Endomorphins and activation of the hypothalamo–pituitary–adrenal axis

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Abstract

Endomorphin (EM)-1 and EM-2 are opioid tetrapeptides recently located in the central nervous system and immune tissues with high selectivity and affinity for the μ -opioid receptor. Intracerebroventricular (i.c.v.) administration of morphine stimulates the hypothalamo–pituitary–adrenal (HPA) axis. The present study investigated the effect of centrally administered EM-1 and EM-2 on HPA axis activation. Rats received a single i.c.v. injection of either EM-1 (0.1, 1.0, 10 μ g), EM-2 (10 μ g), morphine (10 μ g), or vehicle (0.9% saline). Blood samples for plasma corticosterone determinations were taken immediately prior to i.c.v. administration and at various time points up to 4 h post-injection. Trunk blood, brains and pituitaries were collected at 4 h. Intracerebroventricular morphine increased plasma corticosterone levels within 30 min, whereas EM-1 and EM-2 were without effect. In addition, pre-treatment of i.c.v. EM-1 did not block the rise in corticosterone after morphine. Corticotrophin-releasing factor (CRF) mRNA and arginine vasopressin (AVP) mRNA in the paraventricular nucleus (PVN) and POMC mRNA in the anterior pituitary were found to be

unaffected by either morphine or endomorphins. Since release of other opioids are elevated in response to acute stress, we exposed rats to a range of stressors to determine whether plasma EM-1 and EM-2 can be stimulated by HPA axis activation. Plasma corticosterone, ACTH and β -endorphin were elevated following acute restraint stress, but concentrations of plasma EM-1-immunoreactivity (ir) and EM-2-ir did not change significantly. Corticosterone, ACTH and β -endorphin were further elevated in adjuvant-induced arthritis (AA) rats by a single injection of lipopolysaccharide (LPS), but not by restraint stress. In conclusion, neither EM-1 or EM-2 appear to influence the regulation of the HPA axis. These data suggest that endomorphins may be acting on a different subset of the μ -opioid receptor than morphine. The failure to induce changes in plasma EM-ir in response to the chronic inflammatory stress of AA, the acute immunological stress of LPS, or the psychological stress of restraint, argues against an important role for endomorphins in mediating HPA axis activity.

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Introduction

Endomorphins (EM)-1 and EM-2 are endogenous opioid tetrapeptides that have high specificity and affinity for the μ -opioid receptor (Zadina *et al.* 1997). The presence of EM-1 and EM-2 has been detected in various brain regions including the thalamus, hypothalamus, cortex and striatum. EM-2 fibres and terminals exhibit discrete distribution within the CNS in some but not all areas containing dense μ -opioid receptors, including the nucleus accumbens, locus coeruleus, periaqueductal grey and spinal cord dorsal horn (Schreff *et al.* 1998). Recently, EM-1 and EM-2 have been detected in the immune system of the rat (Jessop *et al.* 2000).

Studies in mice have shown that intracerebroventricular (i.c.v.) administration of EM-1 and EM-2 produced potent antinociception with similar potency to morphine.

This analgesic effect was blocked by pre-treatment with the selective μ -opioid receptor antagonists naloxonazine or β -funaltrexamine (Stone *et al.* 1997, Zadina *et al.* 1997, Goldberg *et al.* 1998) which suggests that the antinociceptive properties of the endomorphins exert their physiological effect specifically via the μ -opioid receptor. A similar effect has more recently been demonstrated in rats; i.c.v. injection of EM-1 produced potent spinal analgesia as measured by the tail flick test in a dose-dependent manner (Wang *et al.* 1999). In addition, intrathecal EM-1 and EM-2 caused short-lasting antinociception on tail-flick and carrageenin-induced thermal hyperalgesia (Horvath *et al.* 1999). Morphine is generally considered as an agonist for the μ -opioid receptor and has been shown to exert its effects via the μ -opioid receptor (Matthes *et al.* 1996).

