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ACTH-INDUCED EXCESSIVE GROOMING IN THE RAT

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1. INTRODUCTION

The study of the role peptide hormones and their non-hormonal fragments play in the modulation of animal and human behavior as mainly initiated by David de Wied and his co-workers has opened a new chapter in modern behavioral neuropharmacology. The profound effects of ACTH and vasopressin on behavior are now well known, and numerous investigators have searched for their mechanism of action. We are at the frontier of understanding how such peptides exert their effects on brain physiology and behavior.

Studies of the peptide effects on naturally occurring behavioral repertoires may help to understand the peptide modulation of more complex, cognitive behaviors. Furthermore, such studies aid the neuropharmacologist to identify site of action or neural pathways involved. They also may facilitate to unravel the behavioral patterns which may be regulated by peptidergic pathways in the brain.

In our studies we have chosen the ACTH-induced excessive grooming response as an example of a peptide-modulated naturally occurring behavior of the rat. In this article we will review some of the literature concerning the display of grooming behavior and describe in detail the peptide induction of the behavior. We will analyze the relationship to stress and novelty, to the classical hormonal effects of ACTH and to the minimal structural requirements which enable a fragment of ACTH to induce excessive grooming. Data from implantation and lesion studies will shed light on the neural substrate underlying the peptide-induced grooming. In addition, we will review some of the current literature on the involvement of catecholamine and opiate receptors in this behavioral response to ACTH. Finally, a potential neurochemical mechanism by which ACTH may modulate neurotransmission will be discussed.

As the research on ACTH and behavior—its mechanism of action, its physiological importance, etc.—is a rapidly developing and extending field of research, this review cannot in any way be complete. It is hoped, however, that it suffices in giving a state of the art and inspires further research on peptides, brain, and behavioral mechanisms.

2. GROOMING IN THE RAT

Grooming behavior is a common species characteristic movement pattern with readily definable components (Bolles, 1960; Fentress, 1973). Constituting components are vibration, face washing, body grooming, scratching, paw licking, head shake, body shake and genital grooming (Fig. 1). Vibration is often seen at the onset of a bout of face washing activity. It consists of a brief (1 sec) period of vibrating movements of the

FIG. 1A

FIG. 1B

FIG. 1C

FIG. 1D

FIG. 1E

FIG. 1. Grooming in the Rat A: Face Washing; B: Body grooming; C: Grooming of genital area; D: Scratching; E: Licking paw.

forepaws in front of the snout (Van der Poel, 1967). Face washing is typified by periods of licking of the front paws leading to a series of horizontal single strokes that involve movements of the forepaws along the snout at a high frequency, in turn followed by overbounds of longer duration in which limbs more or less alternate in making large semicircular movements over the top of the head (Fentress, 1968a,b, 1973).

Body grooming indicates the licking of parts of the body. Face washing often is followed by grooming of the side and the back. But also licking of the limbs, tail and the genital area are classified under body grooming. The bouts of body grooming are frequently interrupted by scratching the body by one of the limbs. Such a usually brief

episode is typified by scratching movements of high frequency and is nearly always ended by licking the paw and/or toes used to scratch. Other behavioral elements which can be seen either as an interruption of a grooming bout are head or body shakes. Such shakes are similar to, but of a less intent nature, than the 'wet dog shakes' widely seen in the neuropharmacological studies of the quasi-morphine withdrawal syndrome.

In most studies reported in this review grooming behavior of the rat is observed in solitary situations after some manipulation or treatment.

In the home cage, the individually housed rat has been reported to spend as much as one-third and one-half of his waking time in self-grooming. However, there is a considerable range of individual differences with respect to the incidence of grooming. Some animals groomed three times as much as others (Bolles, 1960). Furthermore, it is difficult to evaluate the Bolles report since the nature of the light-dark cycle is not reported. Other details concerning the procedures would have been useful, e.g. how observations were made during the dark portions of the light cycles. Furthermore, in a second experiment in the same report animals were found to groom only 15% of their waking time.

Even this estimate may be high for rats in most laboratory conditions. For example, Cohen and Price (1979) adapted animals to individual cages in the testing room for two days and then behaviors were observed and recorded on a periodic basis for an hour after spontaneous activity was noted. They report that about 18.25 sec of grooming to occur in the 30 min of actual observation made in the hour. This figure when doubled to provide an estimate for the full hr would be 36.50 sec or about 1% of the time.

In the isolated situation, there seems to be a relation with respect to the type of grooming behavior displayed and the behavior preceding this bout of grooming activity. For example, on awakening, rats tended to scratch themselves, whereas after eating or drinking there was a 'disproportionally' high incidence of face washing (Bolles, 1960). The order of behavioral components of grooming is quite varied over the course of grooming bouts, but it should be noted that bouts of such duration are extremely rare events, at least during daytime hours.

It should be pointed out that the housing of animals in individual cages represents a stressful condition for the animal relative to conditions of group housing. In addition, recent evidence suggests that isolated housing conditions produces a reduction in effectiveness of endogenous opiate systems (e.g. Panksepp *et al.*, 1978) and possible in noradrenergic turnover rates (Nishikawa and Tanaka, 1978). These changes could lead to differences in the amount of grooming demonstrated by animals in the two conditions. Therefore, it is difficult to assert that behavior exhibited in individual cages is representative of true species-typical action. In fact, neither group housing nor individual housing of laboratory rats approximate the natural conditions of the wild members of the species, but the question of what is typical for the highly inbred laboratory rodent is another matter.

Fentress has observed that grooming proceeds independently of its effect on the organism. He found that mice that have had their forelimbs removed painlessly at birth still execute normal grooming movements later in life (Fentress, 1973). Upper arm grooming movements still occur and are coordinated with movements of the head, tongue, and eyes. This implies the existence of a central nervous system generator for behavioral programs that can operate without the normal flow of sensory information. The fact that centrally applied ACTH induces grooming in furless genetic mutant rats (Fig. 2) (Gispén, personal observation) supports such a view.

Jones and Fentress (cited in Fentress, 1977) found that sections of the trigeminal nerve did not abolish grooming exhibited by mice in their home cage and, in fact, after surgery transient increases were observed after the lesions. Sham operations produced increases in grooming afterwards (but not as much as the trigeminal section) and the amount of additional grooming found after the sham operations expended on the strains being tested (DBA/2J, C57/6).

In a previous report (Fentress, 1972) it was noted that the testing of mice in a novel environment produced what seems to be a different sort of grooming behavior than

FIG. 2. Furless genetic mutant rat.

found in the home cage. The grooming in the novel circumstances seemed to occur in short bursts made up of very fast movements. Under conditions of testing in novel circumstances, there were no differences between mice with trigeminal lesions and controls. Fentress found that the removal of afferent information from the forelimbs by appropriate dorsal root section failed to disturb novelty-induced grooming.

Fentress (1978a,b) also has found that the application of mild irritant to the back of a rodent produces a normal sequence of grooming beginning with face washing and gradually working back to the affected area. If the irritant is too harsh, perhaps painful, the animal will direct its attention to that place initially in an intensive and well-directed behavior sequence. The effects of the mild irritant seem to be those of a general but mild stress condition such as found in our novel observation chamber after the necessary handling and transport.

The self-grooming observed in home cages without specific treatment or intervention can be regarded as a baseline condition even though the circumstances of housing may differ both in the number of conspecifics present, in the time of the change from group to individual conditions, and in the vagaries of the laboratory housing situation (e.g. lighting, noise, and so forth). However, it is against this background condition that excessive grooming is measured. As we will use the term excessive grooming represents an increase of grooming beyond that measured under baseline conditions.

3. THE NATURE OF PEPTIDE-INDUCED EXCESSIVE GROOMING

In most studies reported in this review rats with a body weight of 140–150 g were used in the studies conducted in Utrecht, while those conducted at the University of Florida or SUNY-Binghamton were somewhat older and larger (200–250 g). The animals routinely were kept at least for one week in the animal facilities prior to the behavioral experiments. The animal rooms were kept at a normal 12 hr dark/12 hr light schedule (8:00 p.m./8:00 a.m.).

In order to allow intracerebroventricular (i.c.v.) injection into nonrestrained, conscious rats a simple plastic cannula is implanted approximately one week prior to the injection according to the methods described by Brakkee *et al.* (1979). After surgery, all animals are housed individually in plastic boxes without wood shavings. All experiments are performed between 10:00 a.m. and 3:00 p.m. On a typical experimental day a standard grooming test with an i.c.v. injection is run as follows: about 10 cages are transported from the animal room to the behavior observation room. This transport takes 2–4 min in Utrecht, about the same in Florida, and a minute or two longer at SUNY-Binghamton. Then the rats receive an i.c.v. injection after which they are subjected to the behavioral analysis in a dimly lit sound-proof observation room. Observation is carried out through a one-way mirror or by closed circuit TV. After any experimental manipulation (injections or treatments), the rats are placed into individual glass boxes (24 × 12.5 × 14 cm). This moment marks the beginning of the behavioral session.

In order to quantify the display of grooming behavior, a time sampling procedure was developed which results in simple and reliable recording of grooming duration in a given observation period.

An observer scores every animal every 15 sec on the basis of whether or not one of the behavioral components of the grooming act was displayed. If so, a positive grooming score is given for the time interval. Therefore, in a 50 min observation period, the one most routinely used, a maximum of 200 positive grooming scores can be obtained (Gispén *et al.*, 1975).

A disadvantage of such recordings is that much information is lost on the quality of the observed behavior. However, an advantage for neuropharmacological studies is the fact that a trained observer can study as many as 12 rats simultaneously and reliably score the quantity of grooming behavior displayed by each animal during the observation period.

In order to test the validity of the 15th sec time sampling procedure for a given group of 4 rats treated with 2 µg ACTH i.c.v. both the number of the 15th sec grooming scores and the total time spent at grooming during the observation period was recorded by independent observers.

In Table 1, the relative amount of 15th sec grooming scores

$$\left(\frac{x \text{ scores}}{200 \text{ scores}} \times 100\% \right)$$

and grooming duration

$$\left(\frac{x \text{ min}}{50 \text{ min}} \times 100\% \right)$$

per rat are given.

These and other data indicate that the 15th sec grooming score procedure yields data representative of the time spent at grooming behavior under the experimental conditions used. Reliability of recording between individual observers was evidenced by correlation coefficients over 0.9 among different observers.

TABLE 1. Induction of Excessive Grooming by ACTH₁₋₂₄*: Comparison of 15th-Sec-Grooming Scores to Duration of Grooming**

		15th-sec-grooming scores	Grooming duration
		% of total possible	% of total possible
		No.	
Rat	1	78.2	76.7
	2	90.4	89.4
	3	76.6	74.0
	4	79.8	80.2
		$\bar{x} 81.3 \pm 3.1$	$\bar{x} 80.1 \pm 3.4$

*2 µg/5µl intraventr.
**From Gispén *et al.*, 1975.

4. BEHAVIORAL RESPONSE TO A NOVEL GLASS BOX: EFFECT OF I.C.V. SALINE

In most experiments on peptides induced grooming the behavior was analyzed in the novel observation chambers during an interval of 15–65 min after i.c.v. injection. First, it was necessary to establish that i.c.v. injection of the vehicle for ACTH (3 μ l saline) did not initiate a behavioral response different from that seen after the placement of a cannulated or a non-cannulated rat into the observation box after the necessary transport and handling. In a recent experiment eight male rats, all of which had been cannulated one week prior to the experiment, were divided into two groups. Four subjects were injected i.c.v. with saline, while the other four subjects did not receive any injections. Total durations of the various behavioral activities were recorded for each animal in the 15–65 min interval after placement in the observation cage. Four components of grooming were recorded: head washing, bodily grooming, genital grooming, and scratching of the back. In addition, stretching and yawning (SYS), locomotion, sleeping, and quiet sitting were also recorded. After being placed in a novel glass box, these rats display some locomotion and grooming behaviors which may last for some 20–30 min, but then they invariably fall asleep (Fig. 3A). The i.c.v. injection of saline has little effect on the behavioral responses exhibited in the observation box. Furthermore, there was no effect of the saline injection in the several components of the grooming response (Fig. 3B).

