

Comparison of Structural Requirements of α -MSH and ACTH for Inducing Excessive Grooming and Pigment Dispersion

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SPRUIJT, B. M., P. N. E. DE GRAAN, A. N. EBERLE AND W. H. GISPEN. *Comparison of structural requirements of α -MSH and ACTH for inducing excessive grooming and pigment dispersion.* PEPTIDES 6(6) 1185-1189, 1985.— α -MSH and ACTH-like peptides are known to play an important role in the adaptation of many vertebrates to a new environment. These peptides induce pigment dispersion in amphibian melanophores through a receptor-mediated mechanism. In this study we compared the structural requirements of these peptides for melanotropic activity on *Xenopus laevis* melanophores with those for inducing excessive grooming in the rat. With the exception of ACTH₁₋₂₄ there is a close resemblance in structure-activity relationships of the fragments and analogs tested in the two bioassays. [Nle⁴,D-Phe⁷]- α -MSH is extremely active in both assays. Weak agonists such as [Leu⁹]- α -MSH did not possess antagonistic properties either in the melanophore assay or in the excessive grooming test. The data suggest that the mechanism of action of α -MSH-like peptides in rat brain is receptor-mediated like their action on melanophores.

α -MSH receptors Melanotropins Pigment dispersion Excessive grooming Structure-activity

PEPTIDES derived from the precursor proopiomelanocortin are widespread in vertebrates [28]. Despite the phylogenetic distance between different species, the primary structure of these peptides remains remarkably similar. This suggests that peptide-mediated communication both peripherally and in the CNS is a relatively stable phenomenon during the course of evolution [13,28].

α -MSH is among the peptides present in both amphibia and mammals, and has been extensively studied in both phyla. Its classical melanotropic effect in amphibia allows background adaptation through melanophore dispersion [18]. α -MSH and structurally related peptides exert a large variety of behavioral effects in the central nervous system of mammals. Effects on attention, facilitation of learning and excessive grooming have been reported (for reviews see [3, 7, 33]). In both phyla the action of α -MSH can be described as serving to adapt an animal to changes in its environment. The mechanism of action of α -MSH is still largely unknown. According to the classical concept of peptide/hormone action α -MSH exerts its effects on melanophores and melanoma cells through saturable and reversible interactions with specific receptors contained in the outer membranes of its target cells [8]. Extensive structure-activity studies on

amphibian melanophores with α -MSH fragments and derivatives have shown that the α -MSH molecule contains two message sequences, namely 4-9 and 10-13 [11].

Recently, [Leu⁹]- α -MSH has been shown to possess very low melanotropic activity in several biological systems [8,9], but not in *Rana pipiens* [12]. [Nle⁴,D-Phe⁷]- α -MSH appears to be a strong agonist in melanophore assays, but not with respect to grooming behavior [29].

In the present study the structural requirements of peptides for melanophore dispersion were compared to those necessary to induce excessive grooming in the rat, in order to get more insight in the mechanism of action by which α -MSH may interact with brain structures.

METHOD

In Vitro *Xenopus* Melanophore Bioassay

The *in vitro* melanophore bioassay using tail-fins of *Xenopus* tadpoles was applied as described in detail by De Graan *et al.* [6]. Tadpoles of *Xenopus laevis* were used at stage 51-53 according to Nieuwkoop and Faber [26] and adapted to a white background. Pieces of 2×2 mm (containing about 100 melanophores) were excised from the ventral

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tail-fins, equilibrated and subsequently treated with the peptides for 60 min. The average melanophore response was quantified microscopically using the melanophore index of Hogben and Slome [20]. The melanotropic activities of the peptides are expressed as percentage relative to α -MSH. The values are calculated from the EC_{50} values (concentration producing half-minimal dispersion, $MI=3$), obtained from the dose-response curves of the respective peptide. Each peptide was tested at 4 concentrations, each concentration in quadruplicate [6]. Synthetic α -MSH was used as a reference standard. To test $[Leu^9]$ - α -MSH for possible antagonistic properties, the peptide (10 ng/ml or 10 μ g/ml) was added 5 min prior to α -MSH (1.0 ng/ml or 3.0 ng/ml).