Activation of the hypothalamo–pituitary–adrenal (HPA) axis by many different stimuli (stress, immune

challenge, etc.) causes increased secretion of corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) from the median eminence of the hypothalamus and these neuropeptides in turn stimulate the secretion of adrenocorticotrophin (ACTH) from the anterior lobe of the pituitary. Elevated levels of circulating ACTH stimulate production and release of glucocorticoids from the adrenal glands into the circulation, which then exert negative feedback actions at the pituitary and other target areas in the central nervous system, thus regulating HPA axis activation (Harbuz *et al.* 1997).

Opiates are believed to have an important role in the control of the HPA axis. In the rat, acute administration of morphine caused an increase in ACTH, β -endorphin and corticosterone levels in plasma of normal rats (Ignar & Kuhn 1990), and ACTH and corticosterone in animals with adjuvant-induced arthritis (Zubelewicz *et al.* 1999). To date, no studies have examined whether endomorphins affect HPA axis activation. Therefore, the present study consisted of a series of experiments that aimed to investigate the effect of centrally administered EM-1 and EM-2 on HPA axis function in healthy rats. This was achieved by measuring plasma corticosterone in samples taken at regular intervals up to 4 h after i.c.v. injection. Also, pro-opiomelanocortin (POMC) mRNA levels in the anterior pituitary and CRF mRNA and AVP mRNA levels in the paraventricular nucleus (PVN) of the hypothalamus were determined. The release of other opioids such as β -endorphin (Millan *et al.* 1981, Owens & Smith 1987) and met-enkephalin (Boarder *et al.* 1982) are elevated in response to acute stress. Adjuvant-induced arthritis (AA) rats are unable to mount either an ACTH or corticosterone response to acute stress although the response to acute lipopolysaccharide (LPS) and acute morphine remains intact (Harbuz *et al.* 1992, 1999, Zubelewicz *et al.* 1999). We exposed rats to restraint stress to determine whether plasma ACTH, corticosterone, β -endorphin, EM-1 and EM-2 can be stimulated by activation of the HPA axis in control and AA rats, together with EM-1 in control rats.

Materials and Methods

Subjects

All studies were carried out in full accordance with UK Home Office guidelines. All rats (Bantin and Kingman) were kept under heat and humidity controlled conditions in a 12 h light : 12 h darkness cycle (lights on 0700 h), supplied with food and water and allowed to feed *ad libitum*.

Endomorphin i.c.v. experiments

Adult male Sprague–Dawley rats (200–250 g) were housed four per cage and were allowed to acclimatize for

1 week prior to surgery. After surgery for implantation of i.c.v. guide cannulae and i.v. lines, rats were individually housed for the duration of the experiment. On the day of the experiment the cage lid was removed, the i.v. line attached and a basal blood sample withdrawn. The line was flushed through with 0.1% heparin saline and a small quantity injected. The line remained attached. The i.c.v. cannula was then inserted and the i.c.v. injection made over 1–2 min and the cannula left in place for a further minute prior to removal. Following i.c.v. injection the cage lid was replaced such that further blood could be removed remotely.

Stress studies

Adult male Wistar rats (250 ± 20 g) were used for the restraint stress experiment. In addition, adult male Piebald–Viral–Glaxo (PVG) rats (250 ± 20 g) were given an intradermal injection (0.1 ml) of a suspension of ground, heat-killed *Mycobacterium butyricum* (Difco Laboratories, East Mosely, Surrey, UK) in paraffin oil (10 mg/ml) at the tail base for the induction of arthritis (Harbuz *et al.* 1992). Control rats were injected with vehicle alone.

Drugs

Endomorphin-1 and EM-2 (Neosystem, Strasbourg, France) were dissolved on the morning of the experiment in micropore filtered 0.9% sterile saline (with 0.1% ascorbic acid for experiment 3). Morphine sulphate was diluted in filtered 0.9% sterile saline. Vehicle was 0.9% filtered sterile saline (plus 0.1% ascorbic acid for experiment 3). Drugs were kept on ice throughout the experiment. Lipopolysaccharide (LPS; *E.coli*, serotype 055:B5), in experiment 4, was made up to 250 μ g/0.5 ml in 0.9% sterile saline and injected i.p.

