Role of recently discovered opioid neuropeptides in the regulation of Open-Field Behaviour and the Hypothalamic-Pituitary-Adrenal axis

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1 Introduction

1.1 The role of the endogenous opioids in neuroendocrine regulation

1.1.1 The families of opioid neuropeptides; chemical nature, distribution and receptor preference

The opioids have a prominent and versatile impact on numerous physiological parameters. Their analgesic action, the tolerance and dependence they evoke and their side effects have been in the centre of countless studies aiming at the discovery of the ideal "pain-killer" in the past few decades. It is also of vital importance to clearly establish the whole spectra of the effects and feasible side effects of the synthetic opiates and the new endogenous opioid peptides and their analogues to outline their pharmacological potential.

Until the seventies, the physiological background of the diverse actions of morphine, the major alkaloid of the opium poppy (Papaver somniferum) was barely clarified[31]. Numerous potent, synthetic compounds have been identified, but only with the characterisation of the enkephalins[58] did it become clear that it is the endogenous opioid peptides that target the opiate receptors in physiological circumstances.

It is now known that there exist at least five families of endogenous opioids. The first one described in 1975 was that of the enkephalins. Their precursor peptide proenkephalin contains 6 copies of Met-enkephalin and one copy of Leu-enkephalin sequences. They are widely distributed in the brain and the peripheral nervous system, where they function as neurotransmitters and modulators. They are also expressed in the endocrine, reproductive and immune system[31]. Some longer derivatives of these family (like MERF) show especially high expression in the central nervous system (CNS)[130] and appear to possess distinct biochemical[10,161] properties.

The dynorphins (dynorphin-A, dynorphin-B, α-neo-endorphin and some shorter related peptides) derive from prodynorphin. They are also found in neural, endocrine and reproductive tissues, although their concentrations are lower. They are also presumed to function as neurotransmitters in the brain and the spinal cord[74].

The third family consists of β-endorphin and its shorter derivatives, which are synthetized as part of the pro-opiomelanocortin (POMC). POMC products are found in both central and peripheral tissues. In the CNS they are expressed in the cells of the arcuate

nucleus of the hypothalamus and the nucleus tractus solitarius of the medulla. Much higher concentrations are present in the anterior and intermediate lobes of the pituitary and some amount is expressed by the reproductive and immune systems[36,140].

The fourth family is the nociceptin peptide family, product of a reverse-pharmacology[125]. The first described heptadecapeptide orphanin FQ represents the long-sought ligand of a heterotrimeric G protein-coupled receptor that proved very similar in sequence to the opioid receptors and named opioid receptor like 1 (ORL_1)[100]. Nociceptin[109] and its receptor[99] are quite evenly distributed in the CNS. Both the peptide[97,101,118] and the receptor[99] are also expressed at the periphery in peripheral ganglia, smooth muscles, the endocrine and the immune system.

Last but not least the endomorphins (EMs) should be mentioned as the putative endogenous ligands of the μ-opioid receptor. They were isolated from the bovine brain[167]. Besides MERF our efforts were concentrated on revealing their behavioural and endocrine actions.

The five families of endogenous opioid peptides act on 4 classes of opioid receptors for which associated function has been well defined (Table 1.). The are designated as μ, δ, κ and ORL_1 receptors. Moreover, three further opioid receptor classes have been proposed in the past few years. They were named ε, λ and σ receptors, but the functional role of these receptors has not beeen established yet, and they now not considered to be opioid in nature (σ) or do not seem to represent distinct pharmacological entities (ε, λ)[31]. The μ-receptors, selectively targeted by morphine and the EMs, described in the chronic spinal cord model[91], mediate analgesia, miosis, bradycardia and hypothermia. The subtypes (μ_1, μ_2, μ_3) are responsible for different actions since analgesia is mediated by the μ_1, while respiratory depression by the μ_2 receptor. The κ-receptors, described in the same model, are activated ketocyclazocine and the dynorphins appear their endogenous ligands. They are responsible for sedation, depression of flexor reflexes and as many as four subtypes have been identified with radioligand binding assays[27]. The δ receptors[86] mediate analgesia and activate several motor paradigms[30]; they appear to bind preferentially the enkephalins[3]. The ORL_1 is endowed with supraspinal pronociceptive/anti-opioid properties. It has not yet been found to precipitate withdrawal in morphine-tolerant rats nor does it elicit motivational effects, suggesting it lacks abuse liability[97]. Although the σ-receptor is not a typical opiate receptor since it does not show the classical stereospecificity[3] and the receptor activation can not be reversed by naloxone[31], some “non-opioid” actions (CNS activation, mania, pupillary dilatation, tachypnea, tachycardia) of the opioid peptides seem to be transmitted by this

receptor.

The functional assays for studying the opioid receptors revealed that it is the adenylate-cyclase and the intracellular cAMP that are affected by the opioids predominantly. Both the μ and the δ-receptors show a remarkable inhibition of adenylate cyclase[26,31]. Further they also appear to activate potassium conductance and inhibit the voltage-dependent inward calcium current[56,105]. The κ-[138,160] and ORL_1[49] receptors exhibit the same second messenger profile, therefore their distinct pharmacological spectra can be attributed to their different distribution pattern.

1.1.1.1 The endomorphins; the characteristics of the recently discovered endogenous μ-opioid ligands

In spite of the fact that they differ considerably in structure from previously known endogenous opioids[167] (Fig. 1.) radioreceptor binding assays have revealed that these peptides possess high affinity and selectivity for the μ-opioid receptors[47]. It has been concluded that they might be endogenous ligands for the morphine receptors, and they were therefore named endomorphin-1 (EM1) and endomorphin-2 (EM2)[167].

The EMs are widely and densely distributed throughout the rat brain (posterior hypothalamic nuclei, locus coeruleus and amygdala), as demonstrated by immunocytochemical studies[93]. Several physiological effects of the EMs have already been described, too. These results reflect that these opioid peptides have distinct pharmacological profiles, which is in some cases markedly different from that of morphine. They exert a profound spinal analgesic effect[146,167], similarly to morphine, but they even antagonize neuropathic pain, whereas morphine seems ineffective[123]. Further, EM1, like morphine[63,106], has anxiolytic and orexigenic properties[5], while its cardiovascular activity[25] appears to differ from that of morphine[40]. These functional differences may result from the differences in selectivity of the EMs and morphine as concerns the opioid receptor subtypes: the EMs display high selectivity to the μ-opioid receptor, while morphine binds to both the μ-and the δ-receptors[47,103,141,166-168]. Further, the EMs and morphine prefer different molecular forms of the μ–opioid receptor[135], and only the EMs exhibits an ability to internalize their receptor[21]. Taken together the widespread distribution of them in the CNS and the aforementioned physiological phenomena suggests that they might belong to the endogenous peptide mediators of nociceptive, behavioural and autonomous processes, in which opioid regulation plays a well-established and critical role[3].

1.1.1.2 The characteristics of the enkephalin related peptide MERF

Met^5-enkephalin-Arg^6-Phe^7 (MERF) (Fig. 2) is one of the most widespread and abundantly expressed enkephalin derivative in the CNS. Altough it was first isolated from bovine adrenal glands and striatal extracts[145] later proved to be widely distributed in the CNS of different vertebrates[75,130,149]. Despite its high expression and unique and rather ambigouos pharmacological profile, only a few publications have dealt with its physiological function: a chimeric peptide based on MERF displayed prominent antinociceptive characteristics[51] and MERF was also demonstrated to have immunocyte excitatory properties[150]. Besides, contradictory data are still available regarding its affinity for the different subtypes of opioid receptors. *In vitro* receptor binding assays either suggested μ-receptor mediation[76] or demonstrated predominant κ_2 signaling[10,161]. Substantial (50 % in rat cerebrum) naloxone resistant and moderate δ, σ_2 binding was also found in both amphibians and rodents[10,11,14,162]. Biological assays revealed naloxone-reversible antinociception[59] and antitussive effects[70].

