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Effect of chronic *m*-CPP on locomotion, hypophagia, plasma corticosterone and 5-HT_{2C} receptor levels in the rat

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1 The present study examined 5-HT_{2C} receptor agonist-induced behavioural tolerance and 5-HT_{2C} receptor down-regulation in adult rat brain. The effect of chronic subcutaneous infusion of the 5-HT_{2C} receptor agonist, *m*-chlorophenylpiperazine (*m*-CPP, 10 mg kg^{-1}, day^{-1}), for 14 days was examined on daily food intake, the ability of acute *m*-CPP (2.5 mg kg^{-1}, i.p.) to induce hypolocomotion in a novel arena and elevate plasma corticosterone levels and on *ex vivo* cortical [^{3}H]-mesulergine binding and hippocampal 5-HT_{2C} receptor protein levels.

2 Before chronic infusion, *m*-CPP (2.5 mg kg^{-1}, i.p.) attenuated the number of turns and rears made in a novel open field arena. In contrast, while *m*-CPP still elicited this hypolocomotion following 14 days, saline infusion, no such hypolocomotion occurred in rats given chronic *m*-CPP (10 mg kg^{-1} day^{-1}), indicating that almost complete tachyphylaxis of this behaviour occurred with chronic 5-HT_{2C} receptor agonist injection.

3 During chronic infusion of *m*-CPP, rats consumed less food per day than saline-treated controls. Acute challenge with *m*-CPP following two weeks, treatment still attenuated food intake over the next four hours (by 43% and 30%, respectively from that on the previous day) in saline and *m*-CPP infusion groups, showing that only partial tolerance to 5-HT_{2C} receptor agonist-induced hypophagia occurred.

4 In naive home cage rats, plasma corticosterone was elevated in a dose-dependent manner 35 min after *m*-CPP injection (0.5, 1 and 3 mg kg^{-1}, i.p.) but levels were comparable to control values 16 h after *m*-CPP (2, 5 and 10 mg kg^{-1}, i.p.). Sixteen hours after a single *m*-CPP injection (2.5 mg kg^{-1}, i.p.), plasma corticosterone levels were comparable in a group of rats which had received 14 days infusion of *m*-CPP or saline. However, following a similar acute *m*-CPP injection (2.5 mg kg^{-1}, i.p., −16 h) in rats previously infused for 14 days with *m*-CPP, plasma corticosterone levels were lower than those in a separate group which received no chronic infusions (but only acute *m*-CPP injection), even though the plasma *m*-CPP levels were comparable in both groups. The data are consistent with the proposal that chronic *m*-CPP induced some down-regulation of hypothalamic 5-HT_{2C} receptors which contribute, in a tonic manner, to plasma corticosterone secretion under the conditions investigated.

5 Chronic *m*-CPP infusion reduced the amount of [^{3}H]-mesulergine binding (by 27%, without altering the K_D) in membranes prepared from parietal/occipital/temporal cortex (under conditions to exclude binding to 5-HT_{2A} receptors) and 5-HT_{2C} receptor protein-like immunoreactive levels measured by radioimmunoassay in the hippocampus by 38%, confirming that 5-HT_{2C} receptor down-regulation had occurred.

6 Even after 14 days *m*-CPP infusion only partial behavioural tolerance and 5-HT_{2C} receptor down-regulation were observed, which may vary in different brain regions of the rat. Thus the hypophagia produced by *m*-CPP may involve activation of 5-HT_{2C} receptors in the hypothalamus, where there is a greater receptor reserve or which are more resistant to agonist-induced down-regulation than 5-HT_{2C} receptors in limbic areas (striatum and nucleus accumbens) mediating *m*-CPP-induced hypolocomotion.

Keywords: 5-Hydroxytryptamine$_{2C}$ receptors; hypolocomotion; hypophagia; 5-hydroxytryptamine$_{2C}$ antibodies; corticosterone; anxiety

Introduction

5-Hydroxytryptamine (5-HT) has a widespread distribution in the central and peripheral nervous system of mammals and is implicated in a diverse array of behavioural and physiological functions. The effects of 5-HT are mediated through at least 14 different mammalian receptor subtypes, which have been grouped into seven sub-families termed 5-HT_1 to 5-HT_7, although the 5-HT_4, 5-ht_{5A}, 5-ht_{5B}, 5-ht_6 and 5-HT_7 receptors have only recently been sequenced and their functional roles have not yet been fully characterized (Hoyer *et al*., 1994; Branchek, 1995; Lucas & Hen, 1995; Hoyer & Martin, 1997). Within each family 5-HT receptors share several common features. The three 5-HT_2 receptor subtypes (2A 2B and 2C), for instance, are the only ones coupled via G-proteins to phosphatidylinositol hydrolysis (Baxter *et al*., 1995).

Within the 5-HT_2 family, the 5-HT_{2C} receptor has the widest distribution throughout the brain (Pazos *et al*., 1984; Mengod *et al*., 1990; Pompeiano *et al*., 1994; Sharma *et al*., 1997) but is probably not expressed in the periphery. Although a splice variant producing a truncated form of the 5-HT_{2C} receptor has been identified (Canton *et al*., 1996; Xie *et al*., 1996) it lacks both the abilities to bind 5-HT and activate phosphatidylinositol hydrolysis, and so is of unknown functional importance. 5-HT_{2C} receptor polymorphism also occurs (Sodhi *et al*., 1995; Malhoutra *et al*., 1996) yielding a human variant with an altered affinity for 5-HT, but the abundance of this variant in rat is unknown. The diverse CNS effects of 5-HT_{2C} receptor

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agonists support the contention that this receptor mediates numerous central actions of 5-HT (Kennett & Curzon, 1988; Curzon & Kennett, 1990; Fone *et al*., 1991; 1996; Baxter *et al*., 1995). The most commonly used 5-HT$_{2C}$ receptor agonist, *m*-chlorophenylpiperazine (*m*-CPP, Murphy *et al*., 1991; Kennett, 1993) produces hypolocomotion in a novel arena (Lucki *et al*., 1989), hypophagia (Samanin *et al*., 1979; Kennett *et al*., 1987), anxiogenesis (Kennett, 1992; Fone *et al*., 1996) and ACTH and hence corticosterone release (Bagdy, 1996) in the rat, which are prevented by antagonists with high affinity for the 5-HT$_{2C}$ receptor (Curzon & Kennett, 1990; Kennett *et al*., 1995; 1997). Furthermore, early experiments established that *m*-CPP was more effective when given into the cerebral ventricles than systemically, implicating a central site of action for these behavioural and neuroendocrine effects (Kennett & Curzon, 1988). Following chronic administration of *m*-CPP to rats, both the hypolocomotion and the hypophagia appear to attenuate (Sills *et al*., 1985; Freo *et al*., 1992; Kennedy *et al*., 1993), while the plasma corticosterone elevation may remain unaltered (Ulrichsen *et al*., 1992), suggesting that receptor reserve and/or agonist-induced 5-HT$_{2C}$ receptor down-regulation rate may differ in the separate brain nuclei involved in each of these effects. Several groups have used [^{3}H]-mesulergine binding to examine 5-HT$_{2C}$ receptor changes following agonist treatment (Pranzatelli *et al*., 1993). However, the high non-specific binding associated with this ligand makes determination of the precise anatomical localization of 5-HT$_{2C}$ receptor alteration extremely difficult.

