

Food conversion is transiently affected during 4-week chronic administration of melanocortin agonist and antagonist in rats

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Abstract

The central melanocortin system is involved in the regulation of food intake and body weight. In this study, we investigated the effect of a 4-week intracerebroventricular infusion of the melanocortin receptor agonist MT-II and the selective melanocortin-4 receptor antagonist HS024 on food intake and body weight homeostasis. The MT-II-treated rats ate less and lost considerably more weight than the control rats during the first week of treatment. During the second and third week, they gained weight and, by the end of the treatment period, the weight gain was similar to that of the control rats. The HS024 treatment caused hyperphagia and development of obesity during the entire period. Extensive accumulations of fat and a sixfold increase in leptin levels were observed in the HS024-

treated rats, as compared with controls, after the 4-week period. Food conversion ratio, defined as body weight increase relative to weight of ingested food, was clearly increased in the HS024-treated rats, while it was lowered in the MT-II-treated rats compared with controls. The effect on food conversion ratio was transient, being greatest for both experimental groups during the first week and it was then attenuated to reach the level of controls at the end of the study. The results suggest that long-term injection of exogenous melanocortin receptor active substances may have an important transient effect on food conversion.

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Introduction

Maintaining a constant body weight requires the individual to control energy intake according to energy expenditure. Over the past four decades the picture of a physiological system for homeostatic regulation of body weight has been emerging. One of the key factors in this model is the adipose-derived hormone leptin, which seems to function as a circulating indicator of energy stores as well as a mediator of energy balance which acts centrally to inhibit food intake and increase energy expenditure (for review see Ahima & Flier 2000). Centrally, leptin alters the function of a hypothalamic neuronal network mainly originating in the arcuate nucleus. This network includes hypothalamic and brain-stem neurones expressing pro-opiomelanocortin (POMC), hypothalamic neurones co-expressing the endogenous melanocortin antagonist agouti-related peptide (AGRP) and neuropeptide Y (NPY) and the downstream targets of these pathways expressing melanocortin-3 (MC3) and melanocortin-4 (MC4) receptors (Cone 1999, Kalra *et al.* 1999, Schiöth 2001).

It was discovered in the 1980s that POMC peptides, including melanocyte-stimulating hormone (MSH) and adrenocorticotrophic hormone, had anorectic effects (Vergoni *et al.* 2000). However, the melanocortins did not receive much attention in this context until it was discovered that disruption of the MC4 receptor, one of five MC receptor subtypes, caused mice to overeat and turn obese (Huszar *et al.* 1997). In addition, it has recently been shown that inactivation of the mouse MC3 receptor results in increased fat mass and reduced lean body mass without affecting the eating behaviour of the mice (Chen *et al.* 2000). Homozygous MC3 receptor knock-out mice show an approximately 50–60% increase in adipose mass without being significantly overweight (Butler *et al.* 2000). Earlier studies made it clear that the agouti peptide and the structurally related AGRP are endogenous antagonists for the MC receptors (Lu *et al.* 1994, Ollman *et al.* 1997, Wilson *et al.* 1999). Over-expression of the agouti peptide and AGRP causes obesity (Klebig *et al.* 1995, Ollman *et al.* 1997) with similar characteristics to those observed in the MC4 receptor knock-out mice (Huszar *et al.* 1997). Furthermore, it appears that most AGRP neurones also

contain NPY (Cone 1999). While NPY is one of the most potent natural orexigenic substances known, the complexity of this system is enormous, as was clearly displayed when NPY knock-out mice failed to show any abnormalities in growth, body weight or food intake (Palmiter *et al.* 1998).

It is well established that single acute intracerebroventricular (i.c.v.) injections of the non-selective MC3/MC4 receptor agonist MT-II, or the non-selective antagonist SHU9119, or the selective MC4 receptor antagonists HS014, HS024 and HS028, influence food intake in experimental animals (Kask *et al.* 1998a,b, Schiöth *et al.* 1998a,b, Skuladottir *et al.* 1999). It is, however, also clear that the melanocortins act in a complex interplay with other key regulators of food intake, such as leptin and NPY. Leptin, NPY and other hormones may function in counteracting mechanisms during the administration of exogenous melanocortins. This would be likely during chronic administration where the time span is long enough for slow control systems to become activated. The effects of long-term administration of leptin and NPY have been studied intensively (Kalra *et al.* 1999), while further investigations on the long-term exogenous administration of MC4 receptor antagonist and an MC receptor agonist are needed.

