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GABAergic agents prevent alpha-melanocyte stimulating hormone induced anxiety and anorexia in rats

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Abstract

Alpha-melanocyte stimulating hormone (α-MSH) is a hypothalamic peptide believed to play a tonic inhibitory role in feeding and energy homeostasis. Systemic administration of α-MSH is known to produce anorexia and anxiety. Since synaptic contacts between gamma-aminobutyric acid (GABA)ergic terminals and α-MSH neurons in the hypothalamus have been reported, the present work was undertaken to refine our knowledge on the role of GABAergic systems in anxiety and anorexia induced by intracerebroventricular (icv) administration of α-MSH in rats. The anxiety was assessed by elevated plus maze, and spontaneous food consumption was monitored during dark cycle. Prior administration of diazepam and muscimol that promote the function of $GABA_A$ receptors reversed the anxiogenic response and decreased food intake elicited by α-MSH. In contrast, bicuculline, the $GABA_A$ receptor antagonist, not only enhanced the effects of α-MSH but also prevented the influence of GABAergic drugs on α-MSH-induced anorexia and anxiety. These findings suggest that α-MSH-induced anxiety and anorexia are due to its negative influence on GABAergic system.

Keywords: Alpha-melanocyte stimulating hormone; Diazepam; Anxiety; Anorexia; $GABA_A$ receptor complex; Muscimol; Bicuculline

1. Introduction

Alpha-melanocyte stimulating hormone (α-MSH) is a neuropeptide derived from a multifunctional precursor protein, proopiomelanocortin (POMC) (Fan et al., 1997; Grill et al., 1998). Neurons localized in the arcuate nucleus (ARC) are rich in POMC peptide (Bergendahl et al., 1992; Tatro, 1996) and send projections to several brain areas including the paraventricular nucleus (PVN) and dorsomedial and ventromedial hypothalamus (DMH and VMH) that are involved in the regulation of food intake (Mountjoy and Wong, 1997; Harrold et al., 1999). α-MSH is known to play a key role in the regulation of feeding and energy storage (Poggioli et al., 1986; Fan et al., 1997). Central administration of α-MSH and its synthetic analogues suppress eating response and reverse positive energy balance in rodents (Fan et al., 1997; Grill et al., 1998). The receptors that mediate the effect of α-MSH and related melanocortins (MCs) have been identified and cloned (Chhajlani et al., 1993; Gantz et al., 1993a,b). Five melanocortin receptors (MC1–MC5) are currently recognized. They are widely distributed in peripheral tissues and the brain (Mountjoy et al., 1992; Adan et al., 1994). Recently, a body of evidence suggested that the inhibitory effect of α-MSH on feeding is mediated by activation of central MC4 receptors (Harrold et al., 1999; Marsh et al., 1999). Intracerebroventricularly (icv) administered MC4 receptor antagonists (Kask et al., 1999; Benoit et al., 2000) or targeted disruption of MC4 receptor in mice (Marsh et al., 1999; Chen et al., 2000) or humans (Krude et al., 1998; Vaisse et al., 1998) resulted in hyperphagia and obesity. The application of obese phenotype MC4-R knockout mice strongly supported this view (Huszar et al., 1997). Thus, melanocortinergic neurons exert a tonic

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inhibitory effect on feeding behavior and inhibit neuropeptide-Y-induced feeding (Brown et al., 1998; Hansen and Morris, 2002).

In addition to the above, systemic administration of α-MSH has strong anxiogenic effect in rodents (Gonzalez et al., 1996; Vecsernyes et al., 2000). However, the involvement of MC4-R in this action is still debated. The MC4-R antagonists attenuated the stress-induced anorexia (Vergoni et al., 1999), anxiety and depression (Chaki et al., 2003) but not α-MSH-induced anxiety (Kask et al., 1998). Moreover, the anxiogenic effect of α-MSH, but not anorectic effect, was reportedly mediated by corticotropin-releasing factor (CRF) (Vergoni et al., 1999; Vecsernyes et al., 2000).

