

Chronic melanocortin 4 receptor blockage causes obesity without influencing sexual behavior in male rats

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Abstract

We investigated the effects of continuous intracerebroventricular infusion of a melanocortin 4 receptor antagonist HS014 (cyclic [AcCys¹¹, D-Nal¹⁴, Cys¹⁸, Asp-NH₂²²]β-MSH-(11–22)) over 12 days and a subsequent 12-day recovery period on food intake, body weight and copulatory behavior in male rats. The results show that the food intake increased immediately after the start of the infusion of HS014 (0.16 nmol/h) and progressively increased thereafter. No tachyphylaxis was observed. When the infusion of HS014 was terminated, the food-intake levels dropped. The body weights of the rats had increased by 17% by the end of the study, compared with controls. During the recovery period, the body weight decreased towards the levels of the control rats. These results indicate that overeating and the subsequent

increases in body weight caused by blockage of the melanocortin 4 (MC4) receptor are reversible when the blockage is ended. We also tested the copulatory behavior of vigorous male rats in the presence of female rats in estrous. We registered mount latency, the number of mounts, the intromission latency, the number of intromissions, the ejaculation latency and the post-ejaculatory interval three times during the study and also after acute administration of HS014 and α-MSH. The sexual behavior of the male rats was not affected. These results indicate that the MC receptors, in particular the MC4 receptor, may not be a major mediator of effects on copulatory behavior in male rats.

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Introduction

The melanocortin peptides (melanocyte-stimulating hormone (MSH) and adrenocorticotropin (ACTH)) have a broad array of physiological effects. The effects of the melanocortins on peripheral functions, such as steroidogenesis in the adrenal gland and skin or hair pigmentation, are probably those which are most thoroughly characterized. Other peripheral effects of melanocortins, such as those on inflammation (Lipton & Catania 1997) and the cardiovascular system (Guarini *et al.* 1999), are receiving increased attention. The central effects of the melanocortins are, however, much more diverse and complex. One of the reported effects of melanocortins is that on sexual behavior. The first reports are from the mid-1960s: ACTH was reported to cause sexual excitement in male rabbits (Bertolini *et al.* 1968, 1969). The sexual response consisted of penile erection followed by copulatory movements and ejaculations in the absence

of females. α-MSH induced similar responses in rats (Bertolini *et al.* 1975). ACTH can be processed into ACTH(1–13), which has the same amino acid sequence as α-MSH, and it is conceivably the MSH pharmacophore which is responsible for the actions for ACTH in this respect (Schiöth *et al.* 1997).

The melanocortins are also important in the hypothalamic regulation of food intake. It was already discovered in the 1980s (Poggioli *et al.* 1986, Vergoni *et al.* 1986) that melanocortins influence food intake, but it was only recently shown (by knock-out studies in mice) that these effects are mediated through the melanocortin 4 (MC4) receptor (Huzsar *et al.* 1997). The MC4 receptor is one of five subtypes in the family of melanocortin receptors which bind, and are activated by, the melanocortin peptides. The MC4 receptor knock-outs showed characteristics similar to those of mice overexpressing the agouti peptide, which is an antagonist for this receptor (Lu *et al.* 1994). Administration of a MC4 receptor agonist leads to

acute reduction in food intake and body weight, while the reverse effects are observed after administration of a MC4 receptor antagonist (Fan *et al.* 1997, Grill *et al.* 1998, Kask *et al.* 1998, Vergoni *et al.* 1998). The potential use of MC4 receptor agonists against obesity has attracted a great deal of interest. Moreover, as MC4 receptor blockage reduces stress-induced anorexia (Vergoni *et al.* 1999), it has been speculated that MC4 receptor antagonists may become useful in the treatment of different anorectic conditions.

It is still not known which of the MC receptors mediates the effects of melanocortins on sexual behavior. Moreover, it is not known if there are any links between the effects of the melanocortins on food intake and sexual behavior. In this study, we treated male rats with continuous i.c.v. infusion of a selective MC4 receptor antagonist for 2 weeks and monitored the food intake and body weight during this period and additionally for a further 2 weeks after the infusion had been terminated. We also investigated the effects of this treatment, which caused obesity, on sexual behavior. Moreover, in another setting we tested the effect of acute administration of a MC4 receptor antagonist and α -MSH on sexual behavior in male rats.