Excessive Grooming

Male rats of an inbred Wistar strain were used (TNO, Zeist, NL). They were bred at our laboratory and weighed about 145 g at the commencement of the experiments. The animal rooms were kept at a 12 hr light/12 hr dark schedule (8.00 hr a.m./8.00 hr p.m.). One week prior to the experiments the animals received a brain ventricle cannula according to the method of Brakkee *et al.* [4]. All experimental animals were housed individually in plastic boxes with wood shavings and handled every second day. Fifteen min after the injection of either 3.0 μ l saline or the peptide dissolved in 3.0 μ l saline, the recording of grooming behavior was performed as described by Gispen *et al.* [15]. Every 15th sec the observer recorded whether the animal displayed vibrating, washing, body grooming, sexual grooming, tail sniffing, scratching, licking paw (or toe) or other behavior (rest). During a 55 min session a maximum score of 220 was possible.

To investigate possible antagonistic properties of $[Leu^9]$ - α -MSH an injection of this peptide (1 μ g/rat) preceded (5 min) α -MSH treatment (0.3 μ g). The development of single-dose tolerance with respect to peptide-induced excessive grooming is seen within a few hours after the first treatment [23]. To test cross-tolerance between ACTH and α -MSH one group of rats received an injection with α -MSH (0.3 μ g) at $T=0$ and a second group received saline at $T=0$. Both groups received a second treatment with ACTH (0.3 μ g/rat) 4 hours later ($T=4$). The grooming scores of each animal were compared with a *t*-test for paired values.

Peptides

α -MSH and fragments of α -MSH were synthesized as described earlier [10,12]. $[D-Phe^7]$ - α -MSH was purchased from Bachem AG (Bubendorf, CH). ACTH and ACTH fragments were a kind gift from Organon Int. BV (Oss, NL). Oxidation of Met⁴ was performed with standard techniques using H_2O_2 and acetic acid. All peptides were dissolved in 0.001 N HCl neutralized and diluted in saline. Racemization of the peptides was achieved by heating the peptides in 0.01 N NaOH during 40 min at 60°C.

Statistical Analysis

Differences between the average grooming score of several treatment groups were assessed by an analysis of variance followed by a supplemental *t*-test [5]; significance was assigned to the $p<0.05$ level, two-tailed (Figs. 1, 2 and 4). Single dose tolerance was assessed by a paired *t*-test ($p<0.05$; two-tailed, Fig. 3). Structure-activity relationships in the two assays were compared by means of a Spearman-rank correlation test ($p<0.05$ two-tailed, Table 1).