Benzodiazepines and drugs such as muscimol that facilitate GABAergic neurotransmission are known to exert several actions including anxiolysis, sedation and hypnosis, muscle relaxation and stimulation of food intake (Wise and Dawson, 1974; Crawley and Goodwin, 1980; File, 1982; Stratford and Kelley, 1997; Zarrindast et al., 2001). GABA is coexpressed in subpopulations of POMC neurons in ARC (Naftolin et al., 1990), and typical synaptic contacts have also been observed between GABAergic terminals and α-MSH neurons in the hypothalamus (Vincent et al., 1982; Naftolin et al., 1990; Cowley et al., 2001). It has been shown that the endogenous GABA and/or ligands for central benzodiazepine receptors exert negative control on α-MSH neurons (Delbende et al., 1989).

In view of the above, we hypothesize that the anxiety and anorexia displayed by α-MSH may involve interaction with GABA component at the level of the hypothalamus. To test the hypothesis, we administered GABAergic drugs before α-MSH and assessed the anxiety by elevated plus maze and also monitored the spontaneous food intake in rats during dark cycle.

2. Materials and methods

2.1. Subjects

Adult male Sprague–Dawley rats weighing 220–250 g were group housed in polypropylene cages in a temperature- (25 ± 2 °C) and light- (12:12 light:dark cycle, light on at 0700 h) controlled room. However, after icv cannulation and during experiments they were housed individually. They had free access to food (Hindustan Lever, India) and tap water. All experimental protocols were approved by the Internal Ethical Committee for the Purpose of Control and Supervision of Experimental Animals, Ministry of Environment and Forests, Government of India, New Delhi.

2.2. Drugs

α-MSH and muscimol (Sigma, St. Louis, MO) were diluted in artificial cerebrospinal fluid (aCSF) of the following composition (140 mM NaCl, 3.35 mM KCl, 1.15 mM $MgCl_2$, 1.26 mM $CaCl_2$, 1.2 mM Na_2HPO_4, and 0.3 mM NaH_2PO_4, pH 7.4) containing 0.05% bovine serum albumin and injected by the icv route, while diazepam (Sigma) was wetted with 0.5% Tween 80 and uniformly dispersed in saline and injected intraperitoneally (ip). All other drugs including bicuculline (Sigma) were dissolved in saline and injected by ip route.

2.3. Intracerebroventricular administration

Rats were anesthetized with pentobarbitone sodium (60 mg/kg ip) and fixed in a stereotaxic frame. A permanent 22-gauge stainless steel guide cannula (C313G/Spc) (Plastics One, Roanoke, VA) was placed aseptically into the right lateral ventricle (posterior −0.8 mm; lateral from midline +1.2 mm and ventral −3.5 mm, relative to bregma) (Paxinos and Watson, 1998). The guide cannula was secured to the skull using stainless steel mounting screws (Plastics One) and dental cement (Dental Products of India, Mumbai). A 28-gauge stainless steel dummy cannula was used to occlude the guide cannula when not in use. Following surgery, the animals were housed individually to avoid damage to the guide and dummy cannulae. The rats were then allowed to recover for 7 days, during which they were habituated to the experimental protocol to minimize non-specific stress. Intracerebroventricular injections were made using a Hamilton microliter syringe (Hamilton, Nevada) connected by PE-10 polyethylene tubing to a 28-gauge stainless steel internal cannula that extended 0.5 mm below the guide cannula. A volume of 5 μl was injected over a period of 1 min.

At the end of all icv experiments, dilute India ink was injected through the cannula and animals were killed immediately by anaesthetic overdose. The data of only those animals showing uniform distribution of ink into the ventricles were used for statistical analysis. Separate groups of rats were used to measure anxiety and anorexia.

2.4. Spontaneous food intake

Rats fitted with icv cannulae were adapted to eating pelleted food for 7 days to obtain stable baseline intakes. Thereafter, different groups of animals ($n = 5$) were injected 5 μl aCSF icv or 0.2–5 μg α-MSH icv 2 h before the end of the light period; at this time the animal shows its peak feeding activity (Brown et al., 1998). Fresh preweighed food was offered at the beginning and food intake was determined at 2, 4 and 6 h postinjection by weighing the leftover food. Other groups of cannulated rats were administered (1) muscimol (25 ng/rat icv), (2) diazepam (0.5 mg/kg ip), (3) bicuculline (1 mg/kg ip) followed by diazepam (0.5 mg/kg ip), and (4) bicuculline (1 mg/kg ip) followed by muscimol (25 ng/rat icv). Thirty minutes after these respective treatments, all the animals were given α-MSH (1 μg/rat icv) treatment. Separate control groups treated with aCSF and/or α-MSH were also given saline by ip route. Addi-