The aim of the present study was to evaluate the effects of chronic administration of *m*-CPP on locomotor and feeding behaviour and plasma corticosterone levels and, concomitantly, to assess 5-HT$_{2C}$ receptor levels in the cortex and hippocampus to establish whether behavioural tolerance is accompanied by receptor down-regulation.

Methods

Animals

Home cage corticosterone measurement Male Lister Hooded rats (280–320 g, $n=48$ housed in groups of 4–6) were maintained on a 12 h light/dark cycle (lights on at 07 h 00 min) and allowed food and water *ab libitum*. Rats were given saline (1 ml kg^{-1}) or *m*-chlorophenylpiperazine (*m*-CPP, 0.5 to 10 mg kg^{-1}, i.p.) before being returned to their home cage for 35 min or 16 h before being killed by decapitation and mixed arterio-venous blood collected to measure plasma corticosterone. No behavioural analysis was performed on these rats.

Chronic infusion study Male Lister Hooded rats (aged 14–15 months, weighing 340–380 g at the start of the experiment, $n=16$) were individually housed in a temperature controlled room on a 12 h light/dark cycle (lights on at 07 h 00 min) and allowed free access to water but only given access to food during a four hour period each day.

As shown in Figure 1, animals on this restricted feeding paradigm were randomly allocated to one of two groups to receive either chronic infusion of *m*-CPP or saline ($n=8$ each). On day 15, following daily measurement of food intake, rats were anaesthetized with halothane (2% in nitrous oxide) to allow subcutaneous implantation of osmotic mini-pumps (Alzet, model 2002 perfusion rate 4.4 μl h^{-1}), containing either saline (0.154 M) or *m*-CPP (10 mg kg^{-1} day^{-1}). All rats were injected with saline (1 ml kg^{-1}, i.p.) on day 7 and *m*-CPP

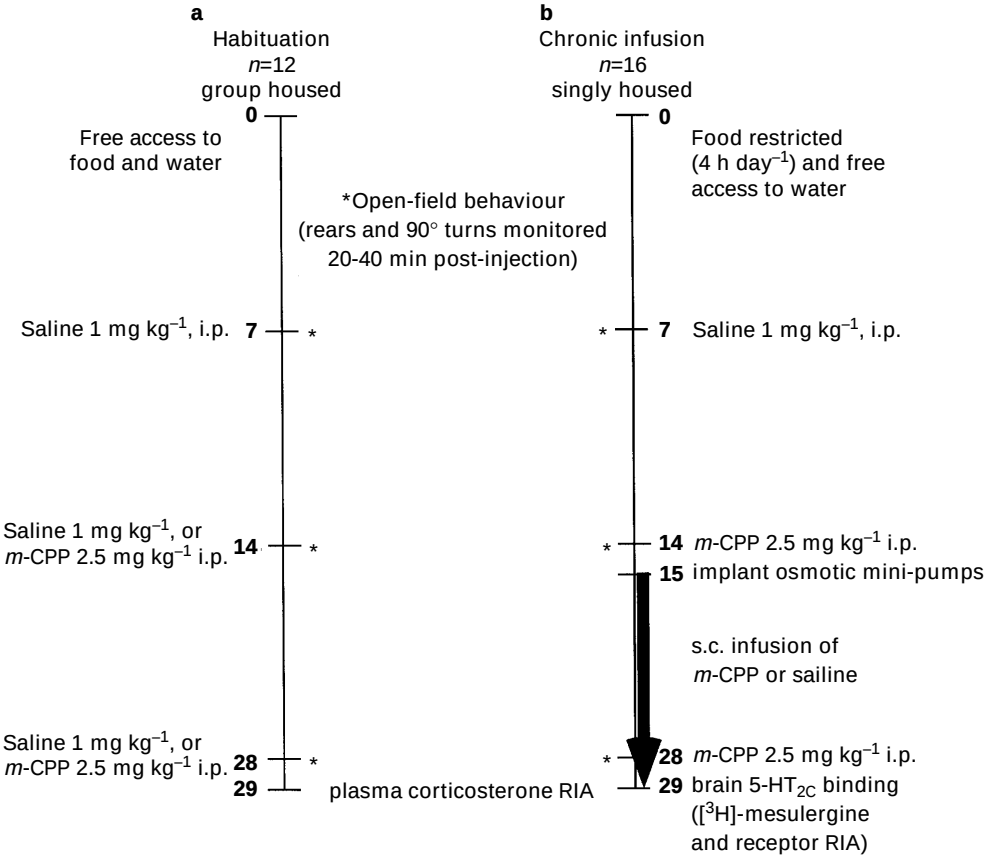

Figure 1 Protocol for both groups of rats used in behavioural studies, indicated against the day (bold numbers) of each procedure.

(2.5 mg kg^{-1}, i.p.) on days 14 and 28 and behaviour monitored as described below.

Habituation study A second (habituation) group of rats (380–400 g, $n=12$), housed in groups of four, were given food and water *ad libitum* but otherwise exposed to the same behavioural testing as the food restricted group (Figure 1). This allowed environmental habituation, which occurs when rats are exposed to the open field on three successive occasions, and *m*-CPP-induced tachyphylaxis to be examined separately. In this habituation study animals were randomly allocated to two groups, both receiving saline (1 ml kg^{-1}, i.p.) on day 7 and either *m*-CPP (2.5 m kg^{-1}, i.p.) or saline ($n=6$ each) on days 14 and 28, i.e. identical to the timing used for behavioural analysis in the food restricted (chronic infusion) study (Figure 1). All behavioural experiments were performed between 09 h 30 min and 16 h 30 min by use of a blind protocol.

Locomotor activity in chronic infusion and habituation studies

On days 7, 14 and 28 in both behavioural studies (Figure 1), rats received *m*-CPP (2.5 mg kg^{-1}, i.p.) or 0.154 M saline (1 ml kg^{-1}, i.p.) and locomotor activity was monitored manually (and recorded by video, Mitsubishi, HS-M34) for 20 min post-injection in a perspex observation box (45 cm^3, 220 lux) without prior familiarization to the arena. During this period the number of rears (simultaneous elevation of both forepaws), turns of the whole body through 90° (an index of locomotion in such a small arena (Fone *et al*., 1989)) and combined head twitches and wet-dog shakes were recorded separately, each minute by hand-held tally counters. In addition, the time spent forepaw-licking and grooming were measured by stop-watch.