5. EFFECTS OF I.C.V. ACTH

If, however, ACTH in a saline vehicle is injected into the cerebrospinal fluid, a remarkable elevation of grooming behavior is quickly seen which lasts throughout the observation period. The amount of grooming elicited depends on the dose used (Table 2). Usually, the grooming is interrupted by occasional episodes of stretching and yawning. Only at the very end of the observation period will these animals adopt a sleeping posture.

In an attempt to see whether or not ACTH-induced grooming differed in composition of saline or novelty-induced grooming, a detailed element-duration analysis of the displayed grooming response was made in thirteen males. Six of these received a saline injection, seven were treated with ACTH_{1–24}/3 μ l. The behavior of the subject was recorded simultaneously by closed-circuit TV followed by analysis of the tape of each

FIG. 3. Behavioral response to a novel cage: effect of i.c.v. saline *A*: Time spent in various behaviors. SYS = stretching and yawning. *B*: Time spent in components of grooming behavior.

TABLE 2. *Excessive Grooming After i.c.v. ACTH: Dose Response*

Saline ACTH ₁₋₂₄ **	⁺ n 6	15th-sec-grooming scores 26 ± 5*
0.01 µg	6	63 ± 10
0.05 µg	5	90 ± 8
0.1 µg	5	114 ± 9
0.5 µg	5	138 ± 7
1.0 µg	5	169 ± 5

*mean ± SEM.

**µg/3 µl.

⁺n number of rats.

subject's behavior in terms of time spent in various components of the grooming behavior (vibration, face washing, body grooming, genital grooming, tail sniffing, scratching, paw licking, body shaking) over a 60 min period immediately following injection and placement in the observation cage (Gispén *et al.*, in preparation). As expected, ACTH-treated subjects spent more time in the display of these grooming elements with the exception of the tail sniffing (which was hardly displayed by either treatment groups). If the data were converted to proportion of time spent per grooming element as a percentage of the total time spent grooming, no significant changes would be detected between the two groups of rats (Table 3). Thus, it appears that ACTH enhances the display of grooming behavior without changing the composition of the behavioral response relative to saline injected or non-injected control animals.

The total grooming score obtained from an animal can be examined in many ways. One way is to count the number of times a grooming bout is initiated regardless of its length. The duration of scored grooming bouts can then be considered independently. The number of times grooming episodes are initiated and the duration of each grooming event can be readily obtained from the records normally used in the time-sampling method devised by Gispén *et al.* (1975).

When observing animals in novel testing situations without any treatment, the number of grooming starts tends to be about 10–14 over the 50 min period. Surprisingly, this number tends to remain constant even when the animals are exposed to environmental manipulations that enhance total grooming scores or after the intercerebral administration of ACTH that induce extreme excessive grooming. The observation of animals in their individual, home cages indicates that fewer bouts are initiated in those circumstances over a comparable time period. This suggests that the effect of environmental conditions and the central administration of ACTH is *not* to produce more grooming episodes but rather to lengthen their duration.

The grooming bouts exhibited by rats in their home cages are of relatively short duration. Using our time sampling methods, the majority of episodes do not extend beyond one or two 15 sec sampling intervals. In order to obtain a more detailed analysis of grooming bout durations, we tabulated grooming scores into the following categories:

TABLE 3. *Time Spent in Various Behavioral Elements as Percentage of Total Grooming Behavior after i.c.v. ACTH**

	Saline (n = 6) %	ACTH (n = 7) %
Vibration	0.4 ± 0.2†	0.8 ± 0.3
Washing	35.1 ± 7.4	21.2 ± 1.9
Grooming	44.3 ± 7.4	55.9 ± 4.1
Grooming genital area	5.8 ± 1.2	7.6 ± 1.5
Scratch/lick paw	13.3 ± 2.8	12.8 ± 2.8
Body shake	0.4 ± 0.3	0.9 ± 0.1
Sniff tail	0.8 ± 0.5	0.8 ± 0.4

* 1 µg/µl.

†mean ± SEM.

FIG. 4. Duration and start analysis of grooming behavior displayed by rats in their home cages, in novel cages or after i.c.v. ACTH₁₋₂₄ (1 μ g/3 μ l). $n = 4$.

Duration 1—grooming scored in one observation period without grooming scored in the observation periods immediately prior to or after scored response. Durations 2–5—grooming scored for 2 to 5 consecutive intervals without grooming being scored prior to the first or after the last period. Similarly, we defined the durations 6–10, 11–20, 21–30, 31–40 and one with a length of over 40 intervals.

A typical pattern of grooming durations from observations made in the home cages is shown in Fig. 4. These data are from a group of 8 intact animals which were merely picked up and then returned to their home cages. In producing the duration curves the percent of each animal's grooming bouts falling into each duration category was calculated and then these category scores are averaged for the entire group. Thus, each animal contributes to the distribution regardless of variations in the number of grooming episodes exhibited. As can be seen over 90% of the bouts scored were of shorter durations than 5 observation periods (Duration 1, 2–5). This means that few bouts lasted longer than 1.25 min.

The effect of handling animals, transporting them to an unfamiliar location, and then placing them in a novel testing chamber is shown in Fig. 4. The change found in the data is an increase in the number of bouts of long duration, that is, those in which grooming episodes lasted over 11 consecutive observation periods.

The effect of intraventricular injection of 1 μ l of ACTH₁₋₂₄ is also shown in Fig. 4. The effect is one of a further reduction of short grooming bouts and an increase in the number lasting for long periods of time. In fact it is not uncommon to find grooming bouts lasting for 60–80 consecutive observation periods (15–20 min). It should be emphasized that the number of bouts *per se* is not altered by ACTH administration, and consequently the increase in total grooming scores is a result of an extension of the duration of the bouts.

The amount of excessive grooming induced by ACTH₁₋₂₄ does not depend on the physical nature of the observation chambers. As much grooming is elicited by i.c.v. ACTH₁₋₂₄ in enriched environments as in the small, stimulation-restricted test chambers most frequently used (Jolles, *et al.*, 1979a). ACTH is also effective in eliciting excessive grooming in an open-field situation in which grooming had previously been shown to have a low frequency of occurrence without the administration of the peptide (Isaacson

A

B

C

FIG. 5. The stretching and yawning syndrome (SYS) in rats. A: Stretching; B: Crouching; C: Yawning.

and Green, 1978). In addition, only extremely high levels of food or water deprivation or intermittent footshock interfere with ACTH-induced excessive grooming.

6. THE STRETCHING AND YAWNING SYNDROME

Although the aim of this review is to cover the area of peptide-induced excessive grooming, some attention will be paid to the stretching and yawning syndrome (SYS). Firstly, because it was the first behavioral stereotopy reported to occur after intracranial injection of ACTH into mammals (Ferrari, 1958; *et al.*, 1963) and secondly, because most ACTH-induced excessive grooming bouts are interrupted by episodes of this syndrome (Izumi *et al.*, 1973; Gispen *et al.*, 1975). In rats, SYS is typified by stretchings, arched-back or crouched positions and yawning (Fig. 5). The SYS was first described in dogs, but could be equally elicited by intracisternal administration of ACTH in monkeys, rabbits, cats, guinea pigs, mice and rats. In rodents, the SYS is preceded by excessive grooming and rabbits yawn even more frequency than dogs (Ferrari *et al.*, 1963). Intrarachidially, ACTH preparation in doses up to $0.02 \mu\text{g/kg}$ in men caused nausea and vomiting, but no stretching (Floris, 1963). In 1967, Gessa summarized the results of the Italian group as follows:

(a) The 'stretching syndrome' may be induced only by peptides possessing ACTH and/or melanocyte-stimulating (MSH) activities; (b) it can be induced also after adrenalectomy; (c) it can be obtained only by injection of the above substances through the CSF route; (d) the effective doses are very low, in the order of micrograms ($0.5 \mu\text{g/kg}$ for the most active purified hormonal preparations); (e) the 'stretching yawning syndrome' can be evoked also in anesthetized animals; (f) this effect can be antagonized by chlorpromazine, atropine, morphine, but is not modified by reserpine, LSD-25, γ -aminobutyric acid, or by monoamineoxidase inhibitors (Gessa *et al.*, 1967).

Gessa *et al.* (1967) then show that each stretching is accompanied by an EEG and behavioral arousal. The most sensitive brain regions studied were the hypothalamic areas, lining the third ventricle. The ACTH injection in these areas induced also sexual excitement (Gessa *et al.*, 1967). In later years the research of this Italian group of investigators has primarily focused on sexual arousal induced by central application of these peptides (Bertolini *et al.*, 1968, 1969, 1975) and was supported by findings of others (Baldwin *et al.*, 1974; Haun and Haltmeyer, 1975).

As discussed elsewhere in this review, brain lesion studies have made it likely to conclude that the neural substrate involved in the induction of SYS and excessive grooming seems to be different (Colbern *et al.*, 1977). In 1963 Ferrari *et al.* reported that the ACTH induced SYS could be blocked among others by anticholinergic drugs (atropine). Recently, Urbà-Holmgren *et al.* (1977) reported findings suggesting that in the rat the act of yawning involves a central cholinergic mechanism, with 'muscarinic' receptors. At variance with a specific cholinergic mechanism seem the data from Huston, who first published that yawning and penile erection in rats could be brought about by cortical spreading depression (Huston, 1971) and subsequently that microinjection of ACTH_{1-24} into neocortex and hippocampus resulted in spreading depression (Jakobartl and Huston, 1977).

Furthermore, there is some indication that drugs stimulating central dopaminergic transmission given subcutaneously in low doses (not producing behavioral excitation) induce yawning in rats. Since DA receptor blockade blocked this yawning response, it was suggested that there is a dopaminergic component in this behavior as well (Mogilnecka and Klimek, 1977). However, Wood *et al.* (1978) reported that the SYS elicited in rats by icv injection of MSH or ACTH_{1-24} was paralleled by a two-fold elevation in the hippocampal turnover rate of acetylcholine. Since this increase was seen only in the hippocampus (and not in parietal cortex, frontal cortex, occipital cortex, striatum, diencephalon, or brainstem) they inferred that these peptides participate in the modulation of septal-hippocampal cholinergic neurons. In a more recent study these authors confirm

these findings and also showed that the 'barrel rotation' seen after somatostatin was paralleled by an enhanced turnover of hippocampal acetylcholine (Wood *et al.*, 1979). Their data point to an interaction of MSH and ACTH₁₋₂₄ with hippocampal receptors and that the septal-cholinergic pathway indeed is necessary to elicit a specific SYS. Our data on the absence of SYS after i.c.v. injection of ACTH₁₋₂₄ in total hippocampectomized rats are in line with this view (Colbern *et al.*, 1977).

7. STRESS AND GROOMING

The increase in grooming scores found after handling, transportation to the area of the observation chambers, and being placed in the novel circumstances of the observation chambers were found to produce an increase in observed grooming relative to home cage grooming after handling. This grooming has been described as novelty-induced grooming.

The first observations of novelty-induced grooming were made by Bindra and Spinner (1958). They systematically varied degrees of novelty between the home cage situation and the testing chambers. The most novel testing conditions produced an increase in grooming and a decrease in sniffing. In their most novel condition, white noise (73 db) was a prominent addition.

Our observation boxes are novel in the sense that their physical features are substantially different from those of the animals' home cages. They are in a new geographic condition, with new lights, sounds, and smells. Colbern *et al.* (1978) noticed the induction of excessive grooming by these conditions and considered it to be a response to these novel circumstances of the observation procedure. The role of various components of procedural and environmental factors was systematically studied by Jolles *et al.* (1979b). In all investigations the novelty-induced excessive grooming habituates with repeated testing. It is susceptible to being blocked by pretreatment with naloxone given systematically (Green *et al.*, 1979).

