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Corticotrophin Releasing Hormone Modulates Morphine Effect on the Met-Enkephalin Activity in the Hypothalamic-Pituitary-Adrenal Axis in Lambs

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The present study evaluated the effects of morphine or morphine together with corticotrophin releasing hormone (CRH) on Met-enkephalin synthesis, secretion, concentration and opioid receptors binding in the hypothalamus, anterior pituitary and adrenal cortex (HPA) in lambs. Lambs received a single i.v. injection of 0.9% NaCl (control) or morphine (MOR) or morphine in combination with CRH (MOR+CRH). Animals were decapitated after 60 min under anaesthesia and fragments of HPA tissues were dissected. Proenkephalin mRNA expression was measured by *in situ* hybridization, Met-enkephalin concentrations by RIA method, opioid secretion by *in vitro* incubation. Specific radioligands were used for each type of receptors - ^{3}H-DAGO for mu, ^{3}H-DPDPE for delta and ^{3}H-EKC for kappa binding sites. Acute injection of morphine affected the proenkephalin mRNA expression, native and cryptic Met-enkephalin concentrations as well as mu, delta and kappa receptors binding. There were multiple cases of the effects of morphine being reversed with CRH effects on the HPA axis level, however the most pronounced changes were observed in the hypothalamus. Interestingly, CRH reversed the effect of the morphine on the proenkephalin mRNA expression in all tested tissues. These results indicated an important role of CRH in the endogenous opioid peptides synthesis and opioid receptors binding.

Key words: PENK mRNA, opioid receptors, *in vitro* enkephalin secretion, HPA.

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Endogenous opioid peptides and their receptors play important roles in the regulation of many physiological processes via nervous, endocrine and immune systems interactions. Opioid peptides, belonging to families of enkephalins, dynorphins and endorphins are synthesized as large peptides (precursors) namely preproenkephalin (PENK), preprodynorphin (PDYN) and proopiomelanocortin (POMC), respectively.

Among opioid peptides, enkephalins have the widest tissue distribution. They are released as native small molecules from multiple central nervous system structures and peripheral organs such as adrenals, gastrointestinal system, pancreas and cardiovascular systems (PIERZCHALA & VAN LOON 1990). Met-enkephalin exists in the blood and tissues in two forms –1. native with five amino acid residues fragments and a very short half-life and 2.

cryptic form in/or associated with large proteins and processed by proteases to active Met-enkephalin and Leu-enkephalin (PIERZCHALA & VAN LOON 1990). Under *in vitro* conditions, native Met-enkephalin might be released from cryptic (bound) precursor by enzymatic hydrolysis with trypsin and carboxypeptidase B.

Endogenous opioids act through specific receptors localized in both the brain and peripheral tissues. Classical pharmacological studies identified three classes of opioid receptors; mu (μ), delta (δ) and kappa (κ) which have been cloned. These classical opioid receptors belong to the family of the guanine regulatory binding (G) protein coupled receptor and are acting through the second messenger systems mainly by inhibition of Gi/Go proteins (BODNAR 2016). Pharmacological and molecular studies suggested that there is more than one type of mu opioid receptor. However, as only a single mu receptor gene has been reported, so the receptor may undergo extensive alternative splicing to generate a group of variants (PASTERNAK & PAN 2011).

Endogenous opioid peptides have been broadly studied as important factors modulating activity of hypothalamic-pituitary-adrenal axis (HPA) (DROLET *et al*. 2001; RUSSELL *et al*. 2008). Opioid-containing neurons have been shown to innervate the median eminence and paraventricular nucleus of the hypothalamus, thereby regulating inputs to ACTH-controlling neurons in the anterior pituitary (STEIN & ZÖLLNER 2009; DI MARZO *et al*. 2007). Thus, the hypothalamic-pituitary-adrenal axis represents a modulatory target for the action of exogenous and endogenous opioid ligands. Indeed, a growing body of evidence suggests that opioids regulate mechanisms activated during the stress response. Conversely, the endogenous opioid system is activated by stressful situations, raising the possibility that activation of the endogenous opioid system may play a role in stress-mediated events. Although stress is often linked to unpleasant events, the stress response can be beneficial. For example, exposure to mild stressors has been shown to activate the HPA axis, which is thought to play an important role in mediating cognitive adaptive changes that promote survival. Previous studies have shown that different stressors – emotional (isolation) and physical (exercise) affect tissue and plasma opioid concentrations and opioids receptors activity in the hypothalamus, pituitary and adrenal (PIERZCHAŁA-KOZIEC *et al*. 2006) indicating a close relation between the opioid systems and corticotrophin releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and glucocorticoids. It was found that restraint stress increased plasma concentrations of Met-enkephalin and corticosterone in rats (VAN LOON *et al*. 1990). Similarly in sheep, the stress of insulin-induced hypoglycemia increased plasma concentrations of both cortisol and Met-enkephalin (OWENS *et al*. 1988). Enkephalins are present in the hypothalamus and interact with the CRH neurones (SMITH & VALE 2006; VAN'T VEER *et al*. 2012; FUNK *et al*. 2014) and probably are secreted in order to modulate the activity of hypothalamic-pituitary-adrenal axis (BRUCHAS *et al*. 2009; LEMERRER *et al*. 2009; LEMOS *et al*. 2012). In addition, the opioid kappa receptor agonist, dynorphin, is co-localized with corticotrophin-releasing hormone (CRH) in hypothalamic neurons (CALOGERO *et al*. 1996).

Intravenous administration of either μ or κ selective opioid receptor agonists increases the circulating concentrations of adrenocorticotropin (ACTH) in fetal lambs (TAYLOR *et al*. 1997). Based on studies with the intracerebroventricular administration of Met-enkephalin and the consequent decrease in plasma concentration of ACTH, it can be assumed that Met-enkephalin can depress ACTH release acting at a central locus (WANG *et al*. 1988).

Morphine, an opium alkaloid acting through μ opioid receptors, has been widely used as an analgesic for decades, however, there are side effects such as respiratory depression, constipation and nausea at the central and peripheral levels (MATTHES *et al*. 1996; GENDRON *et al*. 2006). There is contradictory information on the effects of chronic morphine administration on hypothalamic CRH. Long term treatment with morphine was reported to decrease the CRH synthesis and concentrations in the hypothalamus (LAORDEN *et al*. 2002). In contrast, chronic morphine administration was found to increase hypothalamic CRH mRNA (MCNALLY & AKIL 2002; HOUSHYAR *et al*. 2004). Moreover, morphine administration to rats increased plasma concentrations of ACTH (BUCKINGHAM 1982) and stimulated the release of CRH (CALOGERO *et al*. 1996). In spite of fact that morphine mainly acts through μ receptors it has been demonstrated that acute or prolonged morphine treatment upregulates delta and kappa receptors (PIERZCHALA-KOZIEC *et al*. 2000; GENDRON *et al*. 2006).

Naloxone-induced morphine withdrawal is accompanied by stress-related parvocellular CRH transcription (LIGHTMAN & YOUNG 1988; PASTERNAK & PAN 2011) again supporting cross-talk between opioid receptors and CRH (BRUNTON *et al*. 2005; CHONG *et al*. 2006; LIKAR *et al*. 2007; CHARRON *et al*. 2008; RUSSELL *et al*. 2008).

Recent studies from our laboratory have shown that CRH affects the mu, delta and kappa opioid receptors binding in each level of hypothalamic-pituitary-adrenal cortical (HPA) axis and these effects can be influenced by naltrexone, an opioid receptor antagonist (PIERZCHAŁA-KOZIEC *et al*. 2015).

These results suggest that morphine and CRH are involved into regulation of HPA axis in varying situations but whether their collective effects represent a global mechanism acting to restraint endogenous enkephalin peptides activity remains to be elucidated.

Thus, the goals of the present study were two-fold: 1. to determine whether single injection of morphine affected the synthesis, secretion, concentration of native and cryptic Met-enkephalin and mu, delta and kappa opioid receptor agonist binding in the hypothalamus, anterior pituitary and adrenal cortex of lambs, and 2. to explore the effect(s) of CRH on the morphine-induced changes in these processes.

Materials and Methods

The study employed 3 months old lambs of the Polish Mountain Sheep breed. These were maintained in a controlled environment (photoperiod 14L: 14D with lights on from 7a.m to 7 p.m) and at a room temperature 20°C. The lambs had free access to food and water. The protocol was approved by the First Local Ethical Committee on Animal Testing in Kraków (64/OP/2005/I LKE).