Feeding behaviour in chronic infusion study

Animals on the restricted feeding regime (chronic infusion study) were deprived of food overnight from day 0 and weighed each morning before being given food pellets (50 g, Beekay rodent toxicology diet, gross energy 15.9 MJ kg^{-1}) for 4 h (10 h 00 min – 14 h 00 min or for four hours immediately after behavioural testing, 40 min post-drug injection on days 7, 14 and 28) throughout the study and food (with any spillage) re-weighed.

Tissue collection following behavioural studies

At an equivalent time on day 29 in both behavioural studies, rats were killed by decapitation and mixed arterio-venous blood collected in heparin-treated tubes which were centrifuged (1400 x *g*) and plasma decanted and stored at −20°C to allow corticosterone and *m*-CPP levels to be determined. Following the chronic infusion study in each rat the cortex (whole cortex with the frontal cortex removed to reduce any contribution of 5-HT$_{2A}$ receptors) and hippocampi were rapidly dissected out (at 4°C on a cool tray), frozen in liquid nitrogen and stored at −80°C until required for measurement of 5-HT$_{2C}$ receptor levels by radioligand binding and radioimmunoassay, respectively.

Measurement of 5-HT$_{2C}$ receptor and corticosterone levels

5-HT$_{2C}$ ligand binding was determined in aliquots of membrane prepared from the cortex with [^{3}H]-mesulergine, according to the method of Pranzatelli *et al*. (1992) modified from Sanders–Bush & Breeding (1988). The cortex was homogenized in 20 ml Tris-HCl buffer (50 mM, pH 7.6 at 4°C) with a Polytron. A further 20 ml Tris buffer was added before centrifugation (49640 x *g*, 15 min at 4°C, Sigma 3K20). The supernatant was discarded and this procedure repeated 4 times (except that after the third spin the pellet was incubated in Tris at 37°C for 15 min). The pellet from the fourth spin was resuspended in a final volume of 3.5 ml Tris buffer and stored at −80°C until used. Mesulergine binding was assayed in a final volume of 250 μl, consisting of [^{3}H]-mesulergine (25 μl), ketanserin (10 μM, 25 μl, to prevent 5-HT$_{2A}$ binding as confirmed in preliminary studies) or buffer blank (50 mM Tris-HCl, pH 7.6 at 4°C), buffer (175 μl) and cortical homogenate (25 μl). After 15 min incubation at 37°C (shown in preliminary experiments to attain equilibrium) mixtures were sequentially filtered through filters (FP-100 Whatman GF/B presoaked for ~6 h in 0.05% polyethylenimine to minimize non-specific binding) by use of a Brandel Cell Harvester. For each animal an individual estimate of the amount of [^{3}H]-mesulergine binding (with 1 nM mesulergine) was performed in triplicate and eight point saturation curves on pooled tissue used to determine the apparent K_D. Radioactivity was measured by liquid scintillation spectroscopy in 4 ml of LSC-cocktail (Packard) by use of a beta-counter.

5-HT$_{2C}$ receptor protein levels in the hippocampus were measured in triplicate from the linear portion of a radioimmunoassay curve by use of a polyclonal sheep antiserum raised against the N-terminal decapeptide sequence of the rat 5-HT$_{2C}$ receptor protein (MVNLGNAVRS + YC; Julius *et al*., 1988; see Fone *et al*., 1996; Sharma *et al*., 1997 for details). In brief, serial dilutions of the synthetic 5-HT$_{2C}$ receptor peptide (100–8000 pg/tube in Tris buffer) or tissue homogenates (100 μl aliquots) were incubated (4°C, 24 h) in the presence of a polyclonal sheep antiserum (G241, 1 : 2500, 50 μl) and iodinated 5-HT$_{2C}$ peptide (2000 d.p.m. in 10 s, 50 μl) in radioimmunoassay buffer (0.06 M phosphate pH 7.4, containing 0.01 M EDTA and 0.5% w/v BSA). Free radiolabelled 5-HT$_{2C}$ peptide was precipitated after addition of human plasma (100 μl) and charcoal (500 μl, 5 g l^{-1} in 0.012 M phosphate buffer containing 0.154 M NaCl and 2.5×10^{-5} M dextran) by centrifugation (3000 x *g* for 30 min at 4°C, Mistral 6000) and the pellet counted on an LKB gamma counter (Model 1272 Clinigamma, 180 s). Recovery of 5-HT$_{2C}$-like immunoreactivity (LI) from ventral spinal cord homogenates was 84 ± 6% (mean ± s.e.mean, $n=10$). The intra-assay coefficient of variation measured in cortical homogenates ($n=15$) was 7.5% and the inter-assay coefficient of variation as determined from ED$_{50}$ values ($n=8$) was 14.9% (Fone *et al*., 1996).

Plasma corticosterone levels were measured in duplicate from the linear portion of the standard curve, by use of a double antibody radioimmunoassay kit ([^{125}I]-corticosterone, Immunodiagnostic Systems Ltd).

Plasma m-*CPP determination*

Plasma *m*-CPP was measured by use of a modification of the extraction and high performance liquid chromatographic (h.p.l.c) method described by Suckow *et al*. (1990) with 1-(α, α, α-trifluoro-m-tolyl)-piperazine (TFMPP) as internal standard. In brief, *m*-CPP and TFMPP (100 ng) were extracted from plasma (1 ml) by use of 0.6 M sodium carbonate (pH 9.8) and methyl *tert*-butyl ether (1 : 1 : 6) but back-extracted into 0.1 M potassium dihydrogen orthophosphate (pH 7.2, 250 μl). *m*-CPP and TFMPP were separated by isocratic elution with a

0.05 M potassium dihydrogen orthophosphate mobile phase (pH 3.2, containing 462 μM octane sulphonic acid, 8.7 μM triethylamine and 20% (v/v) methanol) at 1 ml min^{-1} through a column filled with 5 μm trimethylsilyl-bonded silica (25 cm × 4.6 mm, Supelcosil LC1, Supelco, U.K.). Ultraviolet detection (243 nm) was performed with a model 168 photodiode array detector (Beckman) and chromatograms analysed by computer (Elonex PC-466 using Beckman System Gold 7.11 software). Recovery of *m*-CPP was 65–70%.

