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Differential influence of a selective melanocortin MC_4 receptor antagonist (HS014) on melanocortin-induced behavioral effects in rats

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Abstract

We injected i.c.v. the natural agonist α-MSH (melanocyte-stimulating hormone) and the first selective melanocortin MC_4 receptor antagonist HS014 (cyclic [AcCys[11], D-Nal[14], Cys[18], Asp-NH_2^{22}]-β-MSH(11–22) in rats and scored a number of behavioral effects which have been related to the melanocortic peptides. The results showed that HS014 (5 μg/rat) completely blocked α-MSH (3 and 5 μg/rat)-induced grooming, yawning and stretching. Penile erections induced by α-MSH were, however, only partially blocked by HS014. Injections of α-MSH decreased food intake in food-deprived rats, whereas HS014 increased food intake. When the peptides were given together, the food intake was similar to that of saline treated controls. Locomotion/exploration and resting were not influenced by either peptide. Our data show that exogenous α-MSH decreases food intake, and that an endogenous central melanocortinergic inhibitory tone on feeding prevails which can be blocked with HS014, leading to an increase in food intake. Our data also provide evidence that grooming, stretching and yawning in rats may be mediated by the melanocortin MC_4 receptor, whereas penile erections might perhaps be mediated by some other melanocortin receptor.

Keywords: Melanocortin receptor; MSH (melanocyte-stimulating hormone); HS014; Food intake; Grooming behavior

1. Introduction

The natural melanocortin peptides [MSH (melanocyte-stimulating hormone)/ACTH (adrenocorticotrophic hormone)] are known to exert a variety of physiological effects of both peripheral and, perhaps more importantly, central origin. The effects of MSH/ACTH peptides on pigmentation and steroidoneogenesis are fairly well characterized, whereas the mechanisms behind the central MSH/ACTH syndrome (Bertolini et al., 1988), including effects on grooming, stretching, yawning, pyretic control, pain perception, attention, learning and memory, ingestive, sexual and social behaviors, are less well understood (Eberle, 1988; O'Donohue and Dorsa, 1982).

Five melanocortin receptor subtypes have been cloned (Chhajlani et al., 1993; Chhajlani and Wikberg, 1992; Gantz et al., 1993a,b; Mountjoy et al., 1992). The melanocortin MC_1 receptor is found in melanoma cells, where it has a role in mediating pigmentation. The melanocortin MC_2 receptor (or ACTH) receptor is found in the adrenal glands where it mediates the effects of ACTH. The melanocortin MC_3 receptor is primarily found in the CNS (central nervous system) (Gantz et al., 1993a) but its physiological function is still more or less unknown. The melanocortin MC_4 receptor has only been found in the brain, where it is widely distributed in several areas, including the cortex, thalamus, hypothalamus, brain stem and spinal cord (Gantz et al., 1993b; Mountjoy et al., 1994). The melanocortin MC_4 receptor has recently been related to weight homeostasis:-melanocortin MC_4 receptor 'knock-out' mice develop obesity (Huszar et al., 1997) and injection of MSH peptides inhibits feeding behavior (Vergoni et al., 1986, 1990; Fan et al., 1997). The melanocortin

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MC_5 receptor has a wide peripheral distribution and is believed to participate in the regulation of exocrine gland function (Chen et al., 1997).

The natural melanocortin peptides are not selective for any of the melanocortin receptor subtypes, with the exception that α-MSH is somewhat selective for the melanocortin MC_1 receptor and ACTH is selective for the melanocortin MC_2 receptor. The lack of subtype-selective antagonists for the melanocortin receptors has hampered the clarification of the physiological mechanisms behind the various effects of the melanocortin peptides. The development of melanocortin MC_3 and MC_4 receptor antagonists like SHU9119 (cyclic [Nle^4, Asp^5, D-Nal^7, Lys^{10}]α-MSH-(4–10)) (Hruby et al., 1995) and some ACTH-(4–10) analogues (Adan et al., 1994b) has opened new possibilities to clarify the mechanism of action of the melanocortins. However, these substances are not selective for any of the melanocortin receptor subtypes (Hruby et al., 1995; Schiöth et al., 1997b,c). HS014 (cyclic [$AcCys^{11}$, D-Nal^{14}, Cys^{18}, $Asp\text{-}NH_2^{22}$]-β-MSH(11–22) (Schiöth et al., 1998), the first melanocortin MC_4 receptor-selective antagonist, is an additional tool to elucidate the subtype-specific actions of the melanocortin receptors. HS014 is an antagonist of the melanocortin MC_3 and MC_4 receptors, with about 20-fold selectivity for the melanocortin MC_4 receptor above the melanocortin MC_3 receptor, and is a low-affinity partial agonist of the melanocortin MC_1 receptor and the melanocortin MC_5 receptor. HS014 is highly potent in increasing food intake in free-feeding rats (Kask et al., 1998).

