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The role of central melanocortin receptors in the activation of the hypothalamus-pituitary-adrenal-axis and the induction of excessive grooming

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1 In accord with previous studies intracerebroventricular (i.c.v.) injections of $ACTH_{1-24}$ (1 μg) induced a display of excessive grooming, and increased the plasma concentrations of ACTH and corticosterone. Pituitary-adrenal activation was blocked by pretreatment with dexamethasone, indicating that the effect of the (i.c.v.) injected peptide was not caused by a peripheral effect on the adrenal cortex.

2 Doses of 1 and 3 μg of a non-selective melanocortin-3/4-receptor antagonist (SHU 9119), or of 5 and 10 μg of a selective melanocortin-4-receptor antagonist ([D-Arg^8]$ACTH_{4-10}$), coadministered (i.c.v.) with 1 μg $ACTH_{1-24}$, inhibited the $ACTH_{1-24}$-induced activation of the hypothalamus-pituitary-adrenal-axis and excessive grooming.

3 In addition, several doses of the selective melanocortin-3-receptor agonist Lys-γ_2-MSH were centrally administered, but neither neuroendocrine, nor excessive grooming responses were observed.

4 These results imply that the melanocortin-4-receptor, and not the melanocortin-3-receptor, is involved in the $ACTH_{1-24}$-induced rise in plasma levels of ACTH and corticosterone, and excessive grooming.

Keywords: Melanocortin-4-receptor; melanocortin-3-receptor; melanocortin receptor antagonist; ACTH; corticosterone; grooming

Introduction

The multifactorial precursor molecule pro-opiomelanocortin (POMC) is expressed in the anterior lobe of the pituitary gland, in certain peripheral tissues, and in restricted areas of the brain. Differential enzymatic processing of POMC in these tissues yields biologically active peptide end-products. Thus, POMC is the precursor of a large family of melanocortins, including adrenocorticotrophic hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), β-MSH and γ-MSH, that function as hormones in the periphery and as neuromodulators in the brain. As such, the melanocortins play an important role in the regulation of an array of adaptive behavioural and physiological processes (De Wied & Jolles, 1982; Eberle, 1988).

We have previously shown, that intracerebroventricular (i.c.v.) administration of melanocortins induces excessive grooming behaviour in rats (Gispen *et al*., 1975; Gispen & Isaacson, 1986), and in addition activates the hypothalamus-pituitary-adrenal-(HPA-) axis (Wiegant *et al*., 1979). These studies revealed that these effects are both independent of corticosteroidogenic activity of the peptides. In addition, these effects are probably brought about via interaction of the peptides with different targets in the brain, since a reduced effectiveness of a second i.c.v. injection of ACTH (short-tem tolerance) was found for the grooming-response, but not for the activation of the HPA-axis (Wiegant *et al*., 1979).

Recently, the primary structure of the melanocortin (MC) receptor has been established by sequencing of cloned cDNA, and five types of receptor (MC_1 through to MC_5) have now been distinguished that differ with respect to their anatomical distribution and ligand selectivity. This heterogeneity probably underlies the diversity in biological actions of the melanocortin peptide family (Tatro, 1990; Florijin *et al*., 1993; Tatro, 1993; Adan *et al*., 1994a; Low *et al*., 1994; Tatro & Entwistle, 1994).

Expression of the MC_1- and the MC_2-receptors seems to be restricted to peripheral tissues, where the MC_1-receptor on melanotrophs mediates MSH stimulated pigmentation (Chhajlani & Wikberg, 1992), and the MC_2-receptor on adrenocortical cells mediates ACTH-induced steroidogenesis (Mountjoy *et al*., 1992). In the brain, the receptors expressed are predominantly of the MC_3- and MC_4- types (Low *et al*., 1994), although very low levels of MC_5-receptor mRNA have also been detected (Griffon *et al*., 1994; Labbé *et al*., 1994). MC_3-receptor mRNA is primarily found in the hypothalamus (Chhajlani *et al*., 1993; Gantz *et al*., 1993a; Roselli-Rehfuss *et al*., 1993), whereas MC_4-receptor mRNA is expressed in a wide variety of regions throughout the brain (Gantz *et al*., 1993b; Mountjoy *et al*., 1994). The anatomical distribution of these central receptors is consistent with a role in the regulation of neuroendocrine, autonomic and behavioural responses.