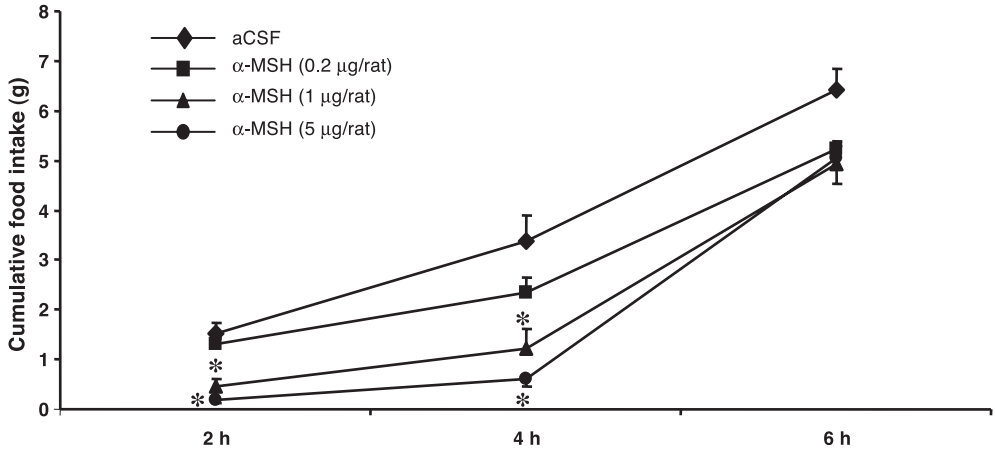

Fig. 1. Cumulative food intake (g) over a period of 6 h in rats treated icv with aCSF (5 μl/rat) and α-MSH 2 h before the start of the dark cycle. Each bar is the mean ± S.E.M. for six rats. $*P<.01$ compared with aCSF-treated rats.

tional group of animals received only bicuculline (1 mg/kg ip) 30 min before subeffective dose of α-MSH (0.2 μg/rat icv). Food intake in all the groups was monitored at the time points mentioned above.

2.5. Elevated plus maze test

A separate group of rats was used to measure anxiety. The apparatus elevated plus maze was made of Plexiglas painted black and consisted of four arms 50 cm long and 10 cm wide. Two opposite arms had walls (38 cm high) on either side (closed arms), while the other two arms were devoid of such walls (open arms). Two open and two closed arms were connected by a central platform (10 × 10 cm) thus making a plus sign. The apparatus was placed 60 cm above the floor. During the experiments, the plus maze was illuminated by a 100-W lamp, which was fixed 2 m above the maze floor. Experiments were conducted using a treatment protocol described above for food intake and the tests took place 1 h after the injection of α-MSH. The rat was placed on the central platform of the maze head facing the open arm. An entry was registered only when all of the four paws of the animal were put on arm. The time spent and number of entries in each arm over a 5-min period were