In this study, we used the natural agonist α-MSH and HS014 to investigate a number of behavioral effects which have been related to the melanocortin peptides.

2. Materials and methods

2.1. Animals and surgery

Adult male rats of a Wistar strain (Harlan Nossan, Correzzana, MI, Italy), weighing 200–220 g, were housed 4–5 per cage (25 × 40 × 15 cm Makrolon cages) in climatized colony rooms (21 ± 1°C; 60% humidity) with food and water continuously available. Stainless-steel guide cannulae (23 gauge) (Plastic Products, Roanoke, VA, USA) were stereotaxically implanted into both lateral ventricles, to a depth of 0.5 mm above the ventricles (measured in millimeters from the bregma: AP = −0.8; $L = 1.4$; $V = 3.25$) (Paxinos and Watson, 1982), under ketamine plus xylazine anesthesia (115 + 2 mg/kg i.p.; Farmaceutici Gellini, Aprilia, Italy and Bayer, Milano, Italy, respectively), and fixed to the skull with screws and dental acrylic. A removable plug, which extended 0.5 mm below the tip of the guide cannula, was kept in place except during drug injections. Correct placement was verified at the end of the experiment by injecting 2 μl of toluidine blue dye through an internal cannula used for drug (or solvent) injection (which extended 0.5 mm below the tip of the implanted guide cannula), followed by decapitation of the rat under ethyl ether anesthesia and dissection of the brain. Data obtained from improperly implanted animals were discarded.

2.2. Drugs and treatments

HS014 was synthesized using a solid-phase approach and purified by HPLC (high performance liquid chromatography) as described earlier (Schiöth et al., 1998). The correct molecular weight of the peptide was confirmed by mass spectrometry. α-MSH was purchased from Sigma, Milano, Italy. The peptides were dissolved in saline and injected into a brain lateral ventricle (i.c.v.) at the doses indicated, in a volume of 5 μl, at the rate of 1 μl/20 s, via the i.c.v. internal cannula connected by polyethylene tubing to a 50-μl Hamilton syringe driven by a micrometric screw.

2.3. α-MSH-induced behavioral syndrome

All the experiments were performed between 0900 and 1300 h, and rats were placed in individual cages. For grooming, animals were scored every 15 s for a period of 50 min according to the procedure of Gispen et al. (1975), starting 10 min after α-MSH (or saline) administration. The observer noted whether or not one of the behavioral components of the grooming act was displayed. If so, a positive score was given for the time interval, so that a maximum of 200 positive grooming scores could be obtained. For stretching, yawning and penile erections, animals were observed continuously for 60 min, again starting 10 min after treatment. Each stretch, yawn and penile erection (spontaneously occurring, not elicited by genital grooming) was scored (Bertolini et al., 1988). Grooming, stretching, yawning and penile erections were scored in the same animals and in the same test by two observers unaware of the treatment. Experiments were performed with non-paired groups, each rat being tested only once, and the behavioral experiment was performed 5–7 days after implantation of the i.c.v. cannulae.