In this study, the effects of a 4-week chronic i.c.v. infusion of the selective MC4 receptor antagonist HS024 and the non-selective MC receptor agonist MT-II on food intake and body weight homeostasis were investigated.

Materials and Methods

Animals and treatment

Thirty male Wistar rats weighing 280–400 g were fed a standard, commercial laboratory diet (Rat and Mouse Maintenance Diet No. 1; Special Diets Services, Witham, Essex, UK) and water *ad libitum* under standard conditions using a reversed 12-h light:12-h darkness cycle (lights off at 1000 h, on at 2200 h). The room temperature was maintained at 22 ± 1 °C and humidity at $50 \pm 5\%$. One week before the administration of drugs, the rats were housed individually in plastic cages. Food intake and body weight were recorded during the last 2 h of the light phase, initially 1 week prior to the implantation of the pumps and then at 1-week intervals during the experimental period. The animals were housed separately from other animals and inspected during the last hour of the light phase every morning, but otherwise left undisturbed. Experimental procedures were carried out in accordance with the guidelines of the European Union, and local legislation and policies.

Peptides and affinities

MT-II (Al-Obeidi *et al.* 1989) and HS024 (Kask *et al.* 1998a) were purchased from Neosystem, Strasbourg,

France. The peptides were dissolved in buffered artificial cerebrospinal fluid (ACSF): NaCl, 138 mM; KCl, 3.37 mM; CaCl₂, 1.50 mM; MgCl₂, 1.0 mM; NaH₂PO₄, 1.45 mM; Na₂HPO₄, 4.85 mM, adjusted to pH 7.4 and stored in aliquots at -20 °C. Stability investigation of these peptides dissolved in the ACSF solution was performed by weekly HPLC and mass-spectrometry methods. This was the same ACSF solution as that used for the *in vivo* injections into the rats.

The affinities of HS024 towards the rat MC3 and MC4 receptors were obtained by a radioligand binding assay in transfected COS (CV-1 Origin SV40) cells as previously described (Schiöth *et al.* 1995). The binding assays were repeated three times. The rat MC3 (Roselli-Rehfuß *et al.* 1993) and rat MC4 (Alvaro *et al.* 1996) receptor clones were generously provided by Dr R. D. Cone, Vollum Institute, Portland, OR, USA and Dr R. Duman, Yale University, MA, USA respectively.

Implantation of pumps

After the 1-week control period, osmotic pumps were implanted subcutaneously in the midscapular region for i.c.v. infusion. The rats were anaesthetised with methohexital sodium (50 mg/kg i.p.; Brielal; Lilly, Indianapolis, IN, USA) and assigned randomly into three groups. The control rats received an i.c.v. infusion of 0.25 µl/h ACSF ($n=11$), while the experimental rats received either an i.c.v. infusion of 0.1 nmol/h HS024 ($n=10$) or 0.1 nmol/h MT-II ($n=9$). The peptides were dissolved in ACSF and infused at 0.25 µl/h. The selection of drug infusion rate (0.1 nmol/h) was based on previous experience (Haynes *et al.* 1999, Kask *et al.* 1999, Skuladottir *et al.* 1999). The infusions were made with an osmotic minipump (Alzet, model 2004; Alza Corp., Palo Alto, CA, USA) which had first been activated by immersion in 0.9% saline at room temperature for 12 h before implantation. Thus the infusion started immediately upon implantation of the pumps which were connected to a brain infusion kit (Alzet) with the cannula opening into the right lateral cerebral ventricle (1.0 mm posterior, 1.5 mm lateral to the bregma). A dental glass ionophore (ChemFil Superior; Dentsupply DeTray GmbH, Konstanz, Germany) and a stainless steel anchor screw were used to secure the infusion cannula in position.