Drugs

meta-Chlorophenylpiperazine (*m*-CPP, Semat) was dissolved in 0.154 M saline. All drugs were randomly allocated to rats and given by use of a blind protocol. [N-6-methyl-^{3}H]-mesulergine (84 Ci mmol^{-1}) was purchased from Amersham, [^{125}I]-sodium iodide (17 Ci mg^{-1}) from NEN Research Products, corticosterone RIA kit from Immunodiagnostic Systems Ltd and ketanserin hydrochloride from Semat, all other drugs were purchased from Sigma.

Statistical analysis

All behavioural and biochemical results are presented as mean ± s.e. mean and ANOVA followed by Duncan's New Multiple Range was used for statistical analysis except where Student's paired and unpaired *t* test are specifically indicated, $P<0.05$ being taken as significant.

Results

Locomotor activity

Habituation study In the group of rats which did not receive subcutaneous drug infusion (Figure 1 and Table 1), there was a significant reduction in the number of rears ($P<0.01$) and turns ($P<0.05$) observed on both second and third exposure to the open field compared with the initial response of the same rats to the novel arena. In addition, acute administration of *m*-CPP significantly reduced both rears and forepaw-licking ($P<0.05$) when given on the second exposure and turns ($P<0.05$), rears ($P<0.01$) and forepaw-licking ($P<0.01$) on the third exposure to the arena compared with those seen in the other group of rats given acute saline injection on the same day. General body grooming was only significantly reduced by *m*-CPP on the third test in the arena (on day 28) compared with that on the first trial following saline. Thus rats partially habituated to the behavioural environment, but the hypolocomotor response to *m*-CPP was still evident even on the third consecutive test in the open field (Table 1).

Chronic infusion study In the separate chronic infusion study, the first acute injection of *m*-CPP (pre-infusion, day 14) significantly attenuated the number of rears and turns (indices of locomotor activity) compared with those observed in the same rats following saline on day 7 (Figure 2). *m*-CPP also significantly reduced the number of rears and turns ($P<0.01$) to a comparable extent pre- (day 14) and post-saline infusion (day 28) in control rats given chronic saline infusion. In contrast, following chronic infusion of *m*-CPP (10 mg kg^{-1} day^{-1} for 13 days) the hypolocomotor response to acute *m*-CPP injection was significantly ($P<0.01$) attenuated compared to that observed on day 14 (second trial) before *m*-CPP infusion (Figure 2). There was no significant change in the few wet-dog shakes/head twitches or grooming observed on any trial following *m*-CPP injection (data not shown).

Table 1 Effect of acute *m*-CPP on behaviour in an open field on three consecutive occasions (Habituation study)

Behaviour	*Day 7*	*Day 14*	*Day 28*
Total turns	33.2 ± 2.8	19.0 ± 4.1†	21.0 ± 5.0†
	33.5 ± 4.0	**12.7 ± 3.8**††	**6.8 ± 2.5**††*
Total rears	27.5 ± 5.0	9.5 ± 2.2††	13.4 ± 3.3†
	32.5 ± 8.9	**2.5 ± 1.9**††*	**0.2 ± 0.2**††**
Forepaw licking	130 ± 30	120 ± 26	111 ± 21
	109 ± 13	**52 ± 13**††*	**47 ± 5**††**
Other grooming	46 ± 15	53 ± 27	57 ± 23
	59 ± 10	**27 ± 17**	**14 ± 9**†

Effect of *m*-CPP (2.5 mg kg^{-1}, i.p. −20 min, lower bold numbers) or saline (1 ml kg^{-1}, upper numbers and both groups on day 7) on (from upper to lower rows) the number of turns through 90° and rears and the time spent (s) forepaw-licking and other grooming excluding forepaw-licking (mean ± s.e.mean, $n=6$ each) over 20 min while in an open field arena (45 cm^3 at 220 lux) on the days indicated without prior exposure to the arena. †$P<0.05$ and ††$P<0.01$ from the same group given saline on day 7 and *$P<0.05$ and **$P<0.01$ from the saline-treated rats on that day, Duncan's New Multiple Range following two-way ANOVA in each case. Note the ability of *m*-CPP to induce hypolocomotion despite habituation to the arena during the three trials.

Feeding behaviour in chronically infused rats

Introduction of a restricted feeding regime to rats initially resulted in a reduction of daily food intake (from 27 ± 4 to 9 ± 1 g on day 0 when rats had free access to food). However, the food intake under restricted access increased steadily, reaching a plateau level, on day 13 (Figure 3, from day 1 to 13, ANOVA $F_{(1,12)}=22.534$; $P<0.001$), which was not significantly less than the original unrestricted food intake and, furthermore, was comparable in both treatment groups. Chronic subcutaneous infusion of *m*-CPP (10 mg kg^{-1} day^{-1}) reduced daily food intake by the third day and reached a minimum intake on day 18 (fourth day of infusion), which was significantly lower ($P<0.05$) than that in saline infused controls on the same day. However, with continued *m*-CPP infusion, although daily food intake remained significantly less ($P<0.05$, except on days 19, 22, 24 and 26 of the study) than that of saline controls, it progressively returned towards that of the saline group (Figure 3). Such that despite continued *m*-CPP infusion the food intake of this group of rats was significantly greater ($P<0.05$) than that on day 18, from day 20 onwards (Figure 3).

Acute administration of *m*-CPP (2.5 mg kg^{-1}, i.p.) on day 14, before drug infusion (Figures 3 and 4), caused a marked, significant ($P<0.01$) reduction in food intake; being 56% and 65% below that on the previous control day in the chronic saline and *m*-CPP infusion groups respectively. Similarly, even after 13 days of chronic *m*-CPP infusion, acute *m*-CPP injection still significantly reduced food intake over the next 4 h in both groups, but the reduction was greater in rats infused with saline than in those which had received *m*-CPP by osmotic mini-pumps (being 43%, $P<0.01$ and 30%, $P<0.05$, respectively, below that on the previous day).