1.1.2 The role of the opioid neuropeptides in the regulation of behaviour and the hypothalamic-pituitary-adrenal axis

The role of the opioid system in the regulation of locomotion[8] is well characterized. Earlier studies demonstrated that opioid peptides influence the locomotor activity through the μ, δ and κ-opioid receptors: the μ and δ-opioid receptors mediate locomotor hyperactivity[98,157], while selective κ-receptor agonists decrease linear locomotion[156]. However, the results of behavioural experiments have been found to depend strongly on the strain[43], the sex[78], the time[60] and the dose[80,142]. Further behavioural phenomena are also under opioid control. The effects of morphine on rearing and grooming are strongly dose- and time-related presumably in consequence of different involvements of opioid receptor subtypes[60,72,117]. Further it is well-established that opioid peptides (mainly enkephalins) participate in the intricate subcortical neuronal networks that regulate locomotion[44] (Fig. 3).

Participation of an opiate mechanism in the corticosterone response previously has been demonstrated too: acute administration of morphine activated the HPA axis in the rat[66] and drew attention to the examination of the role of the opiates in the control of stress response[113]. Further studies indicated that the action of the opioids appears to be species-dependent. In humans, they seem to inhibit HPA activation[28], whereas in rodents their effect

is more probably stimulatory[87,144]. Nevertheless, in both species opioids are the most important mediators of stress-induced analgesia[2,88].

1.2 The purpose of our experiments

The purpose of the present study was to examine the effects of EMs on behavioural responses, and to compare the actions of this endogenous opioid ligand to those of morphine.

In the present work, to further scrutinize the effects of EM1 on the HPA axis, adrenocorticotropic hormone (ACTH) release from isolated anterior pituitary slices and the corticosterone secretion of adrenal slices were measured after EM1 treatment in an *in vitro* perifusion system. The following experiments were designed to shed light on the mediation of the neuroendocrine responses brought about by EM1.

Corticotropin-releasing hormone (CRH) plays a very important role in the activation of the HPA axis[158], and has a marked impact on behavioural phenomena, too. It was reported that microinfusion of CRH into the paraventricular nucleus (PVN) induces locomotion[102], and an increase in rearing has also been described in several publications[143,159]. In the present experiments, therefore the CRH antagonist α-helical CRH_{9-41} was applied before the administration of the EMs, in order to clarify its possible role in mediation of the EMs-evoked neuroendocrinological responses.

As previous studies have indicated that opiates might evoke locomotor hyperactivity through the secretion of dopamine in the nigrostriatal and mesolimbic dopaminergic system[16], the effects of intraperitoneally (ip.) administered haloperidol on the motor activation elicited by EM1 were tested. Since different opioids appear to exert rather diverse effects on the subcortical motor neurons[90,132], the action of EM1 on the basal and stimulated dopamine release from striatal slices were also measured in an *in vitro* superfusion system. Histological studies have demonstrated an interaction between the dopaminergic and opiatergic neurons in the hypothalamus[84], and therefore the effects of haloperidol pretreatment on the endocrine response evoked by EM1 were tested, too.

Several publications have demonstrated that the gaseous neurotransmitter nitric oxide (NO) (Fig. 4.) plays an indispensable role in the mediation of the physiological actions of morphine[48], but conflicting data are available regarding the function of NO in morphine-evoked HPA activation[18,83]. Recent data have provided evidence of the role of NO in the transmission of the vasodilatory action of the EMs[24], though Rialas et al.[126] demonstrated that EMs are unable to release NO through the μ_3-opioid receptor. Therefore, to reconcile these

conflicting data and to investigate whether (beside vasodilation) NO plays a more comprehensive role in the mediation of the actions of the EMs, the effects of the NOS inhibitor N^G-nitro-L-arginine (L-NNArg) on the neuroendocrine responses evoked by EM1 were also examined.

Since MERF was isolated in the highest concentration from locomotor centres and the hypothalamus in the CNS[116,119,130,145], we set out to shed light on its possible role in neuroendocrine control and to elucidate the mediation of its action. The purpose of the present study was to test the effect of MERF on the HPA axis and open-field parameters to observe exploration and stress-related behaviour[77]. Square crossing (horizontal activity), rearing (vertical activity) and grooming were monitored as separate paradigms, since previous publications demonstrated that they can change independently and different neurotransmitters might be involved in their control[73,77,139].

Because *in vitro* data have suggested both opioid and non-opioid binding[10,11], with the help of the non-selective opioid antagonist naloxone we tried to estimate the significance of naloxone sensitive signaling in the *in vivo* actions of MERF. As the heptapeptide is the proposed endogenous ligand for the κ_2–receptors in the rat brain[6,10], the κ-antagonist nor-binaltorphimine (nor-BNI) was also used to investigate the role of κ-mediation in the neuroendocrine effects of MERF.

Previous studies have indicated that opioids might act on locomotive behaviour and the HPA system indirectly, through the secretion of other mediators. Several publications suggested, that the nigrostriatal and mesolimbic dopaminergic circuitries might relay the locomotor hyperactivity elicited by morphine[16,39,122], and histological studies pointed out an interaction between the opioid and the dopaminergic system in the hypothalamus[41,84]. Accordingly, the effect of haloperidol pretreatment on the open-field behaviour and HPA activation brought about by MERF, and the action of this opioid neuropeptide on the basal and stimulated dopamine release of striatal slices were tested. Recent publications have also substantiated that CRH, one of the most potent regulators of stress-related behavioural[96,102] and hormonal processes[158], might mediate the neuroendocrine responses to opioid peptides[19,20]. For this reason, animals were pretreated with α-helical $CRH_{9\text{-}41}$ to investigate the involvement of CRH transmission in the behavioural and hormonal responses elicited by MERF.

2 Materials and Methods

2.1 Materials

2.1.1 The tested peptides

- The EMs were either obtained from the Institute of Biochemistry of the Biological Research Centre, Hungarian Academy of Sciences, Szeged (they were synthesized as described by Tömböly et al.[153]) or purchased from Sigma;
- MERF was synthesized by solid phase peptide synthesis using N-α-butoxycarbonyl (Boc) strategy[161] in the Institute of Biochemistry (Biological Research Centre, Hungarian Academy of Sciences, Szeged).

