THE INDUCTION OF EXCESSIVE GROOMING IN THE RAT BY INTRAVENTRICULAR APPLICATION
OF PEPTIDES DERIVED FROM ACTH: STRUCTURE-ACTIVITY STUDIES

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(Received in final form July 23, 1975)

SUMMARY

Intraventricular administration of synthetic ACTH-like peptides in the rat induces excessive grooming, stretching and yawning. The present study demonstrates that induction of excessive grooming is dose-dependent and independent of the endocrine system. Structure-activity studies show that ACTH₁₋₂₄, ACTH₁₋₁₆-NH₂, ACTH₁₋₁₆, α -MSH and β -MSH are equipotent. Although the presence of the sequence ACTH₅₋₁₀ in the peptides studies seems of importance in the induction of excessive grooming, it appeared that C-terminal elongation is necessary for the expression of the activity. Administration of [D-Phe⁷] ACTH₄₋₁₀ and [D-Phe⁷] ACTH₁₋₁₀ results in appreciable grooming activity of the rat. However, substitution of a D-arginine at the 8 position did not alter the activity of ACTH₄₋₁₀. The structure-activity relationship of these peptides on grooming activity of the rat is compared to that known for retardation of avoidance extinction. Although some similarities exist, it is concluded that the expression of excessive grooming and retardation of avoidance activity is mediated through different mechanisms.

In a variety of mammals after intracranial administration of ACTH or peptides of the N-terminal part of ACTH, a stretching and yawning syndrome (SYS) has been observed (1,2). At least in some species (rat and rabbit) it was noted that the onset of SYS was preceded by an increased display of grooming behavior (1,3). The present study aims at screening peptides derived from ACTH on their capacity to stimulate the display of grooming in the rat after intraventricular administration and to compare such structure-activity relation to that observed for the effects of ACTH-like peptides on avoidance behavior in the rat (4).

Materials and Methods

Animals and surgery

Male rats of an inbred Wistar strain (TNO, Zeist, The Netherlands) were used, weighing approximately 140-160 g. Five days prior to the experimental session, plastic canulas ($\emptyset$ 0.357 mm; depth 4.5 mm) were implanted into the third ventricle using stereotactic coordinates according to König and Klippel (5). The canulas were fixed to the skull with dental cement. Bilateral adrenalectomy was performed 36 h prior to the experimental session and animals got 0.9% NaCl solution instead of water to drink. Hypophysectomy was carried out five days prior to the experimental session using the transauricular route. Castration took place either 3 days or 10 days prior to the experimental ses-

sion. The completeness of the removal of the endocrine glands was verified after the experiment by macroscopic inspection of the sella turcica, the adrenal glands, testes and scrotum.

Peptides

The following synthetic peptides were used: ACTH₁₋₂₄; ACTH₁₋₁₆-NH₂; ACTH₁₋₁₆; [Lys¹⁷,Lys¹⁸]ACTH₅₋₁₈-NH₂; ACTH₅₋₁₆-NH₂; ACTH₇₋₁₆-NH₂; ACTH₅₋₁₄; ACTH₁₋₁₃-NH₂; α -MSH; β -MSH; [Ac-Ser¹]ACTH₁₋₁₀; ACTH₁₋₁₀; ACTH₄₋₁₀; [D-Phe⁷]ACTH₁₋₁₀; [D-Phe⁷]ACTH₄₋₁₀; [D-Arg⁸]ACTH₄₋₁₀; [Met(O₂)⁴,D-Lys⁸,Phe⁹]ACTH₄₋₉; ACTH₁₁₋₂₄. The only "non-ACTH-like" peptide included in the study was synthetic [Des-Gly-NH₂⁹,Lys⁸]Vasopressin (DG-LVP). All peptides were checked for purity, a.o. by thin-layer chromatography; the content of free peptide was determined by amino acid analysis with the help of nor-leucine as an internal standard. The free peptide content usually ranged between 80 and 90%, the rest being composed of water and acetic acid (6).

