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# THE PERIAQUEDUCTAL GRAY: A PREREQUISITE FOR ACTH-INDUCED EXCESSIVE GROOMING

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The periaqueductal gray is known to be involved in the expression of a variety of behaviours such as aggression, β-endorphin-induced immobility and peptide-induced excessive grooming. In order to establish whether the periaqueductal gray (PAG) is indispensible for peptide-induced excessive grooming, lesions were placed in the dorsal part of this structure. Subsequently, the grooming-inducing abilities of adrenocorticotropin$_{1-24}$ (ACTH$_{1-24}$), β-endorphin and bombesin were tested. The lesioned animals did not display excessive grooming after intracerebroventricular injection of ACTH$_{1-24}$. β-Endorphin administration into the lesioned animals resulted in an extreme display of immobility. Local injection of bombesin into the PAG resulted in reduced scratching behaviour followed by immobility. It was hypothesized that excessive grooming (elicited by ACTH) may be mediated through a non-opioid primary target site – situated in the lesioned region of the PAG – while excessive scratching and immobility (elicited by bombesin or β-endorphin, respectively) may be mediated through an opioid primary target site (situated in the remaining part of the PAG). Furthermore, the analysis of social behaviour of lesioned animals revealed that these animals reacted towards an unfamiliar partner predominantly with freezing behaviour. The increase of β-endorphin-induced immobility and socially induced freezing (which is morphologically very similar to β-endorphin-induced immobility) in lesioned animals supports the hypothesis that the release of opioid peptides such as β-endorphin in the PAG plays a role in the regulation of social behaviour.

## INTRODUCTION

There is increasing evidence for a role of the periaqueductal gray (PAG) in the expression of a variety of behaviours. Studies using local electrical stimulation and lesion techniques indicated that the PAG is involved in aggression[1,21]. Several findings suggest a role for the PAG, especially the ventral part, in the expression of fear, as evidenced by decreased latency in passive avoidance[17] and abolishment of hypothamalic-induced escape behaviour[25]. Morphine may exert its analgesic action not only after systemic administration, but also when applied locally into the PAG[13,15]. Larger doses of β-endorphin into the PAG cause a cataleptic state[14], whereas a low dose, (i.c.v.) injected, elicits excessive grooming behaviour[10]. Also morphine in a low dose may induce excessive grooming[3,8]. In a previous study it was shown that adrenocorticotropin$_{1-24}$ (ACTH$_{1-24}$) applied into the dorsal PAG or at least into a structure closely adjacent to the PAG, induced the display of excessive grooming behaviour[27]. The involvement of the PAG in peptide-induced excessive grooming has also been reported by others[11]. Furthermore, it has been

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demonstrated that β-endorphin and bombesin, which both induce excessive grooming, typified by scratching, have binding sites in the PAG[19,20]. In order to investigate whether a difference in morphology of the behavioural reponse to different peptides corresponds with a difference in neural substrate for these peptides, ACTH, β-endorphin and bombesin have been compared in their ability to induce grooming in rats bearing lesions in the PAG.

When it appeared that the lesioned animals reacted with an extreme display of 'opioid-like' immobility upon a low dose of β-endorphin it was hypothesized that opioid receptors in the PAG mediate β-endorphin-induced behaviours. As it is known that opioid characteristics of peptides are dependent on the presence of the first amino acid tyrosine[5], this hypothesis was tested by comparing the scratching-inducing abilities of des-Tyr-β-endorphin with β-endorphin.

As the PAG contains terminals with proopiomelanocortin[30] and since it is known that peptides derived from this precursor[23] may play a role in the regulation of social behaviour, the display of social behaviour may be disturbed by the destruction of peptide-sensitive or peptide-releasing structures in the PAG. Therefore, the social behaviour displayed by lesioned animals in dyadic encounters was studied as well.

## MATERIALS AND METHODS

### *Animals*

Male rats of an inbred Wistar strain were used (TNO, Zeist, The Netherlands). They were bred in our colony and weighed about 145 g at the beginning of the experiments. The animal rooms were kept at a normal day/night cycle of lights on at 08.00 h and off at 20.00 h. After cannulation all animals were housed individually until the onset of the experiments.