Although the restricted food regime and drug treatment caused rats to lose weight (two-way ANOVA $F=3.477$; $P<0.001$, by day) during the course of the experiment, there was no significant difference in the body weight of rats given

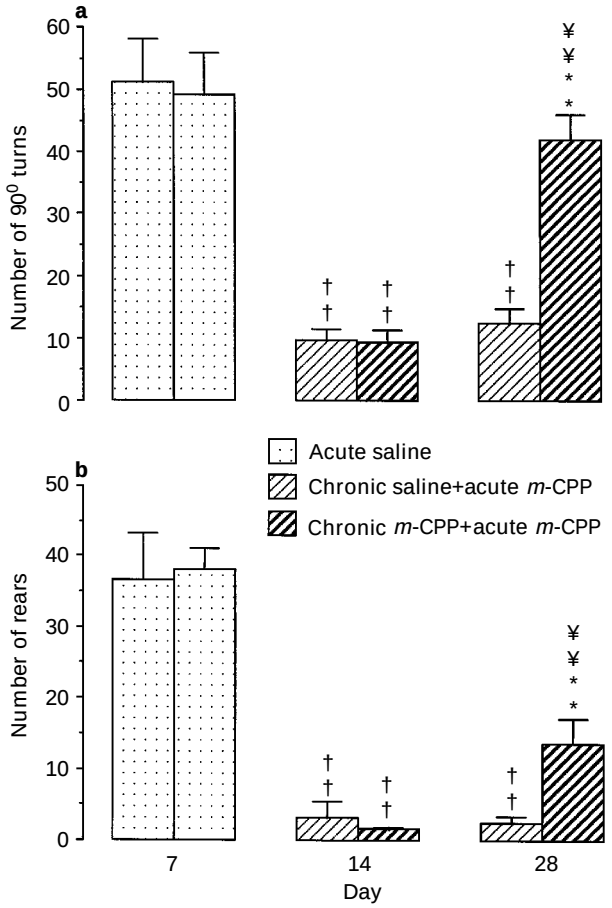

Figure 2 Comparison of the effect of chronic subcutaneous infusion of either *m*-CPP (CM; 10 mg kg^{-1} day^{-1}, $n=8$) or saline (CS; 4.41 μl h^{-1} day^{-1} $n=8$) on indices of locomotor activity ((a) turns of the whole body through 90° and (b) rears; mean$\pm$s.e. mean) induced between 20 and 40 min after saline (0.154 M, 1 ml kg^{-1}, i.p., on day 7, and *m*-CPP (2.5 mg kg^{-1}, i.p.) before (day 14) and again 13 days after (day 28) chronic infusion. ††$P<0.01$ Duncan's New Multiple Range from the response to saline in the same chronic infusion group on day 7. **$P<0.01$ from CM day 14. ¥$P<0.05$ and ¥¥P<0.01 from CS day 28. Following ANOVA; rears $F_{(2,21)}=23.046$, $P=0.0001$, turns $F_{(2m21)}=28.528$, $P=0.0001$.

saline or *m*-CPP infusion on any day ($F=0.443$, $P=0.9829$, by treatment, data not shown).

Corticosterone levels

Acute injections in naive rats Thirty five minutes after the administration of *m*-CPP to naive rats which were subsequently re-housed in their home cage, there was a significant dose-related increase in plasma corticosterone levels (Figure 5a), which were 3.5 fold higher than those in saline controls following the 3 mg kg^{-1}, i.p. dose. In contrast, 16 h after *m*-CPP (up to 10 mg kg^{-1}, i.p.) plasma corticosterone levels were comparable to those in controls given a similar pretreatment with saline (Figure 5b).

Following habituation and chronic infusion behavioural studies Plasma corticosterone levels were measured sixteen hours after the last behavioural test in both food restricted (chronic infusion) and unrestricted (uninfused, habituation study) groups that had received identical acute injections and open field behaviour tests (Figures 1 and 5c). This time of measurement was chosen to prevent any effect of *m*-CPP on the mesulergine binding determination. In the group of rats

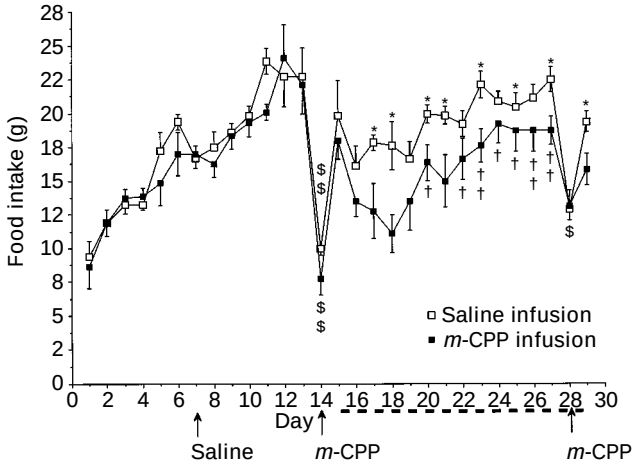

Figure 3 Effect of chronic infusion of *m*-CPP (10 mg kg^{-1} day^{-1}, $n=8$) or saline (4.4 μl h^{-1} day^{-1}, $n=8$, horizontal dotted line) on daily food intake measured over 4 h (between 10 h 00 min–14 h 00 min, or for 4 h starting 40 min after *m*-CPP injection on days 14 and 28) and the hypophagia produced by an acute injection of *m*-CPP (2.5 mg kg^{-1}, i.p., days 14 and 28 as indicated). Points represent means and vertical lines show s.e.mean. *$P<0.05$, Duncan New Multiple Range from the saline infusion group on the same day following ANOVA $F_{(14,209)}=51.379$, $P<0.001$ on data during drug infusion. ††$P<0.01$ and †$P<0.05$ from day 18 food intake in the *m*-CPP-infused group following ANOVA $F_{(14,104)}=2.797$, $P=0.014$. \$\$$P<0.01$ and \$$P<0.05$ from the preceding day.

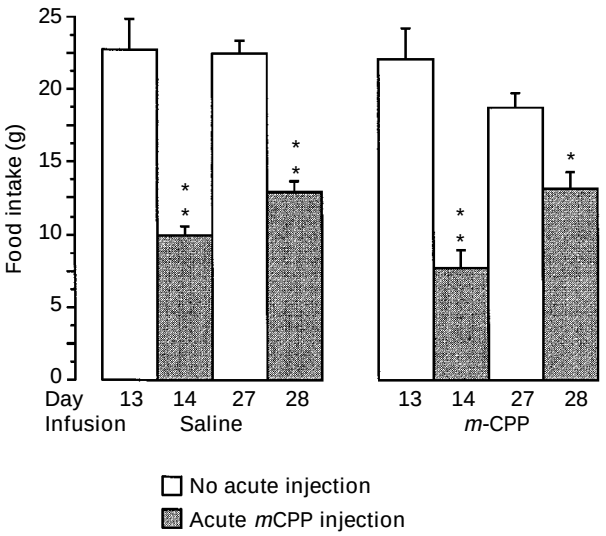

Figure 4 Comparison of the effect of acute injection of *m*-CPP (2.5 mg kg^{-1}, i.p.) before (day 14) and after (day 28) chronic infusion of either saline (4.4 μl h^{-1} day^{-1}, $n=8$) or *m*-CPP (10 mg kg^{-1} day^{-1}, $n=8$) as indicated, on food intake (over 4 h from 40 min after acute *m*-CPP injection) with that on the previous non-injected day. Means$\pm$s.e.mean are shown. Repeated measures ANOVA revealed a significant main effect of acute *m*-CPP ($F=40.670$, $P=0.0001$) but no effect of chronic treatment ($F=3.532$, $P=0.0812$) nor of day$\times$treatment interaction. *$P<0.05$ and **$P<0.01$ from the previous non-injected day, Duncan's New Multiple Range.

which received no chronic drug infusion (habituation study), acute *m*-CPP (2.5 mg kg^{-1}, i.p. -16 h) administration failed to elevate significantly plasma corticosterone levels from that in saline controls, as expected at this time after injection (see Figure 5b). There was also no difference in plasma corticosterone levels in the group of rats that received acute *m*-CPP following either chronic infusion of saline or *m*-CPP.

