British Journal of Pharmacology (2000) 130, 1305–1314

www.nature.com/bjp

Comparative effects of continuous infusion of *m*CPP, Ro 60-0175 and *d*-fenfluramine on food intake, water intake, body weight and locomotor activity in rats

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1 The aim of the study was to compare the effects of 14 day subcutaneous infusion of the 5-HT_{2C} receptor agonists, *m*-chlorophenylpiperazine (*m*CPP, 12 mg kg^{-1} day^{-1}) and Ro 60-0175 (36 mg kg^{-1} day^{-1}) and the 5-HT releasing agent and re-uptake inhibitor, *d*-fenfluramine (6 mg kg^{-1} day^{-1}), on food and water intake, body weight gain and locomotion in lean male Lister hooded rats.

2 Chronic infusion of all three drugs significantly reduced food intake and attenuated body weight gain. In contrast, drug infusion did not lead to significant reductions in locomotor activity in animals assessed 2 and 13 days after pump implantation.

3 In a subsequent 14 day study that was designed to identify possible tolerance during days 7–14, animals were given a subcutaneous infusion of *m*CPP (12 mg kg^{-1} day^{-1}) or *d*-fenfluramine (6 mg kg^{-1} day^{-1}) for either 7 or 14 days. During the first 7 days both drugs significantly reduced body weight gain compared to saline-infused controls; however, from day 7 onwards animals withdrawn from drug treatment exhibited an increase in body weight such that by day 14 they were significantly heavier than their 14-day drug-treated counterparts.

4 Both *m*CPP and *d*-fenfluramine reduced daily food intake throughout the infusion periods. For 14-day treated animals this hypophagia was marked during the initial week of the study but only minor during the second week. In light of the sustained drug effect on body weight, the data suggest that weight loss by 5-HT_{2C} receptor stimulation may be only partly dependent on changes in food consumption and that 5-HT_{2C} receptor agonists may have effects on thermogenesis.

5 These data suggest tolerance does not develop to the effects of *d*-fenfluramine, *m*CPP and Ro 60-0175 on rat body weight gain.

British Journal of Pharmacology (2000) **130,** 1305–1314

Keywords: Serotonin; 5-HT_{2C}; Ro 60-0175; *d*-fenfluramine; *m*CPP; feeding; chronic administration; locomotor activity; tolerance; thermogenesis

Abbreviations: *m*CPP, *m*-Chlorophenylpiperazine; PCR, polymerase chain reaction; RNA, ribonucleic acid; Ro 60-0175, (*S*)-2-(6-chloro-5-fluoroindol-1-yl)-1-methylethylamine); RT–PCR, reverse transcription-polymerase chain reaction

Introduction

Serotonin (5-HT) has been extensively implicated in an array of behavioural and physiological functions including the control of ingestive behaviour (Dourish, 1995); indeed, clinically effective obesity treatments such as the 5-HT releasing agent *d*-fenfluramine, and the 5-HT and noradrenaline re-uptake inhibitor sibutramine, both increase brain 5-HT levels (Guy-Grand, 1992; Weintraub *et al*., 1991; Sabol *et al*., 1992; Gundlah *et al*., 1997).

Serotonin mediates its effects through at least 14 different receptor subtypes which are organized into seven major families designated 5-HT_1 to 5-HT_7 (Boess & Martin, 1994; Martin & Humphrey, 1994). There is increasing evidence for an important role of the 5-HT_{2C} receptor in the control of ingestive behaviour (Dourish, 1995). Hence, acute administration of the 5-HT_{2C} receptor agonist, *m*CPP, induces hypophagia in rats (Kennett & Curzon, 1988a,b) and mutant mice lacking functional 5-HT_{2C} receptors exhibit obesity (Tecott *et al*., 1995). Furthermore, there is evidence from studies using these 5-HT_{2C} receptor mutant mice that *d*-fenfluramine-induced hypophagia is mediated, at least in part, by the 5-HT_{2C} receptor (Vickers *et al*., 1999). Similar conclusions have been reached from studies using 5-HT_{2C} receptor antagonists in rats (Hartley *et al*., 1995). The 5-HT_{1B} receptor has also been reported to have a role in mediating fenfluramine-induced hypophagia in the rat (Neill & Cooper, 1989) though two preliminary studies using selective 5-HT_{1B} receptor antagonists do not support these findings (Hartley *et al*., 1995; Trail *et al*., 1998). Interestingly, a recent study reported that the hypophagia induced by racemic fenfluramine administration was almost entirely abolished in 5-HT_{1B} receptor knockout mice (Lucas *et al*., 1998). In man, the hypophagia observed after acute *d*-fenfluramine administration is completely blocked by the 5-$HT_{2A/2C}$ receptor antagonist ritanserin (Goodall *et al*., 1993). Such data suggest that a selective 5-HT_{2C} receptor agonist may be clinically useful for the treatment of obesity and this hypothesis is reinforced by the finding that *d*-norfenfluramine, the major metabolite of *d*-fenfluramine is a 5-HT_{2C} receptor agonist (Mennini *et al*., 1991; Porter *et al*., 1999).

If 5-HT_{2C} receptor agonists are to be useful in man, one important issue is whether their effects on food intake and body weight are maintained after chronic drug treatment. There are reports that the hypophagia and hypolocomotion observed after *m*CPP (Sills *et al*., 1985; Freo *et al*., 1992; Kennedy *et al*., 1993) or fenfluramine (McGuirk *et al*., 1992;

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Rowland & Carlton, 1986a) are reduced upon chronic administration of the drugs. Such studies have generally not found (McGuirk *et al.* 1992) or reported (Sills *et al.*, 1985; Kennedy *et al.*, 1993) effects of chronic treatment on body weight and have typically utilized a serial injection technique whereby animals were injected with drug once or twice per day either by the subcutaneous or intraperitoneal route; however, in contrast to these studies, daily oral administration of 20 mg kg^{-1} racemic fenfluramine has been reported to reduce rat body weight gain (Stallone & Levitsky 1994). Due to the short half lives in rats of *m*CPP (Caccia *et al.*, 1981) and *d*-fenfluramine (Rowland & Carlton, 1986b), intraperitoneal and subcutaneous injection regimens are likely to cause marked fluctuations of drug concentrations in plasma. Interestingly, in a recent study in which animals were implanted with an osmotic mini-pump that continuously released *m*CPP subcutaneously, animals showed little or no tolerance to the hypophagic effect of the drug but complete tolerance to its sedative effects (Fone *et al.*, 1998). In addition, at least one study using osmotic mini-pumps has demonstrated the maintained effect of *d*-fenfluramine on rat body weight (Rowland, 1986). Such findings (Fone *et al.*, 1998; Kennedy *et al.*, 1993) indicate that different drug exposure regimens can induce differential tolerance to 5-HT_{2C} receptor-mediated functions.