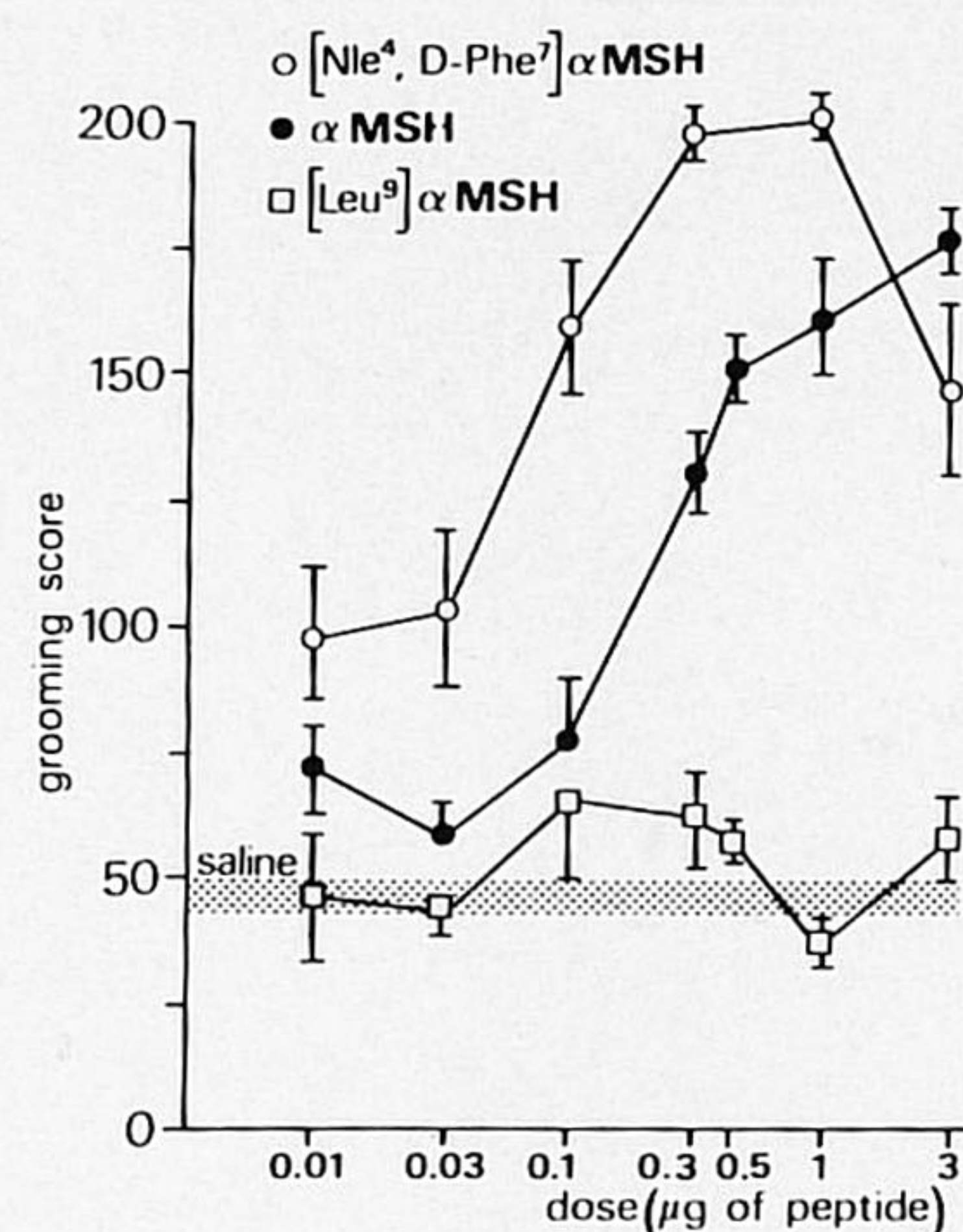


FIG. 1. Dose-response relationships of peptide-induced excessive grooming ($n=6$ per test dose).