Eighteen female lambs were assigned to 3 groups (n=6): control (C), morphine (MOR) and morphine plus corticotrophin releasing hormone (MOR+CRH). Each lamb received one intravenous injection of saline (0.9% of NaCl in the control group), morphine (MOR) (1 mg/kg b. w.) or morphine and CRH (1 µg/kg b.w. Sigma, St. Louis, MO, USA). Blood samples were taken to the heparinized tubes just before (0 time) and 10, 20, 30 and 60 minutes after the injection. The samples were immediately centrifuged and the plasma was stored at -80°C. The site of injection was left jugular vein, the blood was taken from the right jugular vein. Animals were decapitated after anaesthesia. Hypothalami, anterior pituitary glands and adrenal cortex were dissected out one hour after treatment injections. Tissues were divided into four parts and directed to: 1. *in situ* hybridization to estimate proenkephalin gene expression, 2. estimation of native and cryptic Met-enkephalin by radioimmunoassay, 3. opioid receptors binding and 4. *in vitro* secretion of native Met-enkephalin.

Proenkephalin mRNA gene expression was estimated by modified method described by LIGHTMAN & YOUNG (1987). Briefly, the frozen fragments of hypothalamus, anterior pituitary and adrenal cortex were sliced (14 µm sections) using a Leica cryostat microtome (-22°C). The sections were thaw-mounted on gelatin-covered microscopic slides, and stored for 3 days at -20°C before the assay. Then, tissue sections were thawed and fixed in 4% formaldehyde in phosphate buffered saline (PBS; pH 7.4) for 10 min. Then, sections were acylated for 10 min in triethanolamine/acetic anhydride (0.25%). Sections were dehydrated by immersion through graded ethanol (70%, 80%, 95%, 100%) and in air dried.

After pre-hybridization, a synthetic deoxyoligonucleotide, complementary to the fragment of rat proenkephalin (PENK), was labeled using ^{35}S-dATP (1200 Ci/nmol) to obtain a specific activity about 4×10^6 cpm/µl. The probes were diluted in a hybridization buffer (formamide, dextran sulfate, Saline-Sodium Citrate buffer (SSC), Denhardt's solution, yeast tRNA, herring sperm DNA). Hybridization occurred during 20 h in humidified chamber at 37°C. Then, the sections were washed once in SSC for 10 min, then four times for 15 min, each in SSC/ 50% formamide at 40°C, rinsed in SSC and distilled water at room temperature and air-dried. The sections were exposed to Kodak film for four weeks (-80°C). The photo-stimulated luminescence (PSL) density of the irradiated plates was measured with BAS-1000 readout system. The PSL/mm^2 at the resultant film images was determined using computer image analysis system.

Native and cryptic Met-enkephalin concentrations

Native and cryptic Met-enkephalin concentrations in the tissues and plasma were estimated by the radioimmunoassay method of PIERZCHAŁA & VAN LOON (1990). Briefly, fragments of tissues were homogenized in phosphate buffer, pH 6.5, centrifuged (4000×g, 4°C, 20 min) and supernatants were stored at -80°C until further processing. Enkephalin containing peptides (cryptic enkephalin) were hydrolyzed with trypsin (1 mg/ml, 37°C, for 30 min) followed by carboxypeptidase B (5 mg/ml) plus trypsin inhibitor (2.5 mg/ml) for 15 minutes.

Native and cryptic enkephalins were purified on PorapakQ (Waters,100-120 mesh) in 2 ml of absolute ethanol, lyophilized and assayed after reconstitution in 100 µl of 0.06 M phosphate buffer (pH 6.5, 0.2% bovine serum albumin, 0.002% sodium azide). The assay entailed the addition of 50 µl antiserum (rabbit, 1:10,000) and 50 µl of ^{125}I-Met-enkephalin (~1500 cpm) and incubation at 4°C for 24 h. Bound and free opioid was separated after 24 h by the addition of 50 µl of rabbit γ-globulin (1%), incubation for 30 min at 4°C, addition of 250 µl of 25% polyethylene glycol (PEG 8000), incubation for 30 min of incubation samples and finally centrifugation (2000×g, 4°C, 20 min). The supernatants were discarded and the pellets were counted in a γ-counter (Wizard).

Receptor binding assays

Receptors binding assays were performed according to procedures reported by BELCHEVA *et al*. (1994) and HYTREK *et al*. (1996) with some modification (PIERZCHAŁA-KOZIEC *et al*. 2015). Briefly, the dissected tissues were homogenized in ice-cold buffer 50 mM Tris-HCl, pH 7.4 and the homogenate was centrifuged at 20,000 xg for 15 min. Cells membrane preparations (1 ml, 1 mg of protein) were incubated at 30ºC for 30 min with tritiated agonists for each type of opioid receptors: for mu receptors – 26.0 nM ^{3}H-DAGO [D-Ala2, MePhe4, Gly(ol)5]enkephalin, for delta receptor – 6.80nM ^{3}H-DPDPE [[D-Ala2-,N-Me-Phe4,Gly-ol] and for kappa receptor – 59.18 nM of ^{3}H-EKC ([ethylketocyclazocine]). Radioligands were purchased from Amersham International (^{3}H-DAGO, ^{3}H-DPDPE) and from New England Nuclear (^{3}H-EKC). Nonspecific binding was estimated with 10 μM of unlabeled ligands: Met-enkephalin for delta, Leu- enkephalin-Arg for kappa and morphine for mu receptors (Sigma, St. Louis, MO, USA). Free ligand was separated from membrane bound radioligand by filtration under reduced pressure through GF/B Whatman glass filters. Protein concentrations were determined by the bicinchoninic acid (BCA) method (OLSON & MARKWELL 2007).

In vitro Met-enkephalin secretion

Met-enkephalin secretion from fragments of tissues was estimated according to the method of KOWALSKI & GIRAUD (1993) with some modifications. Briefly, fragments of tissues (20-30 mg) sliced by microtome were placed into 24-well plates with 1 ml of Krebs-Ringer bicarbonate buffer (medium). After a 20 min preincubation period, tissues were incubated at 37°C for five successive 20 min periods in 500 μl medium according to the sequence: 1. basal medium; 2. stimulating medium with 100 nM of naltrexone; 3. basal medium; 4. basal medium; 5. stimulating medium with 56 mM KCl. Stimulation with KCl served to validate survival of the tissue through the experiment. The concentration of Met-enkephalin in the basal media were not significantly different so the results were pooled and presented as Met-enkephalin release under basal conditions.

Statistical analysis

Results are presented as means ± SEM. The analysis was performed using the O Dell Statistica, ver.13, (1984-2016 Dell Inc.). Repeated measures ANOVA or paired t-test followed by Fisher test were used to determine the effects of morphine and CRH on the synthesis, secretion, plasma levels of Met-enkephalin as well as mu, delta and kappa receptor binding in the HPA axis.

Results

Proenkephalin

There were some differences (P<0.001) in proenkephalin (PENK) expression between each of the three tissues: hypothalamic (464 ± 44 PSL/mm^2), anterior pituitary (761 ± 66 PSL/mm^2) and adrenal cortical tissues (663 ± 49 PSL/mm^2). Figure 1 summarizes the effects of morphine or morphine in combination with CRH on PENK mRNA concentrations in the hypothalamus, anterior pituitary gland and adrenal cortex of sheep.

There was no effect of morphine on hypothalamic PENK mRNA (Fig. 1). In contrast, an injection of morphine was followed by decreased (P<0.001) PENK mRNA expression by 47% for the anterior pituitary and by 55% in the adrenal cortex (Fig. 1). Administration of morphine together with CRH influenced PENK expression. PENK mRNA was increased (P<0.001) in the hypothalamus, anterior pituitary gland and adrenal cortex in lambs receiving CRH and morphine compared to either control lambs or animals receiving morphine alone (Fig. 1).

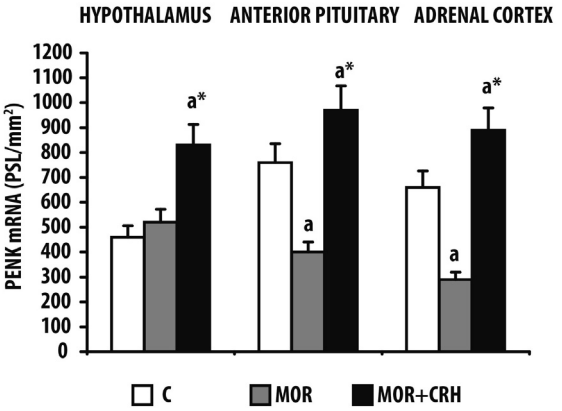

Fig. 1. Effects of morphine and CRH on proenkephalin mRNA expression in the hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in PSL/mm^2 ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

Met-enkephalins concentrations

Native Met-enkephalin

There were much higher concentrations (P<0.001) of native Met-enkephalin in the anterior pituitary gland (138.3±8.92 pmol/g wt.) than either hypothalamus (29.1±1.9 pmol/g w.t.) or adrenal cortex (33.5 ± 1.9 pmol/g wt.). The effects of morphine or morphine in combination with CRH on concentrations of native Met-enkephalin in the

hypothalamus, anterior pituitary gland and adrenal cortex of lambs are shown in Fig. 2.