Surgery

Seven days after arrival into the animal facility, rats were anaesthetized with 1–1.5 ml sodium pentobarbital (Sagatal) (12 mg/ml) and placed into a stereotaxic frame for insertion of an i.c.v. guide cannula into the lateral ventricle which was capped to maintain patency. Following i.c.v. cannulation, the right jugular vein was catheterized for intravenous removal of blood using 0.5 mm fine bore polythene tubing (Portex Ltd, Hythe, Kent, UK). All rats were allowed to recover from surgery for 3 days before any experimental manipulation. Patency of the venous catheter was checked daily and a small volume of 0.1% heparin was flushed through the intravenous lines. Caps from the i.c.v. guide cannulae were removed the day before the experiment.

Experiment 1: the effect of EM-1 on HPA activity

A 400 µl volume of blood was withdrawn from each rat by connecting a length of fine bore tubing to the venous catheter. Blood was immediately stored on wet ice prior to centrifugation. The tubing remained attached to the animal so that further blood could be taken at 30 and 60 min after i.c.v. injection. Rats were randomly divided into four groups and received an i.c.v. injection of either vehicle, 0.1, 1.0 or 10.0 µg/4 µl of EM-1 at time 0. Drugs were administered through a cannula inserted into the guide cannula using a 10 µl volume Hamilton syringe over a period of 1–2 min. The cannula was left in place for approximately 1 min following drug administration. At 240 min, animals were killed by decapitation, trunk blood collected and centrifuged to separate plasma. Plasma was then measured for corticosterone levels at each time point by radioimmunoassay. Brains and pituitaries were removed, immediately frozen on dry ice and stored at –80 °C prior to determination of CRF and AVP mRNAs in the parvocellular part of the PVN and POMC mRNA in the anterior pituitary using *in situ* hybridization.

Experiment 2: the effect of EM-2 on HPA activity including morphine as a positive control

The protocol was similar to experiment 1. Rats received an i.c.v. injection of either vehicle, morphine sulphate (10 µg/4 µl) or EM-2 (10 µg/4 µl). Blood was taken immediately prior to i.c.v. injection and 30, 60 and 120 min after i.c.v. injection. At 240 min, rats were decapitated and trunk blood, brains and pituitaries collected as above.

Experiment 3: the effect of any interaction between EM-1 and morphine on HPA activity

Rats were randomly assigned to three treatment groups and received two i.c.v. injections of either saline+saline, saline+morphine or EM-1+morphine. At time 0 rats received their first i.c.v. injection of saline or EM-1, followed by the second i.c.v. injection of saline or morphine 30 min later. Rats were killed 90 min after the first injection and trunk blood collected for measurement of corticosterone levels in plasma.

Experiment 4: the effect of restraint stress on endomorphin levels

A One group of Wistar rats ($n=8$) was exposed to restraint stress (introduced into a plastic tube in which they remained for 10 min), and control rats ($n=8$) were not restrained. Rats were decapitated immediately following restraint and trunk blood collected. Corticosterone

levels were measured directly in plasma, EM-1, EM-2, ACTH and β-endorphin levels were measured in plasma following Sep-Pak extraction.

B Following the development of hind-paw inflammation at day 14, PVG rats ($n=5-6$) were exposed to restraint for 30 min and killed or received an intraperitoneal injection of lipopolysaccharide (LPS; 250 µg/0.5 ml; *E. coli*, serotype 055:B5) and killed 90 min after injection. These times were chosen as representing peak times of ACTH and corticosterone concentrations after the respective challenges. Plasma concentrations of corticosterone, EM-2, ACTH and β-endorphin were determined as above.