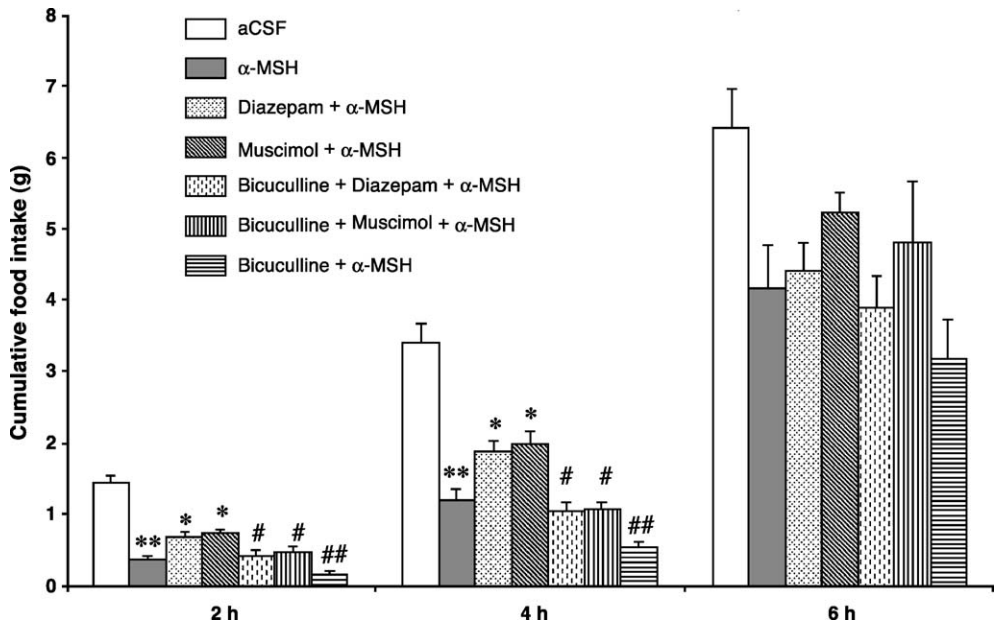

Fig. 2. Effect of α-MSH (1 μg/rat icv) on cumulative food intake (g) over a period of 6 h and its modification by diazepam (0.5 mg/kg ip) or muscimol (25 ng/rat icv) and bicuculline (1 mg/kg ip). α-MSH (1 μg/rat) was injected icv 2 h before starting the dark cycle while diazepam (0.5 mg/kg ip), muscimol (25 ng/rat icv), bicuculline (1 mg/kg ip) + diazepam (0.5 mg/kg ip) and bicuculline (1 mg/kg ip) + muscimol (25 ng/rat icv) were injected 30 min before α-MSH (1 μg/rat icv). Bicuculline (1 mg/kg ip)-treated animals were administered with α-MSH (0.2 μg/rat icv). Food intake was measured at 2, 4 and 6 h post α-MSH injection. Each bar represents the mean ± S.E.M. for five rats. $*P<.05$ vs. α-MSH, $**P<.01$ vs. aCSF, $^{\#}P<.05$ vs. diazepam + α-MSH and muscimol + α-MSH, $^{\#\#}P<.05$ vs. α-MSH.

recorded manually. Separate groups of animals were used for each treatment and each subject tested in this procedure was given a single 5-min trial.

2.6. Data analysis

The data are presented as mean ± S.E.M. Statistical significance was determined by one way analysis of variance (ANOVA) followed by post hoc Student–Newman–Keuls test. Differences were considered significant at $P < .05$.

3. Results

3.1. Effect on spontaneous food intake

Administration of α-MSH resulted in a dose and time dependent inhibition of food intake in free feeding rats. Fig. 1 shows the 2, 4, 6 h food intake all that varied as a function of time and dose. α-MSH (1.0 and 5.0 μg icv) significantly inhibited food intake at 2 and 4 h [$F(3,23) = 18.6$, $P < .0001$ and $F(3,23) = 11.3$, $P < .0001$]. The doses lower than 1 μg of α-MSH failed to exhibit any significant effect. As compared with vehicle-treated rats, icv administration of 1 μg of α-MSH suppressed the food intake by 75% (1.449 ± 0.08 vs. 0.361 ± 0.06 g; $P < .01$) at 2 h postinjection. This effect was evident up to 4 h (reduced by 65%) postinjection, after which it tends to recover to that of vehicle control groups (6.428 ± 0.55 vs. 4.194 ± 0.59 g; $P > .05$). Prior administration of diazepam (0.5 mg/kg ip) or muscimol (25 ng/rat icv) reversed the α-MSH-induced suppression of food intake at 2 h [$F(6,34) = 35.1$, $P < .0001$] and 4 h [$F(6,34) = 32.0$, $P < .0001$] (Fig. 2). However, administration of bicuculline (1 mg/kg ip) before diazepam (0.5 mg/kg ip) plus α-MSH (1 μg/rat icv) or muscimol (25 ng/rat icv) plus α-MSH (1 μg/rat icv) showed feeding comparable to that of α-MSH alone at all the time points (2, 4 and 6 h). Thus, diazepam and muscimol in the presence of bicuculline could not reverse the α-MSH-induced suppression of food intake. On the other hand, administration of bicuculline (1 mg/kg ip)

Table 1
Effect of α-MSH and GABAergic agents in elevated plus maze in rats

Treatment	Percent time in open arms	Percent open-arm entries	Number of closed-arm entries
aCSF/saline	20.54 ± 1.45	36.31 ± 3.27	3.33 ± 0.21
α-MSH (0.2 μg icv)	18.45 ± 1.31	31.24 ± 3.50	3.00 ± 0.36
α-MSH (1 μg icv)	14.75 ± 1.75 *	20.33 ± 2.49 *	4.16 ± 0.47
α-MSH (5 μg icv)	13.21 ± 1.40 *	17.71 ± 3.00 *	3.66 ± 0.42
Bicuculline (1 mg/kg ip)	16.24 ± 1.80	30.92 ± 2.96	2.83 ± 0.47
Diazepam (0.5 mg/kg ip)	24.13 ± 1.56	39.99 ± 2.84	4.00 ± 0.51
Muscimol (25 ng icv)	22.45 ± 1.32	38.23 ± 2.77	3.83 ± 0.65

Percent time in open arms and percent open-arm entries calculated from 5-min trial period. The data represent the mean ± S.E.M. for six rats in each group.
* $P < .05$ vs. aCSF/saline.