2.4. Microstructural analysis of animal behavior

All the experiments took place in the home cage of the rat between 0900 and 1400 h. α-MSH, HS014 or equivolume saline was injected i.c.v. after an 18 h fast. The animals were replaced in their home cages immediately thereafter, pre-weighed food pellets were provided 10 min later and the behavior of each animal was then recorded for 60 min. Behavior was categorized under five headings (Blundell, 1986). (1) Feeding: biting, gnawing or swallow-

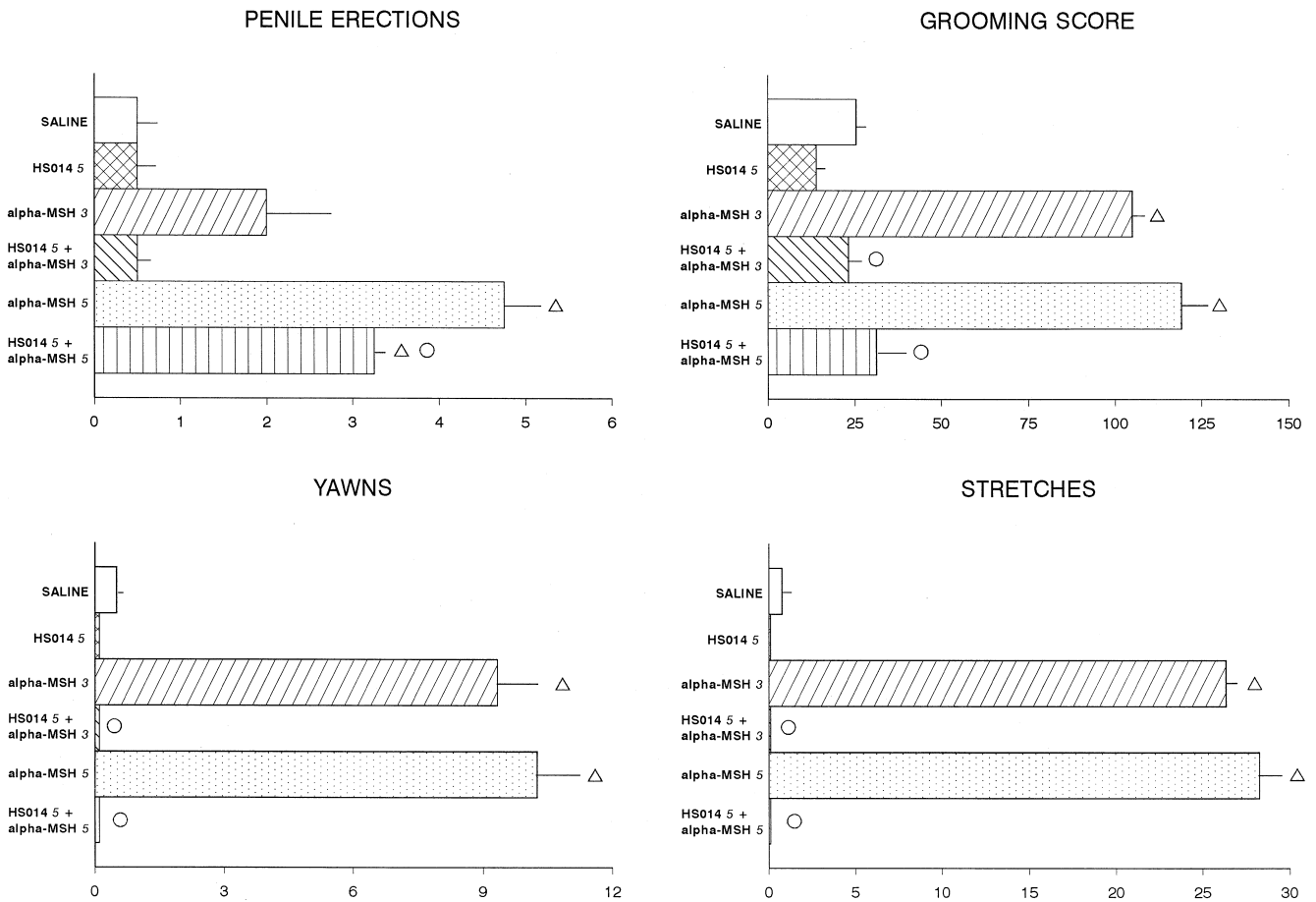

Fig. 1. Influence of HS014 on α-MSH-induced behavioral syndrome in Wistar rats. The peptides were i.c.v. administered at the doses indicated in italic (μg/rat). Grooming behavior was registered for 50 min, starting 10 min after treatment and scored every 15 s (maximum possible score: 200). Penile erections, yawns and stretches were continuously recorded for 60 min, starting 10 min after treatment. Δ $P < 0.05$ vs. Saline; ○ $P < 0.05$ vs. α-MSH at the corresponding dose level (ANOVA followed by Student–Newmann–Keuls test).