### *General procedure*

*Implantation of cannulas.* For experiment I, II and IV stainless-steel cannulas were implanted bilaterally into the PAG according to the coordinates of König and Klippel[16]: A, 1.2; L, 1.0; d, 0.3; in addition, the rats in experiment I and II received a stainless steel cannula unilaterally into the third ventricle[4]. After a recovery period of 3 weeks the animals were injected with the respective peptide in study and the grooming and scratching responses were measured.

*Behavioural tests.* Grooming behaviour was recorded using a time-sampling method as described previously[8]. Shortly, 15 min after injection, every fifteenth second, the display of an element of the grooming repertoire of each animal was recorded during 55 min, allowing a maximum grooming score of 220. The following elements were scored: head washing, body grooming, anogenital grooming, scratching, tail sniffing and body shake. Scratching was of special interest, since β-endorphin and bombesin are known to induce primarily this element.

Social behaviour: the dyadic interactions of the lesioned animals with the sham-operated animals were individually observed for 10 min. The ethogram existed of the following elements: (1) approach, (2) sniffing, (3) sexual investigation, (4) walking away, (5) defense, (6) freezing, (7) parry, (8) autogrooming, (9) aggression, (10) exploration and (11) a rest category for all other occurring behaviour. These behaviours have been extensively described by others[29]. Frequencies and all occurring combinations of behavioural elements – sequences – of these dyadic interactions were recorded[26]. The sequential ordering of behaviour is denoted in so-called transition matrices; subsequently, adjusted residuals were calculated from these matrices (see ref. 26). The width of the arrows in Fig. 5 represents the value of the adjusted residual and is related to the number of the transitions between the two connected behaviours. Thus, an arrow can be read as 'is followed by'. Such a pathway diagram is regarded as reflecting the structure of behaviour. Both the sequential structure of intra-animal behavioural elements and the way they reacted towards each other (inter-animal sequential structure) are taken into account. An extensive description of the statistical techniques applied for composing the pathway diagrams is given in a previous study[26].

*Lesions.* Kainic acid – a cytotoxic agent related to glutamate – was used, since it proved to be a useful tool for cell body destruction, while sparing

the afferents impinging on those cell bodies. Passing myelinated fibres also remain intact[6,18,22]. Kainic acid, 0.25 $\mu$g in 0.5 $\mu$l phosphate buffer (pH 7.1), was administered into the PAG via the implanted cannulas. In order to allow slow administration (30 s) of the kainate and to prevent behavioural hyperactivity, the injection took place under mild anaesthesia (natrium pentobarbital). To prevent, as much as possible, kainate-induced brain damage distant from the site of injection, diazepam (10 mg/kg, s.c.) was given 5 min before the kainic acid treatment. After termination of the behavioural experiments all animals were histologically checked for appropriate cannulation. The presence of gliosis below the tip of the cannula, discovered by using a thionine colouring method, served as an indication of the efficacy of the kainic acid treatment. In Fig. 1 the sites of the injection and the injection volume of the kainic acid treatment are shown schematically; gliosis was always found within the black area. Furthermore, the display of immobility primarily in the presence of a conspecific of all lesioned animals served as a behavioural control of the effectiveness of the lesion procedure.

*Experiment I*

In order to assess a possible effect of the lesion on ACTH-induced grooming behaviour, a grooming test (n = 10) with ACTH (0.3 $\mu$g dissolved in 3 $\mu$l saline) was performed one week before and 3 weeks after the kainic acid treatment.

