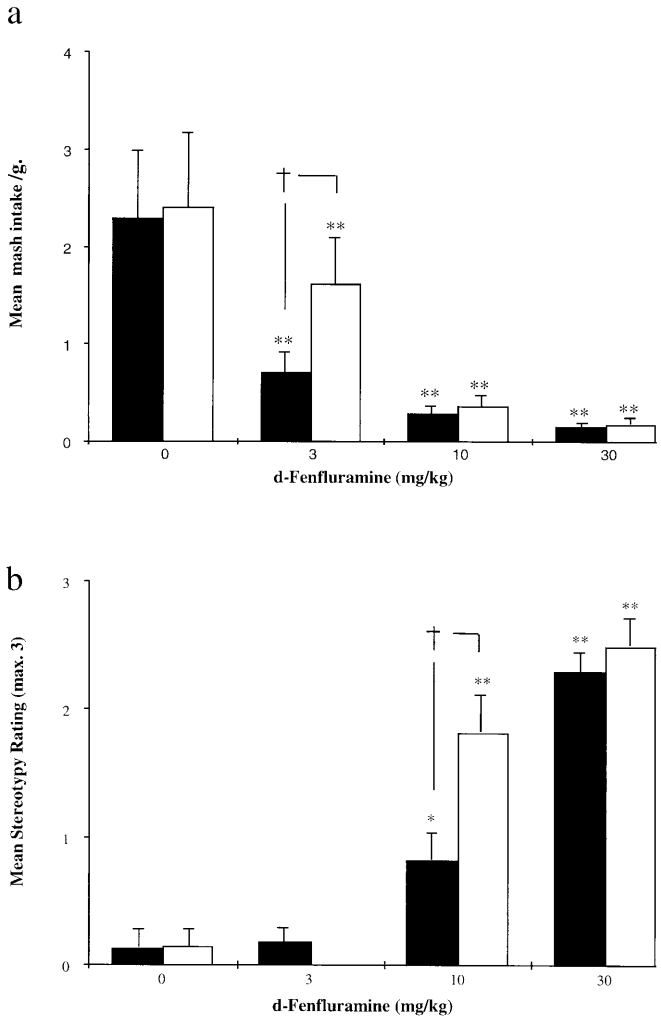

Fig. 1 a The effect of *d*-fenfluramine on food consumption in wild-type and 5-HT_{2C} knockout mice. †P<0.01 comparing the difference in food intake between wild-type (*filled bars*; n=11) and mutant (*open bars*; n=11) animals at the same dose of drug. *P<0.05, **P<0.01 compared to corresponding vehicle-treated group. **b** Mutant mice (*open bars*; n=11) exhibit a significantly increased stereotypy response upon 10 mg/kg *d*-fenfluramine administration compared to wild-type mice (*closed bars*; n=11). †P<0.01 comparing the difference in the stereotyped response to *d*-fenfluramine between mutant and wild-type mice. *P<0.05, **P<0.01 compared to corresponding vehicle-treated group

Fig. 1a]. In wild-type mice, treatment with 3 mg/kg *d*-fenfluramine led to a 70% reduction in the amount of food ingested. In contrast, the same dose led to a 33% reduction in intake by mutant animals. This difference led to a significant dose×genotype interaction [F(3,57)=3.47, P<0.05] and demonstrates that the hypophagic effect of *d*-fenfluramine is substantially reduced in mice lacking 5-HT_{2C} receptors (Fig. 1a). Higher doses of *d*-fenfluramine led to a greater reduction in feeding that was similar in both genotypes. Thus, in comparison to vehicle, food consumption by mutant and wild-type mice was reduced by 86% and 88%, respectively, after administration of 10 mg/kg *d*-fenfluramine (Fig. 1a).