ing of fragments of chow pellets; (2) drinking: licking at water bottle spout; (3) grooming: scratching, washing or licking of coat and paws; (4) locomotion/exploration: standing looking at or sniffing the environment, movements involving all four limbs; and (5) resting: standing, sitting or lying, sleeping with occasional change of position. The time spent on each behavior and the number of episodes, defined as period lasting at least 30 s, for each element were recorded.

2.5. Statistics

For each evaluated parameter, data were submitted to an overall analysis of variance followed by the Student–

Table 1
Influence of intracerebroventricular injection of α-MSH (2 μg/rat, i.c.v.), or of HS014 (10 μg/rat, i.c.v.), or both, on the amount of food ingested

Treatment	Food intake 1 h (g/100 g BW)	Food intake 2 h (g/100 g BW)	Food intake 3 h (g/100 g BW)
Saline	1.87 ± 0.16	2.12 ± 0.13	2.59 ± 0.25
α-MSH	1.23 ± 0.12[a]	1.62 ± 0.08[a]	1.87 ± 0.16
HS014	2.18 ± 0.36	3.02 ± 0.32[a]	3.26 ± 0.25
α-MSH + HS014	1.61 ± 0.14	2.42 ± 0.20	2.63 ± 0.28

Values are means ± SEM. $N = 8–10$ per group. Data were analyzed by means of ANOVA followed by Student–Newmann–Keuls test for individual comparisons.
[a] $P < 0.05$ vs. Saline.
The Wistar rats had a permanent stainless steel cannula in a lateral ventricle and had been starved for 18 h.

Table 2
Behavioral profile following intracerebroventricular injection of α-MSH (2 μg/rat, i.c.v.), or of HS014 (10 μg/rat, i.c.v.), or both

Treatment	Feeding time (s)	Feeding episodes	Drinking time (s)	Drinking episodes	Grooming time (s)	Grooming episodes	Locomotion/exploration time (s)	Locomotion/exploration episodes	Resting time (s)	Resting episodes
Saline	1319.3 ± 162.1	7.5 ± 0.7	252.8 ± 39.4	4.0 ± 1.1	174.2 ± 54.6	1.7 ± 0.2	959.8 ± 200.0	10.0 ± 1.5	893.8 ± 163.1	1.7 ± 0.5
α-MSH	646.1 ± 72.3[a]	5.6 ± 0.9	110.1 ± 14.9[a]	1.9 ± 0.3	1287.5 ± 316.3[a]	5.5 ± 0.1[a]	1159.7 ± 226.9	12.2 ± 1.6	403.3 ± 133.7	2.0 ± 0.7
HS014	2087.7 ± 179.6[a]	7.0 ± 0.5	279.7 ± 31.9	3.7 ± 0.6	128.7 ± 34.6	2.2 ± 0.3	582.5 ± 98.2	5.5 ± 0.7	521.2 ± 114.2	2.2 ± 0.3
α-MSH + HS014	1846.0 ± 144.9	6.8 ± 1.3	144.4 ± 47.6[a]	1.7 ± 0.6	119.0 ± 46.6	1.9 ± 0.7	754.7 ± 114.4	8.4 ± 2.1	735.2 ± 270.6	3.3 ± 0.6

Values are means ± SEM. $N = 8–10$ per group. Data were analyzed by means of ANOVA followed by Student–Newmann–Keuls test for individual comparisons.
[a] $P < 0.05$ vs. Saline.
Rats starved for 18 h were observed continuously for a period of 60 min.

Newmann–Keuls test for individual comparisons between groups, when F values indicated a significant difference among treatments.

2.6. Animal ethics

Experimental procedures were carried out in accordance with the guidelines of the European Community, local laws and policies (D.L.vo 116/92).