In vitro studies with membrane binding assays and expression systems have shown that α-MSH, ACTH and γ-MSH-peptides are the most potent naturally occurring agonists for the MC_3-receptor (Chhajlani *et al*., 1993; Gantz *et al*., 1993a; Roselli-Rehfuss *et al*., 1993; Low *et al*., 1994). The MC_4-receptor is activated by ACTH and α-MSH, but not by γ_2-MSH (Gantz *et al*., 1993b; Low *et al*., 1994; Mountjoy *et al*., 1994). For the MC_5-receptor, α-MSH and $ACTH_{1-24}$ are the agonists with highest potency and affinity, whereas γ_2-MSH is a weak, low affinity agonist for this receptor (Griffon *et al*., 1994; Labbé *et al*., 1994). Thus, the MC_3-receptor is the only known melanocortin receptor in the brain that can be activated by physiological levels of γ_2-MSH.

Recently, we have shown that melanocortin-induced excessive grooming is mediated by the MC_4-receptor in the brain (Adan *et al*., 1994b). In view of our previous findings

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that induction of excessive grooming and activation of the HPA-axis following i.c.v. administration of melanocortins involve different mechanisms (Wiegant *et al*., 1979), it was investigated whether or not different melanocortin receptors underly these effects. Neither selective antagonists for the MC_3-receptor, nor selective agonists for the MC_4-receptor are available at present. Therefore, a non-selective antagonist for the MC_3-receptor and the MC_4-receptor, that retains agonistic activity for the MC_5-receptor (Hruby *et al*., 1995), and a selective antagonist for the MC_4-receptor (Adan *et al*., 1994b) were tested in rats for their ability to antagonize the plasma ACTH and corticosterone response and the display of excessive grooming behaviour following i.c.v. injection of $ACTH_{1\text{-}24}$. In addition, a selective agonist for the MC_3-receptor, Lys-γ_2-MSH, was tested (i.c.v.) for its ability to stimulate HPA-activity and evoke excessive grooming.

Methods

Animals

Male Wistar rats (GDL; Utrecht, The Netherlands) of approximately 130 g were housed individually under standard laboratory conditions (21 ± 1°C; 60% humidity; lights on from 7 h 00 min until 19 h 00 min) and received food and water *ad libitum*. The experiments were performed between 10 h 00 min and 12 h 00 min. Experimental procedures were approved by the Committee on Animal Experiments of the Faculty of Medicine, Utrecht University.

Surgery, treatment, and assay procedure

I.c.v. injections of the peptides and/or antagonists were given in a volume of 3 μl saline. When coadministered, the peptide and antagonist were injected as a mixture. For i.c.v. injections, the brain ventricular system of the rats was cannulated as previously described (Brakkee *et al*., 1979). The rats were anaesthetized by intramuscular injection of 1.0 ml kg^{-1} bodyweight Hypnorm (fentanyl citrate 0.135 mg ml^{-1} and fluanisone 10 mg ml^{-1}, Janssen Pharmaceutica, Beerse, Belgium). During the recovery period (at least ten days) the animals were frequently weighed, handled and trained to avoid handling-induced stimulation of the HPA-axis. The rats were transported to a low noise room at least three days before the experiments were performed. Injections were given by hand with a modified 10 μl Hamilton syringe.

The occurrence of grooming (Gispen & Isaacson, 1986) was recorded in the home cage with a 15th s time sampling procedure as described by Gispen *et al*. (1975). Since we expected the neuroendocrine response to reach a maximum at 30 min after i.c.v. application of ACTH (Wiegant *et al*., 1979), grooming was scored for 29 min starting from 1 min and continued until 30 min after the i.c.v. injection.