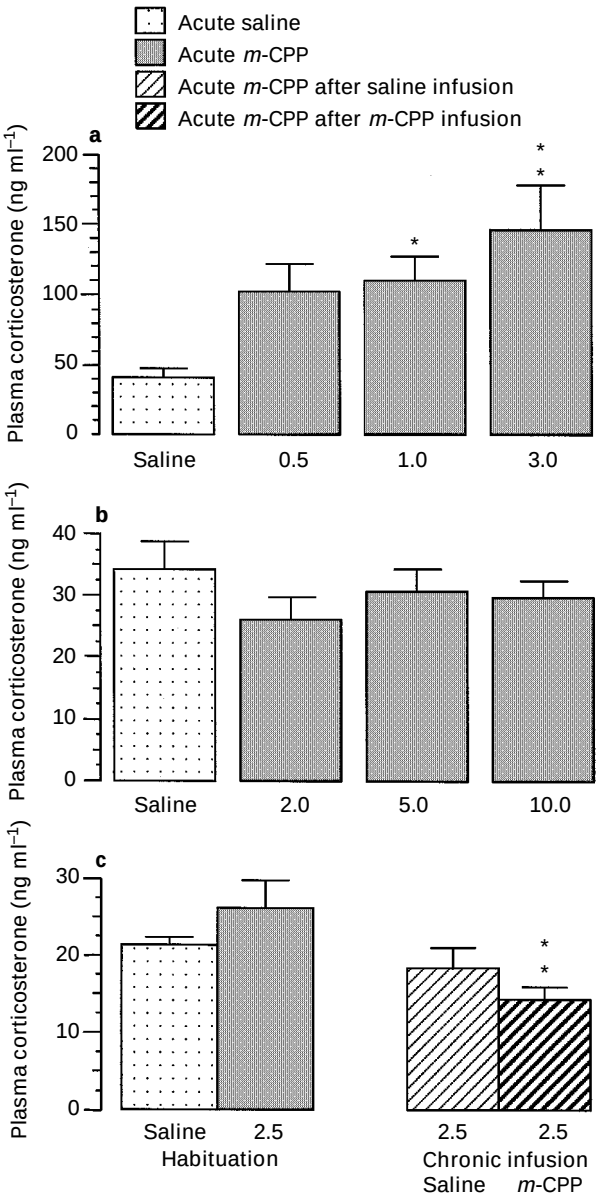

Figure 5 Comparison of the plasma corticosterone levels (ng ml^{-1}) measured (a) 35 min ($n=7-8$ each) or (b) 16 h ($n=4$ each) after injection of either *m*-CPP (0.5 to 10 mg kg^{-1}, i.p., as indicated) or saline (0.154 M, 1 ml kg^{-1}, i.p.) in naive home cage animals or (c) 16 h after the last acute injection of *m*-CPP (2.5 mg kg^{-1}, i.p.) or saline (0.154 M, 1 ml kg^{-1}, i.p.) on day 29 following chronic behavioural studies (see Figure 1 and methods for details). Note that there was a dose-related increase in plasma corticosterone 35 min after *m*-CPP. Means$\pm$s.e.mean are shown. $^{*}P<0.05$ and $^{**}P<0.01$ from saline; Duncan's New Multiple Range following ANOVA $F_{(3,28)}=3.899$, $P=0.0191$ in (a) while there was no significant change 16 h after *m*-CPP even at higher doses in (b). In (c) the two groups of rats either received 14 days infusion (Chronic) of *m*-CPP (10 mg kg^{-1} day^{-1}, CM, $n=8$) or saline (4.41 μl h^{-1} day^{-1}, $n=8$) or (Habituation) no chronic infusion. Both groups received an acute injection of saline (0.154 M, 1 ml kg^{-1}, i.p.) on day 7 and *m*-CPP (2.5 mg kg^{-1}, i.p.) on days 14 and 28 (except Habituation saline, which received saline on all three days). $^{**}P<0.01$ from the Habituation group given *m*-CPP, Duncan's New Multiple Range following ANOVA, $F_{(3,21)}=3.644$, $P=0.0293$.

However, when chronic infused and uninfused groups were compared, plasma corticosterone was significantly higher ($P<0.01$) following acute *m*-CPP in the uninfused (habituation study) group (Figure 5c), despite the fact that plasma *m*-CPP levels (45.0$\pm$12.2 and 30.2$\pm$8.6 ng ml^{-1}, respectively) were similar in these two groups.

5-HT$_{2C}$ receptor levels after chronic m-*CPP infusion*

Chronic *m*-CPP infusion significantly reduced the B_{max} of [^{3}H]-mesulergine binding in cortical homogenates by 27% (from 14.81$\pm$0.96 in saline controls to 10.80$\pm$1.24 fmol mg^{-1} protein, $P<0.02$, Student's *t* test) without altering the K_D, confirming that 5-HT$_{2C}$ receptor down-regulation had occurred. Furthermore, by use of a novel radioimmunoassay to measure 5-HT$_{2C}$ receptor protein-like immunoreactivity, a similar 38% reduction (from 1.91$\pm$0.46 fmol mg^{-1} wet weight in saline controls) was found in the hippocampus, but levels in the hypothalamus were too close to the detection limit to be determined in this study.

Discussion

The arylpiperazine compound *m*-CPP is currently the agonist of choice to examine 5-HT$_{2C}$ receptor function (Murphy *et al*., 1991; Kennett, 1993; Baxter *et al*., 1995). However, from ligand binding studies the affinity of *m*-CPP for the rat 5-HT$_{2C}$ receptor (pK_i 7.7) is only five to ten fold greater than that at 5-HT$_{2B}$ (pK_i 7.3) and 5-HT$_{2A}$ (pK_i 6.7) and ten to fifteen fold more than that at 5-HT$_{1B}$ (pK_i 6.5), 5-HT$_{1D}$ (pK_i 6.6) and 5-HT$_{1A}$ (pK_i 6.6) receptors and studies have also suggested *m*-CPP to be an antagonist at 5-HT$_3$ receptors (Hoyer, 1988; Kennett, 1993; Baxter *et al*., 1995). Therefore, without the simultaneous use of a selective 5-HT$_{2C}$ antagonist it is impossible to ascribe definitively any *m*-CPP-induced changes to the 5-HT$_{2C}$ receptor. Despite this relatively poor pharmacological profile, considerable evidence shows that *m*-CPP mediates hypolocomotion in a novel arena, anxiogenesis, elevation of plasma corticosterone and hypophagia by activation of central 5-HT$_{2C}$ receptors (Sills *et al*., 1985; Kennett & Curzon, 1988; Freo *et al*., 1992; Kennett *et al*., 1995; 1997; Fone *et al*., 1996).