Injection of morphine alone or in combination with CRH was not accompanied by changes in the concentrations of native Met-enkephalin in the hypothalamus (Fig. 2). Morphine administration was accompanied by a 23% decline ($P<0.05$) in the concentration of native Met-enkephalin in the anterior pituitary gland (Fig. 2). Moreover, injection of both morphine and CRH was followed by a larger (80%) decrease ($P<0.001$) in the concentration of native Met-enkephalin in the anterior pituitary gland (Fig. 2). Morphine administration also depressed ($P<0.01$) the concentration of native Met-enkephalin in the adrenal cortex by 27% from 33.5 ± 1.9 to 24.3 ± 1.6 pmol/g wt. (Fig. 2). Native Met-enkephalin concentrations after treatment with morphine and CRH were intermediate of the concentrations seen in control and morphine but did not differ significantly from either (Fig. 2).

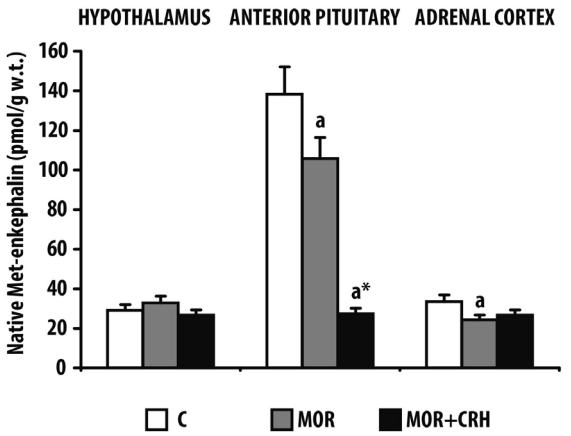

Fig. 2. Effects of morphine and CRH on the native Met-enkephalin concentration in the hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in pmol/g ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

Cryptic Met-enkephalin

There were much higher concentrations ($P<0.001$) of cryptic Met-enkephalin in the anterior pituitary gland (216.9 ± 19.7 pmol/g wt.) than the hypothalamus (43.1 ± 2.7 pmol/g wt.) with adrenal cortex intermediate (160.9 ± 18.3 pmol/g wt.). Figure 3 summarizes the effects of morphine or morphine together with CRH on the concentrations of cryptic Met-enkephalin in the hypothalamus, anterior pituitary gland and adrenal cortex of lambs.

Acute administration of morphine was accompanied by increased ($P<0.01$) concentrations of cryptic Met-enkephalin in the hypothalamus and anterior pituitary by, respectively, 35% and 33% (Fig. 3). In contrast, the concentration of cryptic Met-enkephalin in the adrenal cortex was reduced ($P<0.001$) by 78% in lambs receiving injections of morphine (Fig. 3). Administration of CRH in combination with morphine reversed the effect of morphine on hypothalamic concentrations of cryptic Met-enkephalin (Fig. 3). Anterior pituitary concentrations of cryptic Met-enkephalin did not differ between lambs receiving morphine alone or morphine together with CRH (Fig. 3). Adrenal cortical concentrations of cryptic Met-enkephalin were reduced in lambs receiving morphine plus CRH with the decrease less ($P<0.05$) compared to morphine injected group (Fig. 3).

Opioid receptors binding

Figure 4, 5 and 6 summarizes the effects of morphine or morphine plus CRH on the hypothalamus, anterior pituitary gland and adrenal cortex examining, respectively, mu (μ) opioid receptors (MOR), delta (Δ) opioid receptors (DOR) and kappa (κ) opioid receptors (KOR).

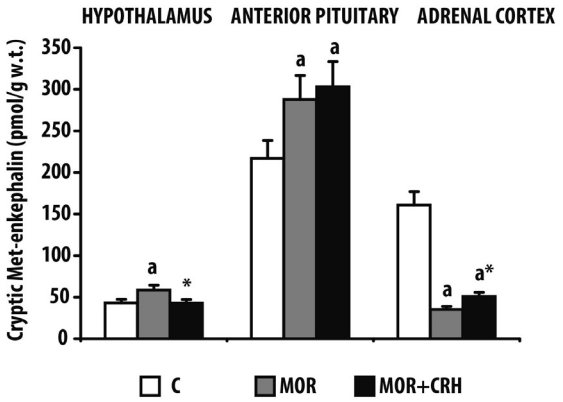

Fig. 3. The effects of morphine and CRH on the cryptic Met-enkephalin concentration in the hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in pmol/g ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

Mu (μ) opioid receptor binding (Fig. 4)

There was markedly greater ($P<0.001$) MOR as indicated by binding (of ^{3}H-DAGO) in adrenal cortex (9.30 ± 0.82 fmol/mg protein) than hypothalamus (5.16 ± 0.28 fmol/mg protein) and, in turn, than the anterior pituitary gland (2.70 ± 0.24 fmol/mg protein). Neither hypothalamic nor adrenal cortical MOR were influenced by morphine treatment (Fig. 4). Anterior pituitary MOR (^{3}H-DAGO binding) was decreased ($P<0.001$) by 63% in lambs receiving morphine administration compared to control lambs (Fig. 4). Hypothalamic μ opioid receptor concentrations were increased

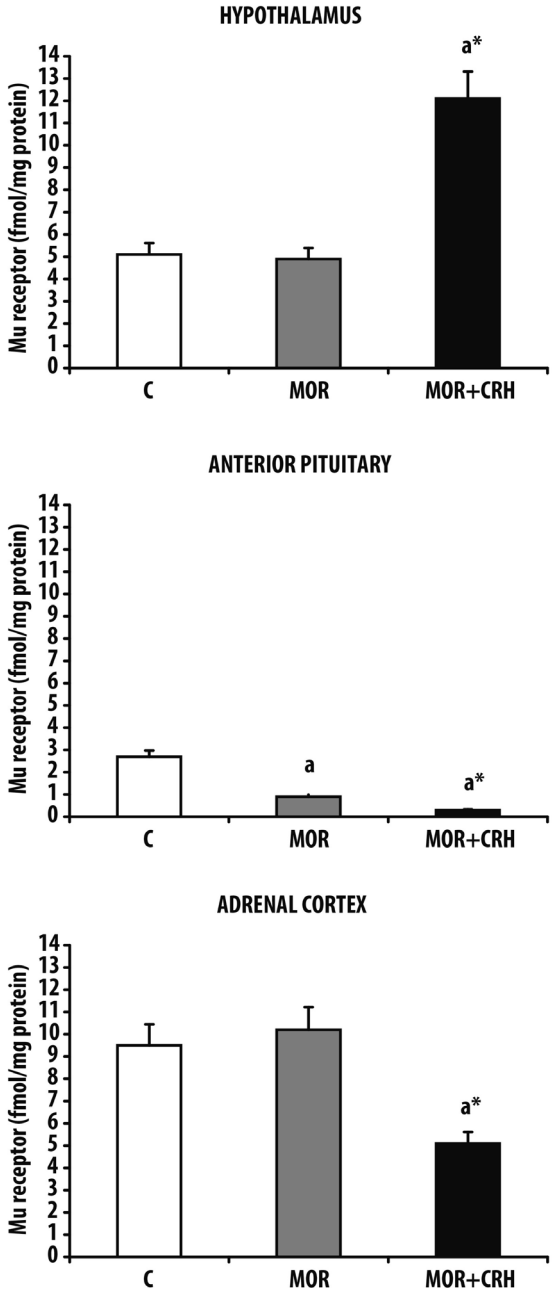

Fig. 4. Mu receptor receptors in the hypothalamus, anterior pituitary and adrenal cortex as estimated by ^{3}H-DAGO binding to cell membrane preparations from lamb hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in fmol/mg protein ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

(P<0.001) by 96% in lambs receiving morphine together with CRH (Fig. 4). In contrast, anterior pituitary concentrations of MOR were lower in lambs receiving morphine and CRH than either morphine alone (89%) or than in control lambs (98%) (Fig. 4). Administration of morphine together with CRH depressed (P<0.001) the μ opioid agonist binding in adrenal cortex tissue by 46% compared to tissue from control sheep.