2.1.2 Substances used in the *in vivo* experiments; the materials used for corticosterone assay

- CRH antagonist α-helical CRH_{9-41}.
- Haloperidol (Richter, Budapest, Hungary)
- L-NNArg (Sigma)
- Naloxone hydrochloride (Sigma)
- Nor-BNI-dihydrochloride (Sigma)
- Saline (sodium chloride inj. of 0.9 %, Biogal, Hungary)
- Ethyl alcohol, methylene chloride and sulfuric acid of analytical grade (Reanal, Budapest) for corticosterone assay

2.1.3 Substances used in the *in vitro* experiments

- The components used for the Krebs' buffer preparation: NaCl, KCl, $MgSO_4$, $NaHCO_3$, glucose, KH_2PO_4 and $CaCl_2$ of analytical grade (Reanal, Hungary)
- (^{3}H)dopamine (Amersham Pharmacia Biotech UK)
- Mixture of 5% CO_2 and 95% O_2 for continuous gassing
- Scintillation fluid (Ultima Gold, Packard)

2.2 Animals

Male CFLP mice of an outbred strain (LATI, Gödöllő, Hungary) weighing 25-35 g were used at the age of 5 weeks. The animals were kept in their homecages at a constant room temperature on

a standard illumination schedule with 12-h light and 12-h dark periods (lights on from 6.00 a.m.). Commercial food and tap water were available *ad libitum*. The animals were kept and handled during the experiments in accordance with the instructions of the University of Szeged Ethical Committee for the Protection of Animals in Research.

2.3 *In vivo* experiments

2.3.1 Surgery

The mice were allowed a minimum of 1 week to acclimatize before surgery. Subsequently, the animals were implanted with a polyethylene cannula (3 mm long; 0.4 mm in inner and 1.8 mm in outer diameter) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg, ip.) anaesthesia. The stereotaxic coordinates were 0.5 mm posterior and 0.5 mm lateral to the bregma, and 3 mm deep from the dural surface, and the cannula was secured to the skull with acrylate. The mice were used after a recovery period of at least 5 days. All experiments were carried out between 8:00 and 10:00 a.m. At the end of the experiments, the correct position and the permeability of the cannulae was checked. In the behavioural studies, each mouse was sacrificed under pentobarbital anaesthesia, and the heads were also collected after decapitation in the endocrinological experiments. Methylene blue was injected via the implanted cannula and the brains were then dissected. Only data from animals exhibiting the diffusion of methylene blue in all the ventricles were included in the statistical evaluation.

2.3.2 Behavioural testing, the open-field apparatus

The mice were removed from their home cages and placed in the centre of a square, wooden, white-coloured open-field box consisting of 49 squares (5 x 5 cm each). The standard source of illumnation was a 60 W bulb from 80 cm. The horizontal locomotor activity was characterized by the total number of squares crossed during a 3-min test session (square crossing). The vertical locomotor activity was characterized by the number of rearings (standing on the hind legs). Groomings (face washing, forepaw licking and head stroking) were also observed.

2.3.3 Corticosterone assay

The mice were decapitated 30 min after treatment, and trunk blood was collected in heparinized tubes. The plasma corticosterone level was determined by fluorescence assay[124,170].

2.3.4 Experimental protocols

2.3.4.1 Investigation of the action of the endomorphins or MERF on open-field behaviour and the activation of the HPA axis

Different doses of EM1 (from 250 ng to 5 μg), EM2 (from 250 ng to 1 μg) or MERF (from 500 ng to 5 μg) dissolved in 0.9 % saline was administered intracerebroventricularly (icv.) in a volume of 2 μl into conscious mice with a Hamilton microsyringe over 30 s, immobilization of the animals being avoided during handling. Control mice received saline alone. Thirty min after EMs administration the animals were subjected to behavioural tests or were sacrificed to obtain blood samples for the corticosterone assay.

2.3.4.2 Combined treatment with peptide (α-helical CRH_{9-41}), opiate (naloxone and nor-BNI) or non-opiate (haloperidol and L-NNArg) antagonists and the opioid neuropeptides

For this experimental setting, the animals were subjected to combined treatment with an antagonist and an opioid peptide. L-NNArg, haloperidol and α-helical CRH_{9-41} were tested with EM1, α-helical CRH_{9-41} with EM2, while naloxone hydrochloride, nor-BNI-dihydrochloride and α-helical CRH_{9-41} with MERF. The opiate antagonists, the CRH antagonist and L-NNArg were dissolved in 0.9% saline and injected icv. in a volume of 2 μl, while haloperidol (dissolved in 0.9% saline) was administered ip. in a volume of 0.3 ml. The opiate receptor blockers were applied in equimolar concentration with a view to finding the most effective dose. The dose of the non-opiate antagonist was the concentration that had proved most effective in our previous experiments, and *per se* does not affect the endocrine and behavioural paradigms[64,115,152]. Thirty min after the antagonist pretreatment, the animals were treated icv. with the dose of the EMs or MERF that had proved to be most effective in the previous protocol. Control mice received saline alone. Thirty min after the peptide administration, the animals were subjected to behavioural tests or were decapitated to obtain blood samples for the corticosterone assay.

2.4 *In vitro* experiments

2.4.1 The superfusion system

2.4.1.1 Investigation of the action of EM1 and MERF on the basal and stimulated dopamine release of striatal slices

The mice were decapitated, the brains were rapidly removed and the striata were dissected in a Petri dish filled with ice-cold Krebs solution. The dissected tissue was cut with a McIlwain tissue chopper and slices of 200-300 μm were produced. The slices were preincubated for 30 min in 5 ml of Krebs solution as an incubation medium, submerged in a water-bath at 37 °C and gassed through a single-use needle (30 G; 0.3 x 13) with a mixture of 5% CO_2 and 95% O_2; the pH was maintained at 7.4. The slices were labelled with (^{3}H)dopamine during the preincubation: the medium was supplemented with 0.15 mM (^{3}H) dopamine (spec. act. 14 Ci/mmol). The superfusion apparatus consisted of four cylindrical perspex chambers (Experimetria Ltd, Budapest, Hungary). The upper half was fitted with an inlet and the lower half with an outlet, and a circular piece of nylon net was placed just below the outlet and above the inlet. When fitted together, the halves enclosed a compartment of about 150 μl (5 mm long and 5 mm in diameter).Gold electrodes were attached to both the upper and the lower half-chamber and the electrodes were connected to an ST-02 stimulator (Experimetria Ltd, Budapest, Hungary). After preincubation, the labelled slices were transferred to the superfusion chambers and washed for 45 min, using a multichannel peristaltic pump (Gilson Minipuls 2), to allow tissue equilibrium and to remove the excess radioactivity from the labelled samples. Before transfer of the slices into the chambers, all the tubing and the lower half were filled with oxygenized superfusion medium, and during the washing-out phase care was taken that all air bubbles came out from the chambers. The chambers were superfused with Krebs buffer at a rate of 200 μl/min from a reservoir kept at 37 °C and gassed with a mixture of 5% CO_2 and 95 % O_2. In the antagonist studies with EM1, L-NNArg was added to the buffer during the initial washing-out period. Afterwards, the slices were superfused for 30 min and the superfusates were collected in tubes in 2-min fractions by means of a multichannel fraction collector (Gilson FC 203B). Chambers I and II were superfused with Krebs solution, and chambers III and IV with Krebs solution containing EM1 (10 μM) or MERF (1 or 5 μM). Two samples were taken as a baseline and electrical stimulation was then delivered to chambers II and IV for 2 min. The stimuli consisted of square-wave impulses (voltage: 25 V, pulse length: 25 ms, frequency: 2 Hz). At the end of the experiment, the slices were solubilized in 200 μl Krebs solution, using an ultrasonic homogenizer (Branson, Sonifier 250). The radioactivity in the fractions and the homogenized tissue samples was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard) after addition of the appropriate scintillation fluid (3 ml Ultima Gold). The fractional release was calculated as a percentage of the radioactivity present in the slices at the sample collection time.