In situ hybridization

In situ hybridization (ISH) was performed using 48-mer oligonucleotides complementary to part of the exonic mRNA sequence coding for AVP, CRF or POMC (Perkin Elmer, Warrington, Cheshire, UK), as previously described (Young *et al.* 1986, Harbuz & Lightman 1989). Briefly, 12 µm-thick sections were taken on a cryostat through the pituitary and the paraventricular nucleus of the hypothalamus of the brain and thaw mounted onto gelatin-coated slides, dried and stored at –80 °C prior to ISH. On the day of the assay, sections were transferred to room temperature, air-dried and fixed in 4% formaldehyde for 5 min, washed twice in 1 × PBS and transferred into 0.25% acetic anhydride in 0.1 M triethanolamine–0.9% saline for 10 min. Sections were then dehydrated through graded ethanol washes, delipidated in chloroform for 5 min and washed in ethanol. All control and experimental sections for each experiment were hybridized in the same hybridization reaction. Probes were labelled with ³⁵S-dATP using terminal deoxynucleotidyl transferase (Boehringer Mannheim, Mannheim, Germany) to a specific activity of approximately 2×10^{18} d.p.m./mol for each of the three probes. A 45 µl volume of hybridization buffer containing labelled probe was added to the slides and coverslipped. After hybridization overnight in a humid chamber at 37 °C with 10^5 c.p.m./slide, coverslips were removed and slides washed four times at 55 °C in 1 × SSC, twice at room temperature in 1 × SSC and after two brief dips in distilled water, to remove salts, they were dried and exposed to Amersham HyperfilmMP (Amersham International, Amersham, Bucks, UK) together with ¹⁴C standards for 2 days for AVP and POMC and 14 days for CRF. For AVP the autoradiographic images of probe bound to the medial parvocellular region of the PVN were analysed according to the method of Kinoshita *et al.* (2000). Corticotrophin-releasing factor mRNA in the parvocellular PVN and POMC mRNA in the anterior pituitary (excluding neurointermediate lobe) were analysed using Image 1.22 software developed by Wayne Rasband (NIH, Bethesda, MD, USA) and run on an Apple Macintosh computer.

Table 1 Plasma corticosterone levels (ng/ml) in rats immediately prior to (pre-i.c.v.) or 30, 60 and 240 min following i.c.v. injection of vehicle or EM-1 (0.1, 1.0 or 10.0 µg/4 µl). Values are expressed as mean ± S.E. (n=7–9)

	Pre-i.c.v.	30 min	60 min	240 min
Group				
Vehicle	156.8 ± 50.2	385.0 ± 119.8	115.2 ± 48.5	338.0 ± 77.2
0.1	135.1 ± 26.1	264.3 ± 39.3	223.3 ± 110.0	274.4 ± 82.7
1.0	231.0 ± 77.8	184.2 ± 39.0	284.3 ± 56.4	143.5 ± 69.1
10.0	118.6 ± 28.3	319.0 ± 83.7	156.0 ± 78.1	186.2 ± 102.4

Radioimmunoassays (RIAs)

Total plasma corticosterone was measured directly in plasma (10 µl diluted in 500 µl buffer) using antiserum kindly supplied by G Makara (Institute of Experimental Medicine, Budapest, Hungary). The tracer was ¹²⁵I-corticosterone (ICN Biomedicals, Irvine, CA, USA) with a specific activity of 2–3 mCi/µg. The assay limit of detection was 5 ng/ml.

Plasma samples (200 µl) for EM-1, EM-2, ACTH and β-endorphin were extracted on Sep-Pak cartridges with 1-propanol (35%) acidified with formic acid (1%), dried and reconstituted in phosphate buffer for RIA. Radioimmunoassays for EM-1 and EM-2 have been fully described elsewhere (Jessop *et al.* 2000). Antisera against C-terminally amidated EM-1 and EM-2 were raised in rabbits and supplied by Advanced Chemtech (Louisville, KY, USA). Cross-reactivity of EM-1 antiserum with synthetic EM-2 was 0.5% and cross-reactivity of EM-2 antiserum with synthetic EM-1 was 0.01%. Neither antiserum cross-reacted with synthetic opioid peptides β-endorphin, dynorphin A, methionine enkephalin or orphanin FQ. The assay limit of detection for both EM-1 and EM-2 was 10 pg/ml. In-house RIAs were used to measure ACTH (Jessop *et al.* 1989) and β-endorphin (Jessop *et al.* 1994). The assay limit of detection was 10 pg/ml for both ACTH and β-endorphin.

Experiment 5: HPLC and endomorphin immunoreactivity

A pool of plasma (3.2 ml) was obtained from an unstressed group of Wistar rats and extracted on Sep-Pak columns prior to reversed-phase HPLC. Extracts were reconstituted in HPLC solvent, injected into a Bondapak C18 column (Waters, Milford, MA, USA) and eluted with a linear gradient running from 8 to 80% acetonitrile in 0.1% trifluoroacetic acid at 1% per min and a flow rate of 1 ml/min. Fractions (1 ml) were collected in polystyrene tubes containing 0.1% (w/v) bovine serum albumin (0.1 ml). After drying in a Speedvac concentrator (Savant), fractions were re-dissolved in phosphate buffer (1 ml) for measurement of EM-1 and EM-2 by RIA.