Peptide solutions were freshly prepared by dissolving the peptides in 10 μ l 0.001 M HCl and further dilution with 0.9% NaCl. Peptide solutions were injected by free hand into the IIIrd ventricle of the conscious rat (approximately 1 μ l per 4 sec).

Behavioral procedure

After injection into the IIIrd ventricle, the rats were placed individually into glass boxes (24/12.5/14 cm). Eight boxes at the time were placed into a low noise room and 15 min after the injection recording of behavior began and continued until 65 min after the injection. In most experiments, one observer recorded every 15th sec whether or not the rat displayed maintenance behavior, while the other observer registered the SYS episodes. The following elements of maintenance behavior were distinguished: vibrating, washing, grooming, scratching, licking paw, licking tail (7). If one of these elements was observed every 15th sec, a positive score was given and since the predominant element recorded, appeared to be grooming we prefer to refer to grooming, in keeping with previous reports (1,3). Thus, in a 50 min observation period a maximum of 200 positive grooming scores can be obtained provided one scores every 15th sec. The validity of this 15th sec sampling procedure was tested by simultaneous measurement of duration of grooming display and the amount of positive 15th sec grooming scores for one given rat. Therefore, in a control experiment, the behavior of 4 rats during the whole observation period was recorded on video tape (Philips, N 1520). The tape was independently analysed for 15th sec grooming scores and for the time spent at grooming, using electronic timers. In the tables results are expressed as mean number of positive grooming scores per treatment group and a difference between groups was assigned to be significant for values of $p < 0.05$ (Student t-test, two tailed).

A rat was considered to have displayed SYS if it had exhibited more than three stretchings and/or yawnings (within the observation period). In the tables the incidence of SYS in a given treatment group is expressed as the amount of rats showing SYS out of the total amount tested.

Results

Qualitative description of observed excessive grooming and SYS after intraventricular ACTH₁₋₂₄ and validity of 15th-sec-grooming scoring technique

Rats injected intraventricularly with 1 or 5 μ l saline and placed in a novel glass box usually display exploratory and grooming behavior, which may last for some 15-20 min but then invariably fall asleep and therefore show very little grooming during an observation period of 50 min, which starts 15 min after the intraventricular injection (fig.1). In contrast, rats injected similarly with 2 μ g ACTH₁₋₂₄ display a completely different behavioral pattern during the observation period. They spend most of their time grooming vigorously interrupted by short episodes of SYS.

FIG. 1

The effect of intraventricular injection of ACTH₁₋₂₄ (2 µg/5 µl n = 2) and saline (5 µl n = 2) on grooming in the rat. The recording of the behavior began 15 min after the injection. Since no SYS was observed in the saline treated rats, only the incidence in time of a SYS episode in the peptide treated rats is indicated by the arrows (S).

In fig. 1 mean grooming scores per 5 min of 4 rats tested with 2 µg ACTH₁₋₂₄ are plotted and the figure clearly shows that a dip in grooming activity is accompanied by the occurrence of a SYS episode. In order to test the validity of the procedure used to score the grooming in a group of 4 rats treated with 2 µg ACTH₁₋₂₄/5 µl both the number of 15th-sec-grooming scores and the time spent at grooming activity during the observation period was recorded as described above. In table I the relative amount of 15th-sec-grooming scores ($\frac{\bar{x}}{200} \times 100\%$) and grooming duration ($\frac{\bar{x}}{50 \text{ min}} \times 100\%$) per rat are given. The data indicate that 15th-sec-grooming scores ($81.3 \pm 3.1\%$) are a good representation of the time spent at grooming activity ($80.1 \pm 3.4\%$) under the present experimental conditions.

TABLE I

Induction of excessive grooming by ACTH₁₋₂₄* : comparison of 15th-sec-grooming scores to duration of grooming

rat #	grooming 15th-sec scores % of total possible	grooming duration % of total possible
1	78.2	76.7
2	90.4	89.4
3	76.6	74.0
4	79.8	80.2
	$\bar{x} \quad 81.3 \pm 3.1$	$\bar{x} \quad 80.1 \pm 3.4$

* 2 µg/5 µl intravent.