2.4.2 The perifusion system

2.4.2.1 Investigation of the action of EM1 on the ACTH release of pituitary and corticosterone secretion of adrenal slices

We used the *in vitro* system described by Saffran and Schally[131] as a starting-point in developing our experimental design. Mice were sacrificed by decapitation and the adrenals and the pituitary were removed. The adrenals were cleaned from the adhering fat and capsule and the posterior lobe of the pituitary was dissected and discarded. The adrenals and the anterior lobe were weighed on a micro torsion balance and were immediately transferred to separate Petri dishes containing ice-cold, Krebs solution (113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, pH=7.4) as incubation medium. Both the adrenals and the pituitaries were rapidly cut with a McIlwain tissue chopper and slices of 200-300 μm were produced and placed in separate plastic flasks. The flasks, containing 5 ml incubation medium, were submerged in a water-bath at 37 °C and constantly and gently gassed through a single-use needle (30 G; 0.3 x 13) with a mixture of 5% CO_2 and 95% O_2; the pH was maintained at 7.4. The slices were preincubated for 1 hour, at the end of which the medium was sucked out and discarded. In 5 ml of fresh medium, 10 μM EM1 or the medium alone as a control was added. The samples were incubated for half an hour, after which 200 μl aliquots of the medium were transferred for ACTH or corticosterone determination. The ACTH concentrations of the samples were determined by RIA: ELSA-ACTH (CIS-bio International, France) is a solid-phase two-site immunoradiometric assay (ACTH radiolabelled with iodine 125). Corticosterone concentrations were determined by fluorescence assay.

2.5 Statistical analysis

Values are presented as means ± S.E.M. Statistical analysis of the results was performed by analysis of variance (ANOVA). For the perifusion experiments and to evaluate the dose-response curves of the opioid peptides one-way ANOVA was applied followed by Tukey's *post hoc* test for multiple comparisons with unequal cell size (Spjotvoll-Stoline), when test prerequisites were fulfilled. If the test of homogeneity of variances had not been passed nonparametric ANOVA on ranks (Kruskal-Wallis) was performed followed by Dunn's test for multiple comparison. Two-way ANOVA was applied for the antagonist studies and two-way ANOVA with repeated measures for the superfusion experiments to detect overall treatment effect and to evaluate treatment x treatment or time x treatment interactions. For

pairwise multiple comparisons Bonferroni's method was carried out. A probability level of 0.05 was accepted as indicating a statistically significant difference.

RESULTS

3. 1. The actions of the EMs

3.1.1. Effects of the EMs on behaviour and the HPA system

3.1.1.1 Effect of the EMs on open-field parameters

The administration of EM1 (0.25-5 μg) into the right lateral brain ventricle caused a significant increase in locomotor activity (Fig. 5). A dose of 0.5 μg increased the number of squares explored ($F(5, 58)$, $p<0.01$ vs. control) but the most effective dose was 1 μg ($p<0.001$ vs. the control). The higher doses of the peptide (2 and 5 μg) did not give rise to further increases in locomotion ($p=0.075$ and 0.08 vs. the control, respectively). The tetrapeptide dose (1 μg) that led to the most significant response in locomotor activity also elevated the number of rearings ($p<0.001$ vs. the control) (Fig. 5). As concerns grooming, EM1 elicited only a tendency to an increase, which did not prove to be statistically significant (Table 2).

Different doses of EM2 (0.25-1 μg) also caused significant changes in the behavioural tests (Fig. 6). A dose of 0.25 μg increased the number of squares explored ($F(3, 48) = 5.97$, $p<0.05$ vs. control), but the most effective dose was 0.5 μg ($p<0.01$ vs. the control). A higher dose of the peptide (1 μg) did not give rise to a further increase in locomotion ($p=0.28$ vs. the control). The tetrapeptide dose (0.25 μg) that led to a significant response in locomotor activity failed to elevate the number of rearings ($p=0.1$ vs. the control) (Fig. 6), but higher doses (0.5 and 1 μg) elicited significant increases in the number of rearings ($p<0.05$). In the case of grooming, no difference was observed between the control and the EM2-treated group (Table 3).

3.1.1.2 Effect of the EMs on basal corticosterone release

EM1 induced a significant increase in plasma corticosterone level. A dose of 5 μg elevated the corticosterone level by 157% as compared to the control ($F(3, 35)=4.48$, $p<0.05$) (Fig. 7).

EM2 in a dose of 0.25 μg elevated the corticosterone level by 142% as compared to the control, though this response was not statistically significant. ($F(3, 44)=4.03$, $p=0.2$ vs. the control). A higher dose (0.5 μg) of EM2 elevated the corticosterone level in a statistically significant

manner ($p<0.05$), but further elevation of the dose (1 μg) did not result in an additional increase ($p=0.99$ vs. the control) (Fig. 8).

3.1.1.3 Effect of the receptor antagonists (α-helical CRF_{9-41}, haloperidol and L-NNArg) on the behavioural responses evoked by the EMs

Preliminary administration of the CRH antagonist α-helical CRH_{9-41} (1 μg) completely abolished the increases in both locomotion ($F(3, 46)$, $p<0.001$ vs. EM1) and the number of rearings elicited by EM1 ($p<0.05$ vs. EM1) (Fig. 9).

The CRH antagonist pretreatment also diminished the EM2-induced locomotor response in a dose-dependent manner. The dose of 1 μg furnished only a tendency to attenuation, but a higher dose (2 μg) brought about a statistically significant inhibition ($F(5, 73)=8.6$; $p<0.01$ vs. EM2) (Fig. 10). The dose of 1 μg of α-helical CRH_{9-41} inhibited the rearing activity induced by EM2 in a significant manner ($p<0.05$ vs. EM2) and the higher dose of the CRH antagonist completely abolished the effect of EM2 ($p<0.001$ vs. EM2) (Fig. 10).

Haloperidol pretreatment inhibited both the square crossing ($p<0.05$ for haloperidol+EM1 vs. EM1) and rearing ($p<0.05$ for haloperidol+EM1 vs. EM1) brought about by EM1 ($F_{1, 32}=28.80$; $p<0.0001$ for square crossing and $F_{1, 32}=17.22$; $p<0.001$ for rearing vs. the control) (Fig. 11). L-NNArg also mitigated the behavioural ($p<0.05$ for square crossing and $p<0.05$ for rearing for L-NNArg+EM1 vs. EM1) effects of EM1 (Fig. 12).

3.1.1.4 Effect of the receptor antagonists (α-helical CRF_{9-41}, haloperidol and L-NNArg) on the endocrine responses evoked by the EMs

The corticosterone response induced by either EM1 (Fig. 13) or EM2 (Fig. 14) was also inhibited by pretreatment with α-helical CRH_{9-41} (($F(3, 24)=7.96$, $p<0.05$ vs. EM1), $F(3, 47) = 10.88$, $p < 0.01$ vs. EM2).

While L-NNArg significantly inhibited the corticosterone release evoked by EM1 ($p<0.05$ for L-NNArg+EM1 vs. EM1) (Fig. 15), the pretreatment with haloperidol proved to be completely ineffective (Table 4).

3.1.2 Effects of EM1 on the transmitter release of isolated tissue samples

3.1.2.1 Effect of EM1 on the corticosterone secretion of adrenal slices and the ACTH release of pituitary slices

The EM1 treatment did not have a statistically significant impact on the corticosterone secretion from the adrenal slices ($F_{1, 22}$=0.32; p=0.58 vs. control) and moderately inhibited the ACTH release from the pituitary slices ($F_{1, 6}$=21.2; p<0.01) (Fig. 16).