The present study evaluated the effects of chronic, continuous, administration of 5-HT_{2C} receptor agonists and *d*-fenfluramine on body weight gain, food and water intake, and locomotor activity in rats. Furthermore, since the hypothalamus has been extensively implicated in the control of ingestive behaviour (Bernadis & Bellinger, 1996), the effect of chronic drug administration on 5-HT_{2C} receptor mRNA levels in the hypothalamus was assessed using the reverse transcription-polymerase chain reaction (RT–PCR).

Two preferential 5-HT_{2C} receptor agonists were used, *m*CPP and Ro 60-0175. Receptor binding studies and functional models have demonstrated that *m*CPP has approximately 10 fold selectivity for the 5-HT_{2C} receptor over the 5-HT_{1A} and 5-HT_{1B} receptors where it acts as a partial agonist (Kennett, 1993). In similar models, *m*CPP has 10 fold selectivity for the 5-HT_{2C} receptor over the 5-HT_{2A} receptor where it may act as an antagonist (Kennett, 1993). Such a hypothesis is reinforced by the finding that peripheral administration of *m*CPP dose-dependently blocks the head twitch response induced by 5-HT_{2A} receptor agonists such as DOI (Schreiber *et al.*, 1995), though this is discrepant to at least one other study reporting increased head twitches after central *m*CPP administration (Willins & Meltzer 1997). In a recent characterization study at cloned human receptors, *m*CPP was reported to be only 3 fold selective for the 5-HT_{2C} receptor over the 5-HT_{2A} receptor and approximately equipotent at the 5-HT_{2C} and 5-HT_{2B} receptor (Porter *et al.* 1999). However, the relative efficacy of *m*CPP at 5-HT_{2A} (0.22) and 5-HT_{2B} receptors (0.24) was considerably lower than that at the 5-HT_{2C} receptor (0.65; Porter *et al.*, 1999). Such weak partial agonism at the 5-HT_{2A} receptor may explain the finding that central administration of *m*CPP into the prefrontal cortex induces a head twitch response that is blocked by the 5-HT_{2A} receptor antagonists ketanserin and MDL 100,907 (Willins & Meltzer 1997). With the exception of the 5-HT_3 receptor (6 fold), which has little documented role in rodent feeding (Dourish, 1995), *m*CPP has greater than 100 fold selectivity for other 5-HT receptors (Kennett, 1993). Ro 60-0175 is a high efficacy agonist at 5-HT_{2C} receptors and is claimed to have 100 fold selectivity over other 5-HT receptors with the exception of the 5-HT_{2B} receptor at which it also has high affinity and efficacy (Martin *et al.*, 1998). However, a recent study suggests that the compound may only be 14 fold selective for the human 5-HT_{2C} receptor over the human 5-HT_{2A} receptor where, unlike *m*CPP, it exhibits high efficacy (Porter *et al.*, 1999). Ro 60-0175 has been reported to significantly decrease rat food intake (Martin *et al.*, 1998) and to have a similar effect on the microstructure of feeding behaviour to *d*-fenfluramine (Dourish *et al.*, 1998).

In a subsequent 14-day study, designed to test whether tolerance to drug treatment occurred during days 7–14 of drug administration, the effect of *m*CPP and *d*-fenfluramine withdrawal after 7 days on body weight and daily food intake was assessed. In the absence of tolerance it was predicted that after drug withdrawal on day 7 the body weight of animals would increase towards control levels and become significantly greater than animals treated with drug for 14 days. Doses of drugs were chosen on the basis of previous reports with these compounds (Rowland, 1986; Dourish *et al.*, 1998; Fone *et al.*, 1998).

Methods

Animals

All work reported in this study was performed in accordance with Home Office regulations as outlined in the Animals (Scientific Procedures) Act 1986. Two experiments were performed. In the initial study, male Lister Hooded rats (Charles River; 180–200 g at the onset of the experiment) were singly housed in an experimental room maintained under a 12 h light/dark cycle (lights on: 0800 h). Ambient temperature was $21 \pm 1^{\circ}C$. A red light was the sole source of illumination during the dark period. Animals had continuous access to standard rodent diet (Bantin & Kingman U.K. Ltd., Hull, U.K.) and tap water. Experimental conditions were identical in the second study with the exception that animals' initial weights were 250–290 g.

Osmotic mini-pump implantation and experimental procedures

Animals were singly housed at least 48 h prior to surgery and were randomly allocated to drug treatment groups. At the time of mini-pump insertion the body weights, and food and water intakes were not significantly different between groups. Osmotic mini-pumps (ALZET model 2ML2 (14 day) or 2ML1 (7 day), supplied by Charles River U.K. Ltd. Margate, Kent, U.K.) were implanted subcutaneously beneath the dorsal skin under isofluorane anaesthesia (3% with 1.5 l min^{-1} oxygen). The pumps remained in place throughout each experiment. Animals were monitored for state of health at least once each day for the study duration.

In the initial experiment animals received a pump (ALZET pump 2ML2) designed to continuously deliver the test compound for a 14 day period. Each pump contained either vehicle (PEG 300), Ro 60-0175 (36 mg kg^{-1} day^{-1}), *m*CPP (12 mg kg^{-1} day^{-1}) or *d*-fenfluramine (6 mg kg^{-1} day^{-1}). Each morning (typically 1000 h) rats, food baskets and water bottles were weighed. Accordingly, a daily record of body weight, food consumption and water consumption was obtained for each animal. This was repeated each day for 14 days. The locomotor activity of each animal was assessed 2 and 13 days after pump implantation. In a second experiment animals received a pump designed to deliver either *m*CPP (12 mg kg^{-1} day^{-1}) or *d*-fenfluramine (6 mg kg^{-1} day^{-1}) for

either 7 (ALZET pump 2ML1) or 14 days (ALZET pump 2ML2). A control group of animals received vehicle for 14 days (sterile saline; ALZET pump 2ML2). As in the previous experiment, body weight, and food and water consumption were recorded daily for 14 days. The locomotor activity of each animal was assessed 2 and 7 days after pump implantation.

Assessment of locomotor activity

On the days stated above, locomotor activity was assessed using automated apparatus (Activity Monitor AM1052, Benwick Electronics). Animals were placed individually into clear Perspex cages (40 cm × 20 cm, Techniplast U.K. Ltd.) and the number of cage transits was recorded for the subsequent 20 min period. A transit was recorded when an animal traversed along the length of the cage from one end to the other breaking each of the seven photocell beams in succession. A maximum of 16 animals could be individually assessed simultaneously in this apparatus. Animals were in visual, but not auditory, isolation for the test. Animals, assigned to drug treatment at random, were run in the locomotor apparatus in numerical order. All locomotor activity testing was performed at approximately 1400 h and all locomotor data were collected in under 90 min.