ACTH₁₋₂₄ induced excessive grooming: dose-response relation and independence of the endocrine system

In this experiment the effect of various doses of ACTH₁₋₂₄ on grooming activity and incidence of SYS is studied. As can be seen in Table II, saline treated control animals show very little grooming activity (20 ± 3) and no signs of SYS. The effects of intraventricular administered ACTH₁₋₂₄ on grooming activity appears to be dose-dependent and is already significant at a dose of 0.01 µg/5 µl

TABLE II

Dose-response relation of ACTH₁₋₂₄ on excessive grooming in the rat

	n	grooming positive 15th-sec scores	incidence SYS
saline 5 μ l	10	20 $\pm$ 3*	0/10
μ g ACTH ₁₋₂₄			
0.01	4	59 $\pm$ 11	1/4
0.1	4	114 $\pm$ 14	4/4
1.0	4	141 $\pm$ 18	4/4
2.0	5	152 $\pm$ 8	5/5
20.0	4	170 $\pm$ 5	4/4

* mean $\pm$ SEM

(59 $\pm$ 11). From 0.1 μ g/5 μ l on, all doses induced the occurrence of SYS. The rats treated with ACTH₁₋₂₄ and showing relatively low grooming scores (0.01 and 0.1 μ g groups) first display the same vigorous grooming activity as the other dosage groups. However, they stop at an earlier point of time in the observation period and then fall asleep like saline treated rats do.

The data in Table III show that the effect of ACTH₁₋₂₄ on grooming is independent of the endocrine system since in adrenalectomized, hypophysectomized or castrated male rats 1 μ g ACTH₁₋₂₄ induces sufficient grooming activity (ranging from 120 to 169) and incidence of SYS. The effect of injection of saline into these operated rats resulted in an activity of grooming in the order of 20-30 and no signs of SYS were observed.

TABLE III

Effect of removal of endocrine glands on the ACTH₁₋₂₄* induced grooming behavior in male rats

		n	grooming positive 15th-sec scores	incidence SYS
Adrenex	3 days	4	148 $\pm$ 10**	4/4
Hypox	5 days	6	120 $\pm$ 11	5/6
Castrated	3 days	4	169 $\pm$ 12	4/4
Castrated	10 days	3	161 $\pm$ 26	3/3

* 1 μ g/5 μ l intraventr.** mean $\pm$ SEM

ACTH and MSH induced excessive grooming: structure-activity relationship and comparison to peptide effect on conditioned avoidance behavior

In a series of experiments first the effect of gradual shortening of the ACTH₁₋₂₄ sequence on the excessive grooming inducing capacity was studied. For all peptides at least a dose equimolar to 3 μ g ACTH₁₋₂₄ was tested. At this dose level the peptides ACTH₁₋₁₆-NH₂, ACTH₁₋₁₆, α -MSH and β _p-MSH showed an acti-

vity comparable to that of ACTH₁₋₂₄. In order of decreasing potency the following peptides also induced the grooming response: [Lys¹⁷, Lys¹⁸]ACTH₅₋₁₈-NH₂, ACTH₅₋₁₆-NH₂, ACTH₁₋₁₃-NH₂ and ACTH₅₋₁₄. The peptides ACTH₁₁₋₂₄, ACTH₇₋₁₆-NH₂, ACTH₁₋₁₀ and [Ac-Ser¹]ACTH₁₋₁₀ gave no response in a dose equimolar to 3 µg ACTH₁₋₂₄. The hepta peptide ACTH₄₋₁₀ was found inactive up to a dose of 40 µg/rat.

Next the effect of introducing some structural modifications was studied. Inversion of configuration of the phenylalanine residue in position 7 caused a remarkable potentiation for the heptapeptide [D-Phe⁷]ACTH₄₋₁₀. A similar substitution in the decapeptide [D-Phe⁷]ACTH₁₋₁₀ appeared to be less effective. On the other hand introduction of a D-arginine residue in position 8 did not alter the activity of the heptapeptide ACTH₄₋₁₀.