3.1.2.2 Effect of EM1 on the basal and stimulated dopamine release of striatal slices and the effect of L-NNArg on the EM evoked response

In the superfusion experiments, all channels displayed stable baseline with minimal leakage of dopamine (2-3 %/min) after the washing-out period, reflecting the viability of the slices. Electric impulse elicited an approximately 300 % increase in dopamine release. Although EM1 did not influence the basal release from the striatal slices, it considerably augmented the dopamine release evoked by electric impulses ($F_{3, 288}$=4.25; p<0.05 vs. control). L-NNArg alone did not have an impact on dopamine release but the effect of EM1 was significantly inhibited by L-NNArg pretreatment (p<0.05 for L-NNArg+EM1 vs. EM1) (Fig. 17).

3.2. The actions of MERF

3.2.1. Effects of MERF on behaviour and the HPA system

3.2.1.1 Effect of MERF on open-field parameters

MERF elicited an increase in square crossing at 1, 2 and 5 μg doses (ANOVA, $F_{4, 47}$ = 8.5, p<0.001; Tukey's *post hoc*, p<0.005, p<0.001 and p<0.0005, respectively) (Fig. 18). An increase in rearing activity was also brought about by MERF, although only the highest dose proved to be effective (ANOVA, $F_{4, 47}$ = 3.5, p<0.05; Tukey's *post hoc*, p<0.05) (Fig. 18). In contrast, MERF did not have a considerable impact on grooming (Table 5).

3.2.1.2 Effect of MERF on basal corticosterone release

MERF evoked an almost 100 % elevation of plasma corticosterone level at the dose of 5 μg (ANOVA on ranks, Kruskal-Wallis H=14.5; p<0.01; Dunn's pairwise comparison, p<0.05) (Fig. 19).

3.2.1.3 Effect of the receptor antagonists (naloxone, nor-binaltorphimine (nor-BNI), α-helical CRF_{9-41}, and haloperidol) on the behavioural responses evoked by MERF

Naloxone inhibited the MERF-induced increases in square crossing and rearing (Fig. 20) at the highest antagonist dose (two-way ANOVA, overall effect of antagonist treatment $F_{2, 80}$=4.9 for square crossing, $p<0.01$; treatment x treatment interaction $F_{2, 80}$=5.8 for square crossing and 6.7 for rearing, $p<0.005$; Bonferroni's pairwise comparison $p<0.05$ vs. MERF). On the other hand, nor-BNI exhibited only a tendency to attenuation of square crossing, which did not prove statistically significant and did not affect the number of rearings (Table 6).

Haloperidol produced a marked inhibition of the MERF-evoked increase in square crossing (two-way ANOVA, overall effect of haloperidol treatment $F_{1, 35}$=27.3, $p<0.001$; treatment x treatment interaction $F_{1, 35}$=14.0, $p<0.001$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 21). The MERF-induced rearing activity was also diminished by haloperidol pretreatment (two-way ANOVA, overall effect of haloperidol treatment $F_{1, 35}$=8.3, $p<0.01$; treatment x treatment interaction $F_{1, 35}$=4.8, $p<0.05$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 21).

The increase in square crossing was attenuated by the preadministration of the CRH antagonist (two-way ANOVA, overall effect of antagonist treatment $F_{1, 31}$=20.8, $p<0.001$; treatment x treatment interaction $F_{1, 31}$=4.3, $p<0.05$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 22). Applying the CRH antagonist similar inhibition was observed in the MERF–induced rearing response (two-way ANOVA, treatment x treatment interaction $F_{1, 31}$=6.8, $p<0.05$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 22).

3.2.1.4 Effect of the receptor antagonists (naloxone, nor-binaltorphimine (nor-BNI), α-helical CRF_{9-41}, and haloperidol) on the endocrine responses evoked by MERF

The HPA response evoked by MERF was almost completely abolished by both 0.4 and 4.0 μg naloxone (two-way ANOVA, overall effect of antagonist treatment $F_{3, 59}$=7.6, $p<0.005$; treatment x treatment interaction $F_{3, 59}$=5.9, $p<0.005$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 23). Also, a clear inhibition of MERF-induced HPA activation was observed with a dose of 10 μg of the κ-antagonist (two-way ANOVA, overall effect of antagonist treatment $F_{3, 84}$= 3.9, $p<0.05$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 24).

While the dopamine antagonist turned out to be ineffective on the HPA response elicited by MERF (Table 7), the HPA response evoked by MERF was completely abolished by the CRH antagonist (two-way ANOVA, overall effect of antagonist treatment $F_{1, 38}$=6.0, $p<0.05$; Bonferroni's test, $p<0.05$ vs. MERF) (Fig. 25).

3.2.2 Effect of MERF on the basal and stimulated dopamine release of striatal slices

The electric impulse elicited an approximately 300 % increase in dopamine release as compared with baseline (two-way ANOVA with repeated measures, overall effect of electric impulse $F_{1, 20}$=16.5, $p<0.001$; time x electric impulse interaction $F_{15, 300}$=24.5, $p<0.0001$). Neither dose of MERF had a significant impact on the spontaneous or the electric impulse-evoked dopamine release (Fig. 26).

4 Discussion

The present experiments clearly demonstrate that intracerebroventricularly (icv.) administered EMs and MERF lead to a marked activation of square crossing and rearing. At a molar basis MERF (2 μg ≈ 2 nmol) evoked a similar response as EM1 (1 μg ≈ 2 nmol). On the other hand EM2 evoked even more prominent behavioural actions than EM1 since considerably smaller concentrations (0.4-0.8 nmol) of EM2 elicited the analogous response.

Our findings are in agreement with those of earlier studies that showed opioids to have a pronounced impact on neuroendocrinological processes[113]. However, the results of behavioural experiments have been found to depend strongly on the strain[43], the sex[78] and the receptor preference of the substance[32,61,85]. Additionally, different opioids produce actions that differ in temporal course[60] and display a very strong dose-dependence[80,142]. Outside the neonatal period[62], the effects of the selective κ agonists, on both locomotion and rearing are inhibitory[61,80,156]. In contrast, morphine activates locomotion[8], possibly acting in the ventral tegmental area[68] and in the nucleus accumbens[55]. Previous studies have suggested that this process is presumably mediated by μ and δ receptors, since both selective μ and δ agonists induce locomotor hyperactivity[85]. Rearing is also enhanced by morphine[72]; μ and δ mediation also play important roles in this phenomenon[32]. Our results, when the receptor specificity of the EMs is taken into consideration, indicate that opioids mainly stimulate locomotion and rearing through the μ-opioid receptor.

Both EMs exhibited a bell-shaped dose-response curve with a downturn phase at higher doses, and the effective concentration range proved to be narrow. Such a phenomenon

has already been described with regard to the effects of other neuropeptides[104,152], the EMs[163] and their derivatives[78]. This feature may reflect functional antagonism in post-receptorial signal transduction[120] such as receptor phosphorylation by a G protein-coupled receptor kinase[42]. The inhibitory action of less-specific receptors[57] at higher concentrations or the postsynaptic down-regulation of the receptor synthesis[7] also might be taken into consideration.

Recently it has been established that, after the formalin test, morphine brought about locomotor hyperactivity exhibiting a downturn phase at the highest dose[142], whereas the EMs did not. Since the doses (1-10 μg) of the EMs tested in the aforementioned study[142] were almost one order of magnitude higher than the effective concentrations in our experiments (0,25-1 μg) and the EMs possess a conspicuously narrow bell-shaped dose-response curve, revealing that in the case of EM2 the response evoked by the dose of 1 μg was not different from the control, our data appear to be consistent at the doses tested with the results of Soignier et al.[142].