In the present study, *m*-CPP was therefore used to examine the effects of chronic infusion of a 5-HT$_{2C}$ receptor agonist on locomotor and feeding behaviour and corticosterone release, while simultaneously measuring *ex vivo* 5-HT$_{2C}$ receptor protein levels in the hippocampus and 5-HT$_{2C}$ ligand binding in the cortex, to determine whether behavioural tachyphylaxis was accompanied by receptor down-regulation.

Chronic exposure of a receptor to an agonist is usually accompanied by decreased response (desensitization) with uncoupling of the receptor from its effector system, followed by loss of receptor protein (down-regulation) by internalization (Lohse, 1993). Conversely, chronic exposure of a receptor to an antagonist can cause supersensitivity and up-regulation. Interestingly, the 5-HT$_{2C}$ receptor undergoes down-regulation following chronic agonist (Sanders-Bush & Breeding, 1990) and, also paradoxically, after chronic antagonist treatment (Blackshear *et al*., 1986; Sanders-Bush & Breeding, 1988; MazzolaPomietto *et al*., 1997), which has been suggested to be due to a lack of endogenous neurotransmitter tone on this receptor subtype. Although up-regulation of the 5-HT$_{2C}$ receptor has been documented in the choroid plexus following neurotoxin-induced destruction of 5-hydroxytryptaminergic neurones (Rocha *et al*., 1993; Sharma *et al*., 1997), less consistent changes occur in other areas, suggesting that brain region specific receptor regulation may occur, as investigated herein.

Habituation to repeated testing in an open field

In preliminary experiments, a group of rats not receiving chronic infusion was exposed repeatedly to an open field, to

determine whether the resultant habituation to the arena would prevent *m*CPP-induced hypolocomotion which requires a high level of exploratory activity, as seen in a novel arena. As expected, the number of rears and turns associated with repeated exposure to the open field reduced with consecutive trials but *m*-CPP further decreased the number of rears (days 14 and 28) and turns (day 28). These data show that *m*-CPP not only attenuates exploration when rats are placed in a novel arena (Kennett & Curzon, 1988) but can maintain this effect on up to three exposures to that arena. In this pilot study, *m*-CPP also attenuated forepaw-licking (days 14 and 28) and whole body grooming (day 28) compared with that observed following saline on the same day, but as the pharmacology of these behaviours has not been thoroughly characterized they were not examined further in this study.

Locomotor activity after chronic m-*CPP*

Following chronic infusion of *m*-CPP by osmotic mini-pumps for fourteen days, acute injection of *m*-CPP failed to attenuate the number of turns compared with those seen in rats receiving chronic saline. In addition, following chronic *m*-CPP infusion on day 28, although the number of rears observed after acute *m*-CPP was reduced compared with the response to saline on day 7, the reduction was not as marked as that obtained in rats infused with saline. Tachyphylaxis of the hypolocomotor activity produced by *m*-CPP has been shown previously following chronic administration of *m*-CPP once daily for 14 days (Sills *et al*., 1985; Freo *et al*., 1992; Ulrichsen *et al*., 1992; Kennedy *et al*., 1993), but complete behavioural tolerance, as observed with turning in this study, has not been achieved before.

It is thought that the ability of *m*-CPP to suppress spontaneous locomotor behaviour in rats involves the 5-HT$_{2C}$ receptor, as antagonists with appreciable affinity for this 5-HT receptor subtype block the hypolocomotor effect (Kennett & Curzon, 1988). In support of this suggestion the recently described selective 5-HT$_{2C}$ receptor antagonist SB 242084 has also been shown to suppress potently *m*-CPP-induced hypolocomotion (Kennett *et al*., 1997), although surprisingly the structurally unrelated selective 5-HT$_{2C}$ receptor antagonist RS-102221 failed to attenuate this behaviour (Bonhaus *et al*., 1997). Furthermore, lesions of 5-HT neurones with 5,7-dihydroxytryptamine potentiate the hypolocomotor effect of *m*-CPP, possibly by development of postsynaptic supersensitivity. In contrast, increasing brain 5-HT levels by administration of monoamine oxidase inhibitors, diminishes *m*-CPP-induced hypolocomotion (Lucki *et al*., 1989). Collectively, this evidence suggests that 5-HT$_{2C}$ receptors, postsynaptic in relation to 5-hydroxytryptaminergic neurones, are involved in the hypolocomotor response to *m*-CPP.

Correlation of the extent of 5-HT$_{2C}$ receptor down-regulation with appropriate brain regions associated with locomotion would provide valuable information pertaining to the nature of the behavioural response. However, the low selectivity of conventional radioligands such as [^{3}H]-mesulergine and the high non-specific binding of this ligand prevents analysis in such small brain areas in individual rats. Nonetheless, in agreement with previous studies, the B_{max} of [^{3}H]-mesulergine in combined temporal, parietal and occipital cortex was reduced by 27% following chronic infusion of *m*-CPP in the present study. By use of a recently developed radioimmunoassay (Fone *et al*., 1996; Sharma *et al*., 1997) a similar 38% reduction in the 5-HT$_{2C}$ receptor protein-like immunoreactivity was also observed in hippocampal membranes, further confirming that 5-HT$_{2C}$ down-regulation had occurred herein.