Materials and Methods

Animals and surgery

Adult Sprague–Dawley male rats (Harlan Nossan, Correzzana, Italy), weighing 175–200 g at the beginning of the experiments, were housed in climatically controlled colony rooms (21 ± 1 °C; 60% humidity) with a reversed light/darkness cycle (12 h/12 h; lights on from 2000–0800 h) and had free access to food and water. After a 3-week period of stabilization to the new light/darkness cycle, the rats were subjected to five preliminary copulatory tests (see below for details) and then either implanted with a stainless steel guide cannula (for acute i.c.v. treatments) or with a cannula connected to a micro-osmotic pump (for chronic administration of a drug). Age-paired females of the same strain served as sexual partners and were housed in the same conditions and subjected to ovariectomy 3 weeks before the start of the sexual activity tests; they were then brought into estrous by sequential treatment with estradiol monobenzoate (s.c., 15 μ g 48 h before testing) and progesterone (s.c., 1000 μ g 4 h before testing). All surgical procedures were done under anesthesia using ketamine plus xylazine (i.p., 115+2mg/kg; Farmaceutici Gellini (Aprilia, Italy) and Bayer (Milano, Italy) respectively). All experiments were performed in accordance with local (D.L.vo 116/92) and international guidelines for animal experiments (European Communities Council Directive 86/609/EEC). For the acute administrations, a stainless steel guide cannula (22-gauge) (Plastic Products Co., Roanoke, VA, USA) was stereotaxically implanted to a depth of 0.5 mm above a lateral brain ventricle (measured in mm from the bregma:

anterior posterior = -0.8 ; lateral = 1.4 ; ventricular = 3.25) (Paxinos & Watson 1982) and fixed to the skull with screws and dental acrylic. A removable plug was kept in place except during the drug injections. Correct placement was verified at the end of the experiment by the injection of 2 μ l toluidine blue dye through an internal cannula (28-gauge) used for drug (or saline) injection (which extended 0.5 mm below the tip of the implanted guide cannula), followed by decapitation, under ethyl ether anesthesia, and dissection of the brain. Data obtained from improperly implanted animals were discarded. For the chronic treatments, a brain infusion cannula (Alzet Brain Infusion Kit, Alza Co., Palo Alto, CA, USA) was stereotaxically implanted intracerebrovascularily at the same co-ordinates as above. The cannula was connected by tubing to the flow moderator of an Alzet micro-osmotic pump (Model 1002). All components were previously filled with saline (NaCl 0.9%) or with drug solution and left in saline at 37 °C for 48 h prior to the implantation. The pump and the tubing extended subcutaneously along the back between the scapulae, so that when the wound was closed all the components were located beneath the skin. The i.c.v. infusion lasted for 12 days and the rats received the solution at a constant rate (0.25 μ l/h). At the end of the infusion period, animals were anesthetized and the pumps were disconnected from the i.c.v. cannulae and then removed. Animals were weighed every day, as was the amount of food eaten during the whole infusion period and for 11 days after the cessation of infusion.

Drugs and treatments

HS014 (Schiöth *et al.* 1998) (cyclic [AcCys¹¹, D-Nal¹⁴, Cys¹⁸, Asp-NH₂²²] β -MSH-(11–22)) was provided by Melacure Therapeutics AB (Uppsala, Sweden) and α -MSH was purchased from Neosystem (Strasbourg, France).

For the chronic treatments, HS014 was dissolved in saline to ensure release at 0.16 nmol/h (0.27 μ g/h). For the acute treatments, the peptides were freshly dissolved in saline and injected intracerebrovascularily, at doses of 5, 7.5 or 10 μ g HS014 per rat and 5 or 10 μ g α -MSH per rat, of in a volume of 5 μ l and at a rate of 1 μ l per 20 s via an i.c.v. internal cannula that protruded 0.5 mm beyond the guide cannula tip, connected by polyethylene tubing to a 50 μ l syringe (Hamilton, Bonadure, Switzerland) driven by a micrometric screw.

Sexual behavior study

All of the copulatory tests (before, during or after the treatment, as indicated in the results section) were performed starting 3 h after lights off under a red light, according to the standard procedure (Dewsbury 1972, Sachs & Barfield 1976). The rats were put in a mating

arena in the presence of a receptive female and the following parameters were recorded: mount-mount with pelvic thrusting; intromission-mount with vaginal penetration; ejaculation-mount with intromission and a final prolonged thrust, slow dismounting, and genital grooming; mount latency and intromission latency time to first mount and intromission, respectively; ejaculation latency time from first intromission to ejaculation; and post-ejaculation interval time from ejaculation to first intromission of a new series. The test was ended after the first post-ejaculatory intromission or when the mount or intromission latency was >15 min, when the ejaculation latency was >30 min or when the post-ejaculation interval was >15 min. Rats were included in the study irrespective of sexual performance; however, all of them satisfied the criteria for sexual vigor (defined as the ability to achieve ejaculation within the cut-off time-limit in at least two out of five preliminary tests).