*Experiment II*

Two groups of 9 rats were cannulated as described under general procedure; one group received kainic acid, the other group the same volume of phosphate buffer. After a recovery period of 3 weeks, a grooming ( = scratching) test

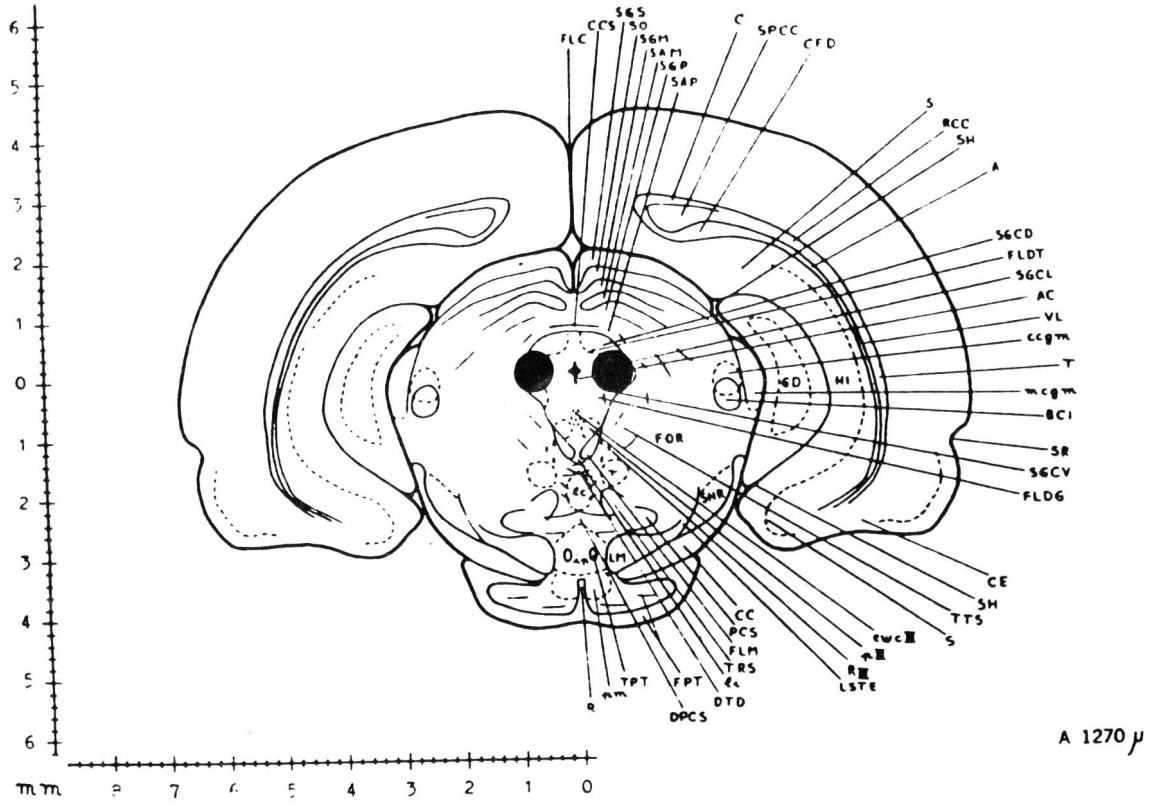


Fig. 1. Schematically representation of the site of injection and the injection volume of kainic acid. Gliosis below the tip of the cannula was always seen within this area.

with β-endorphin was carried out in two different ways. The peptide was administered locally into the PAG (0.03 μg/0.5 μl) and 3 days later β-endorphin was injected via the ventricular system (0.1 μg/3 μl). One week after the experiments with β-endorphin these animals were employed for similar experiments with 0.01 μg/0.5 μl and 0.03 μg/3 μl bombesin, respectively.

*Experiment III*

In a third experiment 4 groups of 5 animals were unilaterally cannulated into the third ventricle. Group 1 was treated with ACTH (0.3 μg/3 μl); group 2 was treated with β-endorphin (0.1 μg/3 μl); group 3 was treated with des-Tyr-β-endorphin (0.1 μg/3 μl) and group 4 with a similar volume of saline. Grooming behaviour was then recorded as described above.

*Experiment IV*

Eight animals bearing a lesion in the PAG and 8 sham-lesioned animals were each placed in the compartment of large cages (100 × 50 × 50 cm) one hour prior to the observation. The compartments (50 × 50 × 50 cm) were separated by a removable partition. To enhance the occurrence of social behaviour the interactions took place during the dark phase of the day/night cycle. The observation started immediately after the removal of the partition and lasted for 10 min.