Feeding behaviour following chronic m-*CPP*

Systemic *m*-CPP administration produces a well documented dose-dependent decrease in food intake in rats (Samanin *et al*., 1979; Kennett *et al*., 1987). In the present study, rats showed the expected marked hypophagia following acute *m*-CPP. Chronic infusion of *m*-CPP also produced a persistent reduction in daily food intake compared with that in saline infused control. However, throughout the two weeks of *m*-CPP infusion food intake gradually returned towards pre-infusion levels, although it remained less than that of saline controls throughout. In addition, in chronic *m*-CPP treated rats, food intake was significantly greater on days 22 to 27 than at the peak effect on day 18 (three days after starting *m*-CPP infusion) demonstrating that some tachyphylaxsis to hypophagia had occurred. Nonetheless, even after 14 day, infusion of *m*-CPP an acute injection of *m*-CPP (2.5 mg kg^{-1}, i.p.) significantly reduced food consumption. However, the hypophagia appeared more marked in rats infused with saline than with *m*-CPP when the response was normalized against the food intake of the group on the previous day. Taken together, the data show that attenuation of the hypophagic action of *m*-CPP was not as marked as the tachyphylaxis to the hypolocomotor effect. This may reflect different receptor reserve or differential down-regulation of 5-HT$_{2C}$ receptors in the different brain areas involved in locomotor and feeding responses. Alternatively, selective involvement of other 5-HT receptors in hypophagia and hypolocomotion, such as 5-HT$_{1B}$ receptors in the former (Curzon & Kennett, 1990) may account for the apparently different extents of tachyphylaxis. Indeed, several drugs have been shown to block *m*-CPP induced hypophagia without affecting locomotor activity (Wilkinson & Dourish, 1991). The hypothalamus has a relatively high density of 5-HT$_{2C}$ receptors (Pazos & Palacios, 1985) and infusion of 5-HT into the paraventricular hypothalamic nucleus reduces food intake in rats (Leibowitz *et al*., 1988). Furthermore, long-term clorgyline treatment antagonizes the hypophagic effect of *m*-CPP (Cohen *et al*., 1983), whilst significantly reducing 5-HT$_{2C}$ binding in the hypothalamus (Hulihan-Giblin *et al*., 1994) consistent with the role of this area in *m*-CPP-induced hypophagia (Dryden *et al*., 1996).

Corticosterone levels following chronic m-*CPP*

In the present study, *m*-CPP produced the expected dose-related increase in plasma corticosterone 35 min after injection (Calogera *et al*., 1990) with no residual effect at 16 h post-injection in naive rats. Likewise, plasma corticosterone was comparable 16 h after acute *m*-CPP injection in rats that had received chronic infusion of either *m*-CPP or saline. Corticosterone levels would be expected to be close to basal so long after acute *m*-CPP but examination at this time was required to ensure that residual *m*-CPP levels did not interfere with 5-HT$_{2C}$ ligand binding measurement. Nevertheless, plasma corticosterone levels were significantly lower after acute *m*-CPP in the chronic infusion than the uninfused rats, even though plasma *m*-CPP levels were comparable and the behavioural and handling protocol experienced by both groups was analogous. *m*-CPP stimulates corticosterone secretion by activation of 5-HT$_{2C}$ receptors in the paraventricular nucleus of the hypothalamus by increasing the release of corticotrophin releasing hormone (CRH) (Calogera *et al*., 1990; Bagdy, 1996). Although activation of 5-HT$_{1A}$ receptors (for which *m*-CPP has some affinity) also elevates coricosterone levels, the effect of *m*-CPP on corticosterone release up to a dose of 1 mg kg^{-1} (i.v.) is mediated by 5-HT$_{2C}$ receptor

activation (Bagdy, 1996). Therefore, these data are consistent with the proposal that mild down-regulation of hypothalamic 5-HT$_{2C}$ receptors, which are tonically involved in mediating CRH release under the conditions used, had occurred following the present chronic *m*-CPP infusion. Unfortunately even with the development of a radioimmunoassay for 5-HT$_{2C}$ receptor protein, levels in the hypothalamus were close to the assay detection limit and could not be measured in the present study to confirm this proposal. One previous study, in which a very similar protocol to the present study was used, showed *m*-CPP-induced plasma corticosterone elevation (30 min post-injection) was unaltered following twice daily injection of *m*-CPP (5 mg kg^{-1}, i.p.) for 14 days (Ulrichsen *et al*., 1992), in agreement with the current suggestion.

5-HT$_{2C}$ receptor down-regulation

Although differential regulation of the 5-HT$_{2C}$ receptor has recently been demonstrated in rat brain areas (Hulihan-Giblin *et al*., 1994), both the hypothalamus and striatum appear to be affected similarly by antidepressants. Thus chronic treatment with the monoamine oxidase type A inhibitor, clorgyline, significantly reduced [^{3}H]-mesulergine binding in both the hypothalamus and striatum (without altering B_{max} in the frontal cortex or hippocampus) (Hulihan-Giblin *et al*., 1994). In separate studies, clorgyline also attenuated both *m*-CPP-induced hypolocomotion and hypophagia (Cohen *et al*., 1983), although the very large dose of *m*-CPP used (10 mg kg^{-1}, i.p.) is not selective for 5-HT$_{2C}$ receptors. However, partial down-regulation of receptors may not elicit any observable functional change if a large receptor reserve exists. It is therefore possible that a smaller 5-HT$_{2C}$ receptor reserve is present in those brain nuclei mediating *m*-CPP-induced hypolocomotion compared to that in the hypothalamus associated with *m*-CPP induced hypophagia and corticosterone secretion. Brain region specific RNA editing of the 5-HT$_{2C}$ receptor transcript in the rat, resulting in isoforms with a 10 to 15 fold alteration in the efficacy of coupling to G proteins, have recently been identified (Burns *et al*., 1997). However, as these post-translational modifications are to the second intracellular loop of the receptor protein, they should not affect agonist-induced down-regulation rates and are thus unlikely to account for the present observations.

From behavioural studies, the 5-HT$_{2A}$ receptor appears to down-regulate more rapidly following agonist treatment than the 5-HT$_{2C}$ receptor. Thus, DOM-induced head twitches and back muscle contractions (5-HT$_{2A}$-mediated behaviours (Fone *et al*., 1991)) are reduced by approximately 30% on the second and 70% on the fourth agonist challenge (Dennis *et al*., 1989; Leysen *et al*., 1989; Fone & Sharma, 1992; Pranzatelli *et al*., 1993). In contrast, *m*-CPP-induced penile erections were unaltered following ten days repeated agonist-injection (Berendsen & Broekkamp, 1991), and *m*-CPP-induced hypolocomotion (Sills *et al*., 1985; Freo *et al*., 1992; Ulrichsen *et al*., 1992) and hypophagia (Kennedy *et al*., 1993) are only reduced by approximately 50% following twice daily *m*-CPP injections for two weeks. Even this slower rate of down-regulation would limit the long-term therapeutic use of 5-HT$_{2C}$ receptor selective agonists should they be developed.

In conclusion, the present study confirms that acute administration of *m*-CPP, a 5-HT agonist with limited selectivity for the 5-HT$_{2C}$ receptor, produces hypolocomotion, hypophagia and elevates plasma corticosterone. However, following 14 days chronic *m*-CPP infusion a marked tolerance to the hypolocomotion occurred whilst the hypophagia was still apparent. Attenuation of these two behaviours to different extents following *m*-CPP infusion is consistent with the proposal that 5-HT$_{2C}$ receptor reserve or down-regulation may occur at variable rates in different brain areas.

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