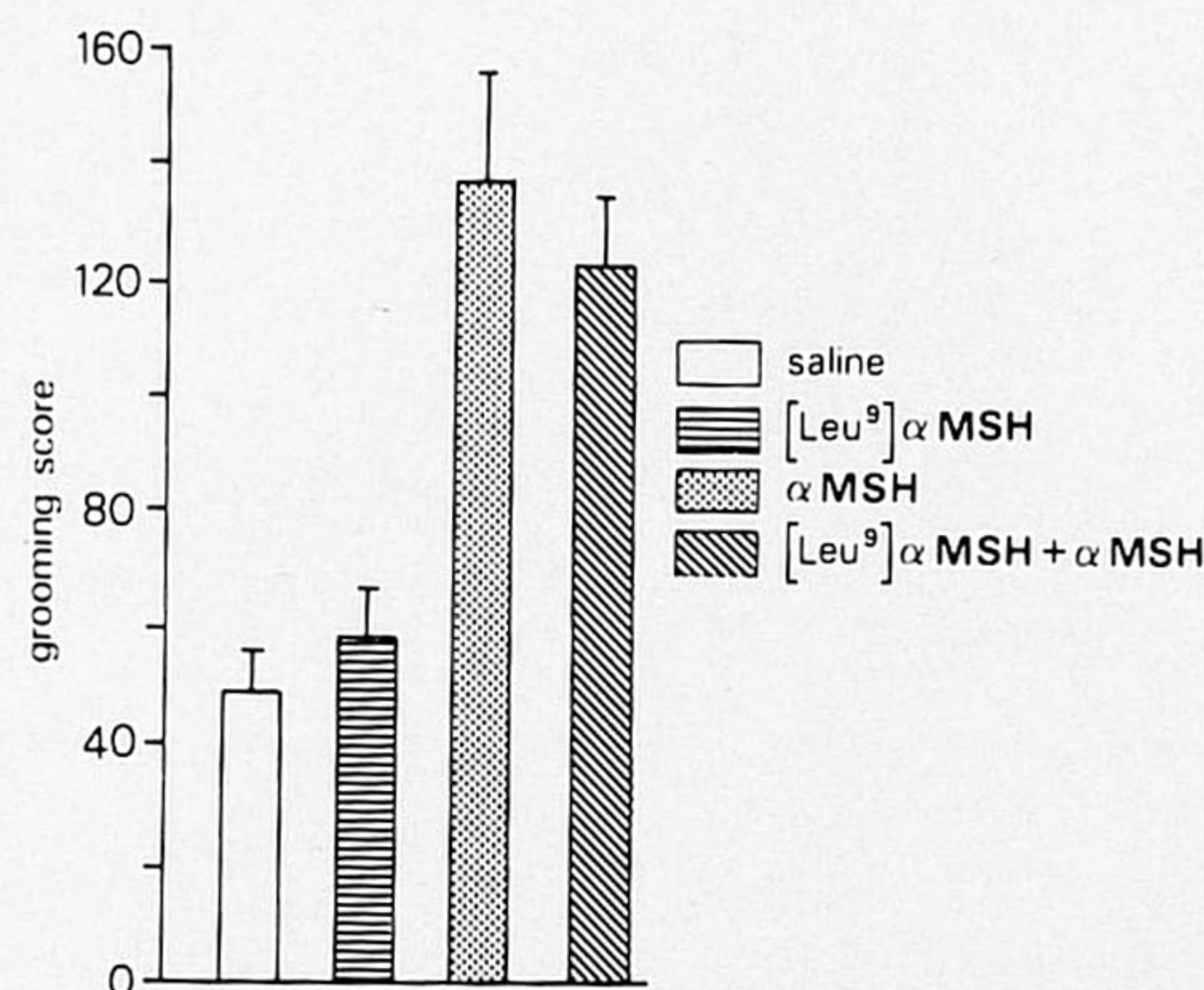


FIG. 2. The effect of $[Leu^9]$ - α -MSH on α -MSH-induced excessive grooming ($n=6$ per group). α -MSH 0.3 μ g, $[Leu^9]$ 1 μ g was injected 5 min prior to α -MSH injection.

RESULTS

To compare the structural requirements of α -MSH and ACTH for inducing pigment dispersion and excessive grooming, the structure-activity data from both bioassays are summarized in Table 1. A Spearman-rank correlation, $r(13)=0.7$; $p<0.05$, demonstrates the similarity in the structural requirements for inducing a biological response in the two assay systems. ACTH is the only exception in Table 1; this peptide is highly potent in inducing excessive grooming, but shows only 5% of the melanotropic activity of α -MSH. In order to facilitate comparison with melanotropic potencies the grooming scores in Table 1 have been corrected for saline treatment; therefore the grooming scores shown in this table cannot be directly compared with the scores in other figures.

α -MSH is a strong agonist in both assay systems. Des-acetyl- α -MSH has reduced activity in both assays. C-terminal truncation of α -MSH results in a gradual decrease in biological activity in both assay systems. N-terminal trun-