Cohen and Price (1979) report the presumed stress of playing back a recording of an adult rat screech to other rats in an observation chamber. The level of amplification was 20 db. This produced bouts of excessive grooming relative to a prior period of observation without the playback. Furthermore, the excessive grooming induced during the sounds was not particularly directed to a 'soiled' side of the body that was, however, the object of attention in animals without the sound stimulation. It is unfortunate that this experiment did not include a white noise condition so that any special effects of the rat screech relative to noise alone could be evaluated. As mentioned above, Bindra and Spinner found that noise, itself, could induce excessive grooming.

The novelty-induced grooming was thought to reflect the behavioral effect of a mild stress experience and its actions in eliciting the release of ACTH from the pituitary (Dunn *et al.*, 1979). In this experiment it was found that the central administration of anti-serum to ACTH reduced novelty-induced grooming, as would be expected from the hypothesis.

In the same study hypophysectomy reduced novelty-induced grooming, but the effects of grooming on this procedure are more complicated than originally thought and will be discussed in a subsequent section of this review. In general the notion that being placed in a novel observation induces a stress reaction involving the pituitary adrenal axis.

The studies of Bohus (1975) suggest that corticosteroid levels are increased by any treatment rats receive, especially during the period the presently described grooming test takes place. In general, 4 hr should be allowed for the corticosterone levels to return to basal, after exposure to a novel environment (Pfister and King, 1976).

Pfister (1979) measured the corticosteroid response to repeated presentations of a novel environment. An habituation of the stress response was also reported that corresponds, in general, with the habituation of novelty-induced grooming.

Novelty-induced grooming has been further studied by Jolles *et al.* (1979b). Several groups of animals were studied that had two common treatments: surgical insertion of a

chronic cannula into the third ventricle and 5 sec of handling before being placed in the observation box. The groups were subjected to various combinations of handling, transport and injection. The results indicate that the procedures used to make an intraventricular saline injection of 3 μ l produced more grooming than found with handling alone when the animals were tested in their home cages, but it did not make any difference if there was also transport involved or transport *and* novel environment, as indicated earlier in the chapter. Either transport or injection, alone, increased grooming to about the same extent.

Another undoubtedly stressful experience for rats is that of immersion in water. If rats are immersed in water for a minute or longer, they will engage in grooming at levels well above both home cage and baseline novel environment-induced grooming. Immersion for less than one minute is inconsistent in producing excessive grooming. Furthermore, while the grooming exceeds that produced by novelty, it is still less than that produced by interventricular ACTH. Following initial observation of this phenomena in Utrecht, (Brakkee and Gispen, in prep) extensions of this work was undertaken at SUNY-Binghamton (Colbern, Gispen, Hannigan, Radin, and Isaacson, in preparation). As part of this study, animals were immersed in water for one minute prior to being observed in the novel observation boxes. The animals engaged in excessive grooming, amounting to about twice the grooming seen in animals tested under 'dry', non-water immersed conditions. Once again, this excessive grooming produced by the water could be reduced by prior treatment with naloxone.

While the stress of water immersion induces excessive grooming, the stress of electrical footshock does not. Jolles, *et al.* (1979b) found that 1.0 mA footshocks of 2 sec duration delivered periodically throughout the observation period suppressed excessive grooming induced by intracerebral administration of ACTH₁₋₂₄. The footshocks also suppressed the 'novelty-induced' grooming exhibited by animals that received saline through intraventricular cannulae.

A different paradigm was used at SUNY-Binghamton to approach the same problem. Instead of applying footshock at different times throughout the test period, footshocks were delivered after the animals had been placed in the observation chamber for one-half hour. After the period of footshock the animals were observed for another 30 min. In one study using Swiss-Webster mice, no effect was found of footshocks when animals were tested over 7 consecutive days (Hannigan, Colbern, Radin, and Isaacson, in preparation). Using Wistar rats subjected to a similar testing paradigm, it was found that strong footshocks suppressed grooming in the second 30 min period relative to rats treated in the same way without the footshock. However, it was also possible to show that grooming could be produced as an anticipatory, or conditioned, response to the test situation. For example, after several days of habituation to the observation chambers, footshock was given between the first 30 min and the last 30 min of a test day. On the subsequent days of observation, grooming was enhanced in the first halves of the observation period relative to all preceding days. This indicates that while footshock itself inhibits grooming the anticipation of it can lead to enhanced grooming. The suppression of grooming by footshock does not occur because of the elimination of competing responses since in both procedures, namely those used in Utrecht and New York, there is abundant time for excessive grooming to be exhibited. The animals do not show constant immobility or exhibit interfering responses in the non-shock periods.

The effects of another type of stress was studied by Hannigan, Colbern, Radin and Isaacson (in preparation). Animals were placed in close bodily restraint and inverted for 5 min before testing. This produced intense struggling and signs of severe stress, including urination, defecation and nasal hemorrhage. The restraint procedure, however, did not induce excessive grooming in the hour subsequent to the restraint when animals were placed in the novel observation boxes. In fact, grooming scores were reduced below the amount usually found in the 'novel' observation boxes after transport and handling.

This study is of special interest because the restraint procedures produced a substantial disruption of the animals' fur, and indeed they had usually fouled themselves in other

ways. Nevertheless, grooming was not observed afterwards. Therefore, excessive grooming cannot be thought of as responses initiated by the physical distortion of the animal's coat.

In the study of other stress procedures on excessive grooming, mixed results have been found, depending on the nature of the stress. For example, in the rat, exposure to cold (5°C) for about one-half an hour induces excessive grooming, while exposure to high temperatures (37°C) does not. Rats also fail to exhibit grooming during the period after exposure to ether fumes or after the administration of an anesthetic dose of barbiturates and before hypnosis is effected. Therefore, it seems clear that exposure to certain stress experiences induce excessive grooming while others do not. It is unlikely that these differences can be explained simply on the basis of conflict among behaviors elicited by the stressors or by peripheral disturbances of skin or fur that may occur.

It should also be noted that certain more natural stress conditions lead to the induction of grooming. For example, in his studies with the vole, Fentress (1968a,b) found that the sight of an apparent predator would induce grooming. In addition, when grooming would occur, and indeed whether it would occur at all, depended on the effectiveness of the stimulus presented and the inferred emotional nature of the species studied—the more timorous the animals, the stronger the reaction to the fear-inducing stimulus and the less subsequent grooming.

It is possible that the strength of the stress condition or the magnitude of the response could be related to the amount of grooming exhibited afterwards. The problem, of course, is how to specify the degree of stress. Little work has been done on the evaluation of stress magnitude using sensitive measures of ACTH release in blood plasma or brain and estimates made from induced corticosteroid release are limited by the response characteristics of the adrenal cortex. It may also be the case that differences in the 'strength' of a stress condition may be considered in light of the simultaneous activation of systems that may oppose or antagonize the neural systems responsible for grooming. This possibility will be considered more fully after a discussion of pharmacologic investigations of the grooming response.

8. THE EFFECTS OF HYPOPHYSECTOMY

The observation of intact animals in an unnatural setting produces excessive grooming as mentioned above. The question of the effect of removal of the pituitary was considered important because the answer could shed light on whether neuropeptides released from the pituitary play a role in determining such behaviors. Dunn *et al.* (1979) reported data that made it appear that it did. Animals hypophysectomized at Charles River Laboratories were tested one week after being received. The animals had undergone surgery the day before shipment. They were tested for grooming for three consecutive days in their home cages and after transport from the colony room to the observation room. Then they were tested for four days in the same observation room while in the novel testing chambers. When tested after being placed in the novel boxes, the intact animals showed a considerable increase in grooming, while the hypophysectomized animals, as a group, evidenced a very small increase. In terms of individual subjects only one-half of the hypophysectomized animals showed increases in total grooming scores when first tested in the novel boxes relative to their last testing day in their home cages. All of the intact animals showed increases in grooming when first tested in the novel boxes.

On the other hand Jolles *et al.* (1979b) found substantial increases in grooming when hypophysectomized animals were tested in a novel environment after transport and handling. Their comparison was made between independent groups of hypophysectomized animals each tested once, while Dunn *et al.* (1979) evaluated a change in grooming after a change in conditions. However, in all of the conditions reported by Jolles *et al.*, grooming scores in the hypophysectomized animals were greater than those found by Dunn and his co-workers.

A number of factors might be responsible for the differences in the two studies. These include the genetics of the animals used, although both were 'Wistar' rats. The animals used by Jolles *et al.* were of an inbred strain of the animals (TNO, Zeist, The Netherlands). It is conceivable that age could make a difference, but in these two studies the animals were about the same body weights (about 150 grams, approximately 40 days of age) at the time of surgery. In searching for other, possibly important differences between the Dunn *et al.* and Jolles *et al.* studies, it was apparent that the animals had been subjected to widely different experiences before testing. We felt that these differences could have played an important role in producing the differences in observed grooming in the novel observation boxes.

To help resolve the question of the effects of hypophysectomy on grooming, another study was undertaken. Hypophysectomized animals were divided into two groups after surgery: one group that received experiences thought to be stressful for two days and another group that was undisturbed. The stressors included 30 min in a cold room (4°C), disturbance of fur and restraint with masking tape, forced swimming for 3 min, and bodily shaking for 5 min. These stress procedures were applied for two days. The animals were observed for grooming in the novel situation on the 4th, 7th and 14th days after surgery. There were no differences between the hypophysectomized animals stressed on the two postoperative days and those that weren't on any test day. There were also no differences between stressed and non-stressed sham operate controls. Therefore, other comparisons were made between the total hypophysectomy and sham operated groups. The sham operated control animals did not change over the repeated testing. However, the hypophysectomized animals had higher grooming scores on the 4th and 14th test days than the sham operates. On the test made on the 7th day postoperative, there was a significant decrease in grooming scores for the hypophysectomized animals and their mean grooming score was lower than that of the controls. This difference, however, was not significant. On this second test day the scores of the hypophysectomized animals were not different from those of the controls. Based on these results and other tests given later in the experiment, it would appear likely that testing in the novel grooming test area decreases the tendency of hypophysectomized animals to give high grooming scores on subsequent tests. It is as if the novel situation was not as novel for the hypophysectomized animals if they are tested again within 2 or 3 days. In the study of Dunn *et al.* the animals had been brought to the testing area for three consecutive days before the important test day in the novel box. The box was new but the environment was not.

In our most recent study the animals on the 4th day had been handled, transported and stressed on the two preceding days in different locations but not the environment used for novel cage testing. It appears then that it is not prior stress *per se* that influences the amount of grooming exhibited by the hypophysectomized animals but the prior experience with the specific test situation.

In general it can be said that the induction of excessive grooming does not depend upon the integrity of the pituitary gland. Indeed, on first exposure to the novel testing situation greater than normal grooming is exhibited. But hypophysectomized animals also show an increased effect of habituation to the area in which the 'novel' boxes are located.

9. INDEPENDENCE FROM THE ENDOCRINE SYSTEM

As the peptides ACTH₁₋₂₄, or α -MSH, and β -MSH have complete classical hormonal activity, it is pertinent to show that the peptide-elicited grooming is the result of a direct effect of these peptides on the central nervous system coordinating this behavioral response and not the result of an indirect action mediated by peripheral endocrine glands or hormone-target tissues.

This question has been approached using ACTH as the behaviorally active hormonal peptide. And variety of evidence from such studies suggests a peptide-sensitive central nervous system mechanism underlying the excessive grooming response.