RESULTS

*Experiment I*

In this experiment the effect of the lesion on ACTH-induced excessive grooming was demonstrated. The grooming scores elicited one week prior to the lesion (i.c.v.) served as control values; the grooming score 3 weeks after the lesion is shown in Fig. 2A (right side and left side, respectively). The latter grooming score was drastically reduced for each animal (using a *t*-test for paired values, $P < 0.05$). The suppression of the grooming response was irrespective of the route of administration: i.c.v. or locally via the PAG (results not shown).

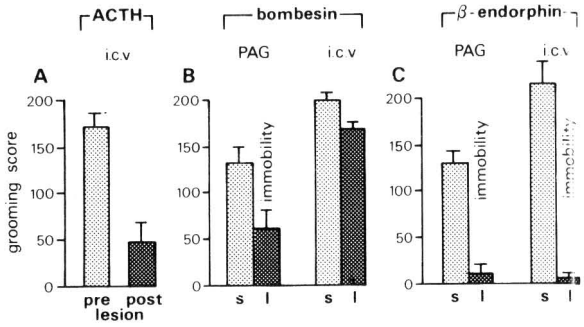


Fig. 2. A: the grooming score of the same animals before and after the lesion (n = 10). B and C: lesioned animals (l) (n = 9) and sham-operated animals (s) (n = 9) have been treated with bombesin and β-endorphin, respectively. The peptides were administered icv, locally into the PAG.

*Experiment II*

In this experiment the effect of the kainic lesion in the PAG on the behavioural activity of β-endorphin and bombesin were tested. After i.c.v. injection of bombesin, excessive scratching was slightly reduced (Fig. 2B) by the kainic acid lesioning of the PAG. However, if bombesin was injected directly into the PAG, a pronounced reduction of the scratching behaviour was seen. Similarly, the cataleptic state was only seen after local administration of the peptide into the PAG; the cataleptic state was less severe than after local administration of β-endorphin. Fig. 2C illustrates that irrespective of the route of administration of β-endorphin (i.c.v. or into the PAG), lesioning of the PAG resulted in a marked display of immobility accompanied by a nearly total suppression of the scratching score. Both the bombesin and β-endorphin treated animals barely showed any other behaviour than scratching and immobility. After the scratching they remained in an immobile position throughout the observation period.

*Experiment III*

In this experiment the significance of the N-terminal residue in the β-endorphin molecule for β-endorphin-induced scratching was investigated. As can be seen in Fig. 3 removal of the Tyr residue results in a loss of scratching activity (Student's *t*-test, $P < 0.05$).

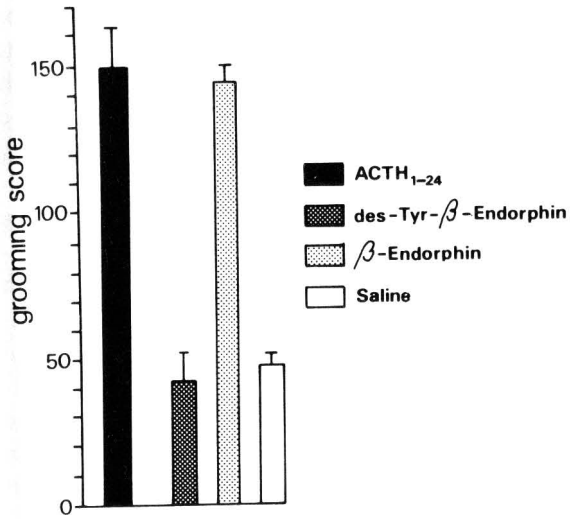


Fig. 3. The induction of grooming by ACTH (n = 5), β-endorphin (n = 5) and des-Tyr-β-endorphin (n = 5) is compared. β-Endorphin differed significantly from saline and its des-Tyr-analogue ($P < 0.05$, Student's $t$-test).