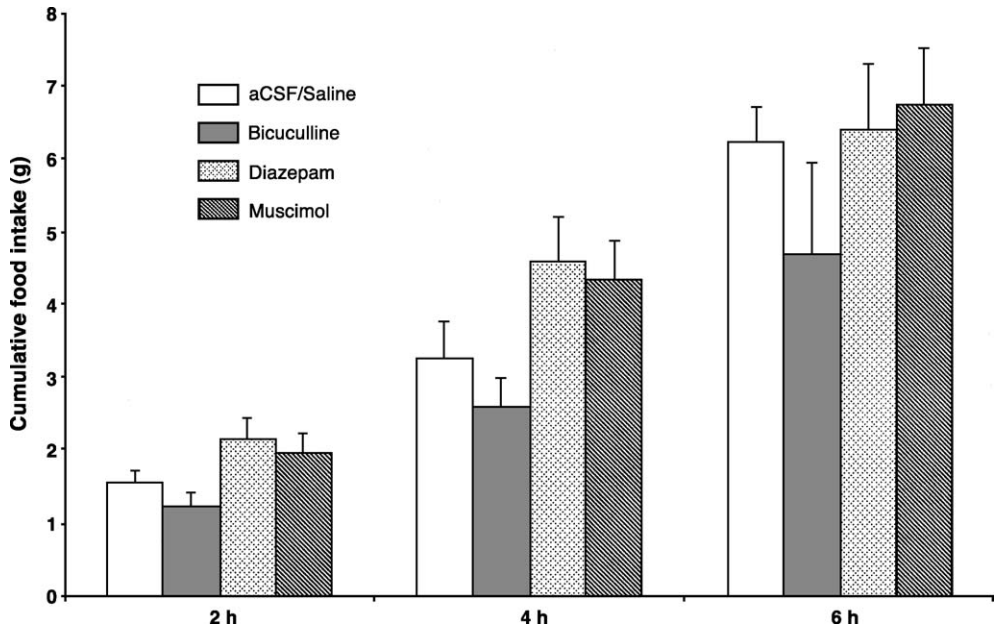

Fig. 3. Effect of aCSF (5 μl/rat icv), bicuculline (1 mg/kg ip), diazepam (0.5 mg/kg ip) and muscimol (25 ng/rat icv) on cumulative food intake (g) over a period of 6 h. Each treatment was given 30 min before vehicle/aCSF (ip/icv) injected 2 h before starting of the dark cycle. Each bar is the mean ± S.E.M. for six rats. All changes are insignificant, $P > .05$ as compared to aCSF/saline at respective time points.

before α-MSH (0.2 μg/rat icv) at subeffective doses showed greater inhibition of food intake (Fig. 2). Administration of bicuculline (1 mg/kg ip), diazepam (0.5 mg/kg ip) and muscimol (25 ng/rat icv) alone at the doses used did not significantly affect normal food intake up to 6 h duration [$F(3,23)=3.17$, $P>.05$] (Fig. 3).

3.2. Effect in the elevated plus maze test

Table 1 shows that administration of α-MSH (1–5 μg/rat icv) significantly decreased the percent time spent