In contrast to the effects of peptides derived of ACTH on adaptive behaviors and amnesia, the excessive grooming response could not be elicited using systemic administration of the same peptide (Ferrari *et al.*, 1963; Gispén *et al.*, 1975). Only intraventricular or intracisternal administration of the peptide results in the display of the grooming response. In our hands, the grooming could be elicited from lateral ventricle, third ventricle, or fourth ventricle injections (Gispén *et al.*, 1975; Colbern *et al.*, 1977). Routinely the behavior is elicited through cannulae placed in the foramen interventricularis (Brakkee *et al.*, 1979), and gives highly reproducible data. Although the short interval between i.c.v. injection and onset of the behavior (a few minutes) is not necessarily indicative of a central action of the peptide, it certainly fits with such a notion.

Some authors suggest that in addition to SYS, intraventricular administration of these peptides also induced sexual arousal in rabbits and rats (Bertolini *et al.*, 1968; 1969; 1975; Baldwin *et al.*, 1974; Haun and Haltmeyer 1975). In fact, evidence has been obtained that suggests a 'permissive' or mediational role of the gonadal steroids in the sexual responses elicited by ACTH (Bertolini *et al.*, 1975). In addition, Haun and Haltmeyer (1975) showed an increase in plasma testosterone (males) or progesterone (females) after intercerebroventricular administration of ACTH into rabbits. Plasma corticosterone was also increased by this treatment in all female rabbits, but only in half of the males. They postulated that the post-injection increases in corticosterone seem to reflect stimulation of the adrenal cortex by the exogenous ACTH which 'escaped' from the cerebrospinal fluid system into the general circulation (Haun and Haltmeyer, 1975).

In a recent study (Wiegant *et al.*, 1979a) we tested the hypothesis that i.c.v. ACTH could 'escape' from the CSF and that in some fashion elevation of adrenal steroids would contribute to the ACTH-induced grooming response. The activity of the pituitary-adrenal system and the induction of excessive grooming was measured over a period of 1-2 hrs after the intraventricular (i.c.v.) or systemic fragments of ACTH. After i.c.v. administration, the fragments ACTH₁₋₂₄ and ACTH₁₋₁₆-NH₂ were equipotent in inducing excessive grooming behavior and in stimulating the release of adrenal corticosterone (Fig. 6). The small fragment ACTH₄₋₁₀, which is active in altering adaptive behavioral processes (De Wied, 1977) was without effect on either grooming or corticosteroid release. As was shown previously (D-Phe⁷)ACTH₄₋₁₀ and ACTH₄₋₁₀ often have different behavioral effects (Bohus and de Wied, 1966; Wiegant *et al.*, 1978a) and indeed the D-enantiomer induced considerable display of excessive grooming and a small but statistically significant rise in plasma corticosterone levels. Thus, it seemed that if a

FIG. 6. Effects of i.c.v. injected fragments of ACTH on plasma corticosterone and excessive grooming. (from Wiegant *et al.*, 1979b). $n = 5$ for each group, sal = saline, 1-24 = ACTH₁₋₂₄ (1 µg) 1-16 = ACTH₁₋₁₆ (1 µg), D-4-10 = [D-Phe⁷]ACTH₄₋₁₀ (5 µg), 4-10 = ACTH₄₋₁₀ (5 µg).

FIG. 7. Effect of i.c.v. ACTH₁₋₁₆ (1 µg) on plasma corticosterone and excessive grooming in hypophysectomized rats (from Wiegant *et al.*, 1979b). ●—● saline, ▼—▼ ACTH₁₋₁₆.

peptide has grooming-inducing potency, intraventricular administration leads to an enhanced release of adrenal corticosterone. However, we were able to demonstrate that the display of excessive grooming was not dependent on circulating steroid hormones. i.v.c. ACTH-induced grooming behavior was successfully elicited in hypophysectomized, adrenalectomized, or gonadectomized rats (Gispen *et al.*, 1975; Wiegant *et al.*, 1979a). In addition rats hypophysectomized 24 hrs prior to the behavioral test groomed as vigorously as intact rats, without a concomitant rise in plasma corticosterone levels after i.c.v. ACTH administration (Fig. 7, Wiegant *et al.*, 1979a).

This experiment demonstrated that the increase in corticosterone after i.c.v. ACTH depends upon an intact pituitary gland. Leakage from the cerebrospinal fluid into the general circulation as a route by which i.c.v. ACTH could activate the adrenal cortex is further ruled out by the differential effect of subcutaneously administered ACTH fragments. ACTH₁₋₂₄ has full corticotrophic activity, whereas ACTH₁₋₁₆ and (D-Phe⁷)ACTH₄₋₁₀ do not stimulate adrenal cortical steroid release (Sayers *et al.*, 1974). Although no excessive grooming behavior was observed after peripheral administration of ACTH₁₋₂₄ and ACTH₁₋₁₆, in the case of ACTH₁₋₂₄ treatment a dramatic rise in plasma corticosterone level was observed (Wiegant *et al.*, 1979a).

The question then arises what, if any, relation exists between the effect of ACTH on grooming and on the pituitary-adrenal axis. It could be envisaged that the behavioral response induced by the peptide would trigger the activation of the endocrine axis. As concluded above, it is unlikely that the rise in plasma steroid levels is responsible for the induction of excessive grooming. On the other hand, it could be that ACTH independently activates two neural substrates, one responsible for the effects on grooming behavior, and the other involved in the regulation of the pituitary function. As was shown before, there is good evidence that there is a refractory period in which a second injection of peptide is remarkably ineffective in producing the behavioral response. Detailed analysis of this phenomenon led us to presume that the neural substrate temporarily had become tolerant to the peptide (Jolles *et al.*, 1978; Wiegant *et al.*, 1978b). This property of the grooming response was used to study the relation of the two effects brought about by central application of ACTH. It was found that a second injection of ACTH 4 hr after a first administration is still a strong stimulus for the activation of the pituitary-adrenal system despite its inability to produce the behavioral response. In view of this differential effect on hormone release and grooming behavior, it is suggested that the two responses are brought about by two different interactions of ACTH and the central nervous system (Wiegant *et al.*, 1979a). Such a conclusion is in accordance with the working hypothesis previously formulated, describing the central effects of ACTH in terms of multiple neural substrates with possible multiple receptors (Gispen *et al.*, 1977).

10. STRUCTURE-ACTIVITY STUDIES

In a series of experiments the effect of gradual shortening of the ACTH₁₋₂₄ sequence (Fig. 8) on the capacity of the peptide to induce grooming was studied. For all peptides at

β -Endorphine β -MSH δ -MSH α -MSH ACTH	β -Endorphine																			
	TYR VAL — GLY — ASP ARG PHE GLY										GLY LYS LYS ARG									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
ASP SER GLY PRO TYR	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80
	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100
TYR GLY GLY PHE MET	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
GLN THR PRO LEU VAL	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
THR SER GLU LYS SER	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
ALA ILE VAL LYS ASN	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
PHE LEU PHE LYS ASN	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
GLU PHE GLU PHE	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60

FIG. 8. Aminoacid sequence of lipotropin (41-91), β -MSH, γ -MSH, α -MSH and ACTH.

least a dose equimolar to $3 \mu\text{g}/3 \mu\text{l}$ ACTH₁₋₂₄ was tested (Gispen *et al.*, 1975). At this dose level, the peptides ACTH₁₋₁₆NH₂, α -MSH and β -MSH were as active as the sequence ACTH₁₋₂₄. In order of decreasing potency the following peptides also induced grooming: (Lys¹⁷, Lys¹⁸) ACTH₅₋₁₈-HN₂, ACTH₅₋₁₆-NH₂, ACTH₁₋₁₃-NH₂ and ACTH₅₋₁₄. The peptides ACTH₁₁₋₂₄, ACTH₇₋₁₆-NH₂, ACTH₁₋₁₀ and (Ac-Ser¹) ACTH₁₋₁₀ were found to be inactive. Interestingly, i.c.v. injection of the combination of ACTH₁₋₁₀ + ACTH₁₁₋₂₄ did not result in the display of excessive grooming, indicating that a certain sequence of amino acids or stereoconformation of a peptide chain was essential and not the mere presence of amino acids (Gispen *et al.*, 1975). The fact that ACTH₁₋₂₄, α - and β -MSH were effective was ascribed to the common presence of the sequence (4-10): Met-Glu-His-Phe-Arg-Trp-Gly (Fig. 8).

When Nakanishi and co-workers published their pioneering study on the nucleotide sequence of cloned cDNA for the bovine corticotropin β -lipotropin precursor (Nakanishi *et al.*, 1979) it became apparent that in the 'cryptic' portion of the precursor protein molecule in addition to the β - and α -MSH sequence, a third melano-tropin sequence was present, labeled γ -MSH. This peptide consists of 12 amino acid residues with the sequence γ -MSH₃₋₈ (Met-Gly-His-Phe-Arg) very similar to the sequence ACTH₄₋₉, having a Gly residue instead of a Glu (Fig. 8). Surprisingly, however, when tested over a wide range for grooming-inducing capacity, this peptide did not induce excessive grooming in the rat, underscoring the structure-specificity of the peptide-induced excessive grooming response (Gispen, unpublished).

Ferrari *et al.* (1963) who selected the dog for his studies on the effect of ACTH on SYS as being the most susceptible for central actions of ACTH, reported that high doses of ACTH₄₋₁₀ induced the display of SYS. Baldwin *et al.* (1974), on the other hand using rabbits, reported only marginal effects of ACTH₄₋₁₀ on SYS and sexual behavior after i.c.v. administration. We reported that intraventricular ACTH₄₋₁₀ even in doses up to $40 \mu\text{g}$ was unable to induce excessive grooming in the rat (Gispen *et al.*, 1975; Wiegant and Gispen, 1977; Wiegant *et al.*, 1978a). This finding is supported by observations of Rees *et al.* (1976) studying excessive grooming in the mouse who reported no effect of ACTH₄₋₁₀.

However, in a later study, the grooming-inducing potency of short sequences of amino acid within the ACTH₄₋₁₀ peptide was examined (Wiegant and Gispen, 1977). It was found that like ACTH₄₋₁₀, the sequences (4-9), (4-8), (4-6), (5-7), and (7-10) were inactive. The only sequence with grooming inducing activity was the sequence (4-7). On an equimolar basis this peptide had only 30% of the activity as compared to ACTH₁₋₂₄.

Studies using analogs of the ACTH₄₋₁₀ peptide also pointed to latent grooming-induction activity within the (4-10) sequence. Substitution of the L-enantiomer of phenylalanine by its D-enantiomer at position 7([D-Phe⁷] ACTH₄₋₁₀) resulted in a peptide with 50-60% of the activity of an equimolar dose of ACTH₁₋₂₄ (Gispen *et al.*, 1975; Rees *et al.*, 1976). Similar substitution in the inactive sequence (1-10) to a lesser extent also resulted in an enhancement of induced grooming. In contrast, D-enantiomer substitution of Arg in position 8 did not potentiate the activity of the (4-10) sequence (Gispen *et al.*, 1975), pointing to a specific role of the phenylalanine at position 7. The [D-Phe⁷] substitution, however, was only effective when the Phe-residue was within a given sequence, as the activity of (4-7) or (7-10) could not be potentiated by D-Phe substitution (Wiegant and Gispen, 1977). Furthermore, i.c.v. injection of D-phenylalanine alone, over a wide dose range, was ineffective in inducing excessive grooming in the rat (Gispen unpublished). Interestingly, combined i.c.v. treatment of ACTH₄₋₁₀ and [D-Phe⁷] ACTH₄₋₁₀ did result in a grooming response as happened when the rats were treated with the D-analog alone. Even an excess of 10 times more ACTH₄₋₁₀ than [D-Phe⁷]ACTH₄₋₁₀ did not interfere with the effectiveness of the D-peptide (Wiegant *et al.*, 1978a).