Our behavioural findings with MERF corroborates the results of earlier histological studies. As previous publications revealed abundant expression of MERF in the striatum of different species[119], and its marked release from striatal slices[116] our findings suggest that the heptapeptide might function as an endogenous opioid regulator of locomotive behaviour. This hypothesis is in line with previous histological data demonstrating enkephalinergic projections to form prominent subsets of both the striatonigral and striatopallidal pathways[44]. Both behavioural phenomena were diminished dose-dependently by the nonselective opioid antagonist naloxone suggesting, that at least in these processes, non-opioid binding does not play a significant role. On the other hand, nor-BNI did not have a considerable impact on the behavioural responses elicited by MERF, and it is worth-mentioning that nor-BNI alone displayed a tendency to activate the motor parameters. It implies that κ–mediation does not play a significant role in the behavioural actions of MERF, and together with the results of Kuzmin et al.[80], assigns a tonic inhibitory function to the κ receptors in the regulation of motor parameters.

Neither the EMs nor MERF elicited a significant alteration in grooming in the doses that brought about marked changes in both locomotion and rearing. Opiates seems to exert quite ambiguous effects on grooming, depending on the dose and the testing schedule[60,117]. Despite the contradictory nature of the available data, it appears that in the setting of acute administration, grooming is activated through the δ receptors[9,157]. Since the EMs in the given

dose-range prefers the μ subtype of the opioid receptor family[133,134], our data support the hypothesis that μ–opioid mediation does not play a significant role in the mediation of grooming. Although, previous experiments revealed[78], that topical administration of an enzyme-resistant analogue, D-Pro^2-EM-2 in a dose of 50 μg into the ventrolateral periaqueductal gray, evoked a sex-dependent activation of grooming in the rat. This difference can be attributed not only to the structural, but also to species differences, or may reflect a pharmacological rather than a physiological action of this endomorphin derivative. Further, as circumstantial evidence, our data reinforce the hypothesis proposed by *in vitro* studies[6,10], that inspite of being an enkephalin derivative, MERF behaves as a κ or μ rather than a δ–agonist.

Centrally administered EMs and MERF also stimulated corticosterone secretion in our experiments. This is in harmony with previous studies revealing a prominent but species-specific action of morphine and other opiates on the HPA axis[113]. Their action, in rodents, is predominantly stimulatory[18-20], although reflects species and age-related differences[19,20,29,46,66,83,95,148,]. Similar species-related differences have been observed regarding the action of opiates on locomotive behaviour[79] and food intake[54], which might be related to differences between rats and mice in the expression and function of the opioid receptors[165].

Earlier studies also pointed out that the opioid system and the stress response are strongly interwoven in another respect, too. It is the opioid peptides that mediate the decreased pain responsiveness upon stress. Cold-swim stress[13], food deprivation[94] and footshock[1] all produce naloxone reversible analgesia. Such a relief was demonstrated to depend on the release of such endogenous opioids as β-endorphin and the enkephalins[2,129]. The two phenomena might form a reinforcing positive feed-back loop at the beginning of the stress reaction that, increasing pain-threshold, helps the individual to cope with the stressful condition.

Both naloxone and nor-BNI pretreatment inhibited the MERF evoked HPA activation. Present findings are consistent with those of previous studies[18,23,112,151] indicating that κ and μ–receptor mediation activates the HPA system. Since the hypothalamus displays high expression of MERF[119], our data support the hypothesis that this endogenous μ and κ_2-opioid ligand might function as a physiological regulator of stress response, and are in agreement with previous data, which demonstrated the selective κ-agonist MR 2034 to stimulate CRH secretion[111].

On the other hand, in the perifusion experiments EM1 did not influence the corticosterone secretion from the adrenal slices and slightly inhibited the ACTH secretion from the pituitary slices, which argues for the central action of the EMs-evoked HPA activation. The hypothalamic site of action of the opioids is also suggested by literature data. They do not activate the corticotrop cells in the pituitary[164], fail to elicit direct adrenocortical activation *in vivo*[34] and have rather an inhibitory impact on the corticosterone release from dispersed adrenal cells[50]. Morphine has also been demonstrated to act on the hypothalamus increasing its CRH content *in vivo*[147] and to activate the CRH release from isolated hypothalami[17]. These findings are in agreement with the antagonist studies: in our experiments α-helical CRH_{9-41} preatreatment completely abolished the endomorphin evoked corticosterone release[19,20] and previous publications revealed that the HPA activation elicited by opioid peptides could be blocked by CRH antiserum preatreatment[111]. Consequently, the effect of the EMS on the HPA axis is likely to be mediated through the action of CRH, and other neuropeptides such as vasopressin[12], neurotensin[110] or thyrotropin-releasing hormone[137] do not seem to play a relevant role in the HPA response to the EMs. This finding is supported by previous data[35] that demonstrated EM1 to inhibit the vasopressin and oxytocin neurones in the hypothalamus. The CRH antagonist also significantly attenuated the corticosterone release brought about by MERF, which results argue for the central action of the heptapeptide, too. However, as complete inhibition was not observed, the involvement of other activators of ACTH secretion (vasopressin and neuropeptide Y) cannot be excluded in the action of MERF on the HPA axis. Taking the distribution[93,116,119] and receptor specificity[6,10,47,76,167] of the EMs and MERF into account our results suggest that they might act as endogenous opioid regulators of the HPA axis.

Not only the endocrinological but also the behavioural responses evoked by the the opioid peptides could be completely blocked by α-helical CRH_{9-41} preatreatment. Since increase in locomotion may reflect not only exploration but also fear, our results might demonstrate that the opioid-induced anxiety (mediated by CRH release) can evoke the increase in locomotion and acts a stress paradigm, too. However, in our experiments the differences between the effective opioid doses on behaviour (0.25-1 μg) and on plasma corticosterone (0.5-5 μg) might suggest that different neural mechanisms might be involved in these actions, despite the fact that both effects can be attenuated or blocked by the CRH antagonist. Indeed, several publications showed that CRH evokes a characteristic locomotor activating response through its action on the PVN[96,102] and the limbic system[81]. The