Data collection

After the 4-week long treatment period the rats were anaesthetised as described above and weight, length and impedance were recorded. The total body fat of the experimental animals was estimated by means of a modified tetrapolar bioelectrical impedance analysis (Hall *et al.* 1989) using an impedance cardiograph (Minnesota Impedance Cardiograph, model 304A; Surcom Inc., Minneapolis, MN, USA). Self-adhesive foil ECG

electrodes (Blue Sensor BS3500; Medicotest A/S, Istrykke, Denmark) were placed on the paws of the animals, with the constant current-inducing source (100 kHz) connected to the paws on one side and the detectors connected to the paws on the other side of the animal. Two replicate recordings were made before switching source and moving detectors from one side to the other with two repeated replicate recordings. The average of the four recordings was used for calculation of resistivity for each animal. The resistivity was calculated by multiplying the measured impedance (Ohm) by the body weight (g) scaled to the power of 2/3 ($[\text{surface area (cm}^2\text{)}]/[\text{volume (cm}^3\text{)}]$) divided by the body length (cm) of the animal ($\text{resistivity} = \text{Ohm g}^{0.67}/\text{cm}$). The femoral vein was catheterised, the rats heparinised (330 IU/kg; Heparin Leo; Lövens kemiske fabrik, Ballerup, Denmark) and blood samples were collected for analysis of plasma leptin and glucose. Blood samples were immediately cooled on ice until centrifuged and plasma was then kept at -20°C until analysed. The anaesthetised rats were decapitated and the weights of visceral fat pads, liver, gastrocnemius muscle and femoral bone from the left leg were recorded. Body mass index (BMI) was calculated as the ratio between body weight (g) and the square of body length (tail excluded) (cm^2). Following an injection of a small amount of a dye through the infusion kit the brains were dissected and positive staining of the cerebral ventricles was verified.

Determination of plasma glucose and leptin

An automated clinical diagnostic system, Vitros (Ortho-Clinical Diagnostics, Rochester, NY, USA), was used to determine plasma glucose levels. Plasma leptin levels were determined by a rat leptin radioimmunoassay kit (Linco Research, St Louis, MO, USA). The sensitivity of the assays was 0.5 ng/ml. Both the intra- and interassay coefficients of variation were less than 10% in all the assays.

Data and statistical analysis

Body weight gain and food intake for each animal during the week before the operation (control period), as well as for every week during the 4-week long treatment period, were recorded. Food conversion ratio was calculated as $[(\text{g weight gain})/(\text{g food consumption})] \times 100$. Group versus time profiles were analysed with analysis of variance for repeated measurements applying Huynh–Feldt adjustments of degrees of freedom. When a significant interaction between the factors was observed, the difference between the groups of rats at each time-point was assessed with the Tukey–HSD method. If Levene's test for homogeneity of variances indicated non-homogeneous variances, a log-transformation was applied to the original values. Pearson's correlation was used to analyse association between parameters. $P < 0.05$ was considered significant in all statistical tests.

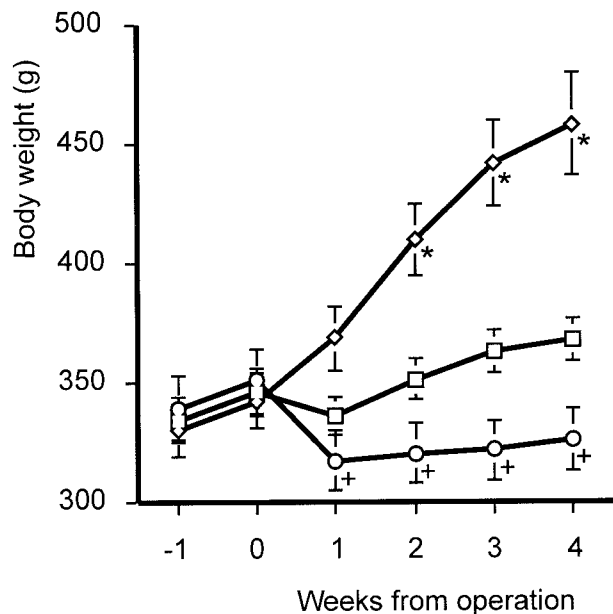


Figure 1 Body weight profile in three groups of rats for 1 week before and during 4 weeks of i.c.v. infusion. Control animals received artificial cerebrospinal fluid (ACSF) (□; $n=11$). The experimental animals received either HS024 (◇; $n=10$) or MT-II (○; $n=9$). Values are means \pm s.e.m. * $P < 0.05$ compared with control rats; + $P < 0.05$ compared with HS024-treated rats (Tukey–HSD test).