RT–PCR assays

On completion of the study, animals were killed in a rising concentration of CO_2. Brains were removed and each hypothalamus dissected and frozen ($n=4$). Total RNA was isolated from tissue samples (RNeasy, Qiagen Ltd, Germany). The RNA concentration was measured by optical density at 260 nm and samples were diluted to give working amounts of 50 ng for use in the RT–PCR reaction. The 5-HT_{2C} receptor primer pair (forward TTG CTG ATA TGC TGG TGG GA; reverse TCC AAT CAC AGG GAT AGC AA) produced a 290 bp product, spanning the region 301–590 that included the splice variant (Canton *et al*., 1996; Xie *et al*., 1996). Ribosomal 18S RNA was used as an internal standard in a relative RT–PCR reaction which was designed to produce a 488 bp product. The 18S primers (Ambion QuantumRNA 18S internal standard kit) comprised the 18S primers and an additional pair of primers (competimers) which had been modified at the 3′ end to prevent extension by the polymerase. The optimal ratio of 18S primers : competimers for 5-HT_{2C} RT–PCR was determined using a superscript one step RT–PCR system (GibcoBRL). A primer : competimer ratio of 3.5 : 6.5 was discovered to be optimal which, when co-amplified with the 5-HT_{2C} gene, yielded both bands of suitable intensity. The reaction parameters that resulted in both the 18S and 5-HT_{2C} products being in the linear range of accumulation was empirically determined. The final RT–PCR reactions included 50 ng total RNA, 18S primers and competimers and 1 pmol forward and reverse 5-HT_{2C} primers in a total reaction volume of 50 μl. The reaction parameters were 48°C for 45 min reverse transcription followed by 94°C for 2 min enzyme inactivation, 94°C for 0.5 min denaturing, 57°C for 1 min annealing and 72°C for 1 min extension. The last three stages were repeated for a total of 30 cycles that resulted in amplification of all products in the linear range of accumulation. 20 μl of PCR product was stained by ethidium bromide on a 1% agarose gel and viewed under UV illumination. Images were acquired using the GeneGenius image analysis system (Syngene, Cambridge, U.K.) under non-saturating conditions, and the intensity of bands processed by densitometric analysis as described previously (Utsugisawa *et al*., 1999; Hager *et al*., 1999).

Drugs

d-Fenfluramine hydrochloride and Ro 60-0175 hydrochloride were synthesized in the Department of Chemistry at Cerebrus. *m*CPP hydrochloride was purchased from Sigma-Aldrich (Poole, Dorset, U.K.). PEG 300, used as vehicle in the initial study, was purchased from BDH-Merck Ltd., Lutterworth, Leics., U.K.

Statistical analysis

All data are presented as mean ± s.e.mean. Body weight and food and water intake data were analysed by two-way ANOVA with drug (between-subjects) and time (within-subjects) as factors. A significant two-way interaction was succeeded by the performance of one-way ANOVA to assess the effect of drug (between-subjects) at each time point. A significant main effect of drug in this ANOVA led to the performance of Newman-Keuls tests (two-tailed) to assess whether drug-treated animals were significantly different from controls. In addition, in the *m*CPP withdrawal experiment unpaired *t*-tests were used to assess whether 7-day treated animals differed significantly from their 14-day-treated counterparts in the second week of the study.

Locomotor activity data were analysed by one-way ANOVA (drug as between-subjects factor). Significant differences from control animals were assessed using Dunnett's

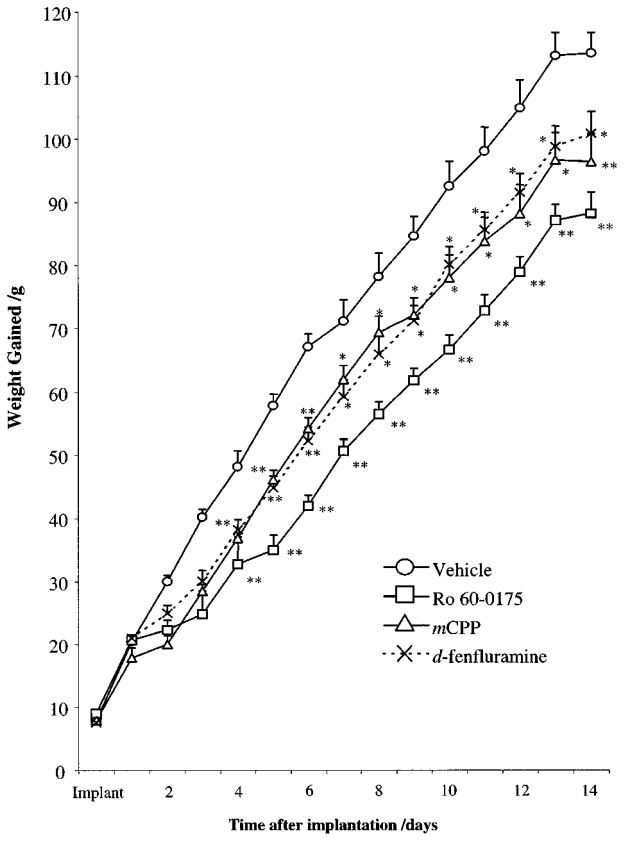

Figure 1 Effect of the preferential 5-HT_{2C} receptor agonists Ro 60-0175 (36 mg kg^{-1} day^{-1}, $n=6$) and *m*CPP (12 mg kg^{-1} day^{-1}, $n=8$) and the indirect 5-HT receptor agonist *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=10$) on the weight gain of lean rats compared to vehicle ($n=7$). Results are treatment group means and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals are denoted by $^{*}P<0.05$ and $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test and ANOVA.

test (two-tailed). A difference of $P<0.05$ was regarded as significant.

Levels of hypothalamic 5-HT_{2C} receptor mRNA were analysed by two-way ANOVA. Drug was a between-subjects factor and product type (either 5-HT_{2C} gene or splice variant) was a within-subjects factor. All data analysis was performed using Statistica (V5.1, Statsoft Inc.).

Results

Experiment 1

Effect of 14 day Ro 60-0175, mCPP, and d-*fenfluramine administration on rat body weight gain and ingestive behaviour*

Body weight All animals gained weight throughout the duration of the study (Figure 1). Over the course of the experiment animals treated with Ro 60-0175, *m*CPP or *d*-fenfluramine exhibited a smaller body weight gain (Figure 1) such that from day 4 after implantation onwards the body weight gain of these animals was significantly less than controls (drug × time, interaction from ANOVA $F_{(42,378)}=4.21$, $P<0.001$). At the end of the experiment animals treated with Ro 60-0175, *m*CPP and *d*-fenfluramine weighed 10, 8, and 5% respectively less than controls.