D-substitution in peptides is one of the means used to improve their metabolic stability. Witter *et al.* (1975) demonstrated a correlation between the half-life of D-potentiated ACTH analogs and the magnitude of the behavioral response in a pole-jumping active-

avoidance extinction test. However, the fact that the peptide [Met (0)⁴, D-Lys⁸, Phe⁹]ACTH₄₋₈—a highly potentiated peptide in avoidance extinction with enhanced metabolic stability—and the peptide [D-Arg⁸]ACTH₄₋₁₀ were both inactive in inducing grooming (Gispen *et al.*, 1975), suggests that improved metabolic stability alone cannot explain the enhancement by the D-Phe⁷ substitution. This data emphasizes the importance of intrinsic activity, affinity for binding site and transport properties of the peptide, which co-determine its ultimate effectiveness for the grooming response.

These ACTH structure/grooming-activity data led us to conclude that the latent activity within the (4-10) sequence resides in the sequence (4-7). In addition the sequence (5-7) seems essential, since there is decreasing potency among the following peptides: [Lys¹⁶, Lys¹⁸]ACTH₅₋₁₈NH₂, ACTH₅₋₁₆-NH₂ and ACTH₅₋₁₄ in their ability to elicit excessive grooming (Gispen *et al.*, 1975). The region adjacent to the C-terminus of ACTH₄₋₇, i.e. 7-24, in itself, does not have the information necessary to induce excessive grooming, since peptides such as ACTH (7-10), (7-16), or (11-24) were without effect. However, C-terminal elongation of the active site ACTH (5-7) to (5-18) led to a marked potentiation indicating that this C-terminal part may be involved in the enhancement of the peptide-grooming receptor interaction, thus providing additional affinity without exerting intrinsic activity itself.

Other ACTH-structure/CNS-activity relationships are not identical to the one observed for excessive grooming, but the general principle of dormant activity and induction of such activity by chain elongation or D-substitution seems to apply (Gispen *et al.*, 1977). With respect to the effect of ACTH on extinction of active avoidance behavior in rats, active sites in ACTH (4-7) in the region C-terminal from (4-7) seems to exist (Greven and de Wied, 1977). Also the structural requirements for ACTH-fragments to displace dihydromorphine from its receptor in brain membranes *in vitro* suggest an active site within ACTH (4-10) and possibly a second active site C-terminal from that site (Terenius *et al.*, 1975; Gispen *et al.*, 1976a, Eberle, personal communication). Similarly counteraction of morphine-induced analgesia in rats by systemic administration of fragments of ACTH points to the importance of the sequence ACTH (4-10) (Gispen *et al.*, 1976a). Effects of ACTH fragments on amino acid incorporation into proteins either *in vitro* or in brain slices also revealed that the situation brought about by ACTH₁₋₂₄ could be mimicked by the short sequence (4-10) (Schotman *et al.*, 1972; Reith *et al.*, 1975, 1977; Dunn *et al.*, 1976; Rees and Dunn, 1977). However, in these studies the [D-Phe⁷] substitutions lead to loss of protein synthesis stimulation or even an inhibition of the protein synthesis rate. Recently Schotman and his co-workers were able to demonstrate that ACTH fragments may modulate brain protein synthesis in a cell-free system *in vitro* (Schotman *et al.*, 1980). The amino acid incorporation rate was stimulated by sequences sharing the ACTH (4-10) region and inhibited by peptides with sequences C-terminal from ACTH₄₋₁₀ (Schotman *et al.*, 1980). Weigant *et al.* (1979b) reported that ACTH₁₋₂₄, ACTH₁₋₁₆-NH₂ and ACTH₄₋₇ inhibited rat brain membrane adenocyclase activity, whereas the sequences (1-10), (11-24), (4-10) and D-Phe⁷ (4-10) were inactive.

So far the best correlation between the structural requirements of an ACTH peptide for grooming activity and another CNS event seems that with the effect of ACTH on rat brain protein phosphorylation (see below, Zwiers *et al.*, 1978).

From the similarities and dissimilarities in the above mentioned ACTH-structure/CNS-activity studies and the multiplicity of the CNS responses modulated or elicited by ACTH, one is tempted to conclude that the CNS contains types of ACTH-sensitive sites (receptors) that are involved in more than one type of function. Yet, it may be that for all ACTH-CNS interactions the crucial information resides in the sequence (4-7), with phenylalanine 7 playing a key role (see de Wied, *et al.*, 1975). Different interactions of the adjacent C-terminal sequence with different ACTH receptors in the brain could be responsible for the discrepancies in the structure-activity relationships as discussed above (de Wied *et al.*, 1975; Gispen *et al.*, 1977). Furthermore, information in a peptide sequence is encoded in multiple forms (Schwyzer, 1968) and therefore often a comparison between peptides on basis of primary structure alone seems hazardous. Induction of

activity by chain elongation of D-enantiomer substitution underscores the importance of knowledge on the stereoconformation of the peptides at their respective receptor site. An important breakthrough in our experimental approach toward the nature of brain cell-ACTH interaction may be the suggestion that at the brain receptor site, ACTH₄₋₁₀ assumes an α -helix conformation with the Met⁴ and the Arg⁸ in close proximity (Greven and de Wied, 1977). Above all, however, identification of ACTH-receptors in rat brain by means of radio ligand assays seems essential for further characterization of ACTH-CNS interaction.

11. ACTH AND BRAIN PHOSPHOPROTEINS: A NEUROCHEMICAL CORRELATE OF GROOMING?

As mentioned in the section on structure-activity studies, the neurochemical event influenced by ACTH most correlated with excessive grooming seems to be the phosphorylation of certain synaptosomal plasma membrane (SPM) proteins.

There is a variety of evidence suggesting that SPM protein phosphorylation may effect the efficiency of neurotransmission (Williams and Rodnight, 1977; Greengard, 1976). Using a neurophysiological approach, two independent research groups have shown, for instance, that repetitive electrical stimulation of pathways in hippocampal slices *in vitro* resulted in synaptic potentiation accompanied by changes in SPM protein phosphorylation (Browning *et al.*, 1979, Bär *et al.*, 1979, 1980). In both studies it was shown that the effects were calcium dependent. The demonstration of calcium dependency of phosphorylation of certain SPM proteins and of the exocytosis of neurotransmitters underscores the potential role such phosphoproteins may have in the information transfer of one neuron to another. Therefore, this process may be a target for modulation of neurotransmission of neuropeptides (Gispen *et al.*, 1979). In a series of studies the effect of ACTH on rat brain SPM phosphoproteins was studied *in vitro* (Zwiers *et al.*, 1976, 1978, 1979, 1980a). It was found that ACTH inhibited the endogenous phosphorylation of at least five phosphoprotein bands after electrophoretic separation of SDS-polyacrilamide slab gels of the SDS solubilized proteins of light synaptosomal plasma membranes. The molecular weight of these bands ranged from 15 to 48 k, and they thus were of a smaller molecular weight than those stimulated by the *in vitro* addition of cAMP (Zwiers *et al.*, 1976). In most of the further research, the effect of ACTH in the phosphorylation of one protein band (B-50, MW48K) was analyzed in more detail. The B-50 protein kinase/B-50 substrate protein complex could be solubilized from the SPM and further purification using DEAE column chromatography, ammonium sulphate precipitation and two-dimensional separation (IEF and SDS) on polyacrylamide gels revealed the presence of an ACTH-sensitive protein kinase phosphorylating the B-50 substrate protein (Zwiers *et al.*, 1979, 1980a).

The apparent molecular weights and isoelectric points for the kinase and the substrate were 70k, 5.5 and 48k, 4.5 respectively. The B-50 protein kinase was sensitive to fragments of ACTH, insensitive to endorphins or cyclic nucleotides and depended on the presence of calcium (Zwiers *et al.*, 1980a; Gispen *et al.*, 1979). Structure-activity studies using ACTH fragments and intact SPM revealed that the sequences (1-24), (1-16), (1-13), (5-18) and to some extent (5-16) were capable of inhibiting the endogenous phosphorylation of the B-50 protein. The sequences (1-10), (4-10), (11-24), (7-16), and the combination of (1-10), (11-24) were ineffective (Zwiers *et al.*, 1978). Therefore, it appears that ACTH fragments capable of inducing excessive grooming after i.c.v. administration *in vivo* (see above) will, *in vitro* inhibit the phosphorylation of a calcium-dependent SPM phosphoprotein (Fig. 9). To our knowledge, this structure-activity relationship is the strongest known between a neurochemical event and a behavioral activity caused by a neuropeptide. Furthermore, preliminary data have been obtained suggesting that i.c.v./ACTH treatment of rats resulting in excessive grooming also led to changes in brain SPM protein phosphorylation as measured in a post hoc *in vitro* assay (Zwiers *et al.*, 1977). More evidence is accumulating pertaining to the notion that excessive grooming

FIG. 9. Structure-activity correlation between ACTH effects on grooming and brain membrane protein phosphorylation. i.c.v.: equimolar to $3 \mu\text{g}/\mu\text{l}$ ACTH₁₋₂₄. In assay: 10^{-4} M peptide. (from Gispen *et al.*, 1979).

and inhibition of B-50 protein phosphorylation are strongly associated. For example, Zwiers and his colleagues have reported the occurrence of a new basic peptide (PIP) which most likely is produced by partial proteolysis of the B-50 protein and which *in vitro* inhibits the phosphorylation of B-50 and *in vivo* induces excessive grooming behavior typified by bouts of scratching and paw licking (Zwiers *et al.*, 1980b). Certainly, more work is needed to assess the exclusivity of the protein phosphorylation and the grooming response relationship. It may well be that the change in SPM protein phosphorylation is a key event in a variety of CNS response modulated by fragments of ACTH.

12. INTERACTION WITH THE OPIATES: GROOMING INDUCTION WITH ENDORPHINS

The dramatic pharmacological effects of opiates on central nervous system functions over the years have inspired researchers to try to unravel what endogenous receptor would recognize the foreign morphine molecule. In the early seventies three independent groups demonstrated in CNS membranes the existence of high-affinity, stereospecific binding sites for opiates (Simon *et al.*, 1973). The search for what endogenous ligand would physiologically occupy and possibly activate this receptor was successfully undertaken and the first ligand was characterized by Hughes, Kosterlitz and their colleagues as being the sequence LPH₆₁₋₆₅ denoted as Met-enkephalin (Hughes *et al.*, 1975). They also reported the existence of a Leu-enkephalin. Rapidly more endogenous opiate-receptor ligands were identified: α -endorphin (Guillenim *et al.*, 1976), β -endorphin (Bradbury *et al.*, 1976) and dynorphin (Goldstein *et al.*, 1979). Zimmermann and Krivoy (1973) had already collected evidence that in *in vitro* spinal cord systems of cat and frog, ACTH could block the morphine-induced depression of spinal reflexes without influencing the reflex, itself. The suggestion of these data that ACTH was recognized by opiate-sensitive sites in the nervous system was confirmed by Terenius (1975), who reported the displacement of dihydromorphine from rat brain opiate receptors by ACTH₁₋₃₉ and ACTH₄₋₁₀. In further studies, it was confirmed that N-terminal sequences of ACTH can interfere with morphine binding to opiate-specific binding sites in rat brain membranes *in vitro* (Terenius *et al.*, 1975). Further analysis of the opiate displacing properties of ACTH led to the conclusion that ACTH should be considered as a partial agonist-antagonist of the opiate receptor (Terenius, 1976). The IC_{50} , however, was in the order of 10^{-6} – 10^{-5} M ACTH, apparently too low to consider ACTH as an important ligand for CNS opiate receptors. Yet, the same authors showed that such fragments when given systemically in

high doses could reduce morphine analgesia in rats (Gispen *et al.*, 1976a). In addition, Katz (1979) recently reported that i.c.v. ACTH₄₋₁₀ antagonized morphine-induced behavioral activation in the mouse.