Statistics

Data from all studies were analysed using one-way ANOVA, followed by *post hoc* Fisher protected least

significant difference test, with the exception of the Wistar stress study. Data from this study were compared using two-tailed Student's *t*-test. *P*<0.05 was considered significant.

Results

Experiment 1: the effect of EM-1 on HPA activity

Plasma corticosterone was measured at each time point following EM-1 (Table 1). Central injection of EM-1 at all three doses did not affect plasma corticosterone levels at any time point up to 4 h post-injection (drug dose × time interaction, $F_{(9,57)}=1.431$, $P=0.197$), main effect of drug dose ($F_{(3,57)}=0.25$, $P=0.860$) and time ($F_{(3,57)}=2.515$, $P=0.067$). *In situ* hybridization data are not shown but revealed that there was no effect of EM-1 on AVP mRNA in the parvocellular subdivision of the PVN as drug treated groups did not differ from vehicle ($F_{(3,19)}=1.165$, $P=0.349$). Corticotrophin-releasing factor mRNA expression in the PVN of the hypothalamus and POMC mRNA expression in the anterior pituitary were also not affected by any of the doses of EM-1 (CRF mRNA, $F_{(3,18)}=0.484$, $P=0.697$; POMC mRNA, $F_{(3,26)}=2.142$, $P=0.119$).

Experiment 2: the effect of EM-2 on HPA activity including morphine as a positive control

The effect of morphine and EM-2 on plasma corticosterone levels are shown in Fig. 1. ANOVA with repeated measures was used to analyse these data, and revealed that there was no significant treatment by time interaction ($F_{(8,52)}=1.833$, $P=0.0918$). However, there was a significant effect of time ($F_{(4,52)}=6.685$, $P=0.0002$) which was the result of the elevation of corticosterone following i.c.v. morphine. Morphine, injected centrally into the lateral ventricle, had the effect of increasing corticosterone levels in plasma within 30 min of injection and was back to baseline levels within 2 h (one-way ANOVA for morphine animals over time, $F_{(4,25)}=5.149$, $P=0.004$).

The expression of CRF mRNA in the PVN of the hypothalamus was unaffected by either morphine or EM-2 ($F_{(2,35)}=1.006$, $P=0.376$). Neither drug had a significant

Figure 1 Plasma corticosterone (ng/ml) in rats immediately before (pre-inj) and at various time points up to 4 h following i.c.v. injection of saline (SAL), morphine sulphate (MOR; 10 µg) or EM-2 (10 µg). Values are expressed as mean±standard error ($n=11-15$). * $P<0.05$, compared with pre-inj.

effect on AVP mRNA in the parvocellular PVN ($F_{(2,31)}=3.03$, $P=0.064$). POMC mRNA was slightly increased following morphine but not with EM-2. However, this effect on POMC mRNA failed to reach significance ($F_{(2,38)}=2.26$, $P=0.119$). Data from *in situ* experiments are not shown.

Experiment 3: the effect of any interaction between EM-1 and morphine on HPA activity

This experiment investigated the effect of prior injection of morphine on plasma corticosterone levels following an earlier injection of either saline or EM-1. ANOVA revealed that the elevation of plasma corticosterone following i.c.v. morphine was unaffected by EM-1, i.e. plasma corticosterone levels remained high when i.c.v. morphine was preceded by an i.c.v. injection of either saline or EM-1 ($F_{(2,16)}=13.279$, $P=0.004$) (Fig. 2).

Experiment 4: the effect of restraint stress on HPA activity

A Plasma corticosterone, ACTH and β -endorphin were significantly elevated following 10 min restraint stress in Wistar rats, but plasma EM-1-immunoreactivity (ir) and EM-2-ir did not change significantly (Fig. 3).