Immediately thereafter, the animals were killed by decapitation in an adjacent room, and trunk blood was collected in ice-cold 10 ml tubes containing anti-coagulant (15 i.u. heparin ml^{-1} blood; Sarstedt, Nümbrecht, Germany). Separate control groups of saline- and $ACTH_{1\text{-}24}$-treated rats were run for each dose of a(nta)gonist.

Dexamethasone sodium-phosphate (Organon, Oss, The Netherlands) was used to block pituitary ACTH release in order to demonstrate that i.c.v. administration of $ACTH_{1\text{-}24}$ induced the neuroendocrine response at the level of the brain and not by leakage to the periphery (Keller-Wood & Dallman, 1984). To that end, dexamethasone (dissolved in Tris buffer; pH = 7.7) was given at a dose of 75 μg/rat s.c. twice, at 2 and 17 h before the experiment.

ACTH and corticosterone radioimmunoassays

Plasma ACTH was determined in duplicate by direct radioimmunoassay (RIA) according to the procedure described by Van Oers *et al*. (1991), by use of a specific rabbit antiserum, directed to the mid-portion of ACTH (code Ft 8514) kindly donated by Dr G.B. Makara (Budapest, Hungary). The antiserum did not cross-react with [D-Arg^8]$ACTH_{4\text{-}10}$, SHU 9119, Lys-γ_2-MSH and dexamethasone sodium-phosphate. Cross-reactivity for $ACTH_{1\text{-}24}$ was 400%. Synthetic human $ACTH_{1\text{-}39}$ (Peninsula Laboratories, Belmont, CA, U.S.A.) was used as standard, and ^{125}I-labelled $ACTH_{1\text{-}39}$ (Iodogen method) as tracer. Sample dilution curves paralleled the standard curve. The sensitivity of the assay, calculated at $B/B_0 = 0.9$, was 10 pg ml^{-1} plasma (0.5 pg/tube). Intra- and interassay variations were 5% and 8%, respectively.

Plasma corticosterone was determined in triplicate after dichloromethane extraction (recovery approximately 100%) by RIA according to the procedure described by Van Oers *et al*. (1991). A specific sheep antiserum, code S-052305676, kindly donated by Dr T.J. Benraad (Nijmegen, The Netherlands) was used. The antiserum hardly cross-reacted with [D-Arg^8]$ACTH_{4\text{-}10}$ (<0.2%), SHU 9119 (<0.2%), Lys-γ_2-MSH (<0.1%), $ACTH_{1\text{-}24}$ (<0.2%) and dexamethasone sodium phosphate (<0.1%). Corticosterone (Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard and ^{3}H-labelled corticosterone as tracer. Sample dilution curves paralleled the standard curve. The sensitivity of the assay, calculated at $B/B_0 = 0.9$, was 0.4 ng ml^{-1} plasma (20 pg/tube). Intra- and interassay variations were 4% and 7%, respectively.

Peptides and antagonists

$ACTH_{1\text{-}24}$ and [D-Arg^8]$ACTH_{4\text{-}10}$ (Table 1) were obtained by Organon (Oss, The Netherlands). The selective MC3-R

Table 1 The amino acid sequences* of the peptides and antagonists used

$ACTH_{1\text{–}24}$	H-Ser-Tyr-**Met-Glu-His-Phe-Arg-Trp-Gly**-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro-OH (non-selective MC_3/MC_4 receptor agonist)
SHU 9119	Ac-Nle-Asp-**His**-D-Nal(2)-**Arg-Trp**-Lys-NH_2** (non-selective MC_3/MC_4 receptor antagonist; MC_5-receptor agonist)
[D-Arg^8]$ACTH_{4\text{–}10}$	H-**Met-Glu-His-Phe**-D-Arg-**Trp-Gly**-OH (selective MC_4-receptor antagonist)
Lys-γ_2-MSH	H-Lys-Tyr-Val-**Met**-Gly-**His-Phe-Arg-Trp**-Asp-Arg-Phe–Gly-OH (selective MC_4-receptor agonist)

*Bold and underlined: original amino acids in the $ACTH_{4\text{–}10}$ sequence. **Lactam bridge between Asp and Lys.

agonist, Lys-γ_2-MSH (Table 1) was synthesized and kindly donated by Dr P. Hoogerhour (RIVM, Utrecht, The Netherlands). SHU 9119 (Table 1) was a gift from Dr R. D. Cone (Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, U.S.A.). Peptides and antagonists were dissolved in saline.