No grooming inducing activity was found for the analogue [Met(O₂)⁴, D-Lys⁸, Phe⁹]ACTH₄₋₉ when tested at the 1 µg/rat dose level, though the same analogue was found a thousand times as active as ACTH₄₋₁₀ when assayed in a pole jumping test on inhibition of extinction of a conditioned avoidance response, a dose of 1 ng/rat given subcutaneously being already effective (4).

Finally a peptide from another group of behaviorally active peptides was tested. DG-LVP is a natural occurring fragment of lysine vasopressin, which retains full behavioral activity of vasopressin when tested on retardation of extinction but is virtually devoid of endocrine effects (8). Application of this peptide in a dose of 0.5 µg did not induce excessive grooming or display of SYS (Table IV).

Discussion

Previous studies have shown that to the exclusion of other peptides ACTH and MSH can induce SYS in a variety of mammals (1). The present paper attempts to measure the potency of peptides in eliciting excessive grooming in the rat, a typical behavior which in time precedes the onset of SYS (Fig. 1). This characteristic property of ACTH- and MSH-like peptides has been noted by others (1,3) although no quantitative data were presented. As can be seen in Tables I and IV, quantification, however, appears to be meaningful in screening the capability of peptides to induce excessive grooming after intraventricular administration. Although the excessive grooming often is accompanied by the occurrence of SYS, the quantitative data on grooming are the most reliable (see also Table I). Smaller numbers of grooming scores reflect shorter time spent at grooming, while the intensity of the grooming activity was similar throughout the treatment groups. The incidence of SYS reflects only frequency of relevant episodes, while intensity and duration differ notably from one episode to another.

It seems highly unlikely that classical endocrine properties of the peptides tested are responsible for the display of excessive grooming (Table III). Therefore, in view of the route of administration and the rather immediate response of the rat, it is tempting to conclude that the observed behavioral response is a result of a direct action of these peptides on central nervous structures. As for the induction of SYS, hypothalamic structures seem of major importance (1,2).

Whether or not the incidence of SYS is related to the display of excessive grooming, is not clear, although a clear parallel was found between the ability of a sequence to elicit excessive grooming and the incidence of SYS. Since also in our hands the latency period of the first episode of SYS was in the order of 35-45 min after the injection (3) it is uncertain that both excessive grooming and SYS are the expression of the same mechanism of action of these peptides. If stretching and yawning are considered to reflect intense neuromuscular activity, it may be that the effectiveness of ACTH- and MSH-like peptides to induce this syndrome, in fact may originate from molecular properties which enable these peptides to facilitate spinal reflexes both *in vivo* and *in vitro* (9,10) and to increase muscle action potential amplitude and contraction height (11,12). The data presented on the structure-activity relationship of the peptide sequences tested are of interest. Within the ACTH series it is important that again frag-

TABLE IV

Effect of intraventricular administered peptides on excessive grooming in the male rat