B Restraint stress (30 min), resulted in a significant increase in ACTH, corticosterone and β -endorphin concentrations in the control animals (Table 2). In contrast, restraint stress in the AA rats did not result in any

Figure 2 Plasma corticosterone (ng/ml) in rats measured 60 min after i.c.v. injection of saline (SAL) or morphine (MOR; 10 µg) that was preceded by a 30-min pre-treatment with either saline or EM-1 (10 µg). Values are expressed as mean±standard error ($n=6-7$). ** $P<0.005$, compared with saline+saline controls.

Figure 3 Plasma concentrations of (A) corticosterone (ng/ml), (B) ACTH (pg/ml), and (C) β -endorphin (Beta-end), EM-1 and EM-2 immunoreactivity (pg/ml) as measured by RIAs in control animals (open bars) or rats exposed to 10 min restraint stress (shaded bars). Values represent mean standard error ($n=8$). * $P<0.05$, compared with unstressed controls.

Table 2 Plasma concentrations of corticosterone (ng/ml), ACTH, β-endorphin and EM-2 (all pg/ml) in untreated PVG rats (Con) or rats with AA that have undergone 30-min restraint stress (Res) or injected with LPS. Values are expressed as mean ± s.e.

	Con	AA	Con/Res	AA/Res	Con/LPS	AA/LPS
Corticosterone	12 ± 5	190 ± 26*	289 ± 60*	166 ± 39	323 ± 36*	253 ± 32§
ACTH	<10	<10	1776 ± 637**	<10	3157 ± 630**	2510 ± 678§§
β-endorphin	66 ± 8	45 ± 11	150 ± 22*	56 ± 13	168 ± 33*	132 ± 24§
EM-2	23 ± 4	39 ± 6	48 ± 13*	25 ± 6	19 ± 3	15 ± 2

* $P<0.05$ and ** $P<0.01$ compared with control animals (Con); § $P<0.05$ and §§ $P<0.01$ compared with arthritic controls (AA).

significant elevation in these parameters compared with levels in the AA unstressed group. Lipopolysaccharide evoked significant increases in ACTH, corticosterone and β-endorphin in both control and AA rats compared with their respective controls. Plasma EM-2 was significantly increased in control animals by 30 min restraint but not altered in any other treatment group.

Experiment 5: HPLC and endomorphin immunoreactivity

Reversed-phase HPLC successfully separated synthetic EM-1 and EM-2, with the peak of EM-1 eluting in fraction 30, while EM-2 eluted in fraction 28. This was a consistent pattern regularly observed. Five peaks of EM-1-ir were present in plasma with only 12% EM-1-ir co-eluting with standard EM-1 (Fig. 4A). Three peaks of EM-2-ir were evident in plasma, with 40% EM-2-ir eluting in the same position as the standard (Fig. 4B).

Discussion

These experiments investigated the effect of endomorphins on activation of the HPA axis and the present results show that neither EM-1 nor EM-2 stimulate this axis. Elevation in circulating corticosterone is the clearest marker of rat HPA activation and in our experiments morphine does indeed produce a rapid increase in corticosterone levels in plasma. This supports previous well documented reports that acute morphine stimulates the HPA axis by increases in corticosterone and ACTH levels (Buckingham & Cooper 1984, Ignar & Kuhn 1990, Zubelewicz *et al.* 1999).

The action of opioids on HPA axis activity are thought to be mediated directly or indirectly by the release of CRF from the parvocellular division of the PVN (Pechnick 1993). However, increases in CRF mRNA or other markers of HPA activation, i.e. increased AVP mRNA in the PVN and POMC mRNA in the anterior pituitary, were not convincingly demonstrated following morphine administration in these studies. This is supported by previous reports that have also shown that although morphine increased plasma corticosterone and POMC

expression in the hypothalamus, the opiate did not affect POMC expression in the pituitary (Zhou *et al.* 1999). Another group reported that acute morphine (30 mg/kg, i.p.) increased both the release of corticosterone and

Figure 4 Reversed-phase HPLC of EM-1-ir (A) and EM-2-ir (B) in rat plasma. HPLC fractions were dried and reconstituted in 1 ml phosphate buffer for RIA of EM-1-ir and EM-2-ir. The elution positions of EM-1 and EM-2 are marked with arrows.