Food consumption Since the drug treatments affected body weight, food consumption data are expressed as a function of that day's weight. Infusion of Ro 60-0175, *m*CPP or *d*-fenfluramine significantly reduced daily food intake (Figure 2). This hypophagia was most marked during the initial week after implantation (drug × time interaction from ANOVA $F_{(42,378)}=3.50$, $P<0.001$); indeed, from day 11 onward drug treatment had no significant effect on food intake (Figure 2).

Water consumption Since the drug treatments affected body weights, water consumption data are expressed as a function of that day's weight. In contrast to the effects on food consumption, chronic infusion of Ro 60-0175, *m*CPP and *d*-fenfluramine had only a modest and inconsistent effect on water intake (drug × time interaction from ANOVA $F_{(42,378)}=1.44$, $P=0.042$; Figure 3a).

Locomotor activity When assessed 2 days after mini-pump implantation (Table 1) animals treated with *m*CPP exhibited a significant increase in cage transits ($P<0.01$). *d*-Fenfluramine treatment also tended to increase spontaneous locomotor activity but this was not significant. Ro 60-0175 administration appeared to decrease locomotion but this effect was also non-significant.

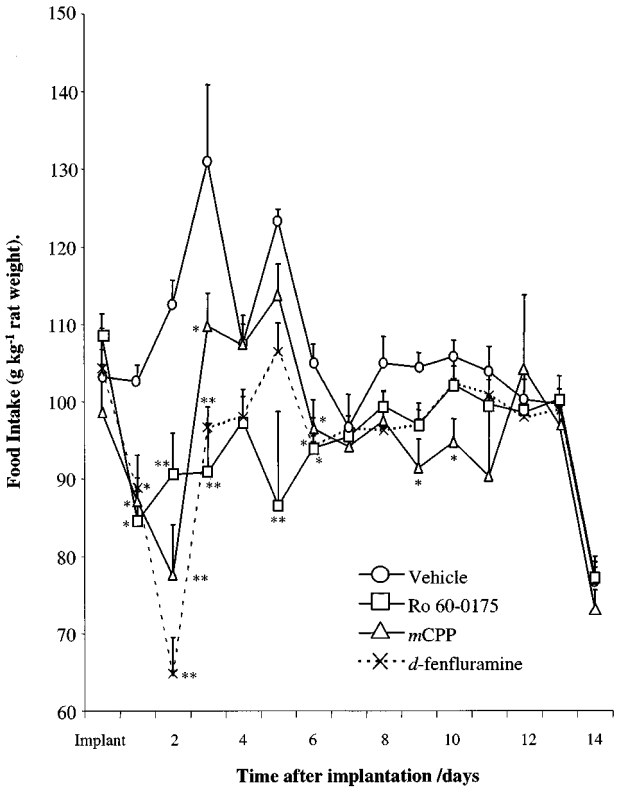

Figure 2 Effect of the preferential 5-HT_{2C} receptor agonists Ro 60-0175 (36 mg kg^{-1} day^{-1}, $n=6$) and *m*CPP (12 mg kg^{-1} day^{-1}, $n=8$) and the indirect 5-HT receptor agonist *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=10$) on daily food consumption (vehicle $n=7$). Results are treatment group means and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals are denoted by $^{*}P<0.05$ and $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test and ANOVA.

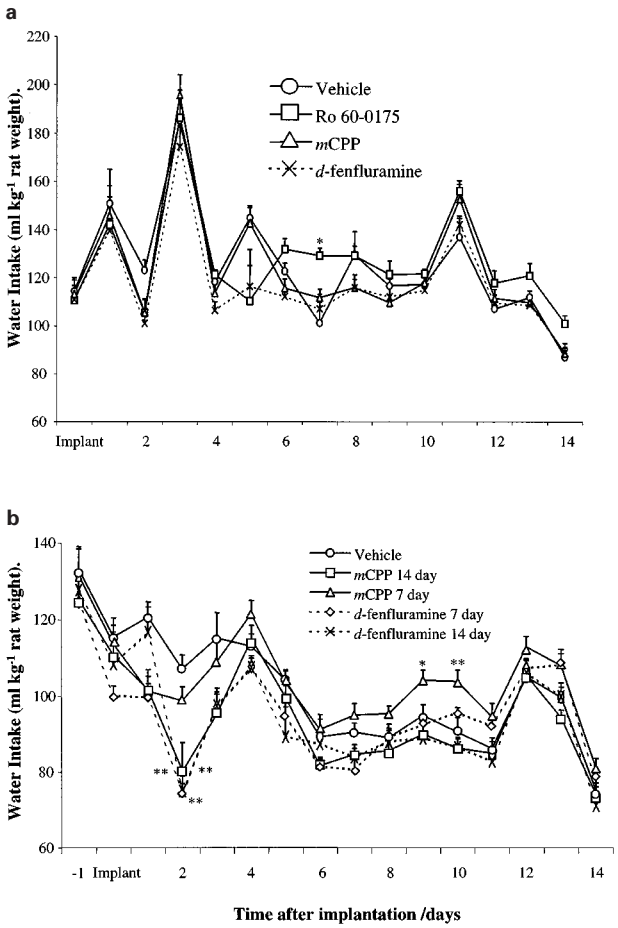

Figure 3 (a) Effect of the preferential 5-HT_{2C} receptor agonists Ro 60-0175 (36 mg kg^{-1} day^{-1}, $n=6$) and *m*CPP (12 mg kg^{-1} day^{-1}, $n=8$) and the indirect 5-HT receptor agonist *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=10$) on daily water consumption (vehicle $n=7$). (b) Effect of 7 and 14 day treatment with the preferential 5-HT_{2C} receptor agonist *m*CPP (12 mg kg^{-1} day^{-1}, $n=9$) and *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=9$) on daily water intake in rats. Results are treatment group means and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals are denoted by $^{*}P<0.05$ and $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test and ANOVA.