peptide	dose in μg	n	15th-sec grooming	incidence of SYS
saline	-	19	31 $\pm$ 5*	0/19
ACTH ₁₋₂₄	3.0	4	169 $\pm$ 8	4/4
ACTH _{1-16-NH₂}	2.0	4	157 $\pm$ 15	4/4
ACTH ₁₋₁₆	0.05	3	127 $\pm$ 15	2/3
	2.0	3	175 $\pm$ 7	3/3
[Lys ¹⁷ , Lys ¹⁸]ACTH _{5-18-NH₂}	1.75	7	134 $\pm$ 18	6/7
ACTH _{5-16-NH₂}	1.5	15	70 $\pm$ 7	10/15
ACTH _{7-16-NH₂}	1.2	8	36 $\pm$ 7**	4/8
ACTH ₅₋₁₄	1.25	16	50 $\pm$ 5	6/16
ACTH _{1-13-NH₂}	1.5	11	69 $\pm$ 10	7/11
	4.5	4	118 $\pm$ 28	4/4
α -MSH	0.5	9	43 $\pm$ 13**	1/9
	1.5	6	157 $\pm$ 15	6/6
β -MSH	2.25	4	153 $\pm$ 11	4/4
[Ac-Ser ¹]ACTH ₁₋₁₀	1.3	4	38 $\pm$ 6**	0/4
ACTH ₁₋₁₀	1.3	7	20 $\pm$ 4**	0/7
ACTH ₄₋₁₀	1.0	5	35 $\pm$ 10**	1/5
	10.0	4	49 $\pm$ 11**	0/4
	20.0	4	57 $\pm$ 11	0/4
	40.0	4	24 $\pm$ 7**	0/4
[D-Phe ⁷]ACTH ₁₋₁₀	1.3	4	64 $\pm$ 14	3/4
[D-Phe ⁷]ACTH ₄₋₁₀	0.3	3	73 $\pm$ 22	3/3
	1.0	4	111 $\pm$ 18	1/4
	10.0	3	169 $\pm$ 7	2/3
[D-Arg ⁸]ACTH ₄₋₁₀	10.0	5	49 $\pm$ 13**	1/5
[Met(O ₂) ⁴ , D-Lys ⁸ , Phe ⁹]ACTH ₄₋₁₀	1.0	8	34 $\pm$ 5**	0/8
ACTH ₁₁₋₂₄	0.5	3	13 $\pm$ 6**	0/3
	1.5	3	28 $\pm$ 13**	0/3
	10.0	4	29 $\pm$ 12**	0/4
ACTH ₁₋₁₀ + ACTH ₁₁₋₂₄	1.3 + 1.3	4	13 $\pm$ 4**	0/4
DG-LVP	0.5	6	28 $\pm$ 6**	0/6

* mean $\pm$ SEM

** not significantly different from saline

ments from the N-terminal part of ACTH can affect central nervous functioning (13). ACTH₁₋₁₆-NH₂, ACTH₁₋₁₆; [Lys¹⁷, Lys¹⁸]ACTH₅₋₁₈-NH₂, α -MSH and β_p -MSH were active in eliciting grooming. To a lesser extent the peptides ACTH₁₋₁₃-NH₂, ACTH₅₋₁₆-NH₂ and ACTH₅₋₁₄ were also able to induce the grooming response. However, the sequences (1-10), (4-10), (7-16) and (11-24) were inactive under the present conditions. The fact that also β_p -MSH was found to be active, confirms the suggestion of others that the sequence (4-10) (which is the common sequence, shared by ACTH₁₋₂₄, α -MSH and β_p -MSH) is the carrier of information for onset of excessive grooming and SYS (1). It is therefore surprising that application of this sequence even in high doses in fact is not effective in eliciting the grooming response in the rat. That Ferrari was able to induce SYS in the dog with ACTH₄₋₁₀ in a dose eight times the minimum active dose of α - and β_p -MSH may possibly be ascribed to species differences, since he selected the dog for his studies as being the species most susceptible to the central effects of ACTH (1). Our data then in part corroborate the findings of Baldwin *et al.* (14) who studied the effect of ACTH₁₋₂₄ and ACTH₄₋₁₀ on the initiation of SYS in the rabbit. They conclude that ACTH₄₋₁₀ may have some stimulating influence on the rabbit's behavior, but is much weaker than the sequence (1-24). Since ACTH₁₋₁₃-NH₂ and ACTH₅₋₁₄ showed only border-line activity (Table IV), one may deduce that in the rat the minimal sequence required for the capacity to induce grooming, is the fragment (5-10) extended at the C-terminal either by the ACTH sequence (11-13)-NH₂, or by a corresponding part of the β -MSH sequence (13-18), possibly to endow the peptide with an improved resistance to metabolic break-down. A similar reasoning may explain the finding that ACTH₁₋₁₃-NH₂ is less active than α - or β_p -MSH: acetylation of the N-terminal serine residue either with an acetyl-group or with the β_p -MSH sequence (1-7) will increase resistance of the active core to degradation by aminopeptidases. In this context the fact that substitution of a D-phenylalanine at the 7th position in the sequence (4-10) and to a less extent in (1-10), generates substantial grooming activity, is less surprising and again (Table IV) points to a crucial role of this sequence in eliciting the behavioral response. Differences in behavioral activity between peptides with L- or D-phenylalanine at position 7 have first been described for the influence of these peptides on extinction of an active avoidance response (15). ACTH₁₋₁₀ and ACTH₄₋₁₀ retard extinction whereas the corresponding 7-D-phenylalanine analogues enhance extinction. However, when tested on passive avoidance behavior, both ACTH₄₋₁₀ and [D-Phe⁷]ACTH₄₋₁₀ inhibit extinction but the effect of treatment with the D-phenylalanine analogue has a much longer duration of action (4). We are inclined to ascribe the increased grooming inducing potency of the 7-D-phenylalanine analogues to improved resistance against enzymatic degradation. Recent work of Witter (16) on the metabolic stability of the ACTH-analogues: ACTH₄₋₁₀ and a.o. [Met(O)⁴, D-Lys⁸, Phe⁹]ACTH₄₋₉ in relationship to behavioral activity in the pole-jumping test, support the suggested correlation between half-life time and magnitude of behavioral response. However, the finding that the exchange of the arginine residue in position 8 for its D-isomer, again aiming at improved metabolic stability, is not accompanied by potentiation, emphasizes the importance of intrinsic activity, affinity for "receptor" site and transport properties, which co-determine the ultimate effectiveness of the peptide.