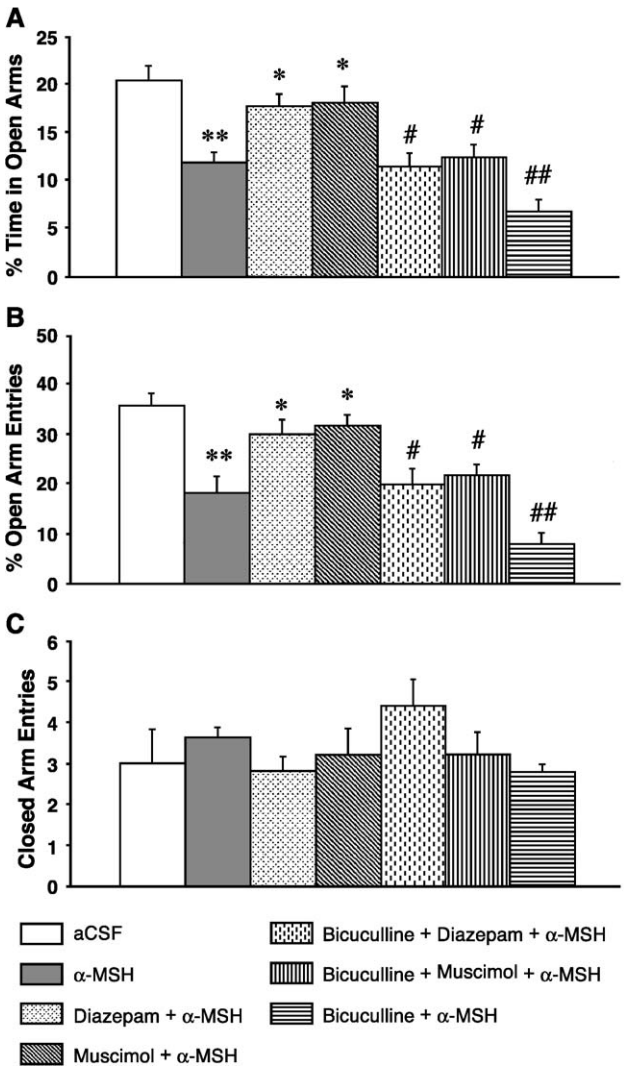

Fig. 4. Effect of α-MSH (1 μg/rat icv) on exploration for 5 min in the elevated plus maze showing (A) percent time spent in open arms, (B) percent open-arm entries and (C) the number of closed-arm entries. Rats were injected with vehicle or α-MSH (1 μg/rat icv) and observations were made at 1 h. Different groups of rats were administered with diazepam (0.5 mg/kg ip), muscimol (25 ng/rat icv), bicuculline (1 mg/kg ip) + diazepam (0.5 mg/kg ip) or bicuculline (1 mg/kg ip) + muscimol (25 ng/rat icv) 30 min before α-MSH (1 μg/rat icv). Separate group of animals was used for bicuculline (1 mg/kg ip) + α-MSH (0.2 μg/rat icv) treatment. Each bar is the mean ± S.E.M. for five rats. $^{*}P<.05$ vs. α-MSH, $^{**}P<.01$ vs. aCSF, $^{\#}P<.05$ vs. diazepam + α-MSH and muscimol + α-MSH, $^{\#\#}P<.05$ vs. α-MSH.

[$F(3,23)=5.05$, $P<.009$] and percent entries into the open arms [$F(3,23)=8.16$, $P<.001$] than those shown by the vehicle-treated group. The lower dose, 0.2 μg, was devoid of any significant effect ($P>.05$).

This anxiogenic effect of α-MSH (1 μg/rat icv) was reversed (Fig. 4A, B) by prior administration of diazepam (0.5 mg/kg ip) or muscimol (25 ng/rat icv); an increase of 49% and 52%, respectively, was observed in the percent open-arm time [$F(6,34)=11.8$, $P<.0001$]. Percent open-arm entries also showed an increase of 66% for diazepam or 77% for muscimol [$F(6,34)=11.4$, $P<.0001$].

Furthermore, bicuculline (1 mg/kg ip) not only prevented the reversal of the effect produced by diazepam and muscimol, but greatly enhanced the anxiogenic effect of α-MSH (0.2 μg/rat icv). The closed-arm entries in all the treatment groups were unaffected [$F(6,34)=0.89$, $P>.05$] (Fig. 4C). At the given doses, bicuculline, diazepam and muscimol, when administered alone, did not produce any significant effect on percent time spent and percent entries on open arms in elevated plus maze test [$F(3,23)=5.20$, $P>.05$ and $F(3,23)=1.74$, $P>.05$] (Table 1).