Behavioural stereotypy

As an initial assessment of the behavioural specificity of *d*-fenfluramine-induced hypophagia, the presence of behavioural stereotypy was quantified. Increasing doses of *d*-fenfluramine led to an increasing incidence and severity of behavioural stereotypy characterised by the presence of a flat body posture and slow stereotyped head shakes [main effect of dose: F(3,60)=66.9, P<0.01; Fig. 1b]. There was no evidence of stereotypy in either genotype after treatment with 3 mg/kg *d*-fenfluramine. Marked stereotypy was evident at the 10 mg/kg and 30 mg/kg doses of the drug (Fig. 1b). The stereotypy observed in mutant mice in response to a challenge of 10 mg/kg *d*-fenfluramine was significantly greater than that seen in wild-type animals (Fig. 1b). Accordingly, the ANOVA revealed a significant dose×genotype interaction [F(3,57)=3.77, P<0.05]. At the 10 mg/kg dose, four wild-type and two mutant animals failed to show any signs of stereotypy whereas administration of 30 mg/kg *d*-fenfluramine led to the development of stereotypy in all animals.

Behavioural satiety sequence

Ingestion of wet mash during the observation period led to the development of a typical satiety sequence in control animals (Fig. 2a, d). Thus, with time, the incidence of feeding declined and an increased incidence of active behaviours and grooming, leading ultimately to the development of resting behaviour, was clearly evident. Interestingly, after vehicle administration mutant animals tended to exhibit a delayed behavioural satiety sequence perhaps indicative of impaired satiety mechanisms. Thus, with the exception of the initial time bin there was a higher incidence of feeding in mutant animals throughout the test period and a postponed onset of resting behaviour. Such a trend is consistent with the mild obesity observed in these mice.

In wild-type animals, treatment with 3 mg/kg *d*-fenfluramine led to a temporal advance in the offset of feeding [drug×time interaction: F(21,420)=16.9, P<0.01] and onset of resting [drug×time interaction: F(21,420) =3.47, P<0.01] whilst preserving the qualitative pattern of behaviour characteristic of the behavioural satiety sequence (Fig. 2b). Hence, this drug treatment led to a significant reduction in the incidence of feeding behaviour throughout the initial 25-min period (P<0.01) and this was coupled with a significant increase in the incidence of resting behaviour (P<0.05). These effects were unlikely to be attributable to the sedative properties of the drug, since active behaviours were significantly increased in the initial time bin (P<0.01).

In contrast to the effect on the behavioural satiety sequence of wild type mice, 3 mg/kg *d*-fenfluramine had a markedly reduced impact in mutant animals (Fig. 2e). Although this drug dose had a modest effect on the initial incidence of feeding behaviour (P<0.05) it had no