Statistics

Data concerning sexual performance were expressed as medians \pm interquartile ranges and were analyzed in the acute experiments using an overall Kruskal–Wallis analysis of variance (ANOVA) for non-parametric data, followed by the Mann–Whitney U test when ANOVA revealed differences among groups; the Mann–Whitney U test was used alone in the chronic experiments since only two groups were tested. Data concerning body weight and food intake in the chronic experiments were expressed as means \pm S.E.M. values and were analyzed with the Student's *t*-test. A *P* value < 0.05 was considered as statistically significant.

Results

We investigated the effects of chronic 12-day administration of HS014 on food intake, body weight and copulatory behavior in male rats. Moreover, we tested the effects of an acute single-dose administration of HS014 on copulatory behavior in male rats. The results on food intake are shown in Fig. 1*a* and those on body weight in Fig. 1*b*. The results show that there was already a significant increase in food intake by the second day of the infusion of HS014, compared with the controls. The difference between the groups increased until the 7th day of the infusion and then the food-intake levels were maintained throughout the infusion period. On the 12th day of the infusion, the HS014-treated rats ate 92% more than the control group. The pumps were disconnected on Day 12 and we carefully monitored the food intake during the additional 12 days. When the infusion was terminated, the food intake gradually decreased in the group that had been treated with HS014. At Day 16, 4 days after the end of the infusion, the food intake was not significantly different between the groups. The HS014-treated rats

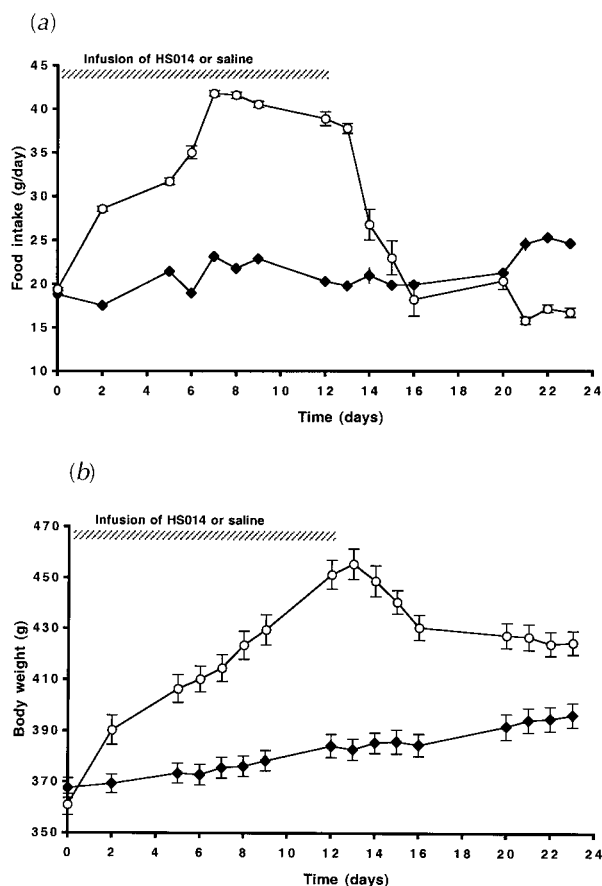


Figure 1 Influence of chronic infusion of (○) HS014 (0.16 nmol/h) or (◆) saline on (a) food intake or (b) body weight in Sprague–Dawley male rats. All values represent mean \pm S.E.M. values; *n* = 14–16 per group.

continued to lower their food intake and at Day 21 the HS014 group had a significantly lower food intake than the control group. This difference was still observed when the study was terminated at Day 24, 12 days post-infusion.

The results on body weight (Fig. 1*b*) reflect the results on food intake. By the second day of infusion the HS014 treated rats were already significantly heavier than the controls. The difference between the two groups continued to increase during the whole period. The HS014 rats weighed 17.5% more than their controls at day 12 of the infusion. The HS014 rats lost weight after termination of the infusion but they were still 7.2% heavier than the controls when the study was terminated.