With most of the information cited above at hand, an effort was undertaken to investigate if ACTH-induced excessive grooming could be suppressed by the specific opiate antagonists (Gispen and Wiegant, 1976). Indeed, subcutaneous injection of naloxone or naltrexone in doses of 0.25–1 mg/kg completely blocked the display of excessive grooming induced by i.c.v. ACTH₁₋₂₄. If indeed opiate-receptor mechanisms were involved, would treatment with opiate agonists then result in the display of such behavior? Previously, Ayan and Randrup (1973) had already reported that peripheral injection of low doses of morphine is followed by, among other behaviors, an increased display of grooming and scratching. Indeed, i.c.v. injection of morphine (0.05–0.5 g/3 μ l) into both hypophysectomized and intact rats resulted in the display of excessive grooming. After higher doses (1 and 5 μ g) invariably the behavior of the rats was depressed and they all sat still during the total observation period (Gispen and Wiegant, 1976). Furthermore, if low doses of β -endorphin (β -LPH₆₁₋₉₁) were injected into the foramen interventriculare of conscious rats an enormous increase in grooming activity was observed (Gispen *et al.*, 1976b). A dose of as little as 30 ng significantly induced excessive grooming and doses in the range of 0.1–0.3 μ g already elicit nearly maximal grooming behavior. Doses from 1–10 μ g resulted in a marked behavioral depression (Wiegant *et al.*, 1978b). Administration of β -endorphin in low doses not only elicits grooming but also excitation in some rats, typified by quick movements of body and head, jumping, gnawing, and body shakes. A further difference with ACTH-induced grooming is that no episodes of stretching and yawning were observed. β -Endorphin was about 10 times more potent than ACTH₁₋₂₄ or α -MSH with respect to the induction of excessive grooming (Gispen *et al.*, 1976b). The nature of the excessive grooming is somewhat different when elicited by ICV ACTH₁₋₂₄ and by i.c.v. β -endorphin. The effect of ACTH is to increase the duration of grooming bouts but not to influence the number of times grooming bouts are begun during the observation period. After i.c.v. β -endorphin, the distribution of grooming durations is unchanged, but the number of bouts is greatly increased. This is shown in Fig. 10.

It has been reported that β -endorphin (30 μ g unto lateral ventricle; 4 μ g into periaqueductal grey) in rats elicits wet dog shaking behavior followed by a marked prolonged muscular rigidity and immobility similar to a catatonic state (Bloom *et al.*, 1976; Jacquet

FIG. 10. Duration and start analysis of grooming behavior induced by i.c.v. ACTH₁₋₂₄ (1 μ g) or β -endorphin (0.1 μ g).

and Marks, 1976). Also with such doses of endorphins profound analgesia is induced (van Ree *et al.*, 1976; Feldberg and Smyth, 1976; Graf *et al.*, 1976). Recently, it has been reported that intraventricular administration of high but sub-cataleptic doses of β -endorphin (7.5 μ g) in rats results in wet dog shaking behavior followed by copious salivation accompanied by clonic, seizure-like state (Holaday *et al.*, 1978). All of these behavioral responses to intracranial administration of β -endorphin can be blocked by pre- or simultaneous treatment with the opiate antagonist naloxone.

It should also be noted that with the low doses of β -endorphin used to induce excessive grooming behavior, there were no alterations produced in body temperature (Gispén, unpublished).

LPH itself does not induce excessive grooming, although it has been reported that intraventricular administration of 2.5 mg sheep β -LPH in the rat elicits some grooming (Izumi *et al.*, 1973). It is conceivable that formation of β -endorphin from this enormous dose underlies the observed activity. In addition, production of the sequences LPH₄₁₋₅₈, (β -MSH) and LPH₄₈₋₅₁ (ACTH₄₋₇) (Gispén *et al.*, 1975; Wiegant and Gispén, 1977) which are located toward the N-terminal of LPH₆₁₋₉₁ may also be factors in initiating any grooming observed after such a massive dose. The peptide with the highest activity and located C-terminal from the 61 residue is β -endorphin itself (Fig. 8). Shortening the sequence to LPH₆₁₋₇₇ (γ -endorphin) or LPH₆₁₋₇₆ (α -endorphin) is accompanied by a dramatic reduction in activity. The sequence LPH₆₁₋₆₉ is the shortest peptide beginning with the 61 residue which has notable activity in grooming. The sequence 61-65 (enkephalin) even in high doses does not induce excessive grooming (Gispén *et al.*, 1976b).

The fact that both sequences N-terminal and C-terminal from the 61st residue can induce the same behavior is not surprising. As with the effects of ACTH on extinction of learned responses and for the MSH effects on melanocytes, more than one active sequence seem to be present in one peptide molecule (Gispén *et al.*, 1977). It may be that the stereoconformation of the molecule at the receptor favors the interaction with only one site. It should be borne in mind, that structure-activity relations as discussed here involve not only peptide-receptor interactions, but also concern metabolic rate, availability, transport, etc.

From the studies on the behavioral responses in quality similar to the quasi-morphine abstinence syndrome, Jacquet (1978, 1979a) suggested that there are two classes of opiate receptors in the periaqueductal region of rat brain. The ligand for one class is thought to be β -endorphin and, for the other, ACTH. Although the term 'ACTH receptor' has been questioned (Amir *et al.*, 1979), Jacquet (1979b) maintains that behavioral effects of opiates are mediated by two receptors, a stereo-specific β -endorphin receptor and a nonstereo-specific ACTH receptor. At present, however, judgment should be reserved with respect to a generalization on this concept to other central nervous system regions. Her studies were limited to the periaqueductal gray region. In addition, extremely high doses of the ACTH fragments were used to induce the abstinence signs (i.e. local injection of 10-50 μ g peptide). Local application of ACTH into the substantia nigra does induce excessive grooming but not jumping described as rapid explosive motor behavior (Wiegant *et al.*, 1977). Similar application of high doses of ACTH into the periaqueductal grey elicits jumping without excessive grooming (since it was not listed under the additional signs of abstinence by Jacquet (1978). Therefore, it appears that the two peptide-induced behaviors are mediated by different neural substrates. Neither from our studies nor from those of Jacquet can the conclusion be drawn that systemic application of naloxone interferes with peptide-induced behaviors at the level of the peptide receptor itself. It may well be that naloxone interferes with an opiate sensitive neural component distal (more toward the effector mechanism) from the site of peptide induction. The fact that certain adaptive behaviors are influenced by both endorphins and ACTH in a similar fashion and not influenced by naloxone (de Wied *et al.*, 1978a; Bohus and Gispén, 1978; Rigter, 1978) and the demonstration of non-opiate endorphin-receptors in the brain (Hazum *et al.*, 1979) further complicate the picture. Therefore, at present, the suggestion of Jacquet is an interesting but certainly not conclusive one.

TABLE 4. Sites of Brain Lesions Suppressing ACTH-Induced Grooming

Lesion area	N°	ACTH-induced grooming ⁺
Sham	8	173 ± 10
Total hippocampus ⁺	4	40 ± 15*
Substantia nigra ⁺⁺	5	70 ± 13*

⁺ 15th-sec-grooming scores mean ± SEM.

[°] number of rats.

⁺ for description of lesion see Colbern *et al.*, 1977.

⁺⁺ for electrode coordinates see Wiegant *et al.*, 1977.

**p* < 0.1 Student-T test two tailed.

13. BRAIN LESIONS AND THEIR EFFECT PEPTIDE-INDUCED GROOMING

One method of trying to locate neural systems associated with the induction of excessive grooming is that of evaluating the effects of lesions in discrete brain regions. The grooming response induced by the intraventricular injection of ACTH₁₋₂₄ has been reported to be reduced by lesions in only two central nervous system areas: the hippocampus (Colbern *et al.*, 1977) and the substantia nigra (Gispen, unpublished). Only large bilateral lesions of the hippocampus were effective in reducing the excessive grooming (Table 4). Smaller lesions of either the dorsal or ventral aspects of the hippocampus were not. The degree of suppression of the peptide-induced grooming was somewhat greater in the animals receiving the large bilateral hippocampal than the substantia nigra lesions, although this result must be interpreted with caution since the animals with hippocampal lesions were tested relatively soon after surgery (8–11 days). It is likely that important neurological and behavioral changes were still occurring at that time. Even though the substantia nigra animals were tested about the same time after surgery, with hippocampal damage there was considerably more total brain damage in the animals. Recovery from the general debilitation following surgery probably takes longer in these animals.

Nevertheless, the effect of either type of brain damage was to reduce the instances of long duration grooming episodes. The number of grooming episodes, *per se*, was no different than intact animals receiving ACTH₁₋₂₄ (1 µg) without the lesion. This is illustrated in Fig. 11. In this figure the number of grooming bouts and the proportional distribution of bout durations are shown. As mentioned above, i.c.v. ACTH prolongs the duration of grooming episodes, and this effect is antagonized by either lesions of the hippocampus or the substantia nigra.

The areas of the brain that do not affect grooming when lesioned are as important as those that do. Colbern *et al.* (1977) found that no effect on ACTH₁₋₂₄ induced grooming produced by lesions in the following areas: rostral, rostral medial, and complete septal areas, medial preoptic area; lateral preoptic area; anterior hypothalamus; mammillary bodies; the amygdala; the parafascicular nucleus; the dorsal neocortex; dorsal hippocampus; ventral hippocampus. The failure of lesions, of the parafascicular nucleus and the rostral septal area emphasizes the difference between grooming and ACTH fragment effects on avoidance behaviors. Both implantation and lesion studies have implicated these areas in the alterations of extinction of conditioned avoidance responses produced by active N-terminal fragments of ACTH (van Wimersma Greidanus, *et al.*, 1974, 1975). Furthermore, lesions of the parafascicular area failed to reduce the grooming produced by (7-d-phe)ACTH₄₋₁₀.

Lesions of the amygdala, mammillary bodies, dorsal hippocampus, ventral hippocampus, but not near-total hippocampus, increased the stretching and yawning response to the injection of ACTH₁₋₂₄. This supports the view that a different set of neural

FIG. 11. Duration and start analysis of grooming behavior induced by i.c.v. ACTH₁₋₂₄ (1 μ g) in rats bearing sham lesions or bilateral lesions in total hippocampus or substantia nigra.

systems serve as the substrate for the grooming and stretching and yawning responses. It should also be noted that lesions of the preoptic region involve the structures that take up ^3H -testosterone and destroyed the ability of centrally applied ACTH to induce erections and ejaculations (but not stretching and yawning) (Bertolini *et al.*, 1975). This could be of interest since grooming is often associated with other signs of pleasure reactions in animals.

Other evidence links destruction of the hippocampus to reductions in grooming. Oades and Isaacson (1977) found that animals with large bilateral hippocampal lesions groomed less during observation during open-field, exploratory tests. In a recent experiment (Bär *et al.*, 1981) we found that novelty-induced excessive grooming was only reduced in the initial 10 min of the observation period but not in the subsequent 50 min. When the animals were observed after water immersion, the animals with hippocampal lesions showed increases in grooming scores that were similar to increases found in the lesioned and non-increased control subjects. The scores of the hippocampally-lesioned group and the control groups were identical. A similar result is found after lesions of the substantia nigra. Lesions of this area also fail to reduce scores obtained after water immersion. Since water immersion-induced increases in grooming can be reduced by pretreatment with naloxone, this indicates that the lesions do not produce neural effects comparable to the effects of the opiate antagonist.

Another effect of the substantia nigra lesions is on the acute tolerance that develops to i.c.v. ACTH administration and to water immersion. In animals with substantia nigra lesions neither two water immersions nor two intraventricular ACTH injections (0.3 μ g ACTH₁₋₂₄) separated by 4 hr show an interaction. Scores obtained on the second test are the same as those obtained on the first. Furthermore, the scores obtained after water immersion are comparable to those of the control animals, while those after ACTH₁₋₂₄ are only about half of the value obtained in control animals (Gispén, in preparation). Because the experiments have not been done, it is not known whether large, bilateral hippocampal lesions would produce similar results.