Statistics

Statistical analysis of data was performed by an one-way ANOVA followed by a *post-hoc* Student-Newman-Keuls test. $P<0.05$ was taken as the level of significance. Data are presented as means $\pm$ s.e.mean for the indicated number of rats.

Results

Effects of $ACTH_{1-24}$ on plasma levels of ACTH and corticosterone

A dose-response curve for 0.03, 0.30 and 3.00 μg i.c.v. injected $ACTH_{1-24}$ was established for the activation of the HPA-axis. The two highest doses (0.3 and 3 μg) significantly increased the plasma concentrations of ACTH and corticosterone as compared to saline (Figure 1). An intermediate active dose of 1 μg $ACTH_{1-24}$ was chosen for further experimentation.

The data in Figure 2 show that the increase of plasma ACTH and corticosterone levels following i.c.v. injection of 1 μg $ACTH_{1-24}$ was completely blocked by pretreatment of the rats with dexamethasone. Since this synthetic glucocorticoid is known to block the HPA-axis at the level of the pituitary and the brain (Keller-Wood & Dallman, 1984), this indicates that the increase in plasma ACTH and corticosterone observed after i.c.v. injection of $ACTH_{1-24}$ was not due to leakage of peptide from the ventricular system into the general circulation. Thus, in accord with our earlier observations (Wiegant *et al*., 1979), we concluded that the effect resulted from an action of the peptide on targets in the brain.

Effects of SHU 9119 on $ACTH_{1-24}$-induced HPA-activation and excessive grooming

I.c.v. injections of 1 μg and 3 μg of the non-selective MC3-/MC4-R antagonist SHU 9119 alone, did not affect the plasma ACTH and corticosterone concentrations, and did not induce excessive grooming (Figure 3). $ACTH_{1-24}$ given i.c.v. at a dose of 1 μg significantly increased plasma ACTH and corticosterone concentrations and induced excessive grooming as compared to the saline-treated animals. Plasma ACTH and

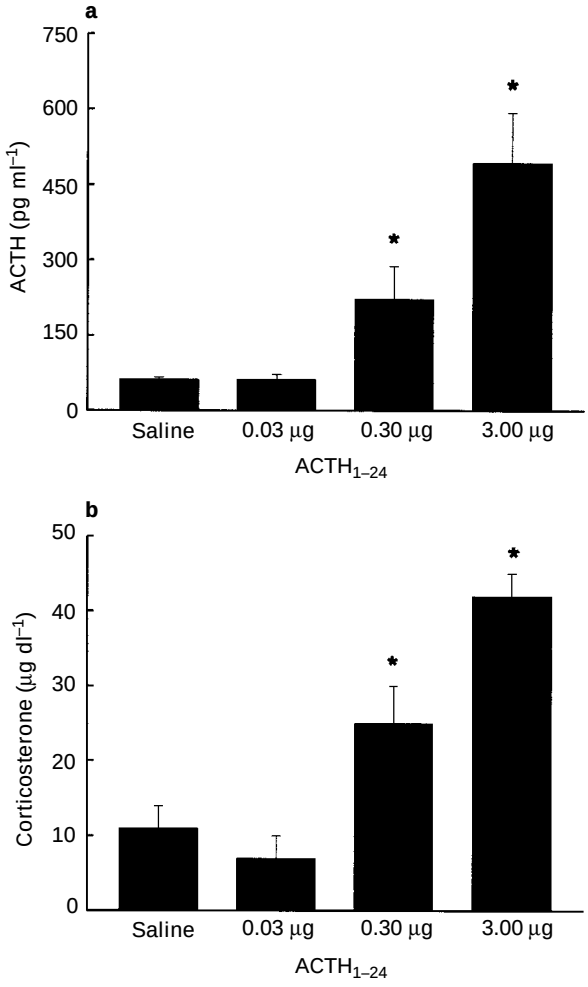

Figure 1 Plasma concentrations of (a) ACTH and (b) corticosterone following i.c.v. application of 0.03 μg ($n=8$), 0.30 μg ($n=8$) and 3.00 μg ($n=7$) $ACTH_{1-24}$ and saline ($n=16$). *$P<0.05$ vs all other groups.