Delta (Δ) receptor binding (Fig. 5)

There were similar DOR concentrations, as indicated by binding of ^{3}H-DPDPE, in adrenal cortical, hypothalamic and anterior pituitary tissue – 39.12±4.11, 53.01±4.78 and 44.40±3.87 fmol/mg protein, respectively. Lambs receiving a single injection of morphine exhibited decreased

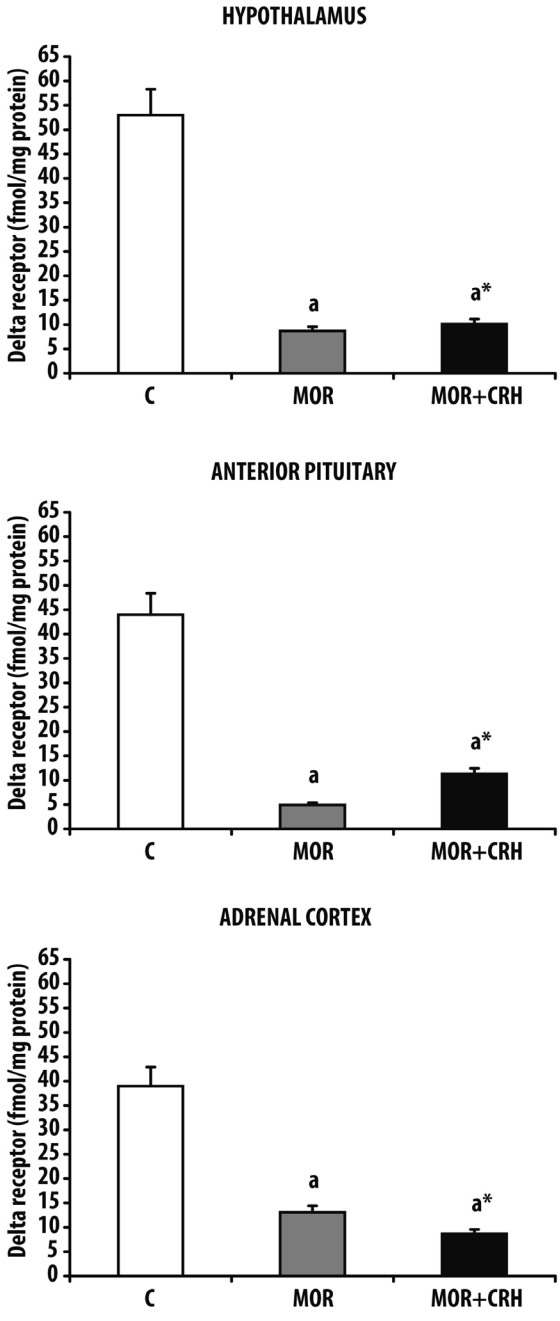

Fig. 5. Delta opioid receptors in the hypothalamus, anterior pituitary and adrenal cortex as estimated by ^{3}H-DPDPE binding to cell membrane preparations from lamb hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in fmol/mg protein ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

(P<0.001) DOR binding in the hypothalamus by 87%, anterior pituitary by 86% and adrenal cortex by 59% (Fig. 5). CRH given together with morphine caused small but statistically significant increases in DOR binding in the hypothalamus and anterior pituitary. In contrast, CRH potentiated the inhibitory effect of morphine on the delta receptor binding in the adrenal cortex.

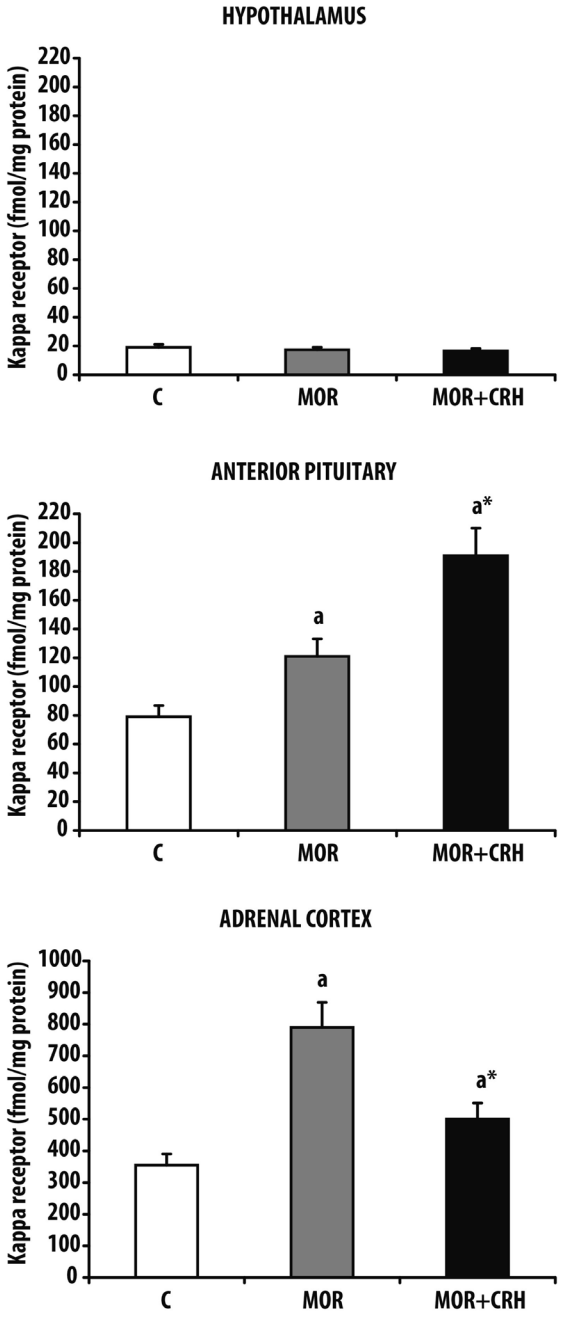

Fig. 6. Kappa opioid receptors in the hypothalamus, anterior pituitary and adrenal cortex as estimated by ^{3}H-EKC binding to cell membrane preparations from lamb hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in fmol/mg protein ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

Kappa (κ) receptor binding (Fig. 6)

There were marked tissue differences (P<0.001) in kappa receptor binding as indicated by ^{3}H-EKC binding. Kappa receptor binding ranged from lowest in the hypothalamus (19.2 ± 1.44 fmol/mg protein), intermediate in the anterior pituitary gland (79.0 ± 6.39 fmol/mg protein) and highest in the adrenal cortex (355.1 ± 37.2 fmol/mg protein in of control lambs). There was no effect of morphine alone or in combination with CRH on the KOR agonist binding in the hypothalamus (Fig. 6). In contrast, κ receptor agonist binding was increased (P<0.001) in both anterior pituitary and adrenal cortical tissue from lambs treated with morphine (Fig. 6); the increases being, respectively, 53 and 122%. Administration of CRH with morphine further increased (P<0.001) κ receptor agonist binding in anterior pituitary tissue. However, injection of CRH with morphine attenuated (P<0.01) KOR binding in the adrenal cortex.

Plasma concentrations of native Met-enkephalin

Plasma concentrations of native Met-enkephalin levels were unchanged in control lambs during the 60 min course of the experiment (Fig. 7). In contrast, injection of morphine was followed rapidly by decreased (P<0.01) plasma concentrations of native Met-enkephalin (Fig. 7) being decreased by 63% after 10 min (P<0.01) and by 79% to a nadir after 20 min (P<0.01) (Fig. 7). Plasma concentrations of native Met-enkephalin remained reduced (P<0.01) at 30 min and after 60 min (Fig. 7). CRH partially overcame the inhibitory effects of morphine on plasma concentrations of native Met-enkephalin (Fig. 7). There was a transitory 44% decrease (P<0.01) in plasma concentrations of native Met-enkephalin 10 min after morphine plus CRH challenge (Fig. 7). After 20 min plasma level of native Met-enkephalin was not different from the value observed in control animals. At 30 minutes Met-enkephalin level was significantly higher compare to control and morphine treated animals (Fig. 7). Administration of morphine plus CRH was followed by what appeared to be a biphasic change in the plasma concentrations of native Met-enkephalin with an initial 10 min decrease (P<0.01), recovery at 20 min, overshoot at 30 min (P<0.05) and a second decrease (P<0.05).