The hippocampus has been implicated in the production of grooming since the mid-1950s when MacLean and his associates found grooming to be a frequent consequence of after-discharges elicited by electrical or cholinergic stimulation of the hippocampus (e.g. MacLean, 1957). The grooming usually occurred just following the termination of the after-discharges and often preceded other signs of pleasure in the stimulated animals. Grooming did not appear during the period of stimulation of the after-

discharges, themselves. Often the grooming was associated with signs of sexual arousal, especially in cats and monkeys, although a partial erection was reported seen when rats were involved with genital grooming (observations of C. Kim, reported by MacLean, 1957).

Grooming can also be found after the termination of electrical stimulation of the brain stem (Waldbillig, 1975) and hypothalamus (Beagley, 1976). The grooming usually only occurred after the stimulation was terminated. This suggests that the behavior may be due to after-discharges produced by the stimulation which can be propagated to numerous, widespread locations. However, Beagley did find a few sites from which grooming could be induced by low frequency, low intensity stimulation. Excessively long grooming episodes followed the termination of the stimulation.

Also, MacLean (1957) mentions a description of grooming given by W. R. Hess and A. E. Meyer (1956) after stimulation of the septal area near the columns of the fornix. Roberts and his co-workers investigated the grooming component of heatloss mechanisms in the opossum and found that the medial preoptic and anterior hypothalamic regions were locations from which certain grooming behaviors could be elicited (e.g. Roberts *et al.*, 1967; 1969). However, the electrical stimulation of these areas induced foreleg and paw grooming most frequently with only infrequent bouts of face washing—the usual initial component of peptide-induced grooming. Furthermore, the studies of grooming as a thermoregulatory response in the opossum are likely to be only remotely related to grooming in the rat in which grooming does not serve a thermoregulatory function.

In sum perhaps the most surprising result from the investigations of brain lesions on excessive grooming is that so few brain regions tend to reduce the response. Furthermore, the brain lesions that are effective only partially affect excessive grooming induced by environmental means, i.e. novelty and water immersion. This contrasts with the ubiquitous effects of naloxone and suggests that the lesions are only influencing a subset of mechanisms responsible for excessive grooming.

14. ACUTE TOLERANCE TO GROOMING BEHAVIOR

The study of grooming induced by ACTH fragments has been furthered by observations on the effects of two treatments that both elicit grooming separated by various periods of time. Colbern *et al.* (1978) found that injections given 24 hr apart over several days produced comparable amounts of grooming. That is, there was no 'carry over' effect from the prior injection(s). Neither tolerance nor sensitization occurred. This observation was confirmed by Jolles *et al.* (1978). In addition, they found that after an injection of 1 μ g ACTH₁₋₂₄ excessive grooming in response to a second administration of the ACTH fragment was reduced for 10–18 hr (Fig. 12). Almost complete suppression of the ACTH-induced excessive grooming was found for 8 hr after the first injection. The suppression was demonstrable over a dose range of 0.01–1 μ g of ACTH, provided the dose of the second injection was the same as used in the first injection of ACTH. Thus, the suppression seems unrelated to the amount of grooming displayed after the first injection (Brakkee and Gispen, in preparation). Also, the effect was not due to a 'fatigue' of the behavioral response, since the reduction in grooming can be found in animals anesthetized at the time of the first injection. The suppression caused by the first injection was not a consequence of the release of corticosteroids as the phenomenon was also demonstrable in adrenalectomized rats (Jolles *et al.*, 1978).

A cross-tolerance among the following substances has been demonstrated using a second i.c.v. injection (ACTH₁₋₂₄) 4 hr after an initial i.c.v. injection: ACTH₁₋₂₄, β -endorphin, [D-Phe⁷]ACTH₄₋₁₀ and morphine (Jolles *et al.*, 1978). In addition, a similar acute tolerance was found when the effect of two injections of β -endorphin were studied. Using this neuropeptide, there is a period of at least 12 hr after the first injection when a second injection of β -endorphin induces greatly reduced grooming activity (Wiegant *et al.*, 1978b).

FIG. 12. Single dose tolerance to ACTH-induced grooming.

Neuropharmacological studies have indicated that the neural substrate mediating the ACTH-induced grooming involves dopaminergic nigro-striatal and nigro-accumbens projections and opiate receptors, most likely at the level of the substantia nigra (see Section 15). It is therefore not surprising that pretreatment with haloperidol (Gispén *et al.*, 1980) or naloxone (Jolles *et al.*, 1978), prior to the first injection, eliminates the attenuating effects of the first injection.

The decreased grooming found with a second treatment 4 hrs after the first reflects a reduced sensitivity to the initiating events. For example, increasing the amount of the active neuropeptide given in the second treatment can restore excessive grooming.

As ACTH and morphine share a number of properties with respect to their effects on the CNS (Wiegant *et al.*, 1977), it is tempting to speculate that the observed reduction of effectiveness of the second injection relates to the acute tolerance which is known to occur to temperature responses to morphine. The time course of both processes seems remarkably similar (Lotti *et al.*, 1966; Rosenfeld and Burks, 1977). The development of acute tolerance to ACTH-induced grooming is also reminiscent of the development of hyposensitivity to catecholamine receptors. Schwartz *et al.* (1978) reviewed the development of hyposensitivity but absence of hypersensitivity to DA agonists in the thermoregulatory system. The single dose tolerance to apomorphine-induced hypothermia (i) does not depend on an increased rate of metabolic disposition of the drug, (ii) can be prevented by pretreatment with haloperidol, and (iii) cross-tolerance to other DA agonists can be demonstrated (Schwartz *et al.*, 1978).

Thus, it may be that ACTH may produce certain effects by activating the dopaminergic nigro-striatal and/or nigro-accumbens pathways, initiating the temporary hyposensitivity of the DA receptors in striatum and/or n. accumbens, necessitating a higher dose of ACTH in the second injection to result in the normal display of excessive grooming behavior. As there seems to develop no long-term tolerance to reported ACTH injections when spaced 24 hr apart, the grooming test may be an excellent model to study the phenomenon of acute tolerance related to CNS receptor mechanisms.

15. NEUROTRANSMITTER SYSTEMS INVOLVED WITH THE GROOMING RESPONSE

15.1. DOPAMINE

Over the years independent studies of several groups of investigators have made it likely that central noradrenergic and dopaminergic transmission may be modulated by

ACTH and its non-hormonal fragments (see Versteeg, 1980; Gispen *et al.*, 1977). Such studies concern measures on neurotransmitter levels, turnover, and synthesis in large and discrete brain area, as well as neurohistochemical approaches. Although there is no clear-cut picture as to what mechanism and what brain region in particular is involved, such studies have pointed out, however, that fragments of ACTH which induce excessive grooming also may alter the impulse flow in the catecholaminergic systems.

The grooming elicited by small doses of morphine administered systematically to rats appears to be dependent on intact noradrenergic and dopaminergic systems (Ayan and Randrup, 1973). Furthermore, it has been shown that the dopamine-containing neostriatum (caudate nucleus) is indispensable for the initiation of certain morphine-induced behaviors (Cools *et al.*, 1974). In view of the opiate sensitivity of the ACTH-induced excessive grooming response (see above) the possible involvement of brain dopamine (DA) in ACTH-induced grooming in the rat was studied.

Low doses of i.p. injected haloperidol (0.1–0.2 mg/kg) did not greatly suppress i.c.v. saline-treated subjects, but did seriously interfere with ACTH-induced grooming. An additional DA antagonist, a long-lasting preparation of flufenanine anatenzol, when administered 60 hr prior to the i.c.v. ACTH-injection, markedly suppressed the display of grooming (Wiegant *et al.*, 1977). To substantiate the notion that a central DA component was involved and to further characterize this component, a series of experiments were undertaken to locally manipulate the activity of nigro-striatal and nigro-accumbens DA pathways (Wiegant *et al.*, 1977; Cools *et al.*, 1978a; Gispen *et al.*, 1980). These studies also were undertaken to gain additional insights about locations in the brain where ACTH might be acting to induce excessive grooming behavior. First, they reported that microinjection of haloperidol (1 μ g) into the neostriatum but not into the substantia nigra suppressed i.c.v. injected ACTH_{1–24} (1 μ g/3 μ l). Furthermore, local application of ACTH_{1–24} (0.2 μ g) into the substantia nigra induced excessive grooming whereas even a high dose (3 μ g) into the neostriatum or n. accumbens was completely ineffective in inducing excessive grooming (Wiegant *et al.*, 1977; Cools *et al.*, 1978a).

Based on anatomical projections of the forebrain dopamine systems, pharmacological evidence and functional studies, Cools (1977a) proposed that at least two distinct dopaminergic systems must be considered: an excitation-mediating dopaminergic system (DA_e receptors) and an inhibition mediating dopaminergic system (DA_i receptors). In the rat DA_e terminal areas are mainly within the neostriatum, although some are also present in the n. accumbens. The reverse appears to hold for the DA_i projections which are found mainly in the n. accumbens (Cools, 1977a). These system are differentially influenced by apomorphine, haloperidol, DPI([3,4-dihydroxyphenylamino]-2-imidazoline-OH) and ergometrine, but equally affected by DA. We were able to show that local stimulation (apomorphine) or inhibition (haloperidol) of DA_e receptors in the neostriatum and similar stimulation (DPI) or blockade (ergometrine) of DA_i receptors in the n-accumbens led to suppression of i.c.v. ACTH-induced excessive grooming. However, using a DA_e antagonist in a region with few DA_e receptors (nucleus accumbens) or a DA_i antagonist in a region with a few DA_i receptors (neostriatum) leads to little if any interference with the behavioral response. Therefore, the hypothesis was put forward that the balance between the DA_e and DA_i systems may underlie ACTH-induced excessive grooming (Cools *et al.*, 1978). Such reasoning is supported by the finding that haloperidol treatment does not potentiate but rather counteracts the ergometrine-induced inhibition of grooming: the counteraction of the locally disturbed balance between DA_e and DA_i activity was paralleled by a partial restoration of the suppressed grooming. Likewise, DA, being unable to affect this balance (Cools, 1977b) did not alter the grooming activity (Cools *et al.*, 1978a).

Morphine produces a characteristic, time-dependent shift in the balance between DA_e and DA_i activity within the feline brain: from predominant DA_e activity during the 'early' morphine effects toward predominant DA_i activity during the 'late' morphine effects (Cools *et al.*, 1978b). The exact nature of the interaction of the two different DA systems in ACTH-induced grooming is at present not understood. Yet, considering the

present results in view of the data concerning morphine it is likely that morphine and ACTH₁₋₂₄ similarly affect the distinct DA systems within the brain, presumably modulating the activity of DA neurons in the substantia nigra.

With the suggestion of central DA mechanisms being involved in ACTH-induced grooming at hand, we recently used the grooming response to assess the notion that (Des Tyr)- γ -endorphin (β -LPH₆₂₋₇₇, DT γ E) has behavioral effects similar to those produced by neuroleptic drugs (de Wied *et al.*, 1978b; van Ree *et al.*, 1980). Recent neuropharmacological studies confirmed such a conclusion. The endorphin fragment, dT γ E, when given subcutaneously, alters the accumulation pattern of radio-labeled spiperone in rat brain (Pedigo *et al.*, 1979). ICV dT γ E alters the nerve input flow of catecholaminergic systems (Versteeg *et al.*, 1979). Indeed, local administration of LPH₆₂₋₇₇ (DT γ E) and not of LPH₆₁₋₇₆ (β -endorphin) into either neostriatum or n. accumbens blocked i.c.v. ACTH-induced excessive grooming. The n. accumbens was extremely sensitive to dT γ E injection. Furthermore, both i.c.v. administration of haloperidol and dT γ E reduced the development of acute tolerance to ACTH-induced grooming (Gispén *et al.*, 1980). Although there are some differences between the effects of haloperidol and dT γ E (de Wied *et al.*, 1978b; Weinberger *et al.*, 1979; Gispén *et al.*, 1980), it was suggested that dT γ E modulates the dopaminergic activity underlying the display of ACTH-induced excessive grooming.