3. Results

3.1. Influence of HS014 on α-MSH-induced behavioral syndrome

We injected α-MSH and HS014 i.c.v. in fed ad libitum rats, and scored grooming, penile erections, yawning and stretching. Data are shown in Fig. 1. As expected, α-MSH significantly increased the number of yawning [$F(5,42) = 21.6$; $P < 0.001$] and stretching episodes [$F(5,42) = 461.85$; $P < 0.001$] and the grooming score [$F(5,42) = 60.75$; $P < 0.001$] at both the doses tested (3 and 5 μg/rat) while the number of penile erections was increased [$F(5,42) = 13.02$; $P < 0.001$] only at the highest dose. HS014 (5 μg/rat) did not affect behavior by itself, but it completely prevented the α-MSH-induced yawning, stretching and excessive grooming. However, penile erections induced by α-MSH were only partially blocked by HS014: the mean value of the group treated with both peptides (5 + 5 μg/rat) was significantly different from that of both the saline and the α-MSH treated groups.

3.2. Influence of HS014 and α-MSH on microstructural analysis of animal behavior

We injected i.c.v. α-MSH and the melanocortin MC_4 receptor selective antagonist HS014 in rats after 18 h of starvation. Animal behavior was categorized into five activities: feeding, drinking, grooming, locomotion/exploration and resting. The total time and the number of episodes of each of the behavioral elements were scored during the first hour after the treatment. The food intake data, given in Table 1, show the cumulative food intake 1 h [$F(3,33) = 3.22$; $P = 0.035$], 2 h [$F(3,33) = 7.56$; $P < 0.001$] and 3 h [$F(3,33) = 4.84$; $P = 0.007$] after treatment. As seen from the table, α-MSH (2 μg/rat) significantly reduced food intake after 1 and 2 h, whereas HS014 (10 μg/rat) significantly increased food intake after 2 h. Food intake was not affected in comparison to that of controls when α-MSH and HS014 were given simultaneously (Table 1).

Data on the microstructural analysis of animal behavior are shown in Table 2. α-MSH significantly reduced the feeding time [$F(3,33) = 16.76$; $P < 0.001$] by approximately 50%, whereas HS014 significantly increased it. When the peptides were given together, the feeding time was similar to that of controls. Thus, HS014 antagonized the anorectic effect of α-MSH on both food intake and feeding time. However, the number of feeding episodes was not different after treatment with any of the peptides. α-MSH significantly reduced the drinking time, whereas drinking was not affected by HS014. When α-MSH and HS014 were administered together, the drinking time was significantly reduced in comparison to that of controls [$F(3,33) = 4.63$; $P = 0.008$]. Thus, HS014 did not antagonize the inhibitory effect of α-MSH on drinking. As expected, α-MSH significantly increased both the grooming time (which reached a value 7-fold higher than of controls) [$F(3,33) = 14.77$; $P < 0.001$] and the number of grooming episodes [$F(3,33) = 16.35$; $P < 0.001$]. HS014 did not affect grooming behavior by itself, but completely abolished the grooming induced by α-MSH. Neither locomotion/exploration nor resting time was significantly affected by the injection of both α-MSH and HS014.

4. Discussion

The melanocortin peptides exert as do many other neuropeptides, a broad array of effects whose underlying mechanisms are not fully understood. It has been known for a long time that multiple melanocortin receptors may be involved in mediating the various central effects of melanocortins. However, the cloning and localization of distinct melanocortin receptor subtypes in the CNS (the melanocortin MC_3 and MC_4 receptors are the melanocortin receptors which are most abundantly expressed in the brain, for review see Mountjoy and Wong, 1997) have provided new targets which can be pharmacologically assessed by using specific selective substances.

The first reports of the anorectic effect of melanocortin peptides (ACTH) came already in the '80s (Poggioli et al., 1986; Vergoni et al., 1986, 1990). Later, it was found that the agouti protein is a melanocortin receptor antagonist (Lu et al., 1994). Since the ectopic over-expression of the agouti protein causes obesity syndrome in mice, the link became more evident. Recent data, showing that 'knock-out' of the melanocortin MC_4 receptor causes obesity in mice (Huszar et al., 1997) with characteristics similar to those found in the agouti mice, clearly indicated that melanocortinergic neurons exert a tonic inhibition of feeding behavior involving the melanocortin MC_4 receptor. Our present data show that α-MSH can reduce food intake in fasting rats and that the selective melanocortin MC_4 receptor antagonist HS014 increases food intake. Moreover, HS014 antagonized the anorectic effect of α-MSH on feeding behavior when the peptides were administered together. The effects were more evident 2 h after the rats had access to food. However, no significant effects were seen after 3 h, perhaps because rats more or less stopped eating after 2 h of intense feeding. We have previously shown that HS014 is a potent antagonist in vitro as evi-