We tested the sexual behavior of the rats three times during the investigation period. The data are shown in Table 1. None of the parameters tested (mount latency, number of mounts, intromission latency, number of intromissions, ejaculation latency, post-ejaculatory interval) differed in the HS014 group compared with the controls at the 5th or 12th days of the infusion or at the 2nd day after

Table 1 Influence of chronic infusion of HS014 (0.16 nmol/h) on the copulatory behavior of vigorous male rats. The values are medians (interquartile range) of 14–16 rats per group.

Treatment	Mount latency (s)	No. of mounts	Intromission latency (s)	No. of intromissions	Ejaculation latency (s)	Post-ejaculatory interval (s)
Saline, 5th day	81 (39.7–122)	3.5 (2–9.2)	89.5 (48.7–225.5)	11 (8.7–14.2)	612 (360.7–730.2)	349 (325–387.2)
HS014, 5th day	50 (30–88.5)	3 (0.2–5.7)	134 (41–237.5)	13 (10.5–17.5)	617 (418–1123.7)	385.5 (237.5–472.2)
Saline, 12th day	33 (21–67)	3.5 (0–7)	62 (21.25–127)	10.5 (5–12.5)	326.5 (218.7–415)	331.5 (312.2–349.5)
HS014, 12th day	54 (11.5–168.5)	1 (0–6.5)	58.5 (18.7–271.5)	12 (7–15)	347.5 (338.7–368.7)	341.5 (308–373)
Saline, 14th day	39.5 (19.7–77.2)	0 (0–0)	39.5 (19.7–80.5)	9 (7.7–16.2)	212.5 (137.7–405.5)	320 (298.2–367.7)
HS014, 14th day	46 (23.7–85.7)	2 (0–4.7)	65.5 (23.7–154.7)	10.5 (8.25–12)	276 (237–309.5)	326.5 (293.7–337.5)

No significant differences were found among groups according to the non-parametric Kruskal–Wallis analysis of variance.

the infusion had been stopped. Moreover, separate tests were conducted to determine if i.c.v. administration of three different single doses of HS014 affected sexual behavior in male rats. The results, which are shown in Table 2, do not show differences between the controls and the treated rats. The mount latency is a little prolonged at the highest dose of HS014 but, as this is not reflected in any other parts of the data, we are not willing to draw any major conclusions from this difference. The results of acute i.c.v. administration of α -MSH are shown in Table 3. The results do not show any differences between α -MSH-treated and saline-treated controls.

Discussion

Our new results show that continuous administration of the MC4 receptor antagonist HS014 causes substantial increase in food intake. It is interesting to note that the increase in food intake was not only observed after the first day of the infusion but progressively increased, reaching a maximum at Day 7. The maximum level of food intake was then maintained throughout the infusion period and additionally for 1 day after the termination of the infusion. On the 2nd day after the end of the infusion, the food-intake levels were drastically reduced and even fell significantly below the control levels on Day 21. We had previously performed a 2-week study with continuous infusion of HS014 in a limited number of animals (Kask *et al.* 1999). In comparison to this earlier study, our present data show a greater increase in the food intake but a similar increase in body weight. In the present study, we also followed the food intake for 12 days after the infusion was stopped. In contrast to our earlier study, in the present study the mini-pumps were actively stopped (in our previous study, the pumps were not disconnected and the end-point of that study was based on the pump manufacturer's given end-point). The results are also different: the rats in the previous study continued to overeat, whereas the rats in our present study stopped overeating 2 days after the infusion was stopped. The same dose of HS014 and the same osmotic pumps were used in both studies. The overeating observed in the previous study after the predicted end-point of the infusion could be related to residual amounts of the substance leaking out of the pumps. Thus we conclude that chronic infusion of the MC4 receptor antagonist for 2 weeks in male rats does not lead to a permanent increase in food intake. It is noteworthy that no tachyphylaxis was observed after 2 weeks of administration of the MC4 receptor antagonist, which is in agreement with our previous studies (Kask *et al.* 1999, Skuladottir *et al.* 1999). The results also indicate that overeating and the subsequent increase in body weight caused by MC4 receptor blockage are reversible when the blockage is stopped.