hypothalamic noradrenaline turnover. However, there was no modification of either CRF or AVP in the PVN (Milanes *et al.* 1997). It seems, therefore, that changes in CRF and AVP mRNA expression are not necessary for the stimulatory effects of morphine on HPA axis function, suggesting mediation of the HPA axis by other releasing factors or extra-hypothalamic mediators. Evidence for extra-PVN derived factors mediating HPA axis function is provided from a recent study where animals bearing PVN lesions were still able to mount a pituitary–adrenal response to chronic inflammatory stress (Makara *et al.* 2001). Whether a similar mechanism mediates activation by opiates remains to be determined.

Endomorphin-1 had no effect on corticosterone release at any of the doses used (0.1–10 µg/4 µl) and EM-2 was ineffective at 10 µg/4 µl. It could be argued that the dose of endomorphin used in our study was insufficient to affect the HPA axis, although this dose has been shown to activate other physiological systems. It is possible that the lack of effect of the endomorphins is due to differences in diffusion and metabolism of i.c.v. administered endomorphins compared with that of morphine. However, this is unlikely as previous studies have demonstrated physiological effects following injections of similar concentrations of endomorphins. The analgesic effect of EM-1 in Sprague–Dawley rats has been shown to be effective at a dose of between 1 and 5 µg i.c.v. (Wang *et al.* 1999) and the higher dose of 10 µg i.c.v. has been shown to produce maximal analgesic response in mice that lasts up to 1 h (Zadina *et al.* 1997). Also, other physiological effects of endomorphins have been demonstrated, e.g. 3–30 nmol i.c.v. produced a dose-related increase in food intake (Asakawa *et al.* 1998), and a similar range, 1–30 nmol/kg (i.v.), of both endomorphins produced a dose-related decrease in systemic arterial pressure (Czapla *et al.* 1998).

As endomorphins were ineffective in our study one could question the biological activity of the drugs. We were unable to test bioactivity of EM-1 and EM-2 in an *in vitro* system. However, the peptides were supplied with amidated C-terminals for full biological activity and as they are only four residues long it is unlikely that bioactivity would have been lost through any structural or sequence defect. However, as discussed, there are many studies that demonstrate bioactivity of these opioids from analgesia to cardiac effects to psychological effects. Generally, these studies do not report any need for special treatment of the drugs to maintain bioactivity, tending to dissolve the drugs in 0.9% saline (Horvath *et al.* 1999, Wang *et al.* 1999) or 0.9% saline containing 0.01% Triton-X-100 (Tseng *et al.* 2000) or sterile water (Sanchez-Blazquez *et al.* 1999).

Our third experiment showed that the stimulatory effects of morphine on plasma corticosterone could not be blocked by pre-treatment with EM-1. In the 30 min period between injection of EM-1 and morphine, all µ-receptors should have been blocked by EM-1. This

suggests that the HPA activation effects of morphine are acting through opiate receptor sites that are not occupied by EM-1. There are reported to be at least two subsets of µ-opioid receptors in the brain, referred to as µ-1 and µ-2 (Pasternak & Wood 1986). µ-1 receptors are thought to be involved in several opioid effects such as supraspinal analgesia, prolactin release and induction of catalepsy. µ-2 receptors are thought to be involved in decreased dopamine turnover and respiratory depression. µ-1 receptors have a much higher affinity for morphine than µ-2 (Minami & Satoh 1995). In opioid receptor binding assays, both EM-1 and EM-2 competed with µ-1 and µ-2 opioid sites potently (Goldberg *et al.* 1998). However, in mice, the analgesic actions of EM-1 and EM-2 have been reported to be acting possibly through different µ-receptors (Tseng *et al.* 2000) with EM-1 acting predominantly as a µ-2 receptor agonist and EM-2 as a µ-1 agonist (Sakurada *et al.* 1999). Furthermore, Carrigan *et al.* (2000) reported that pre-treatment with EM-1 did not block the immunomodulatory effects of morphine, which suggests that EM-1 might be acting at a different µ-opioid receptor subtype than morphine.