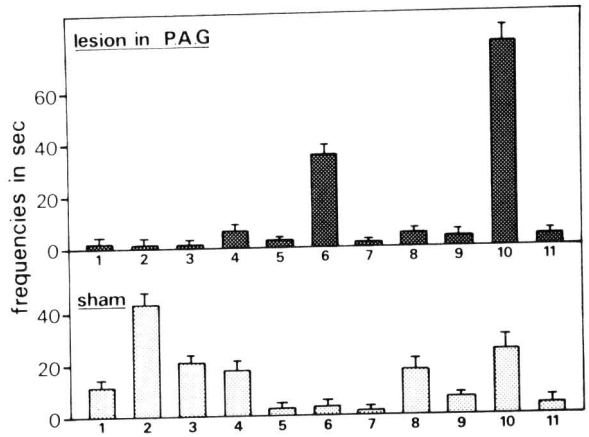


Fig. 4. Frequencies of exploratory (mean ± S.E.M.) and social behaviour performed by lesioned (n = 8) and sham-operated animals. The numbers under the axis refer to the behavioural elements (see Materials and Methods).

*Experiment IV*

In this experiment the change in social behaviour due to the lesion in the PAG was studied. Social behaviour is depicted in Fig. 4, which represents the frequencies of the 11 behavioural elements. The lesioned animals only showed exploratory behaviours and freezing. All other social behaviour (such as approach, sniffing or sexual interest) was almost completely absent. In order to find out whether the freezing behaviour was an unconditioned response of the lesion or a consequence of an interaction of the lesion and the presence of a conspecific, a sequential analysis was performed. The pathway diagram in Fig. 5 shows the pattern of actions and reactions of both animals. Most conspicuous is the vast display of freezing, walking away or to some lesser extent defense behaviours of the lesioned animals in response to social behaviour displayed by the partner: see the large arrows from approach [1], sniffing [2], sexual interest [3] from the partner to freezing and defense of the lesioned animal [5 and 6] (Fig. 5). If the latter rats did show any urge to seek the partners themselves the confrontation was also followed by freezing behaviour. The usual social behaviours seen after approach are negligible compared to the abundant number of transitions of approach to freezing behaviour.

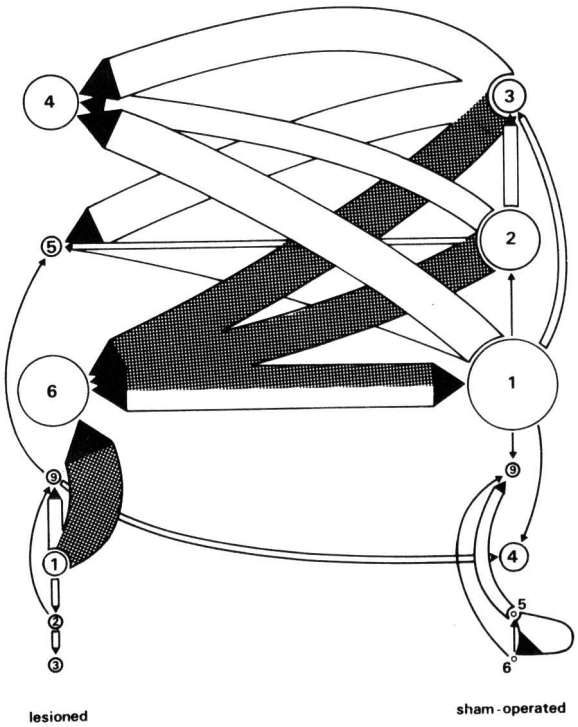


Fig. 5. Pathway diagram of lesioned and sham-operated animals. The size of the circles represents the duration of behaviour. The arrows can be read as 'is followed by'. The grey arrows highlight the most significant transitions; the numbers in the circles refer to the behavioural elements: 1, approach; 2, sniffing the partner; 3, sexual investigation; 4, walking away; 5 defense; 6, freezing; 9, aggression.