Met-enkephalin secretion *in vitro* from the hypothalamus, anterior pituitary gland and adrenal cortex

Figure 8 summarizes *in vitro* secretion of Met-enkephalin from the hypothalamus, anterior pituitary gland and adrenal cortex of control lambs and those treated with morphine or morphine and CRH. *In vitro* secretion of Met-enkephalin was de-

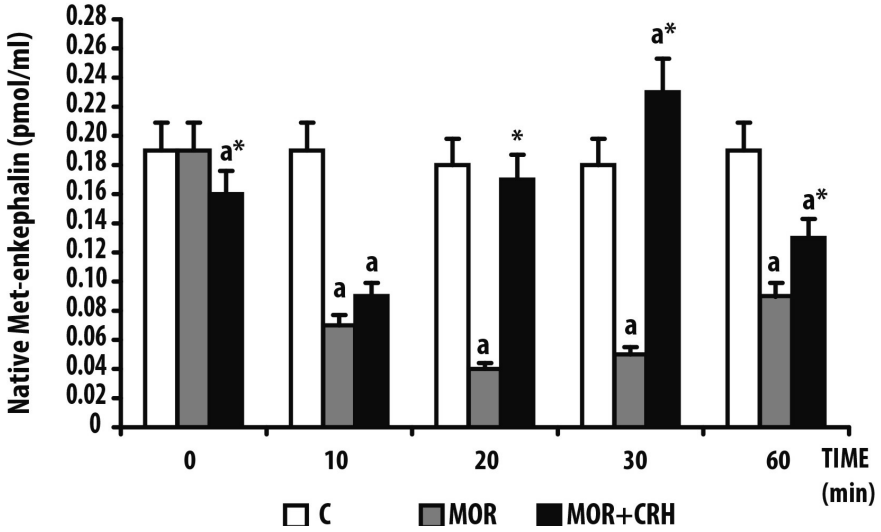

Fig. 7. The effects of morphine and CRH on the plasma concentrations of native Met-enkephalin in lambs. Data is shown as mean in pmol/ml ± SEM (n= 6 animals). Different superscript letter and asterisk (a,*) indicate difference <0.05 compare to control and morphine treated group, respectively. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

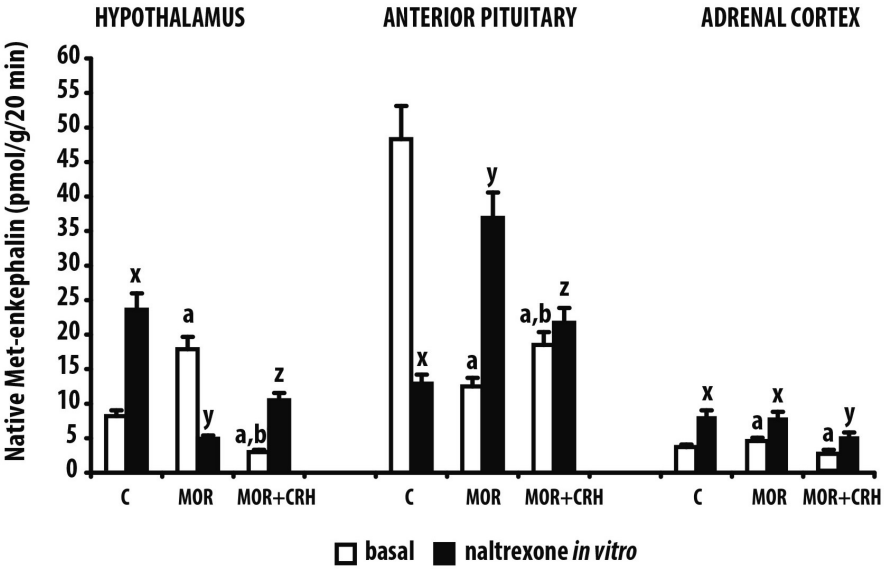

Fig. 8. Effects of *in vivo* treatment (morphine or morphine + CRH) on *in vitro* secretion of Met-enkephalin. Release of native Met-enkephalin was determined *in vitro* in the presence or absence of the opioid receptor antagonist, naltrexone from lamb hypothalamus, anterior pituitary and adrenal cortex. Data is shown as mean in pmol/g/20 min ± SEM (n= 6 animals). Naltrexone was at 100 nM. a, b – different superscript letters indicate difference between *in vivo* treatments P <0.05 x, y, z different superscript letters indicate difference between *in vitro* treatment with naltrexone P <0.05. Treatments – C-control (injection of saline), MOR-morphine injection, MOR+CRH – morphine and CRH injections.

termined in the presence or absence of the opioid antagonist, naltrexone (Fig. 8).

Hypothalamus

Basal release of Met-enkephalin from the hypothalamus *in vitro* was elevated in lambs receiving morphine; increasing from 8.2 ± 0.78 pmol/g/20 min in controls to 17.9 ± 2.1 pmol/g/20 min with morphine treatment (P<0.001) (Fig. 8). In contrast, Met-enkephalin secretion was reduced by 63% (P<0.01) in lambs receiving morphine and CRH (Fig. 8). In the presence of naltrexone, there was markedly increased release of Met-enkephalin from hypothalamus of control animals by 187% (P<0.01) (Fig. 8). However, there was reduced release of Met-enkephalin (by 72%, P<0.001) from tissue from lambs injected with morphine (Fig. 8). In contrast, there was increased secretion (by 250%, P<0.001) of Met-enkephalin from the hypothalamus in the presence of naltrexone in tissue from lambs injected with morphine and CRH (Fig. 8).

Anterior pituitary gland

Basal *in vitro* secretion of Met-enkephalin from sheep anterior pituitary tissue was 5.9 fold greater than from the hypothalamus (Fig. 8). Basal *in vitro* secretion of Met-enkephalin was lower ($P<0.001$) in lambs treated with morphine by 74% and with morphine together with CRH (Fig. 8). The effect of morphine was attenuated somewhat ($P<0.05$) in the presence of CRH with secretion depressed ($P<0.001$) by 62% in tissue from lambs receiving morphine + CRH compared to control lambs (Fig. 8).

In the presence of naltrexone, Met-enkephalin release *in vitro* was decreased ($P<0.001$) by 73% with pituitary tissue from control lambs [from 48.3 ± 3.4 to 12.9 ± 1.6 pmol/g/20 min] but increased ($P<0.001$) by 2.95 fold from tissue of morphine injected animals (from 12.5 ± 1.3 to 36.9 ± 4.5 pmol/g/20 min) (Fig. 8). Naltrexone did not affect Met-enkephalin release *in vitro* ($P>0.05$) from anterior pituitary tissue from lambs treated with morphine and CRH.

Adrenal cortex

Met-enkephalin release *in vitro* from the adrenal cortex tissue is markedly lower than from hypothalamic and anterior pituitary tissue. *In vivo* injection of morphine increased secretion of Met-enkephalin ($P<0.05$) by 22%. In the presence of naltrexone, Met-enkephalin release was increased ($P<0.001$) in adrenal cortical tissue from control lambs (by 121%), from morphine treated animals (by 74%) and from morphine with CRH injected lambs (by 77%).

Discussion

There were novel and marked effects *in vivo* treatment with morphine or morphine combined with CRH on enkephalin related parameters and on opioid receptors (see Figs 1-8). The direct and magnitude of effects results are summarized in Table 1.

The effects of morphine and morphine with CRH on proenkephalin mRNA expression

Acute morphine injection did not change proenkephalin mRNA expression in hypothalamus but significantly decreased it in anterior pituitary and adrenal cortex (Fig. 1). It has been previously reported that morphine administration can acutely change opioid peptide gene transcription in rat brain (BASHEER & TEMPEL 1993) and in specific brain regions (YUKHANANOV & HANDA 1997). Treatment with CRH completely reversed the effect of morphine and increased the proenkephalin mRNA expression in all HPA axis levels.

There is evidence for cross-talk between CRH and the opioid systems. IREDALE *et al*. (2000) found extrahypothalamic CRH to be implicated in anxiety and aversion associated with opiate withdrawal. Type 1 CRH receptor (CRH-R1) antagonists attenuated behavioral signs of opiate withdrawal as well as footshock stress-induced reinstatement of heroin seeking and morphine-conditioned place preference (WANG *et al*. 2006). CRH plays a role in the elevation of withdrawal-induced noradrenergic transmission (FUNADA *et al*. 1994). Moreover, chronic morphine selectively sensitizes locus coeruleus norepinephrine neurons to CRH (XU *et al*. 2004). These mechanisms contribute to the facilitated neuroendocrine stress response seen in morphine dependent rats after withdrawal (HOUSHYAR *et al*. 2004).