The results on body weight show that there was already a clear and significant increase in body weight after 2 days

of infusion of HS014. In line with the data on food intake, the rats continued to gain weight during the whole period and for 1 day beyond termination of the infusion. The amount of weight gain observed after the 12-day infusion is in line with our earlier infusion data for HS014, i.e. an approximately 20% increase in body weight after 2 weeks. After the infusion period, the rats lost weight rather rapidly for 4 days. On the following days, the weight loss was not as rapid as that in the first period, despite the fact that the food intake was lowered below control levels at Day 22. If the weight curves for the last 6 days of the study were to be extrapolated in a linear fashion, the HS014 rats would need approximately one additional week (a total of 3 weeks after the infusion) to reach the same body weight levels as the controls.

There are some earlier pieces of evidence showing that ACTH may affect copulatory behavior in rats (Bertolini *et al.* 1975, Spruijt *et al.* 1985). This effect, however, was reported to be much less pronounced than the clear and undisputed effect of ACTH and α -MSH on penile erection in male rats. Our new results indicate that acute administration of α -MSH does not influence copulatory behavior in male rats. We are not aware of any tests having been conducted on the effects of α -MSH on copulatory behavior prior this study. Most of the central behavioral effects of ACTH have been reproduced using α -MSH. No MC2 receptors (ACTH receptors) have been found in the brain and, as ACTH is processed into α -MSH, it has been assumed that the central effects of ACTH are mediated by the MC receptors. As neither α -MSH nor HS014 was effective after acute administration in our study, we assume that the MC receptors, in particular the MC4 receptors, cannot be linked with copulatory behavior in male rats.

Obesity is also known to interfere with copulatory behavior in some animal models (Young *et al.* 1986, Hemmes & Schoch 1988). Our results show no signs that copulatory behavior is affected by the long-term infusion of HS014 at any of the three investigation points, despite the obvious obesity that was observed in the rats when the infusion was stopped.

We have previously shown that it is unlikely that it is the MC4 receptor that is the mediator of the potent erectogenic effects of α -MSH. It is likely, therefore, that the MC4 receptor is not an important mediator of any of the effects of the melanocortins which have been observed in earlier studies in rats (Argiolas *et al.* 1988, Vergoni *et al.* 1998), rabbits (Bertolini *et al.* 1969) and humans (Wessells *et al.* 1998).

In summary, we have described the effects on food intake, body weight and copulatory behavior in male rats after continuous MC4 receptor blockage for 2 weeks and for 2 weeks after the treatment was stopped. The results indicate that MC receptors, and in particular the MC4 receptor, may not be a major mediator of effects on copulatory behavior in male rats.

Table 2 Influence of acute i.c.v. administration of HS014 (5, 7.5 or 10 µg/rat) on the copulatory behavior of vigorous male rats. The values are medians (interquartile range) of 13–15 rats per group

Treatment	Mount latency (s)	No. of mounts	Intromission latency (s)	No. of intromissions	Ejaculation latency (s)	Post-ejaculatory interval (s)
Saline	44 (29–58.5)	6 (4–8.5)	100 (63.5–167.5)	12 (10–13)	435 (366–537.5)	362 (324.5–397.5)
HS014 (5 µg/rat)	67 (30–88.5)	4 (2–8.5)	78 (37–227.5)	12 (8.5–18.5)	486 (316.5–630.5)	333 (302.5–394)
HS014 (7.5 µg/rat)	69 (35–190)	3 (1–11)	109 (43–249)	12 (11–13)	620 (520–1800)	345 (298–900)
HS014 (10 µg/rat)	215 (59–381)	5 (3–8)	283 (75–527)	10 (7–11)	745 (446–1800)	418 (353–900)

No significant differences were found among groups according to the non-parametric Kruskal–Wallis analysis of variance.

Table 3 Influence of acute i.c.v. administration of α -MSH on the copulatory behavior of male rats. The values are medians (interquartile range) of 8–9 rats per group.

Treatment	Mount latency (s)	No. of mounts	Intromission latency (s)	No. of intromissions	Ejaculation latency (s)	Post-ejaculatory interval (s)
Saline	42 (24.5–47.5)	5 (1.5–7)	68 (43.5–500)	8 (3.5–11)	369 (286–1257)	329 (294–637.5)
α -MSH (5 μ g/rat)	59 (36.7–69)	5 (3.2–8.5)	76.5 (50.7–148.5)	9 (6.2–10)	343 (289.7–508.7)	395 (330.2–454.7)
α -MSH (10 μ g/rat)	41.5 (13.7–348)	1.5 (0.2–7.5)	93.5 (27.5–711.5)	7 (3–8)	240.5 (128.2–838.0)	394.5 (372.5–498.7)

No significant differences were found among groups according to the non-parametric Kruskal–Wallis analysis of variance.

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