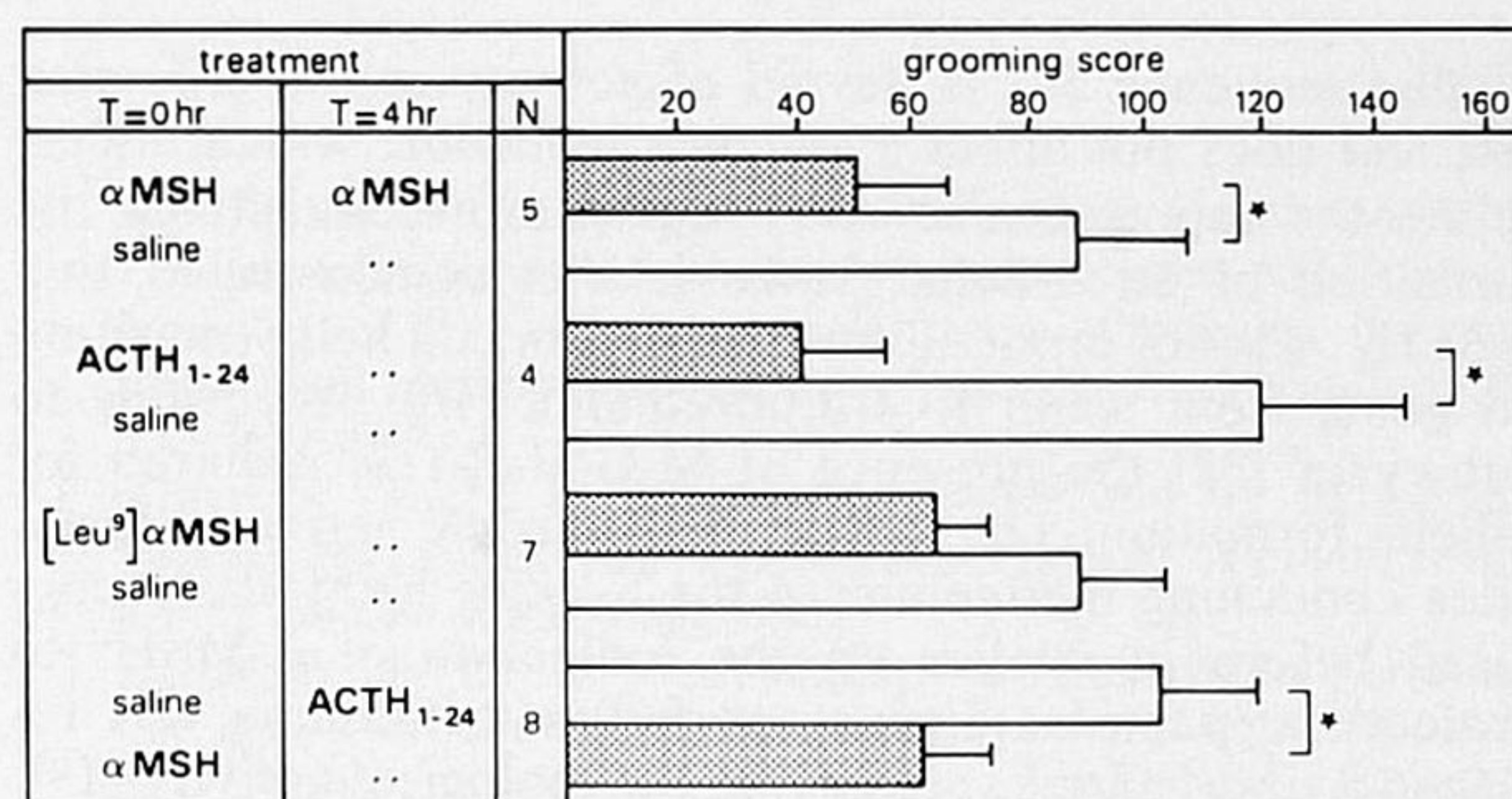


FIG. 3. Single dose-tolerance. The animals received at T=0 either the peptide (0.3 μ g) or saline. At T=4 all animals were treated with the peptide (always 0.3 μ g).