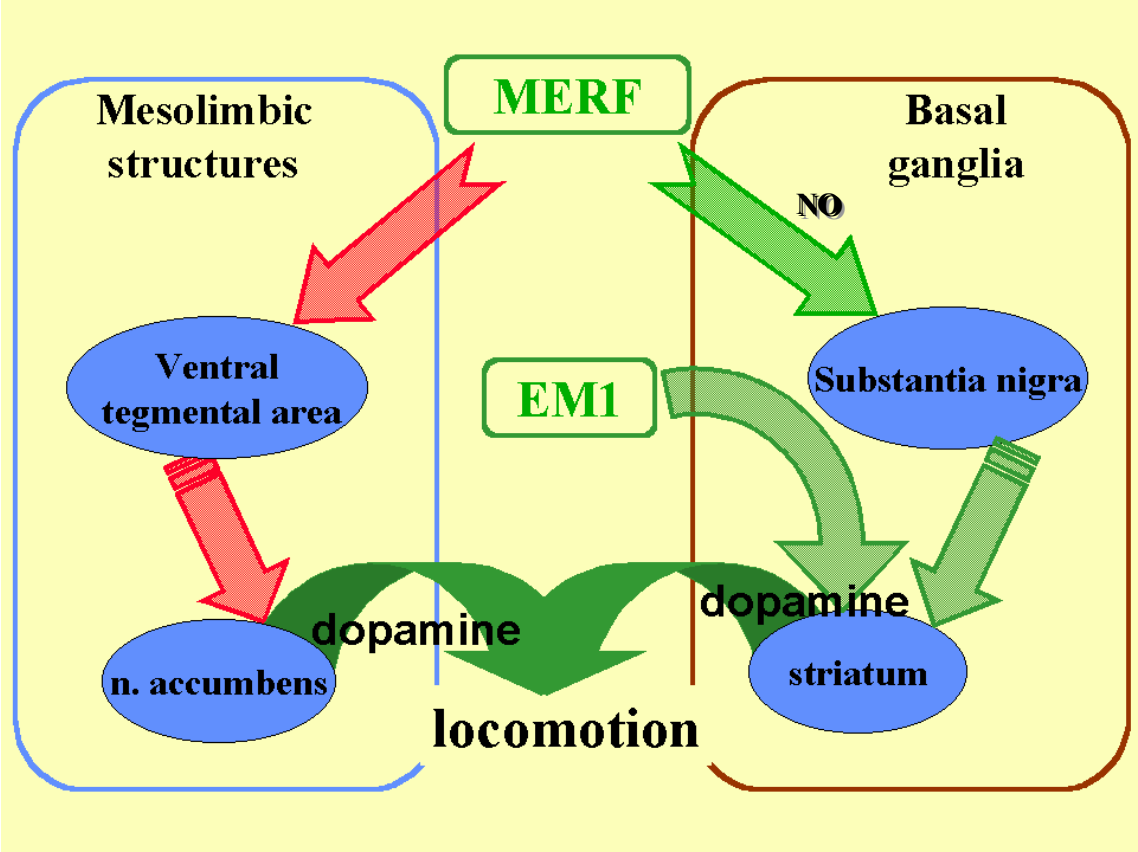
behavioural actions of CRH appear to be independent of its effect on the pituitary-adrenal axis as demonstrated by both indirect[15] and direct[38] evidence: neither hypophysectomy[38] nor dexamethasone blockade[15] of the HPA axis influences the locomotor response evoked by CRH. The opioids presumably induce CRH release at a hypothalamic level and this action can be inhibited by the antagonist at a pituitary level. On the other hand the behavioural effects of them may also be elicited in extrahypothalamic structures. Nevertheless the behavioural response also appears to be mediated by CRH, as it could be inhibited by the CRH antagonist.

The EM2-evoked activation of locomotion, rearing and corticosterone release is quite similar to that of EM1[19]. However, some differences are worth mentioning. The concentrations of EM2 that evoked the analogous response were lower than those of EM1 in all studies. Moreover, while the actions of EM1 could be completely antagonized by 1 μg CRH antagonist[19], twofold concentration was needed to abolish the EM2-evoked responses. Similar differences in effectiveness have been reported by other authors. EM1 seems to exert more profound effects in the tail-flick and hot-plate responses[154], while EM2 appears to be more effective in the formalin test[142] and its cardiorespiratory effects[33] are more pronounced than those of EM1. Histological studies have revealed that their distributional patterns in the CNS differ too[93]: EM1-like immunoreactivity is more widely and densely distributed in the brain, whereas EM2 is more prominent in the spinal cord. The dyscrepancy in their physiological actions can be explained by the putative differences in the signal transduction of EMs. EM1 and EM2 apparently activate different subtypes of the μ-opioid receptors: the actions of EM2 seem to be transmitted by the μ_1 or the heroin/morphine-6βeta-glucuronide subtype of the μ opioid receptor, while the effects of EM1 appear to be confined some isoforms of the μ_2 receptor[108,133,134]; neither of them interact with the μ_3 subtype. The G-protein profiles activated by EM1 or EM2 also appear to differ[135]: the analgesic response evoked by both EM1 and EM2 could be inhibited by the impairment of proteins $G_{i1\alpha}$ and $G_{i3\alpha}$, while the impairment of protein $G_{i2\alpha}$ blocked only the action of EM2. Moreover, there is strong evidence that EM1 activates only the μ-opioid receptors, whereas after initial μ-opioid activation, EM2 may elicit the release of Met-enkephalin and/or dynorphin[154].

Haloperidol pretreatment completely abolished the EM- or MERF-induced increase in both square crossing and the number of rearings suggesting their behavioural actions are mediated through dopaminergic transmission. The present finding corroborates the results of previous studies demonstrating the dopamine-dependent motor actions of opiates[68], and the crucial and species-specific[79] role of the ventral and dorsal striatum in relaying the action of

opiates on locomotion[169]. Further, by *in vitro* studies, the locomotor action of opiates has been demonstrated to depend on the dopamine concentration of the mesolimbic and nigrostriatal structures[89]. However, the apparent dyscrepancies between the effects of the EMs or MERF on the striatal dopamine release suggest that their *in vivo* actions might imply different molecular mechanisms. While EM1 appear to facilitate dopamine release or alternatively decrease dopamine reuptake, the negative results of the superfusion studies with MERF cast doubt on its direct action on the dopaminergic terminals in the striatum; such finding can be explained by the differences in the receptor specificity of EM1[108,133,134] and MERF[6,10,76]. EM1 might act presynaptically on the axons of the nigrostriatal pathway, while the MERF positive neurons may belong to the opiatergic projections of the striatonigral and striatopallidal pathways[44], and they regulate dopaminergic transmission in the substantia nigra[52] rather than in the caudate-putamen. Besides, compelling evidence is emerging, that MERF may also stimulate another monoaminergic circuitry, the mesolimbic dopaminergic cells, through the inhibition of GABAergic[67,82] and serotonergic[121] transmission[37], or through the activation of CRH release[19,20] (Fig. 27.). Nonetheless, it is worth-mentioning that MERF is more susceptible to the action of inactivating peptidases[10] than the EMs[153], which might also

Fig. 27. The role of EM1 and MERF in the control of the subcortical motor system



explain the conspicous difference in the effectiveness of these opioids. Therefore, further experiments with the help of peptidase inhibitors might help to shed light on the direct action of MERF on nigrostriatal axon terminals.