## DISCUSSION

Table I summarizes the main effects of the lesion of this study. The animals with a lesion in the PAG did not respond to ACTH with either excessive grooming or with any other detectable behavioural response different from that seen after saline treatment. Apparently, the substrate mediating the ACTH effect (grooming) is at least partly situated in the region of the PAG that has been destroyed and differs from the substrate sensitive to β-endorphin/bombesin (scratching and immobility), which has not disappeared and is obviously situated elsewhere in or closely adjacent to the PAG. As can be seen in Table I the behavioural effect of β-endorphin was fairly similar to that seen after bombesin. Both peptides elicited immobility after local administration into the kainic acid-lesioned PAG, albeit that the relative amount of immobility versus scratching was larger for β-endorphin. In the case of i.c.v. administration of bombesin, scratching was barely reduced, although immobility was still seen. The ability for β-endorphin to induce immobility in intact animals has been reported before, requiring, however, a much higher dose[14]. Thus, the destruction of the dorsal PAG may lead to a behavioural effect of β-endorphin resembling the effects of higher doses of the peptide in intact animals. The occurrence of immobility in lesioned animals after both bombesin and β-endorphin treatment into the PAG, suggests that the substrate of these peptides might be similar in part. This is in agreement with other observations: both peptides, locally applied into the PAG, induce analgesia[24,28]. β-endorphin-induced scratching and bombesin-induced scratching can be antagonized by nalaxone (ref. 9 and Van Wimersma Greidanus, personal communication, respectively). However, the latter is at variance with reports of Gmerek and Gowan[11]. Since scratching followed by immobility was elicited in the PAG in a much lower dose than after i.c.v. treatment, it was concluded that bombesin might be acting through structures in ventral parts of the PAG. After all, bombesin immunoreactivity and bombesin binding sites are present in the PAG (see Introduction).

At the molecular level evidence is also presented suggesting different substrates for grooming and scratching. The N-terminal Tyr-residue is a prerequisite for β-endorphin-induced scratching, but not for dynorphin-induced grooming[2]. Seemingly, ACTH and dynorphin have an affinity for a non-opioid 'grooming' receptor and β-endorphin has an affinity for an opioid 'scratching' receptor. Thus far, our data are in agreement with the hypothesis of Jacquet[12], who stated that the PAG contains two classes of opiate receptors: one mediating analgesia, catalepsy, sedation with a high degree of stereospecificity and the other possessing a low degree of stereospecificity and mediating hyperreactivity. Destruction of the latter could explain the pronounced expression of the endorphin-sensitive structures (disinhibition). The behaviour of the lesioned animals in a social situation can be reconciled with this concept as well. The notion that proopiomelanocortin peptides may regulate social behaviour has been put forward previously by others[12,23]. Such a regulation may be exerted through the PAG (see Introduction). The extreme display of freezing behaviour seen in lesioned animals – very similar to β-endorphin-induced immobility – suggests a physiological release of this peptide in social situations and, as a result of the destruction of normally inhibiting structures, a pronounced expression of the behavioural effect of the release of this peptide.

TABLE I

*The effects of ACTH, β-endorphin and bombesin on various activities following lesions in the PAG of male Wistar rats*

+, slightly more than the effect in non-lesioned animals; −, slightly less than the effect in non-lesioned animals; + + or − −, much more or much less effect, respectively; =, no different from the effect in intact animals.

| | *PAG-L* | | | | | | |
|---|---|---|---|---|---|---|---|
| | *Social contact* | *ACTH* | | *β-Endorphin* | | *Bombesin* | |
| | | *i.c.v.* | *local* | *i.c.v.* | *local* | *i.c.v.* | *local* |
| Immobility | | | | + | + + | | + |
| Freezing | + + | | | | | | |
| Scratching | | | | − | − − | = | − |
| Grooming | | − − | − − | | | | |

It may be that activation of either of the two ypes of peptide receptors in the PAG underlies he functional antagonism of ACTH versus β-endorphin in several test situations such as, for nstance, analgesia[7].

Summarizing, the conclusion can be drawn hat the PAG contains different peptide systems, one of which mediating scratching and immobility (β-endorphin), may be associated with the opioid receptors with a high degree of stereospecificity and the other (ACTH) with a low degree of stereospecificity mediating grooming and situated in the dorsal part of the PAG.

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