Recently, we have extended the studies on the role of the substantia nigra in ACTH-excessive grooming. Bilateral electrolytic destruction of large parts of the zona compacta and zona inserta of the substantia nigra suppressed in grooming induced by a large i.c.v. dose of ACTH₁₋₂₄ from 170–180 (out of 200 maximal grooming scores) to 80–90. Furthermore, local applications of naloxone into the s. nigra and not in n. accumbens or neostriatum interfered with i.c.v. ACTH-induced excessive grooming. In line with postulating a crucial role of the substantia nigra in mediating the ACTH-induced response, are preliminary data on i.c.v. picrotoxin-induced excessive grooming, as it may block GABA-ergic inhibitory input to the s. nigra (Gispén *et al.*, in preparation). Others have shown that local application of substance P into the A9 area of rats (region of the DA-containing neurones that give rise to the accumbens-mesocortical DA projections) enhances the display of locomotion and exploration. This response could also be blocked by injection of a neuroleptic into the nucleus accumbens (Stinus *et al.*, 1978; Kelly *et al.*, 1979). Previously, these authors had shown that injection of substance P into the substantia nigra induced grooming and rearing which could be blocked by 6-OHDA lesions in the neostriatum (Kelly and Iversen, 1978a,b). These data open the possibility that both peptide-induced behaviors are mediated by the same DA neurons. Whether they activate the same receptors is, of course, difficult to assess at this moment, although for substance P it was also claimed that the peptide would counteract morphine-induced analgesia (Stewart *et al.*, 1976), no displacement of dihydromorphin from its receptor could be demonstrated under conditions which enabled ACTH to do so (Terenius, 1975).

15.2. NORADRENALINE

Research on the neurochemical mechanisms related to excessive grooming has concentrated on the role of the dopaminergic systems. It seems clear that systemic administration of haloperidol, at least at dose levels of 0.2 mg/kg will antagonize excessive grooming produced by 1 μ g of i.c.v. ACTH₁₋₂₄ and partially do so at a dose of 0.1 mg/kg. However, a dose of haloperidol at this level does not only antagonize dopaminergic actions but also affects the noradrenergic system at high dose levels (Andén *et al.*, 1970a). There is evidence that neuroleptics like haloperidol influence serotonergic receptors, as well (e.g. Leysen *et al.*, 1978). Furthermore, as with locomotor behavior, it is likely that the exhibition of excessive grooming depends on a complex interaction of both dopaminergic and noradrenergic systems (e.g. Andén *et al.*, 1973; Maj *et al.*, 1972).

To extend the study of pharmacologic agents that influence excessive grooming, various doses of drugs affecting the noradrenergic systems have recently been studied. Preliminary results obtained by Hertzler (personal communication) indicated that the

tricyclic antidepressant amitriptyline reduced novelty-induced grooming in a dose-dependent fashion at 6 dose levels (range 0.5 to 20 mg/kg). At the lower dose levels, novelty-induced grooming was reduced without a correlated general motoric depression. Since it is likely that many of the effects of amitriptyline are due to the drug's action on the noradrenergic system (Svensson and Usdin, 1978), a more detailed study of adrenergic influences on grooming was undertaken. Hertzler, Hannigan, and Isaacson (in preparation) found that the α -adrenergic agonist, clonidine, had an unusual dose-related effect on novelty-induced grooming. Doses of 0.2 and 10 μ g/kg reduced the grooming scores, whereas the 1 μ g/kg dose did not alter them. With other groups of animals pretreated with clonidine, water immersion failed to influence the excessive grooming produced in this way.

The effects of clonidine on behavior are likely due to its ability to stimulate central α -adrenergic receptors (Anden *et al.*, 1970b), but it should also be noted that the drug also acts as an agonist for H_2 receptors, as well (Sastry and Phyllis, 1977). Therefore, the effects of clonidine on excessive grooming may not reflect adrenergic influences.

Changes in adrenergic activity has been found in the development of tolerance to barbiturates (Tabakoff *et al.*, 1978) and to morphine (Cools, 1977b; Cools *et al.*, 1977), during ethanol withdrawal (Banerjee *et al.*, 1978), and during reduced sensitivity periods after long-term treatment with antidepressants (Crews and Smith, 1978). It has been suggested that changes produced by chronic haloperidol treatment involve components of the noradrenergic system, although different authors favor different ones, Cools (1978) favoring α -like noradrenergic alterations, while Dunstan and Jackson (1979) look toward B_1 adrenergic receptor alterations. It is tempting to consider that the short-term tolerance effects found with repeated i.c.v. injections of ACTH could involve alterations in adrenergic responsiveness, as well.

16. SUMMARY

Excessive grooming is a remarkable behavioral response for several reasons. Probably the most important is that it represents an almost unique behavioral assay for the injection of the longer ACTH fragments. Fractions of a microgram of ACTH₁₋₂₄ or ACTH₁₋₁₆ produce extensions of grooming bout duration to an extent that the animal does little else during the entire observation period. The specificity of the response is remarkable, as well. Few other neuropeptides induce excessive grooming, and those that do (e.g. [7-d-phe]ACTH₄₋₁₀) are only of limited effectiveness. Also of special interest is the limited number of brain regions in which lesions influence the response. Only two such regions have been found: the substantia nigra and the hippocampus. A link to naturally occurring events is now provided by the observations which indicate that certain forms of environmental manipulations also lead to excessive grooming. These are conditions of mild stress, the most completely studied being that of 'novelty'.

While providing a connection to real world events, the study of stress-induced excessive grooming has also led to certain difficult questions. In the study of stress conditions that produce excessive grooming, only certain ones have been found to be effective. These include handling, noise, transport, novelty, the anticipation of footshock and water immersion. Of these, water immersion has the most pronounced effects. From our pharmacologic studies, water immersion produces a type of grooming that is distinctly different from either that produced by novelty or other mild stress conditions and also different from that produced by central ACTH administration. For example, the excessive grooming induced by water immersion is not reduced by either substantia nigra or hippocampal lesions, neither is it reduced by the systematic administration of clonidine. On the other hand, these treatments and procedures reduce both novelty and ACTH-induced excessive grooming.

The stress procedures which do not induce excessive grooming also bear consideration. They include the exposure to ether fumes, bodily restraint, and electrical footshock. In fact, the latter reduces excessive grooming.

Putting aside, for a moment, the effects of water immersion, stressors seem to fall into two classes: those that produce excessive grooming after their termination and those that do not. It seems to us that this could come about in two ways: (1) the stressors that do elicit grooming may produce a different type of distribution of ACTH release in the brain than those that do not; or (2) all stressors may produce ACTH release in a similar fashion, but those that fail to elicit grooming may also elicit antagonistic activity, possibly involving the brain monoamine systems. While there is little evidence to support a differential pattern of release as a consequence of different stress conditions, the possibility cannot be ruled out. The second possibility, the activation of incompatible neural systems by certain stressors, seems more likely. If this is the case, then it could be proposed that the amount of excessive grooming displayed by an animal is positively related to the amount of ACTH affecting certain brain regions involved with the production of excessive grooming, and negatively related to the activity in opposing systems. ACTH could reach the target areas of the central nervous system from the anterior pituitary (de Kloet and de Wied, 1980; Mezey, *et al.*, 1978) or from central ACTH networks.

Beagley (1976) felt that this electrical stimulation at higher intensities may have activated an excitatory system for grooming that did not achieve behavioral expression because of the simultaneous induction of an opposing, inhibitory process. He proposed that the grooming system, however, had a longer afterdischarge than the opposing processes. Thus, when inhibition or the competing response tendencies declined in activity, grooming would be observed. Bolles (1960) came to a similar conclusion. He had found that the induction of motivation for food or water by deprivation decreased grooming as did the induction of fear. He concluded that these 'motivational' procedures did not induce grooming directly but that grooming occurred after the conclusion of goal-directed acts or highly motivated episodes, presumably when competing neural mechanisms declined in activity.

The relatively high levels of excessive grooming seen after water immersion seem to be quite different in some aspects from either novelty- or ACTH-induced grooming. Their resistance to lesions effects and pharmacological intervention suggest a different component or mechanism has been involved. It may be that this is related to increased 'maintenance' grooming (an attempt to remove water from the fur), but if this were the case, it would be necessary to account for the fact that from 1 to 3 min of water immersion is necessary to induce the effect. Exposure to water for less than one min fails to elicit the effect. It is also difficult to explain why the soiling and disturbances of the fur in the restraint procedures fails to elicit a similar amount of grooming on the basis of a maintenance hypothesis.

The involvement of opiate-sensitive systems in the grooming response is based on the ubiquitous blocking effect of naloxone on all excessive grooming, including that induced by water immersion. It is certainly not a response common to all of the known endogenous opiates since the enkephalins and some endorphin fragments are ineffective in producing excessive grooming. In fact, the excessive grooming induced by β -endorphin is qualitatively different from that produced by ACTH. β -endorphin seems to activate the animals into more behavioral activities including grooming, while ACTH acts to extend the duration of grooming episodes.

The neurochemical mechanism by which ACTH induces the grooming behavior is not understood. However, the recent isolation of an ACTH-sensitive protein kinase from rat brain synaptosomal plasma membranes suggests that ACTH may modulate trans-synaptic neuronal activities by altering the degree of phosphorylation of certain synaptic proteins. The insensitivity of this protein kinase to endorphins and the unique ACTH structure-activity correlation between the induction of excessive grooming and inhibition of protein phosphorylation support this notion.

Since excessive grooming is found after the termination of several stressors, it is possible that it is related to restorative neural and chemical processes. ACTH could be thought of and acting as an initiating factor in the mobilization of the organism to meet

the emergency situation while at the same time initiating processes aimed at re-establishing a stable, internal equilibrium. The grooming response, itself, has been considered to play a deactivating role in animal behavior (for example see Delius, 1970; Delius, *et al.*, 1976; Jolles *et al.*, 1979a).

It is also possible to cast the behavioral observations into a related but different context which removes some of the objections that can be made to the view that grooming should only occur before relatively inactive states. This would be that excessive grooming is a correlate of a central state in which behavioral activation may be present but without concomitant demands for fight or flight. This central state may be correlated with a number of classes of responses and the ones selected for exhibition would depend on environmental opportunities, the animals past history and genetic predispositions.

Grooming may occur *after* extremely arousing conditions. This suggests that these acts may be correlated with pleasurable experiences. The slow body stretching and paw extensions found in cats by MacLean (1957) would be one example as would the signs of sexual excitation observed. Bertolini, *et al.* (1975) report similar signs of sexual arousal after injection of ACTH₁₋₂₄ into the lateral ventricle of the rabbit. However, the presumed sexual arousal reflects an activation of the mechanisms related to sexual behavior without an enhanced motivation to copulate. During the peptide-induced stimulation the animals do not seek copulation. For example, the injection of the peptide into the ventricles of male rats does not increase the percentage of males copulating with females. It is as if the animal is 'tuned in' to its own pleasurable bodily reactions that may include those of a sexual nature. Bertolini *et al.* also provided evidence that the sexual activation in male rats or rabbits instituted by ACTH₁₋₂₄ depends on the integrity of the testes.

However, the excessive grooming observed in rats after the intraventricular injection of ACTH₁₋₂₄ or β -endorphin, in the amounts used in our laboratories, as well as the grooming induced by novelty does not concentrate on the genital area. Few signs of sexual arousal can be observed. However, it is possible that a 'pleasurable', positive affective state is induced by these treatments and that at greater magnitudes the pleasurable state could facilitate signs of sexual arousal. This would suggest that in the rat the central state induced by i.c.v. administration of ACTH₁₋₂₄ is closely limited to the grooming response but it may be tied to other, many more varied responses in other species.

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