4. Discussion

The present study replicates earlier findings on dose-related anxiety-like effects in the elevated plus maze test (Gonzalez et al., 1996; Vecsernyes et al., 2000) and inhibition of spontaneous food intake following α-MSH icv administration in rats (Fan et al., 1997; Brown et al., 1998, Vergoni and Bertolini, 2000). In the elevated plus maze, a test based on the natural aversion of rodents to open spaces, α-MSH decreased the percent time spent in open arms. Although several studies support the involvement of MC4 receptors (Poggioli et al., 1986; Vergoni and Bertolini, 2000; Chaki et al., 2003) and CRF (Vecsernyes et al., 2000) in regulation of food intake and/or anxiety, the contribution of other brain neurotransmitters such as dopamine, acetylcholine, GABA or serotonin in the α-MSH-induced behaviors has never been excluded. An interesting finding of our study is that both the anxiety-like and anorexic effects of α-MSH are counteracted by drugs that promote the function of $GABA_A$ receptors, either directly (muscimol) or indirectly (diazepam). The benzodiazepine diazepam, a clinically effective antianxiety agent has been shown to antagonize α-MSH-induced behavior like excessive grooming, locomotion, rearing and stretching/yawning syndrome (Cremer et al., 1995). This indicates that α-MSHergic transmission might be closely coupled to GABAergic system. It has been proposed that two inhibitory factors, GABA and NPY regulate α-MSH production in rat hypothalamus (Delbende et al., 1989; Blasquez et al., 1992, 1995). The endogenous GABA and/or ligands for central benzodiazepine receptors exert negative control on α-MSH neurons (Mabley et al., 1991). A quantitative analysis has shown that GABA-containing terminals account for about half of the total

synaptic inputs in different nuclei of the medial hypothalamus including ARC (Decavel and Van Den Pol, 1991). While a direct innervation of POMC containing neurons by GABAergic afferents has been demonstrated in rat ARC (Naftolin et al., 1990), the same were shown to possess $GABA_A$ receptors (Blasquez et al., 1994). GABA has been reported to participate in the stimulation of feeding in the rat (Olgiati et al., 1980) and benzodiazepines, which promote the Cl^- ion channel openings by $GABA_A$ receptor activation (Obata and Yamamura, 1986), increased food intake (Pecina and Berridge, 1996). While infusions of anxiolytic agents like GABA (Olgiati et al., 1980), muscimol (Tsujii and Bray, 1991; Stratford and Kelley, 1997) or benzodiazepines (Pecina and Berridge, 1996; Higgs and Cooper, 1996) in VMH, elicit eating, the $GABA_A$ receptor antagonist, bicuculline, has been reported to decrease the food intake (Hansen and Ferreira, 1986). In the present study, diazepam (0.5 mg/kg ip) and muscimol (25 ng/rat icv) per se had no effect on spontaneous food intake or exploratory behavior in elevated plus maze. However, these drugs seem to reverse the α-MSH-induced suppression of food intake and the percent open-arm entries and percent time spent in the open arms. Since the closed-arm entries displayed by rats of various groups were in a similar range, the anxiogenic profile of α-MSH and its antagonism with muscimol or diazepam cannot be interpreted as the changes in general locomotor activity.

The hypothalamus is a major center involved in feeding regulation and other physiological functions. The release of α-MSH in hypothalamus is tonically inhibited by GABAergic drugs, and this effect of endogenous GABA is counteracted by bicuculline (Delbende et al., 1989; Mabley et al., 1991), and positively modulated by benzodiazepines (Mabley et al., 1991; Blasquez et al., 1991). In our study, bicuculline abolished the influence of muscimol and benzodiazepine on α-MSH-induced behaviors. Furthermore, at subeffective dose, the α-MSH (0.2 μg/rat icv) exhibited much greater anxiogenic-like and anorectic effects in bicuculline-treated (1 mg/kg ip) animals. Interestingly, this effect was even more pronounced than 1 μg of α-MSH given alone. This further supports our view that α-MSH through inhibition of $GABA_A$ and/or benzodiazepine receptors induces anxiety and anorexia.

Since $GABA_A$ receptors are present in the neuronal circuits involved in the α-MSH-induced behaviors (Naftolin et al., 1990), it is concluded that α-MSH may exert negative influence on GABAergic activity, thus leading to both the anxiogenic-like and anorectic effects. However, the impact of down-regulation of GABAergic systems or $GABA_A$ receptor on other mediators of α-MSH-induced behaviors like CRF (Vecsernyes et al., 2000), MC4-R (Poggioli et al., 1986; Vergoni and Bertolini, 2000) and vice versa remains to be clarified. The present results provide a basis for further investigation aimed at understanding the interaction between these mediators in the control of food intake and anxiety.

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