There is evidence that µ, delta and kappa opioid receptors are involved in stimulation of the HPA axis (Iyengar *et al.* 1987) and that kappa-receptor agonists are most potent (Hayes & Stewart 1985, Taylor *et al.* 1997). However, it is reported that morphine acts primarily through µ-receptors to activate the HPA axis and not through other opioid receptors as the selective µ-antagonist CTOP totally blocked plasma corticosterone increases whereas nor-binaltorphimine (a kappa-receptor antagonist) and naltrindol (a delta-receptor antagonist) were ineffective (Mellon & Bayer 1998). There is no indication from the literature whether morphine exerts its stimulatory effects on the HPA axis via either µ-1 or µ-2 receptors, but if morphine is acting on µ-1 receptors and EM-1 through µ-2 receptors, this may explain why EM-1 did not antagonize the effect of morphine in our experiment. Pharmacological studies have revealed that opioid agonists of peptide and non-peptide classes interact with µ-opioid receptors in a different manner (Sanchez-Blazquez & Garzon 1988) and small non-peptide agonists such as morphine bind to regions of the µ-opioid receptor that are partially distinct from those bound by peptide agonists (Fukuda *et al.* 1995). In addition, different agonists have different patterns of G-protein activation at µ-opioid receptors (Sanchez-Blazquez *et al.* 1999) which may alter physiological outcomes and may account for differences in endomorphins and morphine that we observed.

This is the first report of EM-1 and EM-2 in plasma. Our RIAs detected EM-1 and EM-2 circulating in concentrations around 200 pg/ml in Wistar rats, which are considerably higher than we observed for β-endorphin, but within the range previously reported for β-endorphin (Holtt *et al.* 1978). However, EM-2 concentrations in

PVG rat plasma were much lower than in Wistars, perhaps indicating strain differences in EM-2 secretion or degradation. The HPA axis response to morphine has been compared in different strains of rat (Baumann *et al.* 2000). A decreased response to morphine was observed in the Lewis strain but similar responses were seen in two other strains (Fischer and ASI). We are unaware of any other studies investigating opiate or EM regulation of HPA axis activation in different strains.

We observed heterogeneity of EM-1-ir and EM-2-ir in plasma extracts following reversed-phase HPLC. Peaks of EM-1 and EM-2 which co-eluted with the standard were detected, which demonstrates that genuine EM-1 and EM-2 are present in these plasma samples. However, a number of peaks of EM-1-ir and EM-2-ir which do not co-elute with their respective synthetic standards were also detected. The identity of these generally more hydrophobic compounds remains to be ascertained, although it is likely that they represent precursor polypeptides, post-translational modifications or degradation products of EM-1 and EM-2.

Although plasma corticosterone, ACTH and β -endorphin increased in response to restraint stress, no significant changes in plasma EM-ir concentrations were detected. A significant, albeit modest, increase in plasma EM-2 occurred in control PVG rats in response to 30 min restraint, but the overall levels remained low relative to the increases in β -endorphin and ACTH. In isolation this represents a response to restraint in control animals. However, the lack of consistency within the data group as a whole leads us to question the physiological relevance of this increase in EM-2. The relative lack of response of EM-ir to restraint stress is not altogether surprising given the low levels of EM-ir previously reported in the tissues of the HPA axis (Jessop *et al.* 2000), the principal pathway mediating the response to stress (Buckingham *et al.* 1997). Our observations that corticosterone and ACTH are further elevated in AA by LPS, but not by restraint, confirm previous work (Harbuz *et al.* 1999). A novel feature of the data reported here is that β -endorphin release, like ACTH release, in response to restraint stress does not occur in the AA rats. However, as with ACTH and corticosterone, this response is intact in the AA rats in response to LPS.

In conclusion, the failure to induce changes in plasma EM-ir in response to the chronic inflammatory stress of AA, the psychological stress of restraint, or the immunological stress of LPS, argues against an important role for endomorphins in mediating effects of stress. Under our experimental conditions, neither EM-1 nor EM-2 appear to influence the regulation of the HPA axis. Therefore, endomorphins may be acting on a different subset of the μ -opioid receptor than morphine. This finding may be advantageous as it possibly limits potential unwanted side-effects of the endomorphins if they are likely to be considered as potential candidates as novel analgesics.

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