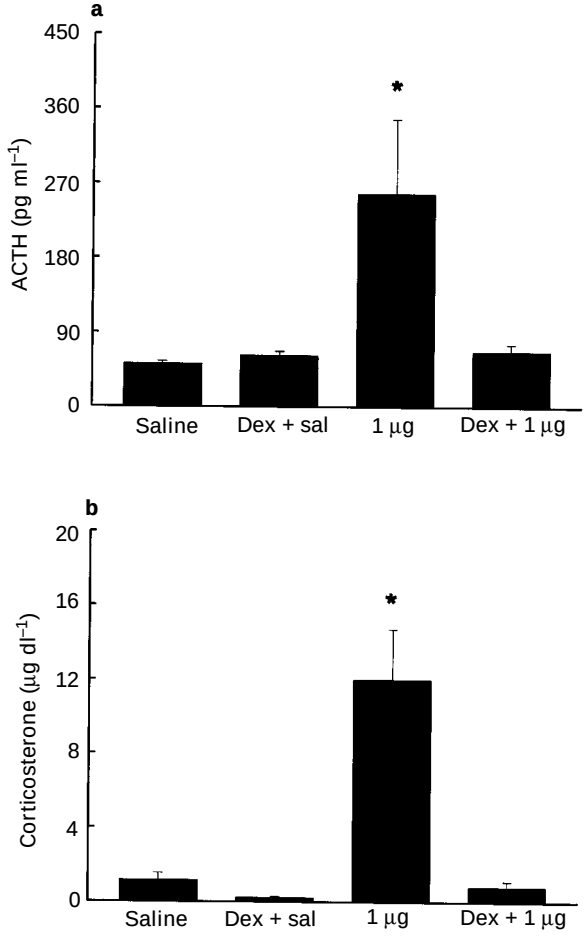

Figure 2 Plasma concentrations of (a) ACTH and (b) corticosterone following i.c.v. injection of saline without dexamethasone pretreatment (saline, $n=15$) or after dexamethasone pretreatment (Dex + sal, $n=7$), and of 1 μg $ACTH_{1-24}$ without dexamethasone pretreatment (1 μg, $n=6$) or after dexamethasone pretreatment (Dex + 1 μg, $n=6$). *$P<0.05$ vs all other groups.

grooming responses following i.c.v. injection of 1 μg $ACTH_{1\text{-}24}$ were significantly antagonized by 1 and 3 μg SHU 9119. The plasma corticosterone response following i.c.v. injection of $ACTH_{1\text{-}24}$ was significantly antagonized by the 1 μg dose of SHU 9119, whereas the effect of 3 μg dose on the corticosterone response did not reach significance. No significant difference between the effect of 1 and 3 μg SHU 9119 in $ACTH_{1\text{-}24}$-treated rats was found. The 3 μg dose of SHU 9119, when coadministered with $ACTH_{1\text{-}24}$, caused barrel rotation or other behavioural abnormalities in some animals (33%). These rats were eliminated from statistical analysis.