Results

Stability and radioligand binding

Stability investigations indicated that HS024 and MT-II were stable in buffered ACSF for at least 4 weeks at 37°C . The affinities of HS024 towards rat MC receptors were 29.4 ± 14.9 nM K_i value for the rat MC3 receptor and 0.225 ± 0.045 nM K_i value for the rat MC4 receptor.

Food intake and weight gain

A 4-week growth curve is given in Fig. 1 and the terminal values of body weight and cumulative food intake over the 4-week treatment period are shown in Table 1. Weekly food intake, weight gain and calculated food conversion ratio are given in Fig. 2. The data show that, before operation, the body weight and food intake in the three groups of rats were almost identical. However, following the implantation of the osmotic mini-pumps and start of treatment, the three groups of rats responded differently.

The food consumption of the control and MT-II-treated rats was similar throughout the period (Fig. 2a). The weekly food intake and the gain in body weight for the HS024-treated rats was higher than in the control and MT-II-treated rats during the whole treatment period (Fig. 2a and b). From the second week, the HS024-treated

Table 1 Physical and plasma parameters: control animals received artificial cerebrospinal fluid (ACSF) while experimental animals received either selective MC4 receptor antagonist (HS024) or MC receptor agonist (MT-II). Values are expressed as means \pm S.E.M.

	ACSF (n=11)	HS024 (n=10)	MT-II (n=9)
Body weight (g)	367 \pm 9	458 \pm 22*	325 \pm 13†
Body length (cm)	24.2 \pm 0.2	24.3 \pm 0.2	23.9 \pm 0.3
Body mass index (g/cm)	0.63 \pm 0.01	0.77 \pm 0.02*	0.57 \pm 0.01*†
Cumulative food intake (g)	582 \pm 10	922 \pm 66*	533 \pm 21†
Resistivity (Ohm \times g ^{0.67} /cm)	163 \pm 4	198 \pm 6*	157 \pm 4†
Gonadal fat weight (g)	6.7 \pm 0.4	14.1 \pm 1.2*	4.9 \pm 0.4*†
Retroperitoneal fat weight (g)	6.8 \pm 0.5	17.1 \pm 1.8*	3.8 \pm 0.5*†
Liver weight (g)	9.8 \pm 0.5	12.4 \pm 0.9*	9.1 \pm 0.4†
Gastrocnemius muscle weight (g)	1.20 \pm 0.04	1.17 \pm 0.06	1.16 \pm 0.05
Femoral bone weight (g)	1.18 \pm 0.03	1.20 \pm 0.03	1.16 \pm 0.05
Plasma leptin (ng/ml) ^a	4.6 \pm 0.8	28.0 \pm 6.2*	2.0 \pm 0.4*†
Plasma glucose (mM)	7.1 \pm 0.4	6.7 \pm 0.2	6.7 \pm 0.3

^aLeptin: Levene test for homogeneity of variances.**P*<0.05 compared with control rats; †*P*<0.05 compared with HS024-treated rats (Tukey-HSD test).

rats were heavier than both the MT-II and control rats (Fig. 1). During the first week of treatment the control and MT-II-treated rats lost weight (Fig. 2b). This effect was more pronounced in the MT-II-treated rats, showing a reduction in body weight compared with the control rats. After the transient decrease in body weight during the first week of treatment, both the control and MT-II-treated rats started to gain weight again, while the body weight of the MT-II-treated rats increased at a slower rate during the second and third week of treatment. During the last week of treatment, the MT-II-treated rats returned to levels similar to those of the control rats, but at a lower level than the HS024-treated rats (Fig. 2b).