Table 1 The effect of chronic drug infusions on cage transits (Experiment 1)

	Vehicle	*Ro 60-0175*	m*CPP*	D-*fenfluramine*
Day 1	22.14±3.21	12.67±2.40	43.25±5.93**	34.40±2.84
Day 13	32.57±5.12	35.00±4.67	34.5±3.17	36.33±4.55

Values are mean cage transits±s.e.mean. Animals received either vehicle ($n=7$), Ro 60-0175 (36 mg kg^{-1} day^{-1}, $n=6$), *m*CPP (12 mg kg^{-1} day^{-1}, $n=8$) or *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=10$) for a 2 week period. Activity was assessed 2 and 13 days after mini pump implantation. **$P<0.01$, Dunnett's test from vehicle-treated group following ANOVA $F_{(3,27)}=9.72$, $P<0.001$. No significant effect on locomotor activity was observed after the 13 day drug infusion period: ANOVA $F_{(3,26)}=0.12$, $P=0.94$ (data for one *d*-fenfluramine-treated animal were lost due to equipment malfunction).

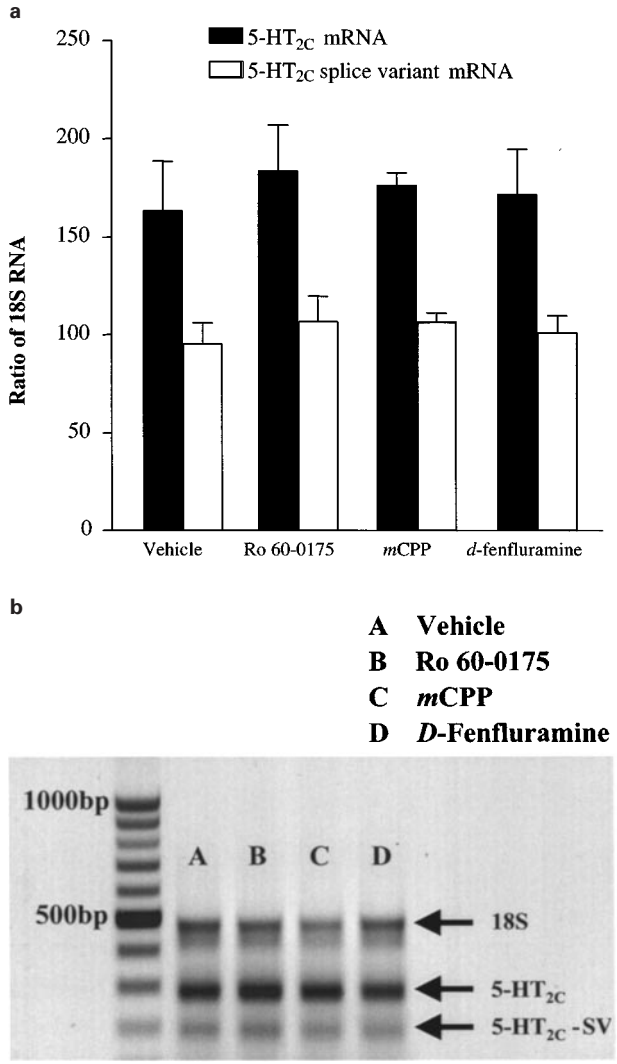

Figure 4 (a) Effect of chronic administration of Ro 60-0175 (36 mg kg^{-1} day^{-1}), *m*CPP (12 mg kg^{-1} day^{-1}) and *d*-fenfluramine (6 mg kg^{-1} day^{-1}) on mRNA levels of the 5-HT$_{2C}$ receptor. Each bar is the mean of four individual animals whose RNA was treated independently and normalized to 18S RNA which was co-amplified in the same tube. The 5-HT$_{2C}$ receptor products are expressed as a ratio of the 18S product which was normalized to 100%. Vertical lines represent s.e.mean. (b) Electrophoretic division of RT–PCR products from a representative animal from each treatment group. The PCR products were run down a 1% agarose gel and stained by ethidium bromide, the gel has been inverted for clarity. A 100 bp ladder is included in lane 1 to confirm the size of the PCR products.

When animals were tested 13 days after pump implantation there was no significant effect of drug infusion on locomotor activity ($F_{(3,26)}=0.12$, $P=0.94$; Table 1).

5-HT$_{2C}$ receptor mRNA levels in the hypothalamus The specificity of the 5-HT$_{2C}$ PCR product was confirmed by sequencing (data not shown). Continuous drug infusion had no significant effect on the relative amounts of mRNA that encode for either the 5-HT$_{2C}$ receptor gene or splice variant (drug × product type interaction $F_{(3,12)}=0.094$, $P=0.96$; Figure 4a). Interestingly, the non-functional splice variant appeared to represent approximately 37% of the mRNA for the 5-HT$_{2C}$ receptor in the hypothalamus. This led to a main effect of product type in the ANOVA ($F_{(1,12)}=130.0$, $P<0.001$). Figure 4b shows the results from a representative animal from each treatment group illustrating the relative intensity of the PCR products. The ratio of the 5-HT$_{2C}$ receptor gene products relative to the 18S bands from the same animals were calculated resulting in each animal acting as its own internal control.

Experiment 2

Effect of drug withdrawal after 7 days on mCPP-, and d-*fenfluramine-induced changes in ingestive behaviour and body weight*

Body weight All animals gained weight throughout the duration of the study (Figure 5a, b). Animals given infusions of *m*CPP or *d*-fenfluramine for 14 days exhibited a reduced increase in body weight over the 2-week period (drug × time interaction from ANOVA $F_{(56,560)}=12.24$, $P<0.001$) replicating the results of Experiment 1. Animals given either *m*CPP or *d*-fenfluramine treatment for 7 days showed a significantly reduced body weight gain compared to vehicle-treated animals throughout the 2-week study; however, during the second week animals treated with drug for only 7 days increased in weight such that the weight gained over the duration of the experiment was significantly greater than animals treated with drug for 14 days. These data are illustrated in Figure 5a, b (for clarity the *m*CPP and *d*-fenfluramine data are presented on separate graphs).

Food consumption Infusion of *m*CPP (Figure 6) or *d*-fenfluramine (Figure 7) significantly reduced daily food intake. As in Experiment 1 this hypophagia was most marked during the initial week of drug administration (drug × time interaction from ANOVA $F_{(56,560)}=9.74$, $P<0.001$). Data for *m*CPP and *d*-fenfluramine are presented on different axes for clarity. Interestingly, during the second week, the food consumption of 7 day, but not 14 day, *m*CPP and *d*-fenfluramine-treated animals increased such that their daily food consumption tended to be greater than controls (Figures 6 and 7).

Water consumption As in experiment 1, chronic infusion of *m*CPP and *d*-fenfluramine led to a significant main effect of drug in the ANOVA ($F_{(4,39)}=4.86$, $P<0.01$) and a significant drug × time interaction $F_{(60,585)}=1.95$, $P<0.01$; Figure 3b). In agreement with the initial study, the effects on water consumption were not as marked as the effects on food consumption. A significant reduction in water intake was only seen at one point and not thereafter.