Although part of the data so far points towards a great similarity in structure-activity relationship for grooming and avoidance activity, important differences occur. Retardation of avoidance extinction can be elicited by systemic or intracerebral administration of ACTH₄₋₁₀ and ACTH₁₋₁₀. These two peptides as such were found to be inactive with respect to grooming. Furthermore, no excessive grooming and stretching is observed after subcutaneous administration of ACTH-like peptides even at doses up to 0.5 mg/100 g b.w. (Gispen, unpublished); (see also Ferrari (17): 1 mg/kg in v. carotid; no effect on SYS). However, preliminary data show a significant increase only in yawning activity after peripheral administration of 0.5 mg/kg ACTH₁₋₂₄ in the rat, which could very well be in accordance with the reported increased yawning of mice after chronic treat-

ment with a high dose of α -MSH (18). That the expression of excessive grooming and retardation of extinction is mediated through different central mechanisms is also apparent from the observation that the analogue [Met(O₂)⁴, D-Lys⁸, Phe⁹] ACTH₄₋₉, a compound which is a 1000 times more effective in retarding extinction of a pole-jump response than ACTH₄₋₁₀, was found inactive with respect to grooming induction and that DG-LVP, a very potent peptide in retarding avoidance extinction in rats, also has no behavioral effect under the present conditions.

The biological significance of the observed effect of ACTH- and MSH-like peptides on grooming activity and the incidence of SYS is unclear. Others have suggested that rabbits intraventricularly treated with ACTH₁₋₂₄ in spite of the induction of SYS would show sexual excitement (19,20,14). We failed to observe penile erections or lordotic behavior in male or female rats after intraventricular administration of ACTH₁₋₂₄ although in both sexes excessive grooming occurred accompanied by a display of SYS. Recent observations of Bohus *et al.* (21) in fact showed that in castrated male rats, maintained on low testosterone therapy, subcutaneous administration of ACTH₄₋₁₀ prolonged latencies of intromission and ejaculation. Further work is in progress on the site of action in the brain and the mechanism of action by which these peptides induce excessive grooming in rats.

Acknowledgements

The authors wish to acknowledge the skillful technical assistance of J.H. Brakkee, J. Leijendekkers and A.P.M. Mangal. Part of this research was supported by a FUNGO-ZWO grant no. 31-13-03B.

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