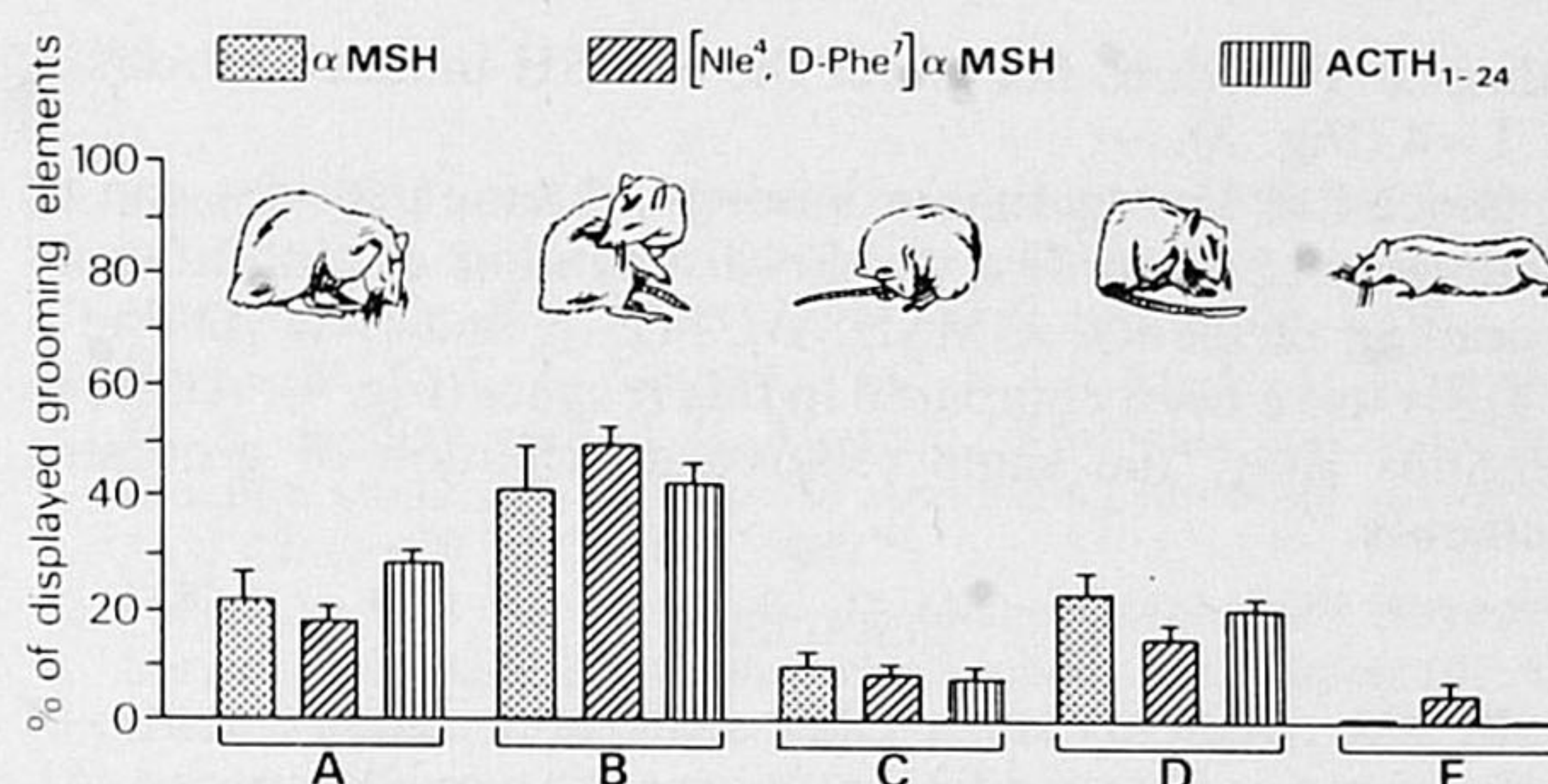


FIG. 4. The distribution of grooming elements for different peptides (n=8 per group) relative to total grooming displayed. A: washing face, B: grooming, C: genital grooming, D: scratching, E: stretching.

TABLE 1
COMPARISON OF PEPTIDE EFFECTS IN TWO BIOASSAY SYSTEMS

Peptide	Pigment dispersion %*	Excessive grooming in rat†	
		grooming score \pm SEM	N†
ACTH ₍₁₋₂₄₎	5	120 \pm 8	16
ACTH ₍₁₋₁₆₎ NH ₂	50	117 \pm 17	6
α -MSH		115 \pm 12	9
des-acetyl- α -MSH	75	35 \pm 19	7
α -MSH(1-12)	50	64 \pm 18	4
α -MSH(1-11)	9	45 \pm 11	4
α -MSH(1-10)	5	27 \pm 12	10
α -MSH(5-13)	0.09	45 \pm 22	6
[Leu ⁹] α -MSH	0.19	0 \pm 5	4
[Met (O) ¹]- α -MSH	25	37 \pm 10	9
[Nle ⁴ , D-Phe ⁷]- α -MSH	3200	160 \pm 11	4
racemized α -MSH	200	113 \pm 19	5
Saline	0	0 \pm 3	26

The grooming score of saline-treated rats has been subtracted from all values in order to have zero values in the control groups.