At the end of the treatment period, the HS024-treated rats had ingested about 65% more food than the control and MT-II-treated rats and weighed about 34% more on average than when they were operated. On the other hand, the control and MT-II-treated rats had hardly gained any weight, the average body weight values being at 106% and 93% respectively of that on the day on which they were operated. Thus the cumulative food intake and final weight of the HS024-treated rats were significantly higher than the control and MT-II-treated rats, while the difference between the control and MT-II-treated rats was not significant (Table 1 and Fig. 1).

The food conversion ratio, defined as body weight increase relative to weight of ingested food, is shown in Fig. 2c. During the first 2 weeks of treatment, this ratio was different between all the three groups, being the highest for the HS024-treated rats and lowest and negative for the MT-II-treated rats. During the third week, the food conversion ratio was still significantly lower for the MT-II-treated rats than for the HS024-treated rats and control rats, but no significant difference was observed during the last week.

Body fat and plasma values

No significant difference between the three groups of rats was observed in body length, gastrocnemius muscle weight or femoral bone weights (Table 1). Liver weight was higher in the HS024-treated rats compared with each of the other two groups. BMI was lower in the MT-II-treated rats and higher in the HS024-treated rats compared with control rats. Resistivity was higher in the HS024-treated rats compared with the control rats, while it was not significantly different between the MT-II-treated rats and control rats. Differences in gonadal and retroperitoneal fat pads were observed between all three groups of rats. Gonadal and retroperitoneal fat weights in the HS024-treated rats were more than twice as high as in the control and MT-II-treated rats. Figure 3 shows the relationship between body resistivity and combined weight of gonadal and retroperitoneal fat pads expressed as % of body weight. A close correlation was observed between individual values of these parameters ($n=30$, $r=0.82$, $P<0.001$).

Plasma glucose and leptin levels are shown in Table 1. The plasma glucose levels were similar in all three groups of rats. The HS024-treated rats showed sixfold higher levels of leptin than control rats, whereas the rats which received MT-II had less than half the leptin levels of the control rats.

Discussion

The present study shows that long-term blocking of the MC4 receptor by the exogenously administered selective antagonist HS024 causes increase in food intake and weight gain which is persistent for at least 4 weeks. Furthermore, a long-term stimulation of the central MC