Effects of [D-Arg^8]$ACTH_{4\text{-}10}$ on $ACTH_{1\text{-}24}$-induced HPA-activation and excessive grooming

[D-Arg^8]$ACTH_{4\text{-}10}$ (i.c.v.) at doses of 5 μg and 10 μg, did not induce either a neuroendocrine or a grooming response (Figure 4). The increase of plasma ACTH and corticosterone

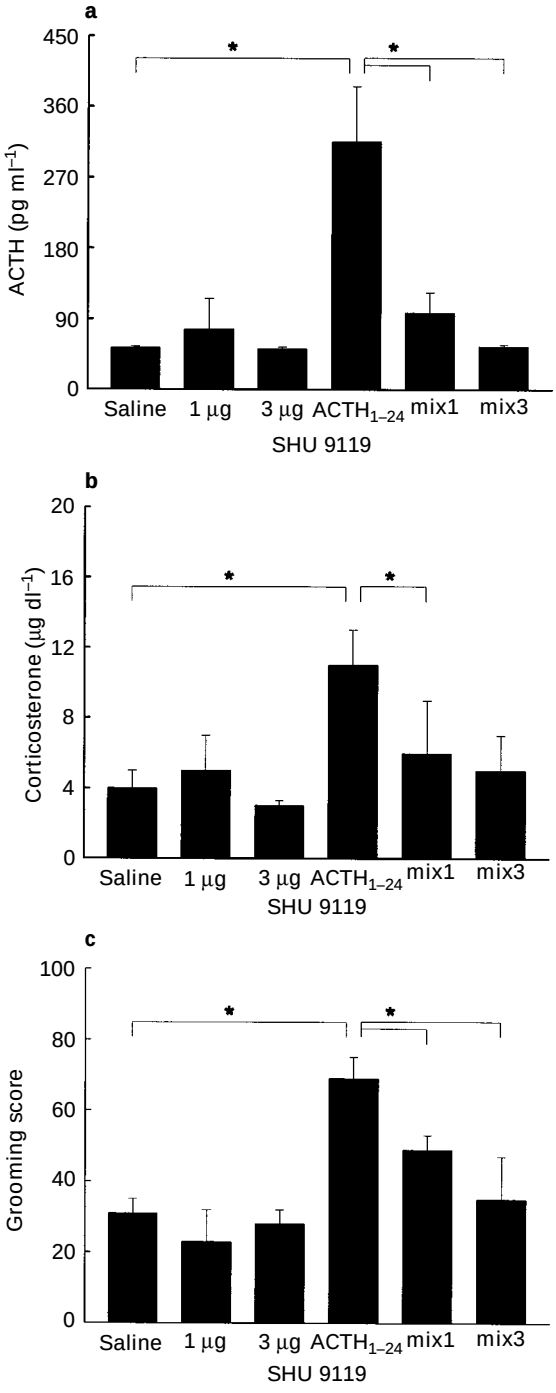

Figure 3 Plasma concentrations of (a) ACTH and (b) corticosterone and (c) grooming behaviour following i.c.v. injection of saline ($n=15$), 1 μg ($n=5$) and 3 μg ($n=6$) SHU 9119, 1 μg $ACTH_{1\text{-}24}$ ($n=11$), and after the administration of the mixture of 1 μg $ACTH_{1\text{-}24}$ + 1 μg SHU 9119 (mix1, $n=7$), and the mixture of 1 μg $ACTH_{1\text{-}24}$ + 3 μg SHU 9119 (mix3, $n=5$). *$P<0.05$.