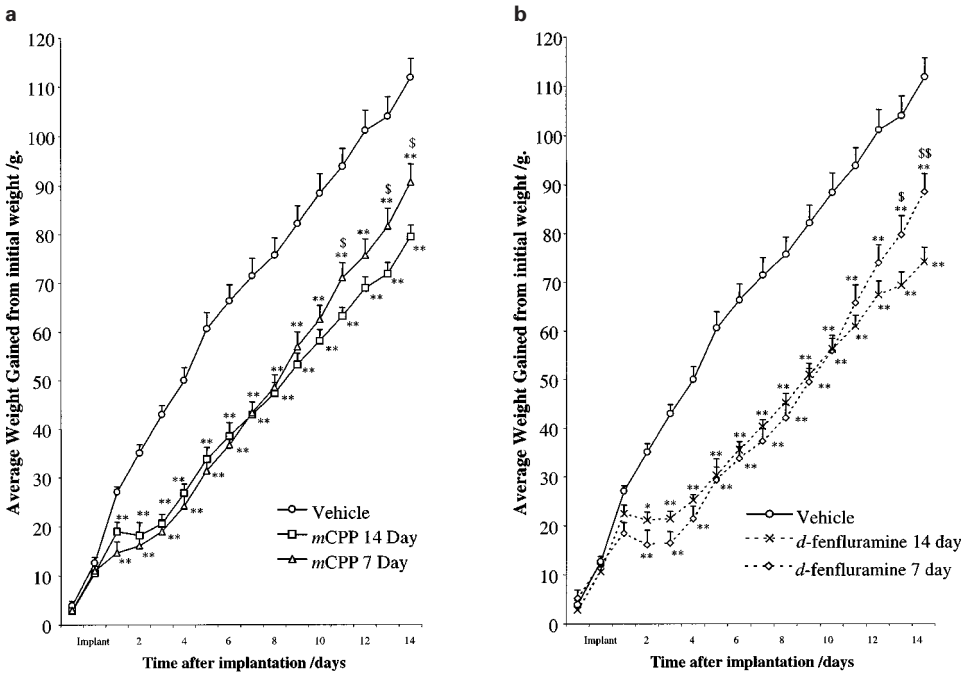

Figure 5 (a) Effect of drug withdrawal after 7 day infusion of *m*CPP (12 mg kg^{-1} day^{-1}) on body weight gain in rats. Results are treatment group means ($n=9$) and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals are denoted by $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test and ANOVA. Significant differences between 14 day and 7 day-treated animals are denoted by $P<0.05$. These planned comparisons were assessed by non-paired *t*-test. (b) Effect of drug withdrawal after 7 day infusion of *d*-fenfluramine (6 mg kg^{-1} day^{-1}) on body weight gain in rats. Results are treatment group means ($n=9$) and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals are denoted by $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test and ANOVA. Significant differences between 14 day and 7 day-treated animals are denoted by $\$P<0.05$ and $\$\$P<0.01$. These planned comparisons were assessed by non-paired *t*-test.

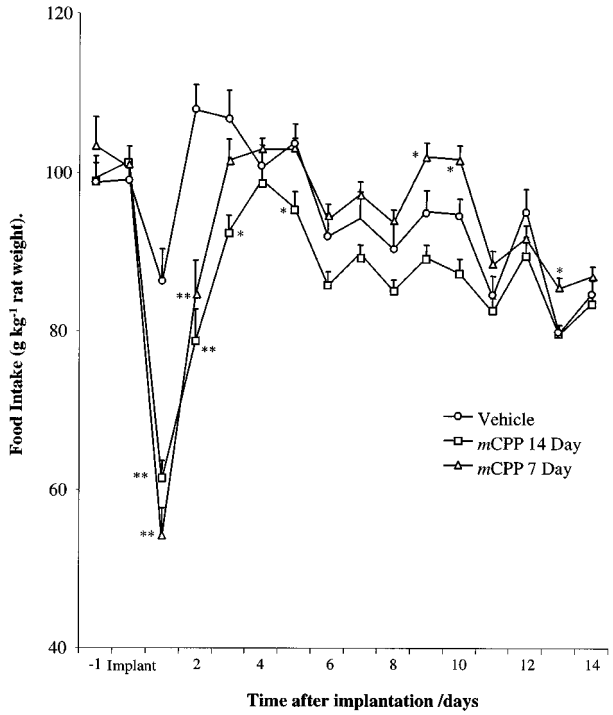

Figure 6 Effect of 7 and 14 day treatment with the preferential 5-HT_{2C} receptor agonist *m*CPP (12 mg kg^{-1} day^{-1}, $n=9$) on daily food intake in rats. Results are treatment group means and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals (saline, $n=9$) are denoted by $^{*}P<0.05$, $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test after significant ANOVA (dose × day interaction $F_{(56,560)}=9.74$, $P<0.001$; results of one-way ANOVAs performed at each level of day not shown).

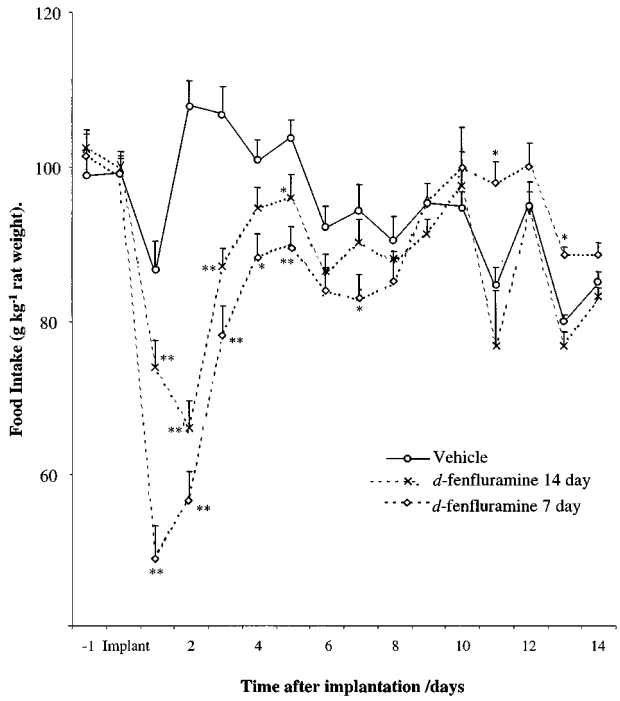

Figure 7 Effect of 7 and 14 day treatment with *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=9$) on daily food intake in rats. Results are treatment group means and vertical lines represent s.e.mean. Significant differences from vehicle-treated animals (saline, $n=9$) are denoted by $^{*}P<0.05$, $^{**}P<0.01$. Differences were assessed for significance by Newman-Keuls test after significant ANOVA (dose × day interaction $F_{(56,560)}=9.74$, $P<0.001$; results of one-way ANOVAs performed at each level of day not shown).