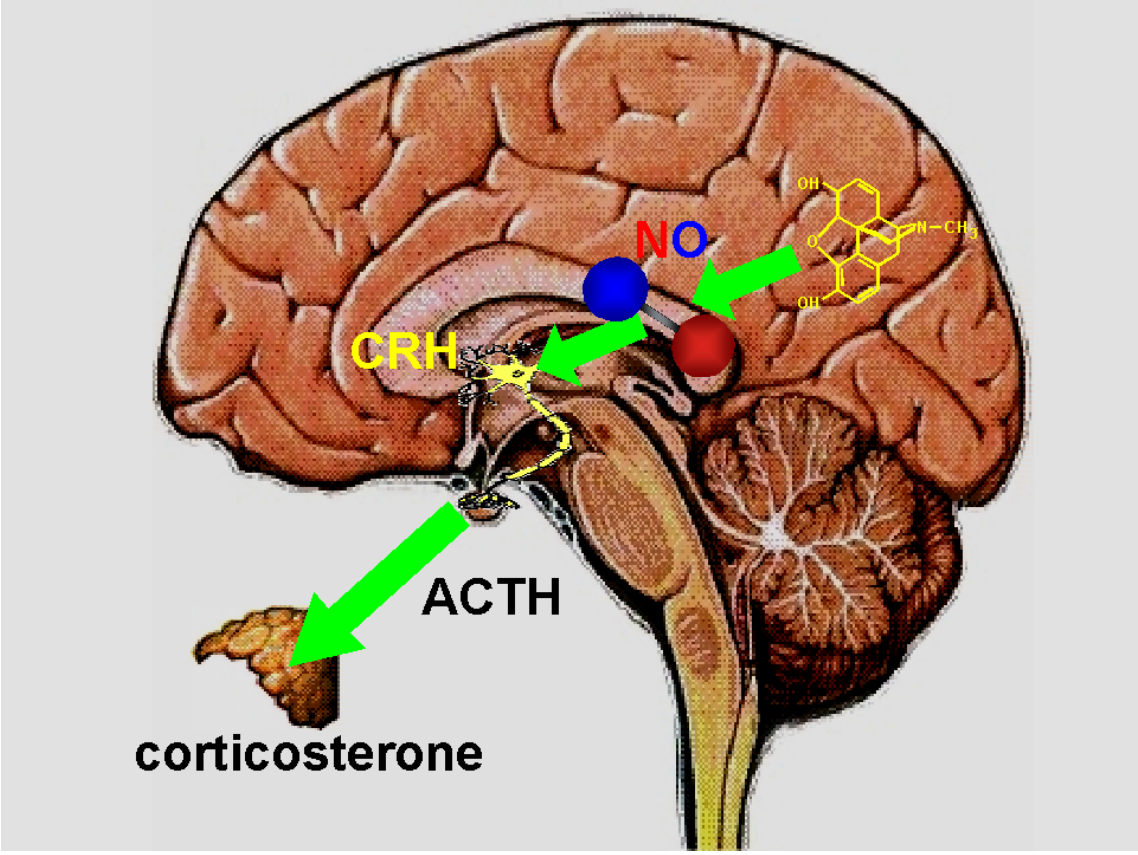
Although conflicting data are available regarding the role of dopaminergic mediation in the HPA response[4,65], the histological evidence suggests an interaction between the opioid and the dopaminergic neurons in the PVN[84]. However, in our experiments, the HPA activation evoked by neither the EMs nor MERF was inhibited by haloperidol pretreatment, which lends support to the view resulting from previous data[92] that the actions of opiates on the HPA system do not involve dopaminergic transmission[92].

Both the behavioural and endocrine responses elicited by EM1 proved to be especially sensitive to L-NNArg pretreatment. Further, L-NNArg successfully inhibited the facilitatory effect of EM1 on the stimulated dopamine release in the superfusion studies. These data are in harmony with the findings of previous authors, clearly establishing that NO and/or the glutamate-NO-cGMP system[114] mediates numerous actions of opiates[45]. Further, our data also broaden the concept of NO mediation in the action of the EMs. It appears not only that the cardiovascular actions of the EMs are transmitted by NO[24], but also that a general role can be

assigned to NO in the mediation of the actions of these opioid tetrapeptides. Moreover, the opioid-NOS system might represent the physiological source of those NO which has been established to stimulate locomotion[22] and striatal *c-Fos* expression[53].

Our results suggest that L-NNArg plays neither an activatory nor an inhibitory role in the basal secretion of corticosterone, which result is in harmony with numerous *in vitro*[71] and *in vivo* data[128]. On the other hand, it proved to inhibit the corticosterone release elicited by EM1. Hence, the present findings in agreement with previous publications, assign a critical role to NO in the mediation of HPA activation. Literature data point to an intricate mechanism, which governs the release of NO in the stress response, which seems strongly dependent on the nature of the stressor. Different neural pathways and mediator profiles

Fig 28. The mediation of the action of EM1 on the HPA axis



belonging to various stress stimuli appear to have strikingly different impacts on the release of this gaseous transmitter. NO proved to stimulate the stress response to physico-emotional stimuli[155], while it displayed a robust inhibition of the HPA activation elicited by immune signals[127]. When our results are taken into consideration, it can be hypothesized that the EMs

and/or other endogenous opioids released by physical stressors, such as electric foot-shocks[107], might activate the hypothalamic NOS[155], which leads to the subsequent activation of the HPA system. This idea is strongly supported by literature data demonstrating the stimulatory action of the NO-cGMP system on hypothalamic CRH release[71] (Fig. 28).

In conclusion, present data signify that both the EMs and MERF might function as a physiological regulator of the HPA axis and behaviour. Our findings confirm the hypothesis that the μ and κ-receptors may relay the actions of MERF but argues against the role of non-opioid mediation in the actions of the heptapeptide. CRH proved to be an important mediator of both the endocrine and behavioural actions of the peptides, and the experiments with haloperidol strengthen the hypothesis that CRH release might lead to the subsequent activation of the mesolimbic dopaminergic structures. This hypothesis is corroborated by previous studies demonstrating that the locomotor activating properties of CRH strongly depend on the dopamine metabolism in the nucleus accumbens[69]. However, on the basis of the *in vitro* data only the EMs appear to act directly on the striatal dopamine release, and according to the results of the endocrinological studies, the HPA activation evoked by the opioids does not involve dopaminergic transmission. Our results also raise the possibility that the EM-NO-CRH system might be one of the prominent stimulators of the HPA axis, and a similar neurotransmitter cascade might be involved in the stress-related motor activation elicited by the EMs.

5 SUMMARY

In the present experiments, the role of the EMs and MERF in the control of open-field paradigms and the HPA response has been established. The main findings of the presented studies:

1. Both the EMs and MERF after icv. administration elicited remarkable horizontal and vertical responses in the open-field system. Their action was dose-dependent and in the case of the EMs the dose-response curve displayed a characteristic bell-shape.
2. Similarly, these opioids activated the HPA axis in a dose-dependent manner and also, at a molar basis, like in the behavioural experiments, EM2 proved to be more effective than EM1 and MERF.
3. Naloxone and nor-BNI pretreatment significantly and dose-dependently attenuated the endocrine response evoked by MERF while in the behavioural experiements only naloxone proved to be effective. These data point to the importance of κ-mediation in the MERF evoked HPA activation, while μ–δ-mediation appear to prevail in the behavioural responses.
4. The previous icv. application of the CRH antagonist α-helical CRH_{9-41} inhibited both the behavioural and the HPA response induced by the opioid peptides. As regards EM1 these data with the findings of the perifusion experiments argues for the central site of action of the EMs.
5. Haloperidol pretreatment completely abolished the behavioural responses evoked by MERF or EM1 but proved to be ineffective on the endocrine responses elicited by the opioids.
6. Both the *in vivo* and *in vivo* effects of EM1 could be inhibited by L-NNArg pretreatment, which findings support the view that NO might mediate the actions of the EMs and other opiates.

We hope that our data have provided further evidence for the importance of opioid neuropeptides in the complex and flexible regulation of the HPA axis and behaviour. Our findings revealed the receptor preference of MERF *in vivo* and outlined the spectra of mediators involved in the transmission of the neuroendocrine actions of MERF and the EMs.

In a further set of experiements we intend to broaden the scope of our studies and try to clarifiy the action of these opiates and their analogues (like Tyr-D-Ala-Gly-Phe-D-Nle-Arg-Phe of MERF) on the regulation of further behavioural (spontaneous locomotion) and autonomic processes (thermoregulation, circulation) in a telemetric system.

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