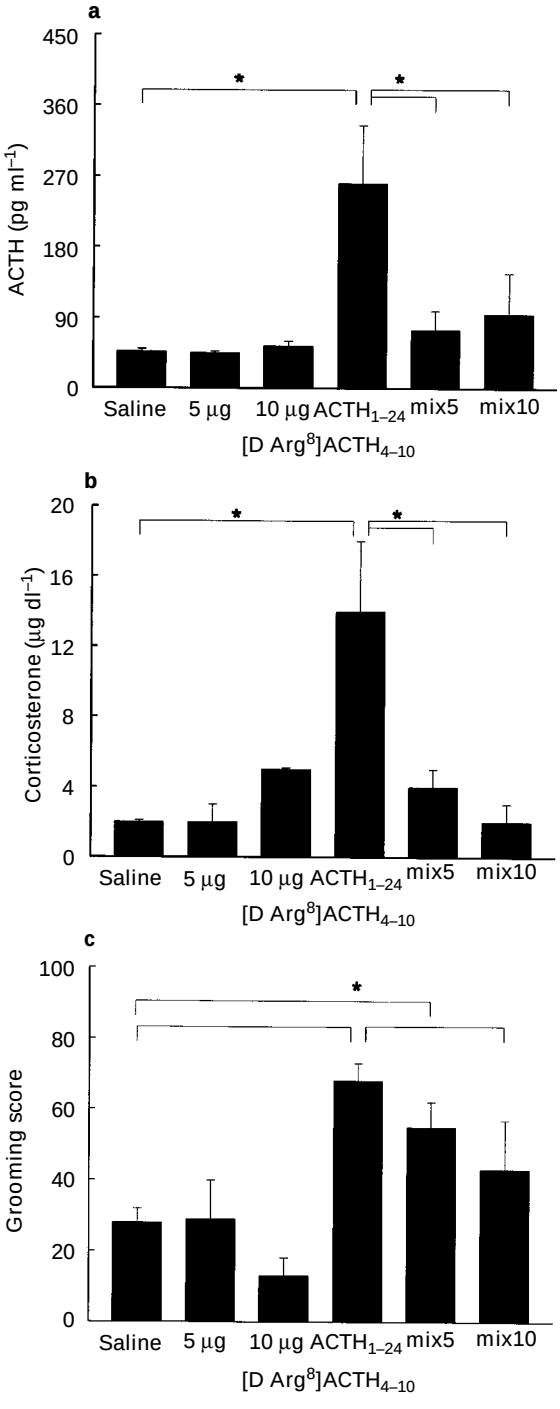

Figure 4 Plasma concentrations of (a) ACTH and (b) corticosterone and (c) grooming behaviour following i.c.v. injection of saline ($n=15$), 5 μg ($n=6$) and 10 μg ($n=6$) [D-Arg^8]$ACTH_{4\text{-}10}$, 1 μg $ACTH_{1\text{-}24}$ ($n=12$), and after administration of the mixture of 1 μg $ACTH_{1\text{-}24}$ + 5 μg [D-Arg^8]$ACTH_{4\text{-}10}$ (mix5, $n=6$), and the mixture of 1 μg $ACTH_{1\text{-}24}$ + 10 μg [D-Arg^8]$ACTH_{4\text{-}10}$ (mix10, $n=5$). *$P<0.05$.

concentrations, and of grooming behaviour following i.c.v. injection of 1 μg $ACTH_{1-24}$ was significant as compared to the saline-treated animals. The $ACTH_{1-24}$-induced increase in plasma ACTH and corticosterone concentration was significantly inhibited by coadministration with 5 or 10 μg [D-Arg^8]$ACTH_{4-10}$. The grooming response following i.c.v. injection of 1 μg $ACTH_{1-24}$ was significantly antagonized by the 10 μg dose of [D-Arg^8]$ACTH_{4-10}$, whereas the inhibition of the grooming response by the 5 μg dose of the antagonist did not reach significance. No significant difference between the effect of 5 and 10 μg [D-Arg^8]$ACTH_{4-10}$ in $ACTH_{1-24}$-treated rats was found for the grooming response.

Effects of Lys-γ_2-MSH on the HPA-axis and on excessive grooming

No significant effects of i.c.v. injection of 10, 20 and 50 μg doses of Lys-γ_2-MSH on plasma ACTH and corticosterone levels and grooming behaviour, were observed (data not shown).

Discussion

The present study confirms and extends our earlier observations that i.c.v. injection of ACTH stimulates the HPA-axis and induces excessive grooming behaviour, and that these effects arise from actions of the peptide within the central nervous system.