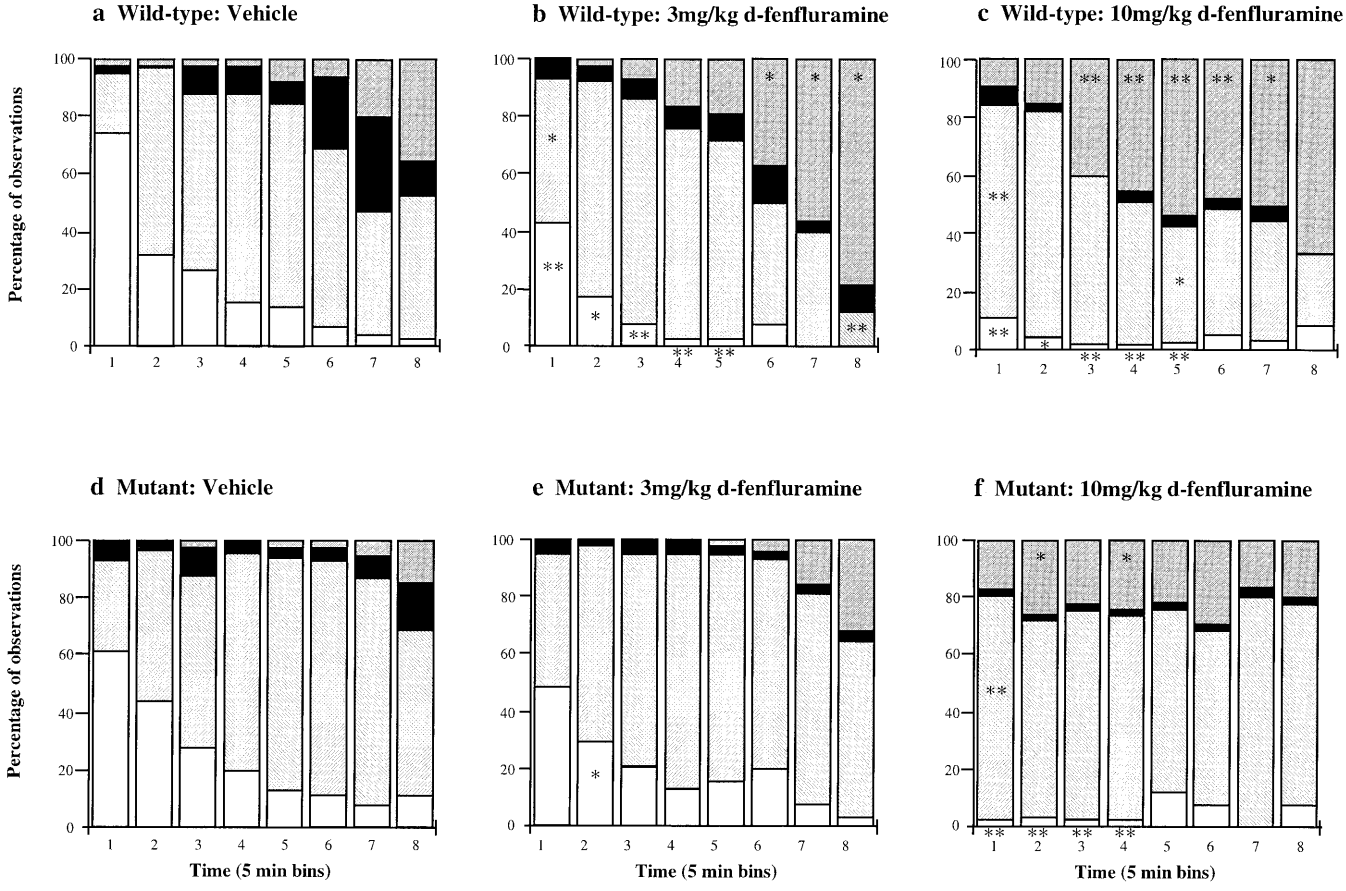

Fig. 2a–f Behavioural satiety sequence in wild-type (*upper panels*; *n*=11) and mutant (*lower panels*; *n*=11) animals treated with *d*-fenfluramine (30 mg/kg data not shown). Mice were individually observed for the incidence of feeding □, exploratory ▒, grooming ■ or resting ▒ behaviour at 2.5-s intervals for a period of 40 min. **P*<0.05, ***P*<0.01 comparison to vehicle-treated group of that particular genotype

significant effect on the incidence of resting, grooming, or active behaviours. Due to the presence of stereotypy, higher doses of *d*-fenfluramine (Fig. 2c, f; 30 mg/kg data not shown) led to a pronounced reduction in the frequency of feeding ($P<0.01$) and a marked increase in the incidence of resting that was present even in initial time bins ($P<0.01$).

Discussion

The present data demonstrate for the first time that the behavioural satiety sequence is observed in mice and that *d*-fenfluramine inhibits mouse feeding behaviour by enhancing satiety. The data also indicate that this hypophagia is mediated in large part by stimulation of the 5-HT_{2C} receptor subtype, since mice lacking these receptors exhibit a reduced response to a satiating dose of *d*-fenfluramine; indeed, at such a dose *d*-fenfluramine had a markedly reduced impact on the behavioural satiety sequence.

These results are compatible with reports in rats that *d*-fenfluramine reduces food consumption in a manner compatible with the enhancement of satiety (Blundell and McArthur 1978; Vickers et al. 1996). However, the data contrast with the observation that post-prandial resting is reduced after fenfluramine treatment in food-deprived rats (Montgomery and Willner 1988; Willner et al. 1990). The present data are also in good agreement with studies that have reported either a complete or partial blockade of *d*-fenfluramine-induced hypophagia by the 5-$HT_{2A/2C}$ antagonist ritanserin (Neill and Cooper 1989; Goodall et al. 1993), the 5-$HT_{2B/2C}$ antagonist SB-200646 (Hartley et al. 1995) and the selective 5-HT_{2C} antagonist SB-242084 (Trail et al. 1998). The findings are also consistent with reports that 5-HT_{2C} receptor agonists decrease food consumption in rats (Kennett and Curzon 1998a,b).