Table 2 The effect of chronic drug infusions on cage transits (Experiment 2)

	Vehicle	m*CPP* (7 day)	m*CPP* (14 day)	D-*fen* (7 day)	D-*fen* (14-day)
Day 2	23.78±4.78	45.44±9.51	38.78±8.95	29.11±5.85	35.88±7.86
Day 7	24.11±2.14	48.22±4.75**	45.78±5.15**	34.89±4.47	35.66±3.52

Values are mean cage transits±s.e.mean. Animals received either vehicle ($n=9$), m=CPP (12 mg kg^{-1} day^{-1}, $n=9$) or *d*-fenfluramine (6 mg kg^{-1} day^{-1}, $n=9$) for a 2 week period. Activity was assessed 2 and 7 days after mini pump implantation. **$P<0.01$, Dunnett's test from vehicle-treated group following significant ANOVA $F_{(4,40)}=5.39$, $P<0.001$. No significant effect on locomotor activity was observed after the 2-day drug infusion period: ANOVA $F_{(4,39)}=1.25$, $P=0.30$ (data for one 14 day *d*-fenfluramine-treated animal were lost due to equipment malfunction).

Locomotor activity When assessed 2 days after pump implantation (Table 2) animals treated with *m*CPP or *d*-fenfluramine (both 7 and 14 day-treated animals) tended to exhibit an increased number of cage transits compared to vehicle. However, these differences were non-significant (ANOVA ($F_{(4,39)}=1.25$, $P=0.30$)). Data for one animal (14 day *d*-fenfluramine-treated) were lost due to equipment malfunction.

When tested 7 days after pump implantation, ANOVA revealed that *m*CPP-treated animals showed a significant increase in the number of cage transits ($F_{(4,40)}=5.39$, $P=0.001$; Table 2). Although *d*-fenfluramine-treated animals tended to exhibit an increased number of cage transits this was not statistically significant.

Discussion

Continuous infusion of the preferential 5-HT$_{2C}$ receptor agonists, Ro 60-0175 and *m*CPP and the indirect 5-HT agonist *d*-fenfluramine, reduced body weight gain in lean rats over a 2-week period. A subsequent experiment was performed in order to test that the effects on body weight in the first study did not simply represent an initial decrease in weight succeeded by a period of normal weight gain where drug-treated animals could never 'catch-up' growing control animals. In this study *m*CPP and *d*-fenfluramine given for 14 days reduced body weight as before but animals which received drug treatment for only 1 week showed a significant increase in weight gain during a week of drug withdrawal compared to rats given drug for the duration of the 14 day study. These data indicate that rats do not show tolerance to the effects of preferential 5-HT$_{2C}$ receptor agonists and *d*-fenfluramine on body weight gain during chronic drug administration.

In both chronic studies each of the compounds tested significantly reduced daily food consumption but the effects on food intake were robust only during the first 10 days of drug treatment. Although a similar effect has been reported after daily oral administration of racemic fenfluramine (Stallone & Levitsky, 1992), our findings with *m*CPP and Ro 60-0175 are novel and significant as the effects of drug treatment on body weight gain were maintained for the duration of the studies. One possible explanation for a maintained body weight reduction in the absence of a robust decrease in food intake may be that the drugs increase metabolic rate and, therefore, promote thermogenesis. This interpretation of the data is consistent with evidence that 5-HT$_{2C}$ receptor agonists can increase rat body temperature under thermoneutral conditions (see Kennett, 1993). Furthermore, there is an extensive literature detailing *d*-fenfluramine as a potent stimulator of thermogenesis in both rats (Rothwell & Le Feuvre, 1992) and obese patients (Scalfi *et al*., 1993). Such a conclusion may be reinforced by the additional finding that, except for 1 or 2 days, animals withdrawn from *d*-fenfluramine treatment consumed a similar amount of food to animals still receiving *d*-fenfluramine infusions despite the *d*-fenfluramine-withdrawal group showing increased weight gain.

Since the hypophagic effects of *m*CPP and Ro 60-0175 are reduced in the latter days of the 2-week period, the present data are in agreement with studies suggesting that rats exhibit almost complete tolerance to the hypophagic effects of preferential 5-HT$_{2C}$ receptor agonists when injected chronically (Sills *et al*., 1985; Freo *et al*., 1992; Kennedy *et al*., 1993). Interestingly, in a recent study which also delivered *m*CPP *via* osmotic mini-pumps (Fone *et al*., 1998), no significant effect of *m*CPP on body weight was observed. However, this finding may have been compromized by the fact that animals were only allowed to feed for a 4 h period each day.

Plasma levels of *m*CPP (Barbhaiya *et al*., 1996) and *d*-fenfluramine (Rowland & Carlton, 1986b) are much more stable in man than they are in the rat; indeed the plasma half life of fenfluramine in man is 18–24 h whereas in the rat it is only 2 h (for review see Rowland & Carlton, 1986b). Accordingly, when investigating the chronic effects of these drugs on rat behaviour, continuous infusion using a mini-pump may be an appropriate method for modelling the effects observed in man. This hypothesis is reinforced by the fact that 14-day administration of *m*CPP (Sargent *et al*., 1997) and 1-year administration of *d*-fenfluramine (Guy-Grand, 1992) lead to weight loss in man, an effect observed in the present experiments. Interestingly, at least one study has reported a reduction in body weight gain in rats receiving a daily oral administration of a 20 mg kg^{-1} dose of racemic fenfluramine (Stallone & Levitsky, 1994). Compared to a single subcutaneous administration where, typically, there is rapid drug absorption and a markedly fluctuating drug concentration in plasma with time, when given orally a drug is generally absorbed more slowly from the gut and its pharmacokinetic profile may, therefore, be similar to that observed using continuous delivery *via* a mini-pump. A study examining the effect of chronic oral *m*CPP in the rat would be of interest.