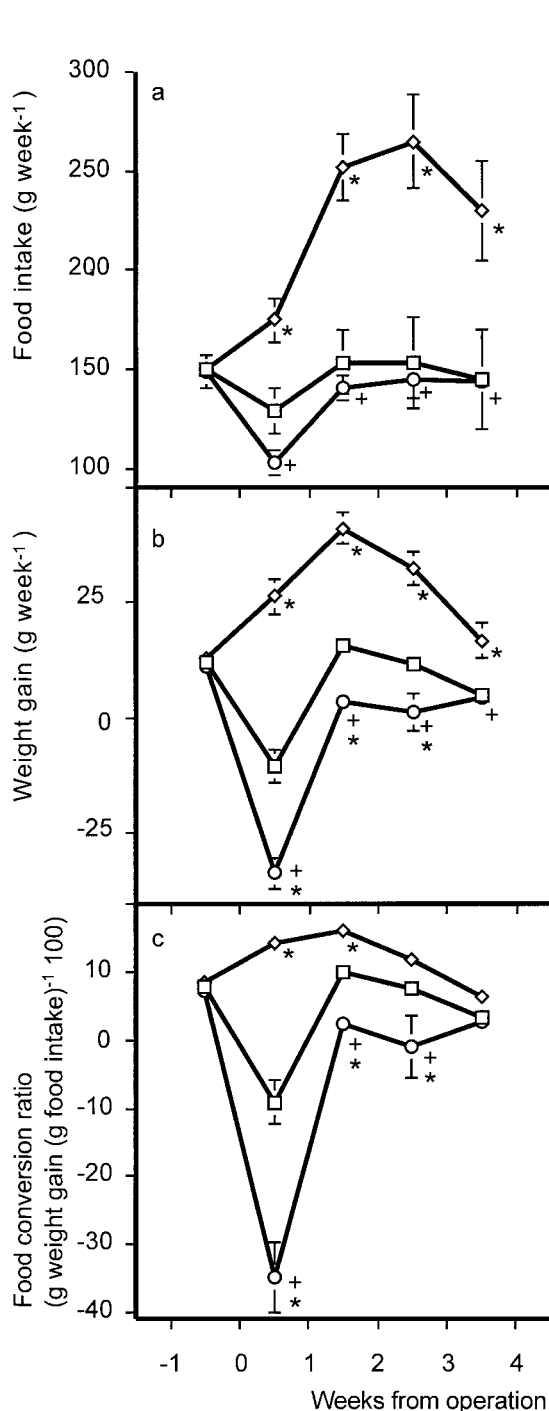


Figure 2 Weekly (a) food intake, (b) weight gain and (c) food conversion ratio in three groups of rats for 1 week before and during 4 weeks of chronic i.c.v. infusion. Control animals received ACSF (□; n=11). The experimental animals received either HS024 (◇; n=10) or MT-II (○; n=9). Values are means ± S.E.M. *P<0.05 compared with control rats; +P<0.05 compared with HS024-treated rats (Tukey–HSD test).

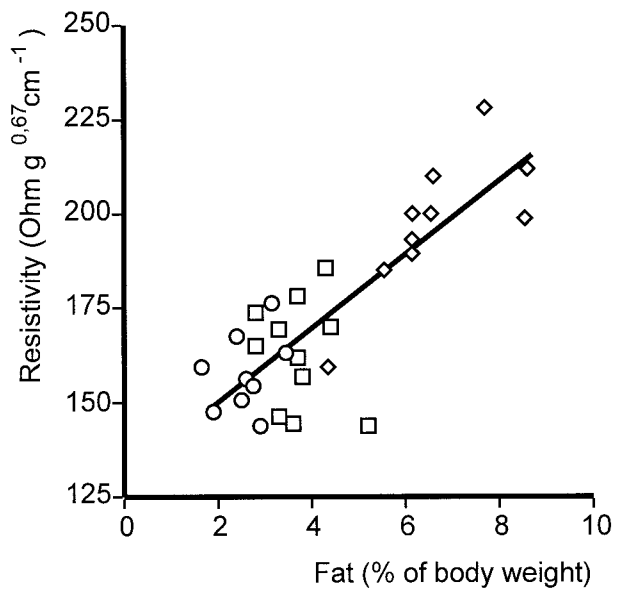


Figure 3 Relationship between body resistivity and combined weight of gonadal and retroperitoneal fat pads expressed as % of body weight. Each point represents an individual subject (n=30) in three groups of rats after 4 weeks of chronic i.c.v. infusion. Control animals received ACSF (□; n=11). The experimental animals received either HS024 (◇; n=10) or MT-II (○; n=9). r=0.82, P<0.001.

receptors by the potent agonist MT-II leads to a clear reduction in food intake and body weight loss during the first week of the treatment. Food intake was back to the control level by the second week and weight gain by the fourth week of treatment.

The control rats were affected by the surgical operation associated with implantation of the osmotic pumps as indicated by a modest reduction in food intake and weight loss during the first week of treatment. The reduced food intake caused by the operation was blocked by the selective MC4 antagonist HS024, which is in agreement with earlier data from a 1-week study on HS028 (Skuladottir *et al.* 1999) and a 2-week study on HS014 (Kask *et al.* 1999), both substances being MC4 antagonists. This is also in agreement with previous results showing that immobilisation-induced anorexia was reversed by administration of the selective MC4 receptor antagonist HS014 (Vergoni *et al.* 1999). At this point it must be stressed that HS024 is 130-fold more selective for the rat MC4 receptor than for the rat MC3 receptor as indicated by respective K_i values (see Results).

The present data show that there is a relatively lower weight gain in the agonist MT-II-treated rats than in the antagonist HS024-treated rats, considering how much they eat. This is shown in Fig. 2c, as there was a lower food conversion ratio for the agonist MT-II-treated rats as compared with both the HS024-treated rats and control rats. It is also intriguing that the food conversion ratio is