denced in assays for recombinant melanocortin MC_4 receptors. The orexigenic effects of HS014 are thus conceivably due to its antagonistic action on the melanocortin MC_4 receptor. The results are also in line with earlier data showing that SHU9119 reduces food intake in fasting mice (Fan et al., 1997) and that HS014 increases food intake in free-feeding rats (Kask et al., 1998). It is interesting to note that α-MSH is a potent inducer of anorexia despite its relatively low affinity for the melanocortin MC_4 receptor subtype (Schiöth et al., 1996). This report is, to the best of our knowledge, the first that shows a direct anorexic effect of α-MSH. ACTH, in particular ACTH-(1–24), commonly used as a ACTH-(1–39) substitute, has activity similar to that of α-MSH for the MC_4 receptor in vitro (Adan et al., 1994a; Schiöth et al., 1996, 1997a). Taken together, these data indicate that some aspects of the so-called ACTH-induced syndrome (Bertolini et al., 1988) can be mediated by the melanocortin MC_4 receptor.

Changes in food intake are often associated with changes in drinking behavior; for example, neuropeptide Y-induced feeding is associated with increased drinking (Stanley and Leibowitz, 1984). Our results show that α-MSH significantly decreased the drinking time and reduced the number of drinking episodes. HS014 did not affect drinking. Moreover, very interestingly, HS014 did not antagonize the reduced drinking behavior induced by α-MSH. These data may indicate that the decrease in drinking behavior induced by α-MSH could be mediated by a melanocortin receptor other than the melanocortin MC_4 receptor.

It is well-known that grooming behavior (Spruijt et al., 1985) is induced by central administration of melanocortin peptides including both α-MSH and ACTH. Our data show that α-MSH is a potent inducer of grooming behavior in both starved and non-starved rats. Interestingly, the effect of α-MSH was even more pronounced in the starving rats. HS014 completely antagonized the α-MSH-induced excessive grooming. An earlier report concerning an ACTH-(4–10) analogue, a low-affinity melanocortin MC_4 receptor antagonist, suggested that grooming behavior in rats is mediated by the melanocortin MC_4 receptor (Adan et al., 1994b). Our data provide further evidence that grooming behavior is indeed mediated by this receptor.

Stretching and yawning are also a well-known feature of the behavioral syndrome elicited by melanocortin peptides (Ferrari et al., 1963). Our data confirm that α-MSH is a potent inducer of both yawning and stretching. This effect of α-MSH was completely antagonized by HS014. Our data indicate that the melanocortin MC_4 receptor mediates stretching and yawning behavior. It is thus possible that selective melanocortin MC_4 receptor agonists, which could be developed for use in the treatment of pathological obesity, might as a side effect unavoidably induce grooming, stretching and yawning behavior, at least in rodents. At present, it is not clear whether activation of the MC_4 receptor in humans would result in similar effects.

Induction of penile erections is a well-known behavioral phenomenon induced by melanocortin peptides. Our data on α-MSH are in line with earlier observations. In contrast to grooming, stretching and yawning behavior, the penile erections induced by α-MSH were not antagonized by HS014. These data quite clearly indicate that penile erections are probably mediated by a receptor other than the melanocortin MC_4 receptor. This other receptor could perhaps be the melanocortin MC_3 receptor, because the melanocortin MC_3 receptors besides the melanocortin MC_4 receptors are the most abundantly expressed melanocortin receptors in the CNS. However, although HS014 is selective for the melanocortin MC_4 receptor it also binds and effectively blocks the response to α-MSH in melanocortin MC_3 receptor-transfected cells in vitro (Schiöth et al., 1998). The slightly lower number of penile erections observed in rats which were treated with both HS014 and α-MSH, compared to those that got α-MSH, may be due to blockade of the melanocortin MC_3 receptor.

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