Our behavioural data showing an apparent absence of tolerance to the effects of chronic administration of preferential 5-HT$_{2C}$ receptor agonists and *d*-fenfluramine on body weight in rats is reinforced by the results of our *in vitro* studies. Thus, chronic treatment with *d*-fenfluramine, *m*CPP or Ro 60-0175 did not affect 5-HT$_{2C}$ receptor mRNA levels in the hypothalamus, a region likely to be involved in the regulation of feeding and drinking behaviour (Bernadis & Bellinger, 1996) and in the mediation of the hypophagia induced by 5-HT$_{2C}$ receptor agonists (Hutson *et al*., 1988). The present data suggest that hypothalamic 5-HT$_{2C}$ receptors were not down-regulated by the treatment regimes used. To our knowledge, no previous studies have examined the effects of chronic 5-HT agonist treatment on the mRNA levels of the 5-HT$_{2C}$ receptor in rat hypothalamus. Interestingly, previous studies have demonstrated that chronic treatment with 5-HT$_{2C}$ receptor agonists and, paradoxically, 5-HT$_{2C}$ receptor antagonists,

causes down-regulation of 5-HT_{2C} receptors both *in vitro* and *in vivo* (Barker & Sanders-Bush, 1993; Pranzatelli *et al*., 1993). One potential explanation for this apparent discrepancy is that different drug exposure regimens may lead to differential effects on 5-HT_{2C} receptors. Hence, 5-HT_{2C} receptors may be less prone to down-regulation during continuous, steady-state, drug infusion than after serial injection regimes which cause fluctuating drug levels in plasma. Alternatively, hypothalamic 5-HT_{2C} receptors may be relatively resistant to agonist-induced down-regulation and this conclusion is in agreement with at least one other study (Fone *et al*., 1998). However, 5-HT_{2C} receptor regulation is complex and although agonist-induced down-regulation has been reported to involve decreases in receptor mRNA (Saucier & Albert, 1997), this may not always be the case (Barker & Sanders-Bush, 1993).

In contrast to studies reporting that acute *m*CPP administration reduces rat locomotor activity (Kennett & Curzon, 1988a; Kennett *et al*., 1997b), in the present study, *m*CPP significantly increased locomotor activity when rats were assessed either 48 h or 1 week after implantation of a mini-pump. Furthermore, in contrast to previous reports (Rowland & Carlton, 1986b; Callaway *et al*., 1993), *d*-fenfluramine also tended to increase the number of cage transits. Only Ro 60-0175 tended to reduce locomotor activity and, although non-significant, this may be due to the relatively high dose used. Despite this trend, all Ro 60-0175-treated animals appeared to be in good health exhibiting no obvious signs of sedation. Furthermore, the reduction in locomotor activity on day 2 was not attributable to decreased food consumption since the other drug treatments had greater effects on consumption that day. One explanation for the apparent discrepancy between the present observations on locomotor activity and the acute effects previously described may be that the 5-HT_{2C} receptor population which mediates *m*CPP-induced hypolocomotion rapidly desensitizes when chronically activated (Fone *et al*., 1998). Accordingly, this receptor population may desensitize after sub-chronic *m*CPP infusion (for example, 2 or 7 days). Since *m*CPP is a preferential 5-HT_{2C} receptor agonist the compound may increase the number of cage transits through activation of other 5-HT receptor subtypes. Candidate receptors include the 5-HT_{1A} and/or 5-HT_{1B} receptor subtypes, for which *m*CPP has affinity and acts as a partial agonist (Kennett, 1993); indeed, this hypothesis is reinforced by data demonstrating that after treatment with the 5-HT_2 receptor antagonist LY53857, *m*CPP causes hyperlocomotion in mice, an effect that is abolished by either the 5-HT_{1A} receptor antagonist WAY-100635 or the 5-HT_{1B} receptor antagonist GR-127935 (Gleason & Shannon, 1998). Similar experiments using selective 5-HT receptor antagonists could test this hypothesis in the current model. After 14 day infusion we observed no significant effect of the drugs tested on locomotion. This finding is in agreement with a similar study (Fone *et al*., 1998) which reported no significant hypolocomotor effect of an acute *m*CPP challenge in animals that had received continuous infusions of *m*CPP for 2 weeks. Although the reason for all the observed effects on locomotor activity are unclear at present, it is evident that the effects of the compounds on ingestive behaviour in the present study are unlikely to be attributable to sedation.

If the hyperlocomotion observed after 2-day *m*CPP infusion is attributable to agonist activity at 5-HT_{1B} and/or 5-HT_{1A} receptors, then the role of these receptors in mediating the effect of *m*CPP on food intake and body weight should also be considered. The effect of both 5-HT_{1A} and 5-HT_{1B} receptor agonists on food consumption is well documented (Dourish, 1995) with 5-HT_{1A} receptor agonists stimulating feeding and 5-HT_{1B} receptor agonists inhibiting feeding. Chronic studies administering both *m*CPP and selective antagonists could resolve this issue. However, it should be noted that the profile observed with *m*CPP was markedly similar to that observed with Ro 60-0175 which has low affinity for either the 5-HT_{1A} or 5-HT_{1B} receptor (Martin *et al*., 1998). Since Ro 60-0175 is efficacious at the 5-HT_{2A} receptor, though not highly potent, and in light of the reports that 5-HT_{2A} receptor agonists inhibit feeding (Simansky 1998), it is possible that the chronic effects of this drug treatment may be attributable, at least in part, to activation of this receptor. Chronic studies using selective antagonists would prove useful to resolve this hypothesis. However, animals were carefully monitored at least once each day and no headshakes or other behaviours indicative of 5-HT_{2A} receptor stimulation were observed throughout the study duration. Ro 60-0175 is also a highly potent and high efficacy agonist at 5-HT_{2B} receptors (Porter *et al*., 1999). Interestingly, 5-HT_{2B} receptor activation has been reported to lead to small increases in food consumption under conditions of low baseline intake (Kennett *et al*., 1997a). Accordingly, such activity of Ro 60-0175 may be expected to counteract the effects of 5-HT_{2C} receptor activation on body weight gain. Again, studies with selective 5-HT_{2B} receptor antagonists would address this hypothesis.

The present data suggest that 5-HT_{2C} receptor agonists may prove to be useful in the treatment of obesity since the maintained effects of the preferential 5-HT_{2C} receptor agonists Ro 60-0175 and *m*CPP on body weight gain were comparable to those of *d*-fenfluramine. Future studies blocking the effects of Ro 60-0175 and *m*CPP with selective 5-HT_{2C} receptor antagonists would reinforce this hypothesis. Evidence in the mouse, rat, and man suggests that the hypophagia observed after acute *d*-fenfluramine administration is mediated, at least in part, through activation of the 5-HT_{2C} receptor subtype (Goodall *et al*., 1993; Trail *et al*., 1998; Vickers *et al*., 1999). The potential therapeutic utility of 5-HT_{2C} receptor agonists has been reinforced with the finding that in a short, 14-day, study *m*CPP administration leads to a modest weight loss in moderately obese human patients (Sargent *et al*., 1997). Thus, the 5-HT_{2C} receptor may provide a useful target for the development of a novel anti-obesity agent.

The authors gratefully acknowledge the assistance of Craig Bass, Dr Mayke Hesselink and Tony Debens.

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(Received October 5, 1999
Revised March 2, 2000
Accepted May 2, 2000)