The concentrations of cryptic and native Met-enkephalin in the hypothalamic-pituitary-adrenal axis

Data in the literature regarding opiate-induced regulation of the brain enkephalin system are controversial. Thus, increases or decreases in enkephalin immunoreactivity were reported to occur in brain tissues after chronic treatment with morphine, whereas in other studies, no change was observed (VAN BOCKSTAELE *et al*. 2000). These differences are probably due to the various experimental protocols used in chronic morphine treatment, the fragments of brain, different methods for enkephalin estimation, autoradiography or *in situ* hybridization and small opioid peptide extraction from tissues (NIETO *et al*. 2002). In the present experiments acute morphine caused increase of cryptic enkephalin in the hypothalamus without any serious changes in the native form of opioid. In spite of lower synthesis of proenkephalin as an effect of decreased gene expression after acute morphine, the concentration of cryptic Met-enkephalin in the anterior pituitary was increased. This higher level of cryptic enkephalin probably was an effect of lower activity of enzymes responsible for processing large precursor to native form what resulted in significantly lower concentration of native Met-enkephalin. Met-enkephalins, cryptic and native, were decreased after morphine injection in the adrenal cortex, probably due to inhibition of synthesis and enzymatic hydrolysis. Corticotrophin releasing hormone potentiated stimulating effect of morphine on the cryptic Met-enkephalin concentration and inhibiting effect of opiate on the native opioid in the anterior pituitary. CRH reversed completely (hypothalamus) and partially (adrenal cortex) morphine effects. It is interesting that administration of CRH with morphine did not influence the concentrations of either native or cryptic Met-enkephalin in the hypothalamus despite the elevated PENK mRNA expression (Table 1,

Table 1

Summary of the acute effects of morphine or combined treatment with morphine and CRH on Met-enkephalin and opioid receptors in the hypothalamus, anterior pituitary gland and adrenal cortex of lambs (based on data in Figs 1-8). The effects of treatments were compared to control column

Parameter	Control	Morphine	Morphine + CRH
Hypothalamus			
Pro-enkephalin expression	→	→	↑
Met-enkephalin release *in vitro*	→	↑	→
Effect of naltrexone *in vitro*	↑↑	↓↓	↑
Cryptic Met-enkephalin concentration	→	↑	→
Native Met-enkephalin concentration	→	→	→
Delta opioid receptor binding	→	↓↓↓	↓↓
Kappa opioid receptor binding	→	→	→
Mu receptor receptor binding	→	→	↑↑
Anterior pituitary gland			
Pro-enkephalin expression	→	↓↓	↑
Met-enkephalin release *in vitro*	→	↓↓↓	↓↓
Effect of naltrexone *in vitro*	↓↓	↑↑	→
Cryptic Met-enkephalin concentration	→	↑	↑
Native Met-enkephalin concentration	→	↓↓	↓↓
Delta opioid receptor binding	→	↓↓↓	↓↓
Kappa opioid receptor binding	→	↑	↑↑
Mu receptor receptor binding	→	↓↓	↓↓↓
Adrenal cortex			
Pro-enkephalin expression	→	↓↓	↑
Met-enkephalin release *in vitro*	→	↑	→
Effect of naltrexone *in vitro*	↑	↑	↑
Cryptic Met-enkephalin concentration	→	↓	↓
Native Met-enkephalin concentration	→	↓	↓
Delta opioid receptor binding	→	↓↓	↓↓↓
Kappa opioid receptor binding	→	↑↑↑	↑
Mu receptor receptor binding	→	→	↓↓
Plasma			
Concentration of Met-enkephalin	→	↓↓	↓

Figs 1-3). It seems probable that mRNA expression did not mirror the proteins concentrations particularly these characterized by short half- life.

Opioid receptors binding

Mu receptor binding in the anterior pituitary gland was decreased in lambs receiving injections of morphine (Fig. 4). However, there were no effects in the hypothalamus and adrenal cortex suggesting an absence of down regulation and probably lack of receptor internalization in the hypothalamus and adrenal. Treatment with CRH along with morphine was accompanied with marked shifts in mu receptors with the suppressive effects of morphine on the anterior pituitary gland augmented, mu receptors binding in the hypothalamus increased and mu receptors binding in the hypothalamus decreased (Fig. 4). Administration of injection alone CRH was observed to increase the mu receptor binding in the hypothalamus (PIERZCHAŁA-KOZIEC *et al*. 2015). It is possible that ^{3}H-DAGO could not bind *in vitro* to receptors that were persistently occupied by morphine or other opioid agonists. KISSIN *et al*. (1991) postulated that the absence of correlation between anal-

gesia and morphine brain concentration both with the constant-rate morphine infusion and after a single injection; suggesting the development of acute tolerance. Data should be interpreted with caution about varied doses of morphine, animal species and methodology of experiments.

Injection of morphine greatly reduced *in vitro* binding of ^{3}H-DPDPE to delta receptors in hypothalamic, anterior pituitary and adrenal cortical tissues (Fig. 5). In contrast, prolonged or acute morphine treatment has been reported to upregulate delta and kappa receptors in the HPA axis (PIERZCHALA-KOZIEC *et al.* 1990; 2000; GENDRON *et al*. 2006). Co-treatment of CRH with morphine partially reversed the effects of morphine on the hypothalamus and adrenal cortex but increased the inhibiting effect of morphine on the ^{3}H-DPDPE binding in the adrenal cortex. Similarly, administration of injection CRH reduced delta receptor binding in each level of the HPA axis (PIERZCHAŁA -KOZIEC *et al*. 2015). These data agree with the report of agonist induced activation of the delta opioid receptor leads to receptor desensitization and finally to internalization (PRADHAN *et al*. 2009; 2012).

The level of kappa receptor binding varied markedly from the lowest in the hypothalamus, intermediate in the anterior pituitary gland and highest in adrenal cortex (Fig. 6). The presence of kappa opioid receptor in the lamb hypothalamus is consistent with the role for kappa receptors in modulating the release of luteinizing hormone (GOODMAN *et al.* 2004; LOPEZ *et al*. 2016), thyrotrophin releasing hormone and growth hormone releasing hormone (DEPAOLI *et al*. 1994; FUNK *et al*. 2014) and the presence of κ-opioid receptors located in GnRH neurones (WEEMS *et al*. 2016).

Dynorphin and alfa-neo-endorphin are the main agonists for kappa receptors. Dynorphin has been found to regulate neuronal excitability broadly in brain and can affect learning, cognition, seizures, nociception, and endocrine function. Recently, activation of the dynorphin/kappa receptor system has also been shown to be necessary and sufficient for stress-induced behavioral responses in animal models of anxiety, depression, and drug seeking behaviors (VANDERAH 2010).

In the present study, acute treatment with morphine alone or in combination with CRH did not influence kappa receptor binding in the hypothalamus of lambs (Fig. 6). This may be due to antagonistic properties of mu receptor agonists at the central level. PFEIFFER *et al.* (1986) reported that kappa receptor agonists are responsible for induction dysphoric and psychomimetic effects in contrast to mu receptors agonists. In contrast, there were increases following morphine injection in ^{3}H-EKC binding to kappa receptor in the anterior pituitary gland and adrenal cortex (Fig. 6). The effect was potentiated by CRH on the anterior pituitary gland but attenuated by CRH with adrenal cortical tissue (Fig. 6). These tissue differences may reflect different class of kappa receptors and this could be investigated using novel and new kappa agonists that never enter the CNS (VANDERAH 2010). The role of CRH-induced activation of the kappa receptors and dynorphin opioid peptides has been implicated as a mediator of adverse responses to stress. Stress-induced release of dynorphins has been reported to potentiate the "reward" effects of drugs e.g. cocaine, ethanol and nicotine (VAN'T VEER *et al.* 2012, 2013). Kappa receptors activity has been linked to circulating concentrations of corticosterone levels as there is a faster rate of increase of corticosterone following stress in mice lacking dynorphin (BILKEI-GORZO *et al*. 2008).

Plasma native Met-enkephalin concentrations

Plasma concentrations of Met-enkephalin were markedly reduced following administration of morphine with the nadir 20 minutes after injection (Fig 7). Interestingly, to our best knowledge it is first report about morphine decreasing plasma concentrations of Met-enkephalin level. Plasma concentrations of Met-enkephalin showed tendency to increase by 60 min following morphine challenge (Fig. 7). The magnitude of the decline in plasma concentrations of Met-enkephalin (Fig. 7) coupled with the short half-life of Met-enkephalin suggests an even greater decrease in the release and/or production of Met-enkephalin. The present results with morphine administration are consistent with the anterior pituitary and/or the adrenal cortex being significant sources of circulating Met-enkephalin.

Combined injection of morphine and CRH evoked a biphasic effect on plasma concentrations of Met-enkephalin with an initial decrease, recovery and then increase up to 30 min. This is arguably analogous to the response of rats to stressors with an immediate increase of native Met-enkephalin (around 1 min), then return to basal levels and second peak of opioid after 30 minutes (PIERZCHALA *et al*. 1987). The response to morphine was completely abolished in the presence of CRH at 20 and 30 minutes following challenge and attenuated at 60 minutes (Fig. 7). It is suggested that CRH is effectively acting as an antagonist. It is further suggested that the loss of effectiveness of CRH in depressing the effects of morphine on plasma concentrations of Met-enkephalin 60 minutes after challenge was due to prolonged clearance of CRH and appears to be responsible for the sustained release of ACTH that occurs after injection of this hormone.

In vitro Met-enkephalin release from HPA

Short-term *in vitro* incubations allowed direct examination of Met-enkephalin secretion (Fig. 8). There were marked tissue differences with hypothalamic and adrenal cortical release of Met-enkephalin (increase) in morphine treated lambs and anterior pituitary decreased release of Met-enkephalin in morphine treated lambs (Fig. 8). Morphine treatment *in vivo* influenced the release of Met-enkephalin *in vitro*; stimulatory for hypothalamus and inhibitory for the anterior pituitary gland. There is evidence that the effects of morphine are direct as they are reversed in the presence of the opioid receptors antagonist, naltrexone, in the incubation media (Fig. 8). It is also likely that basal release of Met-enkephalin from each tissue is influenced by an endogenous opioid receptor antagonist because there are shifts in the release of Met-enkephalin in the presence of naltrexone. Administration of CRH along with the morphine was accompanied by reduced release of Met-enkephalin from the hypothalamus compared to either control or morphine treated lambs (Fig. 8). Moreover, *in vivo* treatment with CRH along with morphine overcame the effects of morphine on release of Met-enkephalin from anterior pituitary tissue (Fig. 8).