The present study further shows that the neuroendocrine, as well as the grooming responses induced by central administration of $ACTH_{1-24}$, can be inhibited by the non-selective antagonist for the MC_3- and MC_4-receptors, SHU 9119. A similar result was obtained with the selective MC_4-receptor antagonist, [D-Arg^8]$ACTH_{4-10}$. These results indicate that the MC_4-receptor is involved in both effects of $ACTH_{1-24}$, but do not exclude a role for the MC_3-receptor. However, the selective MC_3-receptor agonist, Lys-γ_2-MSH, failed to elicit a neuroendocrine or a grooming response, suggesting that the MC_3-receptor is not involved. Furthermore, no agonistic activity of SHU 9119 was observed on the HPA-axis or grooming. Since SHU 9119 retains agonistic activity for the MC_5-receptor, this indicates that a role for the MC_5-receptor in these responses is unlikely. Taken together, the results therefore indicate that the activation of the HPA-axis by central administration of $ACTH_{1-24}$ is probably mediated by the MC_4-receptor, as has been shown previously for the induction of excessive grooming (Adan *et al*., 1994b).

A role for the MC_4-receptor in the activation of the HPA-axis is in line with the distribution of MC_4-receptor mRNA in the brain. Firstly, MC_4-receptor mRNA is found in the dorsal medial parvocellular part of the paraventricular nucleus (PVN) of the hypothalamus (Mountjoy *et al*., 1994), which gives rise to most of the CRH fibres that project to the external zone of the median eminence, and by that means is associated with the regulation of pituitary-adrenal activity (Swanson, 1986). Secondly, MC_4-receptor mRNA is found in several brain areas that are associated with afferent control of the PVN, such as the bed nucleus of the stria terminalis, the subfornical organ, the parabrachial nucleus, the nucleus of the solitary tract, and other hypothalamic areas (Swanson, 1986; Mountjoy *et al*., 1994).

The role of the MC_4-receptor in the excessive grooming response is supported by earlier findings, showing that: (i) MC_4-receptor antagonists inhibit the induction of excessive grooming behaviour induced by central administration of α-MSH (Adan *et al*., 1994b), (ii) excessive grooming can be induced by local injection of $ACTH_{1-24}$ in the periaqueductal gray (Spruijt *et al*., 1984), the periventricular nucleus (Van Erp *et al*., 1991) and the substantia nigra (Wiegant *et al*., 1977), areas where expression of the MC4-receptor has been demonstrated (Mountjoy *et al*., 1994). The absence of (i.c.v.) $ACTH_{1-24}$-induced excessive grooming in animals which are lesioned in the periaquaductal gray suggests a predominant role of the MC_4-receptor in this area in ACTH-induced grooming (Spruijt *et al*., 1986) and (iii) the structure-activity relationships for excessive grooming induction by melanocortins resemble those for the induction of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation in HEK 293 cells expressing the MC_4-receptor (Adan *et al*., 1994a), and in brain slices (Florijn *et al*., 1993) of areas in which the MC_4-receptor has been found (Mountjoy *et al*., 1994).

The observations that SHU 9119 blocked the excessive grooming response at both doses (1 and 3 μg), and that [D-Arg^8]$ACTH_{4-10}$ was only effective at the 10 μg dose, might be explained by the fact that [D-Arg^8]$ACTH_{4-10}$ is a less potent antagonist for the MC_4-receptor than SHU 9119, based on the pA_2-values found *in vitro* (Adan *et al*., 1994b; Hruby *et al*., 1995). However, the difference in effectiveness of these antagonists may also be caused by differences in pharmacokinetic properties.

In an earlier study from our laboratory (Wiegant *et al*., 1979) a dissociation of the neuroendocrine and grooming responses to central administration of ACTH was found, and it was suggested that the two responses are brought about by two different interactions of ACTH and the brain, involving different neural substrates with possibly multiple receptors. In that study a reduced effectiveness of a second i.c.v. injection of ACTH in inducing the grooming response was found, whereas no single-dose tolerance was observed for the effect of i.c.v. ACTH on the HPA-axis. However, the present results clearly indicate that the rise in plasma concentrations of ACTH and corticosterone and the display of excessive grooming are mediated by the same melanocortin receptor type, the MC_4-receptor. Thus, the dissociation between the neuroendocrine and the grooming responses with regard to single dose tolerance is not dependent on the type of melanocortin receptor, but more likely on the localization and the connectivity of the target neurones involved.

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