Since the hypophagic effect of *d*-fenfluramine was not completely abolished in 5-HT_{2C} receptor knockout mice, it is possible that other 5-HT receptor subtypes mediate this residual hypophagia. Indeed, one likely candidate is the 5-HT_{1B} receptor as the effects of *d*-fenfluramine have been reported to be blocked in rats by pre-treatment with the 5-$HT_{1A/1B}$ antagonist (±)-cyanopindolol (Neill and Cooper 1989) and the hypophagic effect of racemic fenfluramine was absent in food-deprived 5-HT_{1B} knockout mice (Lucas et al. 1998). Surprisingly, however, the selective 5-HT_{1B} antagonist GR-

127935 had no effect on the reduction of food intake induced by fenfluramine (Hartley et al. 1995; Trail et al. 1998).

Hypothalamic 5-HT_{2C} receptors may prove to be a crucial neural substrate for mediating, at least in part, the hypophagic effects of *d*-fenfluramine, since in vitro evidence suggests that *d*-fenfluramine is ineffective in stimulating hypothalamic corticotrophin-releasing factor release from the hypothalami of 5-HT_{2C} knockout mice (J. Raber and L. Tecott, personal communication). Furthermore, administration of the 5-HT_{2C} agonist TFMPP into the paraventricular nucleus (PVN) of the hypothalamus dose-dependently decreased food intake in rats (Hutson et al. 1988). However, such a role for hypothalamic 5-HT_{2C} receptors remains speculative, since *d*-fenfluramine inhibits food intake in rats with lesions of the PVN (Fletcher et al. 1993).

The behavioural stereotypy observed in the mice after *d*-fenfluramine administration was similar to the serotonin syndrome which has been extensively described in rats (Jacobs 1976). The stereotypy induced by high doses of *d*-fenfluramine was significantly enhanced in mutant animals and the mechanism mediating this effect is unclear at present. The syndrome resembled that seen after administration of 5-HT agonists and one potential explanation is that developmental or neuroadaptive changes may lead to alterations in serotonergic neurotransmission in the mutant mice. Alternatively, 5-HT_{2C} receptor stimulation may oppose *d*-fenfluramine-induced stereotypy in addition to inducing a hypophagic response.

It is unlikely that the differences observed in mutant and wild-type animals after treatment with *d*-fenfluramine are due to differences in the pharmacokinetics of *d*-fenfluramine in these animals. Thus, no simple pharmacokinetic explanation could account for *d*-fenfluramine having a blunted effect on feeding-related behaviours in mutants yet an enhanced effect on the incidence of stereotypy in the same animals.

The observation that the hypophagic effect of 3 mg/kg *d*-fenfluramine is greatly reduced in mutant mice lacking 5-HT_{2C} receptors suggests that a selective agonist at this receptor subtype may prove to be a novel and useful therapeutic strategy for the treatment of obesity. However, there is evidence that anorectic doses of the non-selective 5-$HT_{1B/2C}$ agonist *m*CPP elicit hypolocomotion and anxiogenesis in a social interaction test (Kennett et al. 1994). Indeed, the selective 5-HT_{2C} receptor antagonist SB-242084 has anxiolytic-like effects in several animal models of anxiety (Kennett et al. 1997). Interestingly, a recent study demonstrated weight loss in moderately obese subjects given *m*CPP at a dose which did not affect ratings of nausea or light-headedness (Sargent et al. 1997). Finally, the use of a selective 5-HT_{2C} agonist could avoid the recently reported cardiac valvular dysfunction observed after fenfluramine administration (Connolly et al. 1997) as 5-HT_{2C} receptors are present in high density in the brain but are present in low density or are absent in peripheral tissues (Hoyer et al. 1994).

Acknowledgements This work was supported by grants from NIDA and NARSAD (L. H. T.).

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