Basal Met-enkephalin *in vitro* release from the adrenal cortex was lower than from hypothalamus and anterior pituitary. Interestingly, morphine injection increased the enkephalin basal release but CRH again reversed this effect. There were consistent *in vitro* stimulatory effects of naltrexone on release of Met-enkephalin from adrenal cortical tissue. These results provide clear evidences for a physiological role for Met-enkephalin in the adrenal cortex. Met-enkephalin and other opioid receptor agonists have been reported to increase production of both aldosterone and corticosterone by rat adrenocortical cells with the effects mediated by, respectively, mu and both mu and kappa receptors (KAPAS *et al*. 1995). Moreover, opioid agonists were reported to increase cortisol production by porcine adrenal cortical cells (KRAZINSKI *et al*. 2011).

Summary

The anterior pituitary gland had consistently higher concentrations of PENK mRNA, native and cryptic Met-enkephalin and basal release of Met-enkephalin *in vitro* than either the hypothalamus or adrenal cortex. Acute morphine injection affected synthesis, concentrations of both forms of Met-enkephalin, agonists receptor binding as well as opioid release in the hypothalamic-anterior pituitary-adrenal cortex axis. However, it must be pointed that the morphine effect on the hypothalamic Met-enkephalin changes were much smaller than in other tested tissues. This contention is supported by morphine decreasing the following: anterior pituitary gland PENK expression in both the anterior pituitary gland and adrenal cortex (Fig. 1), native Met-enkephalin in both the anterior pituitary gland and adrenal cortex (Fig. 2) and cryptic Met-enkephalin in the adrenal cortex (Fig. 3). Moreover, in the case of the anterior pituitary gland, tissue for morphine treated lambs exhibited much reduced *in vitro* release of Met-enkephalin (Fig. 8). Treatment with CRH either attenuated or augmented effects of morphine (Table 1) depending on the tissue and enkephalin activity.

In conclusion, the present data demonstrated acute effects of morphine on the synthesis, secretion and concentrations of native and cryptic Met-enkephalin. The present results provide further support for the cross-talk between mu, delta and kappa receptors and the HPA axis. Furthermore, evidence is advanced supporting the ability of CRH to attenuate some of the effects of morphine and to augment other effects of morphine.

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References

BASHEER R., TEMPEL A. 1993. Morphine-induced reciprocal alterations in G alpha s and opioid peptide mRNA levels in discrete brain regions. J. Neurosci. Res. **36**: 551-557.

BELCHEVA M.M., BARG J., MCHALE R., COSCIA C.J. 1994. Naltrexone-induced down- and upregulation of delta opioid receptors in rat brain regions. Brain Res. Bull. **35**: 69-72.

BILKEI-GORZO A., RACZ I., MICHEL K., MAUER D., ZIMMER A., KLINGMÜLLER D., ZIMMER A. 2008. Control of hormonal stress reactivity by the endogenous opioid system. Psychoneuroendocrinology **33**: 425-36.

BODNAR R.J. 2016. Endogenous opiates and behaviour. 2014. Peptides **75**:18-70.

BRUCHAS M.R., LAND B.B., LEMOS J.C., CHAVKIN C. 2009. CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior. PLoS One. **4**: e8528.

BRUNTON P.J., MEDDLE S.L., MA S., OCHEDALSKI T., DOUGLAS A.J., RUSSELL J.A. 2005. Endogenous opioids and attenuated hypothalamic-pituitary-adrenal axis responses to immune challenge in pregnant rats. J. Neurosci. **25**: 5117-5126.

BUCKINGHAM J.C. 1982. Secretion of corticotrophin and its hypothalamic releasing factor in response to morphine and opioid peptides. Neuroendocrinology **35**: 111-116.

CALOGERO A.E., SCACCIANOCE S., BURRELLO N., NICOLAI R., MUSCOLO L.A., KLING M.A., ANGELUCCI L., D'AGATA R. 1996. The kappa-opioid receptor agonist MR-2034 stimulates the rat hypothalamic-pituitary-adrenal axis: studies *in vivo* and *in vitro*. J. Neuroendocrinol. **8**: 579-585.

CHARRON C., FRÉCHETTE S., PROULX G., PLAMONDON H. 2008. *In vivo* administration of corticotropin-releasing hormone at remote intervals following ischemia enhances CA1 neuronal survival and recovery of spatial memory impairments: a role for opioid receptors. Behav. Brain Res. **188**: 125-135.

CHONG R.Y., OSWALD L., YANG X., UHART M., LIN P.I., WAND G.S. 2006. The mu-opioid receptor polymorphism A118G predicts cortisol responses to naloxone and stress. Neuropsychopharmacology **31**: 204-211.

DE PAOLI A.M., HURLEY K.M., SADA K., REISINE T., BELL G. 1994. Distribution of opioid receptor mRNA in adult mouse brain. An *in situ* hybridization histochemistry study. Mol. Cell Neurosci. **5**: 327-335.

DI MARZO V., BISOGNO T., DE PETROCELLIS L. 2007. Endocannabinoids and related compounds: walking back and forth between plant natural products and animal physiology. Chem. Biol. **14**: 741-756.

DROLET G., DUMONT E.C., GOSSELIN I., KINKEAD R., LAFOREST S.,TROTTIER J.F. 2001. Role of endogenous opioid system in the regulation of the stress response. Prog. Neuropsychopharmacol. Biol. Psychiatry **25**: 729-741.

FUNADA M., SUZUKI T., SUGANO Y., TSUBAI M., MISAWA M., UEDA H., MISU Y. 1994. Role of beta-adrenoceptors in the expression of morphine withdrawal signs. Life Sci. **54**: PL113-PL118.

FUNK D., COEN K., LĘ A.L. 2014. The role of kappa opioid receptors in stress-induced reinstatement of alcohol seeking in rats. Brain & Behav. **4**: 356-367.

GENDRON L., LUCIDO A.L., MENNICKEN F., O'DONNELL D., VINCENT J-P., STROH T., BEAUDE A. 2006. Morphine and pain-related stimuli enhance cell surface availability of somatic-opioid receptors in rat dorsal root ganglia. J.Neurosci. **26**: 953-962.

GOODMAN R.L., COOLEN L.M., ANDERSON G.M., HARDY S.L., VALENT M., CONNORS J.M., FITZGERALD M.E., LEHMAN M.N. 2004. Evidence that dynorphin plays a major role in mediating progesterone negative feedback on gonadotropin-releasing hormone neurons in sheep. Endocrinology **145**: 2959-2967.

HOUSHYAR H., MANALO S., DALLMAN M.F. 2004. Time-dependent alterations in mRNA expression of brain neuropeptides regulating energy balance and hypothalamo-pituitary-adrenal activity after withdrawal from intermittent morphine treatment. J. Neurosci. **24**: 9414-9424.

HYTREK S.D., MCLAUGHLIN P.J., LANG C.M., ZAGON I.S. 1996. Inhibition of human colon cancer by intermitted opioid receptor blockade with naltrexone. Cancer Lett. **101**: 159-164.

IREDALE P. A., ALVARO J. D., LEE Y., TERWILLIGER R., CHEN Y. L., DUMAN R. S. 2000. Role of corticotropin-releasing factor receptor-1 in opiate withdrawal. J. Neurochem. **74**: 199-208.

KAPAS S., PURBRICK A., HINSON J.P. 1995. Action of opioid peptides on the rat adrenal cortex: stimulation of steroid secretion through a specific mu opioid receptor. J. Endocrinol. **144**: 503-510.

KISSIN I., BROWN P.T., ROBINSON C.A., BRADLEY E.L. Jr . 1991. Acute tolerance in morphine analgesia: continuous infusion and single injection in rats. Anesthesiology **74**: 166-171.

KOWALSKI C., GIRAUD P. 1993. Dopamine decreases striatal enkephalin turnover and proenkephalin messenger RNA abundance via D2 receptor activation in primary striatal cell cultures. Neuroscience **53**: 665-672.

KRAZINSKI B.E., KOZIOROWSKI M., BRZUZAN P., OKRASA S. 2011. The expression of genes encoding opioid precursors and the influence of opioid receptor agonists on steroidogenesis in porcine adrenocortical cells *in vitro*. J. Physiol. Pharmacol. **62**: 461-468.