*: α -MSH=100%. Percentage response as calculated from EC₅₀ value of each peptide (see the Method section).

†: Refers to the number of rats used for the grooming assay.

‡: Peptides were tested at least with three different doses; statistical analysis was performed with one dose level, presented in this table, equivalent to ACTH₁₋₂₄ (1 μ g/3 μ l).

cation of the α -MSH sequence with 4 amino acids results in a dramatic drop in the biological activity in the melanophore assay and to a lesser extent in grooming behavior. Modification of position 9 from Trp to Leu reduces the melanotropic activity to 0.2%, whereas the grooming activity is almost completely lost.

In both assays [Nle⁴, D-Phe⁷]- α -MSH shows a prolonged activity as compared to α -MSH (results not shown). Oxidation of the Met¹ leads to a severe loss of activity in grooming behavior and to a lesser degree in pigment dispersion. [Nle⁴, D-Phe⁷]- α -MSH is about 30 times as active as α -MSH with respect to pigment dispersion and also superpotent in inducing excessive grooming. Racemization of α -MSH enhances melanotropic activity, but not the grooming score.

The dose-response relationships depicted in Fig. 1 show

that [Leu⁹]- α -MSH does not induce grooming behavior at any of the doses tested and that [Nle⁴, D-Phe⁷]- α -MSH is more potent than α -MSH.

Despite the structural resemblance between α -MSH and [Leu⁹]- α -MSH, pretreatment with this peptide does not interfere with a subsequent α -MSH-induced grooming response (Fig. 2), nor with α -MSH-induced pigment dispersion (results not shown). Similarly, pretreatment with the weak agonist [Met(O)¹]- α -MSH has no effect on α -MSH-induced grooming (not shown). Figure 3 shows the cross-tolerance between ACTH₁₋₂₄ and α -MSH. The same animals are treated with one of the two peptides at time point T=0 and with the other at time point T=4 (4 hr later). The response to the second peptide treatment is significantly diminished in all 3 combinations tested. Administration of [Leu⁹]- α -MSH or

saline at T=0 does not affect the α -MSH-induced grooming at T=4 (Fig. 3).

Since it is known that grooming-inducing peptides can be distinguished according to the distribution of the different grooming elements, α -MSH, ACTH₁₋₂₄ and [Nle⁴,D-Phe⁷]- α -MSH have been compared in this respect (Fig. 4). All three peptides show the same relative distribution of grooming elements.

DISCUSSION

In the present study the structural requirements of melanocortins in two different bioassays were compared. Although for a number of peptides classical dose-response curves could be established in the grooming assay (see Fig. 1), the complexity of peptide-brain interactions limits the use of a number of fragments studied. For, at high doses other peptide-induced behavioral syndromes, such as stretching and yawning (ACTH₁₋₂₄, [15]) and barrel rotation (α -MSH₅₋₁₃, data not shown), compete with the display of grooming behavior. Therefore the comparison between the two assays was performed with the highest dose at which only or primarily an effect on grooming behavior was seen, i.e., equivalent to ACTH (1 μ g/3 μ l). The spearman rank-correlation analysis demonstrated striking similarity in structure-activity of melanocortins in both assays.

Apparently, N-terminal acetylation enhances the biological activity of the MSH peptides. Reduced grooming activity of desacetyl derivatives has been shown previously [15, 27, 28] and reduced melanotropic activity has been shown in *Anolis carolinensis* and in *Rana pipiens* [9]. Whether the decrease in activity of α -MSH₅₋₁₃ must be explained by the lack of the potentiating N-terminal part [11] or by the fact that Met¹ is also part of the message sequence has not been established. Reduction of the C-terminal message sequence leads to a decrease in biological activity in both assays. The indispensability of the classical MSH message sequence 4-9 is indicated by the low activity of derivatives, in which only one amino acid in this sequence is substituted or modified. [Leu⁹]- α -MSH was only slightly active in the *Xenopus* assay and completely inactive in inducing grooming. Our results are in line with the results of Eberle and Schwyzler [12], demonstrating a low biological activity of [Leu⁹]- α -MSH on melanophores of *Anolis carolinensis* and on tyrosinase activity in melanoma cells [9].