much higher for the antagonist HS024-treated rats as compared with the control rats. The present data give further support to the notion that the MC4 receptor is not only involved in a satiety response but also mediates an important influence on the metabolic status of the animals. A recent study shows that MC4 receptor-null mice have enhanced caloric efficiency (Ste Marie *et al.* 2000), similar to that seen in the agouti obesity syndrome and in MC3 receptor-null mice (Butler *et al.* 2000, Chen *et al.* 2000). It is thus likely that both the MC3 and MC4 receptors are involved in the long-term effects that were observed on the food conversion ratio in this study. It is interesting that the positive influence of the antagonist on the food conversion ratio and the negative influence of the agonist is greatest during the first week of treatment, attenuates during the next 2 weeks and disappears during the last week of the study. These results indicate that long-term administration of MC receptor active substances is likely to directly affect metabolic rate in a transient manner. The metabolic influence is also demonstrated in increase in liver weight of the HS024-treated rats compared with the control rats (Table 1). The transient effects on the food conversion ratio could be due to changes in expression of the MC receptors or as mentioned above for the food intake, through other hormones like leptin which is elevated in the HS024-treated rats at the end of the experimental period.

Considering the data on the physical and the plasma parameters, it appears that the body weight increase during the long-term MC4 receptor antagonist treatment is mainly due to extensive increase in fat deposits, since the body length, gastrocnemius muscle and femoral bone weights were not affected. The electrical impedance recordings used to assess the body composition are based on the fact that adipose tissue, owing to its high content of lipids, may be regarded as an electric insulator as compared with other tissues which contain more electrically charged particles dissolved in the body fluid (Cunningham *et al.* 1986). The body composition has thus been estimated by impedance measurements in the rats (Hall *et al.* 1989). In order to avoid placing the electrodes in the head and neck region, where scar tissue due to previous implantation of brain infusion kits and osmotic mini-pumps could be anticipated, the electrode placement was moved from the dorsal mid-line to the paws of the animals. Resistivity was clearly increased in the HS024-treated rats while it was similar for the MT-II-treated and the control rats. We conclude that the increased resistivity in the HS024-treated rats is due to the increased proportion of total body fat in the animals. This is based on the high degree of linear correlation between resistivity and relative cumulative weight of retroperitoneal and gonadal fat and assuming a proportionally similar increase in fat deposits elsewhere. This conclusion is also supported by a significant increase in BMI in the HS024-treated rats, compared with the control rats.

It is well established that there is a direct correlation between plasma leptin levels and mass of adipose tissue (Maffei *et al.* 1995, Clayton & Gill 1997). This is in agreement with the findings of the present study. The level of plasma leptin was highly elevated in the HS024-treated rats, while it was significantly lower in the MT-II-treated rats compared with the control rats. Furthermore, the HS024-treated rats were severely obese, while the MT-II-treated rats had less fat than the control rats. Another putative explanation might be that a blockage of the melanocortin signalling, which may interfere with the anorectic signalling of leptin, could turn on a positive feedback signal to the fat cells in order to counteract the MC receptor blockage and vice versa for the agonist treatment.

It has been shown that MT-II acts on both the main central MC receptors, i.e. the MC3 and MC4 receptors (Fan *et al.* 1997). It is, however, unlikely that MT-II influences food intake through the MC3 receptor as it has been shown that γ -MSH, a peptide with a preference for the MC3 receptor, does not influence food intake, while both α -MSH and β -MSH are effective (Kask *et al.* 2000) and that the MC3 knock-out mice eat normally (Chen *et al.* 2000). We have shown the peptides to be stable in ACSF for at least 4 weeks at 37 °C. The recovery of food intake and body weight increase in the MT-II-treated rats after the first week of treatment is thus not likely to be due to degradation of the peptides. Adaptive processes, such as down-regulation of MC receptors (Harrold *et al.* 1999, Lindblom *et al.* 2000), are more likely to be involved. Such adaptive processes might be acting concurrently with other controlling mechanisms beyond the melanocortin system, such as reduced leptin secretion following the decline in body fat (Maffei *et al.* 1995) during the first week of treatment, or effects of the NPY system which is integrated with the melanocortins (Cone 1999). Similar adaptation has been described regarding the inhibitory effect on feeding caused by long-term administration of corticotropin-releasing hormone (Krahn *et al.* 1990) and leptin (Cone 1999).

Our results indicate that the MC receptors may mediate an important transient influence on food conversion and the results provide an insight into how long-term external administration of MC receptor active substances may affect body weight homeostasis.

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