LAORDEN M.L., NUŃEZ C., ALMELA P., MILANÉS M.V. 2002. Morphine withdrawal-induced c-Fos expression in the hypothalamic paraventricular nucleus is dependent on the activation of catecholaminergic neurones. J. Neurochem. **83**: 132-140.

LE MERRER J., BECKER J.A.J., BEFORT K., KIEFFER B.L. 2009. Reward processing by the opioid system in the brain. Physiol. Rev. **89**: 1379-1412.

LEMOS J.C., ROTH C.A., MESSINGER D.I., GILL H.K., PHILLIPS P.E.M. 2012. Repeated stress dysregulates k-opioid receptor signaling in the dorsal raphe thorough a p38MAPK-dependent mechanism. J. Neurosci. **32**: 12325-12326.

LIGHTMAN S. L., YOUNG W. S. III. 1988. Corticotropin-releasing factor, vasopressin and pro-opiomelanocortin mRNA responses to stress and opiates in the rat. J. Physiol. **403**: 511-523.

LIGHTMAN S.L., YOUNG W.S. 1987. Changes in hypothalamic preproenkephalin A mRNA following stress and opiate withdrawal. Nature **328**: 643-645.

LIKAR R., MOUSA S.A., STEINKELLNER H., KOPPERT W., PHILIPPITSCH G., STEIN C., SCHÄFER M. 2007. Involvement of intra-articular corticotropin-releasing hormone in postoperative pain modulation. Clin. J. Pain **23**: 136-142.

LOPEZ J.A., BEDENBAUGH M.N., MCCOSH R.B., WEEMS P.W., MEADOWS L.J., WISMAN B., COOLEN L.M., GOODMAN R.L., HILEMAN S.M. 2016. Does dynorphin play a role in the onset of puberty in female sheep? J. Neuroendocrinol. **28**. https://doi.org/10.1111/jne.12445.

MATTHES H.W., MALDONADO R., VALVERDE O., KITCHEN I., BEFORT K., DIERICH A., LE MEUR M., DOLLÉ P., TZAVARA E., HANOUNE J., ROQUES B.P., KIEFFER B.L. 1996. Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature **383**: 819-823.

MCNALLY G.P., AKIL H. 2002. Role of corticotropin-releasing hormone in the amygdala and bed nucleus of the stria terminalis in the behavioral, pain modulatory, and endocrine consequences of opiate withdrawal. Neuroscience **112**: 605-617.

NIETO M.M., WILSON J., CUPO A., ROQUES B.P., NOBLE F. 2002. Chronic morphine treatment modulates the extracellular levels of endogenous enkephalins in rat brain structures involved in opiate dependence: A Microdialysis Study. J. Neurosci. **22**: 1034-1041.

OLSON B.J., MARKWELL J. 2007. Assays for the determination of protein. Curr. Prot. Protein Sci. **48**: 14-17.

OWENS P.C., CHAN E.C., LOVELOCK M., FALCONER J., SMITH R. 1988. Immunoreactive methionine-enkephalin in cerebrospinal fluid and blood plasma during acute stress in conscious sheep. Endocrinology **122**: 311-318.

PASTERNAK G., PAN Y.-X. 2011. Mu opioid receptors in pain management. Acta Anaesthesiol. Taiwan **49**: 21-25.

PFEIFFER A., BRANTL V., HERZ A., EMRICH H.M. 1986. Psychotomimesis mediated by kappa opiate receptors. Science **233**: 774-776.

PIERZCHALA K., HOUDI A.A., VAN LOON G.R. 1987. Nicotine-induced alterations in brain regional concentrations of native and cryptic Met- and Leu-enkephalin. Peptides **8**: 1035-1043.

PIERZCHAŁA K., VAN LOON G.R. 1990. Plasma native and peptidase-derivable Met-enkephalin responses to restraint stress in rats. Adaptation to repeated restraint. J. Clin. Invest. **85**: 861-873.

PIERZCHAŁA-KOZIEC K., DZIEDZICKA-WASYLEWSKA M., OELTGEN P. 2000. Corticotrophin releasing hormone modulates opioid receptor binding in the hypothalamic-pituitary-adrenal axis in the ewes. Soc. Neurosci: 434.2.

PIERZCHAŁA-KOZIEC K., ZUBEL J., RZĄSA J. 2006. Effect of prolonged progesterone treatment on the proenkephalin

mRNA gene expression and enkephalins concentration in the sheep brain. Reprod. Biol. **6**: 37-46.

PIERZCHAŁA-KOZIEC K., DZIEDZICKA-WASYLEWSKA M., OELTGEN P., ZUBEL-ŁOJEK J., LATACZ A., OCŁOŃ E. 2015. The effect of CRH, dexamethasone, and naltrexone on the mu, delta and kappa opioid receptor agonist binding in lamb hypothalamic-pituitary-adrenal axis. Folia Biol. (Kraków) **63**: 187-193.

PRADHAN A.A., BECKER J.A., SCHERRER G., TRYOEN-TOTH P., FILLIOL D., MATIFAS A., MASSOTTE D., GAVÉRIAUX-RUFF C., KIEFFE B.L. 2009. *In vivo* delta opioid receptor internalization controls behavioral effects of agonists. Plos ONE **4**: e5425.

PRADHAN A.A., SMITH M.L., KIEFFER B.L., EVANS Ch.J. 2012. Ligand-directed signaling within the opioid receptor family. Brit. J. Pharmacol. **167**: 960-969.

RUSSELL J.A., DOUGLAS A.J., BRUNTON P.J. 2008. Reduced hypothalamo-pituitary-adrenal axis stress responses in late pregnancy: central opioid inhibition and noradrenergic mechanisms. Ann. N Y Acad. Sci. **1148**: 428-438.

SMITH S.M., VALE W.W. 2006.The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues Clin Neurosci. **8**: 383-395.

STEIN C., ZÖLLNER C. 2009. Opioids and sensory nerves. Handb. Exp. Pharmacol. **194**: 495-518.

TAYLOR C.C., WU D., SOONG Y., YEE J.S., SZETO H.H. 1997. Opioid modulation of the fetal hypothalamic-pituitary-adrenal axis: the role of receptor subtypes and route of administration. J. Pharmacol. Exp. Ther. **281**: 129-135.

VAN BOCKSTAELE E.J., PEOPLES J., MENKO A.S., MCHUGH K., DROLET G. 2000. Decreases in endogenous opioid peptides in the rat medullo-coerulear pathway after chronic morphine treatment. J. Neurosci. **20**: 8659-8666.

VANDERAH T. W. 2010. Delta and kappa opioid receptors as suitable drug targets for pain. Clin. J. Pain **26**: S10-S15.

VAN LOON G.R., PIERZCHALA K., HOUDI A.A., KVETNANSKÝ R., ZEMAN P. 1990. Tolerance and cross-tolerance to stress-induced increases in plasma Met-enkephalin in rats with adaptively increased resting secretion. Endocrinology **126**: 2196-2204.

VAN'T VEER A., CARLEZON W.A. Jr. 2013. Role of kappa-opioid receptors in stress and anxiety-related behavior. Psychopharmacology (Berl.). **229**: 435-452.

VAN'T VEER A., YANO J.M., CARROLL F.I., COHEN B.M., CARLEZON W.A. Jr. 2012. Corticotropin-Releasing Factor (CRF)-induced disruption of attention in rats is blocked by the kappa-opioid receptor antagonist JDTic. Neuropsychopharmacology **37**: 2809-2816.

WANG J., FANG Q., LIU Z., LU L. 2006. Region-specific effects of brain corticotropin-releasing factor receptor type 1 blockade on footshock-stress- or drug-priming-induced reinstatement of morphine conditioned place preference in rats. Psychopharmacology **185**: 19-28.

WANG X.M., TRESHAM J.J., SCOGGINS B.A., COGHLAN J.P. 1988. Met-enkephalin and the enkephalin analogue FK-33824 centrally inhibit adrenocorticotrophic hormone secretion in sheep. Clin. Exp. Pharmacol. Physiol. **15**: 865-873.

WEEMS P.W., WITTY C.F., AMSTALDEN M., COOLEN L.M., GOODMAN R.L., LEHMAN M.N. 2016. κ-Opioid Receptor Is Colocalized in GnRH and KNDy Cells in the Female Ovine and Rat Brain. Endocrinology **157**: 2367-2379.

XU G. P., VAN BOCKSTAELE E., REYES B., BETHEA T., VALENTINO R. J. 2004. Chronic morphine sensitizes the brain norepinephrine system to corticotropin-releasing factor and stress. J. Neurosci. **24**: 8193-8197.

YUKHANANOV R.Y., HANDA R.J. 1997. Effect of morphine on proenkephalin gene expression in the rat brain. Brain Res. Bull. **43**: 349-56.