In our experiments a 5 min pretreatment with [Leu⁹]- α -MSH did not suppress the subsequent response to α -MSH either in the grooming assay or in the melanophore assay. Apparently, this analog has no antagonistic properties. Similar negative results were obtained in the grooming assay with [Met(O)¹]- α -MSH (not shown). The classical message sequence 4-9 contains the tetrapeptide 4-7, which has also been identified as the crucial site for induction of excessive grooming [15] and facilitation of avoidance behavior [35]. C-terminal elongation of 4-9 is required for full expression of activity in the grooming test and for pigment dispersion in melanophores but not in the avoidance paradigm [14], since ACTH₄₋₁₀ and Org2766 are full agonists in the latter paradigm. α -MSH contains two message sequences for the induction of pigment dispersion in melanophores [11]. The similarity in structure-activity relationships of fragments tested in the two assays cannot be ascribed to the second C-terminal message sequence, since ACTH₇₋₁₆ is not effective in the grooming assay, whereas ACTH₅₋₁₄ is [33].

The sequence 5-7 is devoid of activity in the grooming test and does not affect avoidance behavior, which underscores the importance of Met¹. Met¹ is a prerequisite for the formation of an α -helix, since it was demonstrated that ACTH₅₋₁₀ is not prone to form a random coil helix transition on going from water to trifluoroethanol [16]. According to Schwyzler [32] the presence of M-G-H-P-T is required for α -helix formation. The loss of melanotropic activity of peptides containing methionine in the S-oxide form was recognized before in studies on the iodination of α -MSH; to protect the peptides against oxidation methionine was replaced by norleucine, preserving the biological activity [19]. With an additional modification at position 7 one can create long-lasting, highly potent analogs. Prolonged biological activity has been reported for [Nle⁴,D-Phe⁷]- α -MSH [17,21], which was tested in both assay systems. Our findings confirm that this sticky and "long-lasting" MSH derivative is extremely potent in the melanophore assay [29]. The grooming score induced by this peptide is higher than the grooming score reported by Kobobun *et al.* [24], who found α -MSH and [Nle⁴,D-Phe⁷]- α -MSH to be equipotent. Strain differences may be explanatory in this respect. The moderate grooming response of Sprague-Dawley rats in comparison to Wistar rats after administration of ACTH₁₋₂₄ has been observed by others (Meyerson, personal communication). Substitution of a L-Phe⁷ by its D-enantiomer increases the potency not only in the melanophore assay and the grooming test [15], but also in certain learning tasks [3].

The cross-tolerance between α -MSH and ACTH and the fact that the effects of both peptides can be blocked by pretreatment with naloxone (data not shown), indicate that similar neural systems are involved in α -MSH and ACTH-induced excessive grooming. The lack of effect of [Leu⁹]- α -MSH (4 hr in advance) on a subsequent response to α -MSH confirms that this analog is biologically inactive. However, based on the "cross-tolerance" phenomenon alone, it cannot be concluded that ACTH and α -MSH share a similar primary site of action, since the involvement of the opioid system, presumably responsible for the tolerance [23], is not the first of the series of events leading to grooming behavior [2].

The structure-activity relationships of ACTH-like peptides in brain have not only been studied at the behavioral level, but also at the molecular level on the adenylate cyclase activity [36] and on the degree of phosphorylation of a presynaptic membrane protein B-50 [22]. The structural requirements for inhibition of B-50 phosphorylation show a striking similarity with those for inducing excessive grooming [22]. Effects of α -MSH on cAMP levels have also been reported in melanocytes of *Rana* [1], *Xenopus* [34], melanoma cells [25,30] and in steroidogenesis [31].

Thus, a remarkable similarity in structure-activity relationship of α -MSH-like peptides in excessive grooming, pigment dispersion, adenylate cyclase activation and inhibition of B-50 phosphorylation is apparent. The data presented here can be considered as strong evidence in favor of the hypothesis that similar peptides (α -MSH-like) and similar mechanisms of action may be involved in different processes of adaptation in very different species. The combined use of these different species may be a useful tool in characterizing " α -MSH" receptors in the central nervous system of mammals.

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