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The Effect of CRH, Dexamethasone and Naltrexone on the Mu, Delta and Kappa Opioid Receptor Agonist Binding in Lamb Hypothalamic-Pituitary-Adrenal Axis*

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The aim of the study was to evaluate changes in the opioid receptor binding (mu, delta and kappa) in the hypothalamus, anterior pituitary and adrenal cortex (HPA) of lambs treated *in vivo* with corticotrophin releasing hormone (CRH), naltrexone, an opioid receptor antagonist (NAL), and dexamethasone, a potent cortisol analog (DEX). Experiment was carried out on 3 months old female lambs of polish mountain strain. Lambs received a single i.v. injection of NaCl (control), CRH (alone or in combination with naltrexone), naltrexone or dexamethasone. One hour later animals were decapitated under anaesthesia, tissues were dissected out and receptor binding assays were performed with radioligands for each type of opioid receptors – ^{3}H-DAGO, ^{3}H-DPDPE and ^{3}H-EKC for mu, delta and kappa receptor, respectively. Coexistence of specific binding sites for each type of opioid receptor was demonstrated in all levels of HPA axis of control lambs, however their distribution was uneven. Acute treatment with CRH, DEX and NAL caused downregulation or upregulation of mu, delta, kappa receptor binding in each level of HPA axis. CRH effects on mu, delta and kappa opioid receptor binding varied within the HPA axis and were modulated by naltrexone. Treatment with naltrexone increased *in vitro* mu, delta and kappa receptor binding in most tested structures except delta receptor binding in adrenal (decrease by 52%) and kappa receptor binding in pituitary (decrease by 41%). Dexamethasone significantly decreased the mu, delta and kappa opioid receptor binding in adrenal cortex but differentially affected opioid receptor binding in hypothalamus and pituitary. It seems probable that endogenous opioid peptides acting through mu, delta and kappa receptors interact with the hormones released from the hypothalamic-pituitary-adrenal axis in physiological and pathophysiological situations.

Key words: Opioid receptors binding, HPA axis, antagonist, lambs.

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Endogenous opioid peptides and their receptors are broadly expressed throughout peripheral and central nervous systems and have been investigated for several decades. The opioid system plays a central role in nociception and analgesia, and also regulates numerous physiological functions as respiration, gastrointestinal transit and response to stress, as well as endocrine and immune functions (BODNAR 2013). Since 1992 when the first opioid receptor gene was identified by expression cloning several homologous genes were discovered. The opioid receptor gene family consists of three members encoding mu (MOR, Oprm1), delta (DOR, Oprd1), kappa (KOR, Oprk1) receptors and recently the

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nonopioid orphaninFQ/nociceptin (NOR, Oprl1) receptor was included. These receptors are membrane receptors with a seven-transmembrane topology and belong to the large G protein-coupled receptor superfamily (BODNAR 2013).

The first endogenous ligands for delta receptors characterized by HUGHES *et al*. (1975) were two pentapeptides, Met- and Leu-enkephalin. Since then, many peptides forming the opioid peptide families of enkephalins, dynorphins, and β-endorphin were discovered. Opioid peptides are synthesized as large protein precursors named as preproenkephalin (PENK), preprodynorphin (PDYN), and proopiomelanocortin (POMC), respectively. However, in spite of many research on the opioid receptors cloning, distribution in the nervous system and peripheral tissues, only then in 2012 four papers reported crystal structures that provide the first direct evidence for the binding mode of opioids to their receptors (GRANIER *et al.* 2012; MANGLIK *et al.* 2012; THOMPSON *et al.* 2012; WU *et al.* 2012).

Maintenance of homeostasis in the presence of variety of challenges requires activation of a complex responses of the endocrine, nervous, and immune systems, known as the stress response. Disturbance of the stress response has been linked to a wide array of pathologies mainly autoimmune disease, hypertension, affective disorders, and major depression (KOOB 1999; BODNAR 2013).

Opioid peptides are also involved into mechanism activated during stress response, such as the regulation of the hypothalamic-pituitary-adrenal axis (HPA) activity. Opioid-containing neurons (mainly endorphins and enkephalins) innervate the paraventricular nucleus, the median eminence and affected adrenocorticotrophin hormone (ACTH)-controlling neurons (SMITH & VALE 2006; LEMERRER *et al.* 2009; VANVEER *et al.* 2012). Dynorphin, an endogenous ligand for kappa receptors, as well as endorphin (mu receptor ligand) and enkephalins (delta receptor agonists) are localized with corticotrophin releasing hormone (CRH) in hypothalamus and probably are simultaneously secreted to modulate ACTH release (BRUCHAS *et al.* 2009; LEMOS *et al.* 2012; FUNK *et al.* 2014). Our previous experiments showed that emotional stress evoked by isolation of animals from the herd, without acoustic and visual contact, significantly changed the concentration of opioids in the all levels of hypothalamic-pituitary-adrenal axis and decreased delta receptor binding in the hypothalamus (PIERZCHAŁA-KOZIEC *et al.* 2006). Simultaneously, increased plasma levels of ACTH and cortisol were observed what might suggest an interaction of the opioid system and stress hormones. Sheep are useful animal model for the studying the effects of emotional stress due to their vulnerability to such stressors, however, no complex analysis of opioid receptor binding in stressed sheep has been performed so far. In order to minimize the side effects of environmental stress factors (induced for example by lamb isolation) direct treatment with known concentrations of HPA axis hormones is much suitable and may give much faster response of endogenous opioid peptides and their receptors.

Thus, the aim of the study was to evaluate mu-, delta- and kappa-opioid receptor binding in the hypothalamus, anterior pituitary and adrenal cortex of lambs treated *in vivo* with corticotrophin releasing hormone, dexamethasone, a potent cortisol analog, and with opioid receptor antagonist-naltrexone.

Material and Methods

Animals

Experiment was carried out on thirty 3 months old lambs (females) of polish mountain breed (Polish Mountain Sheep). Animals were housed in individual cages under constant conditions of temperature (18ºC), humidity and 12L:12D cycle (light on 7a.m. to 7 p.m.) for one week before the study. Animals had free access to water and feed.

The protocol was approved by the First Local Ethical Committee on Animal Testing in Kraków (64/OP/2005/I LKE).

Lambs were divided into 5 groups (n=6) and injected intravenously at 9 a.m. with either 0.9% NaCl (control), or naltrexone hydrochloride (3 mg/kg b.w. SIGMA), corticotrophin releasing hormone (CRH, rat, 1 μg/kg b.w. SIGMA), CRH with naltrexone and dexamethasone (Dexaven, 0.1 mg/kg b.w. PHARMASWISS Czech Republic). One hour later, lambs were decapitated under anaesthesia and fragments of hypothalamus, anterior pituitary and adrenal cortex were dissected out. Tissues were immediately frozen on dry ice and stored at -70ºC until receptor binding assays were performed.

Receptor binding assays

Receptor binding assays were performed according to procedures reported elsewhere (BELCHEVA *et al.* 1994; HYTREK *et al.* 1996) with some modification. According to results of pilot study establishing K_d and B_{max}, optimal levels of specific ligands for mu, kappa and delta receptors were chosen (data not shown). Briefly, the dissected tissues were homogenized in ice-cold buffer 50 mM Tris-HCl, pH=7.4. The homogenate was centrifuged at 20000xg for 15 min at 4°C and cell mem-

brane preparations (1 ml, 1 mg of protein, in triplicate) were incubated at 30ºC for 30 min with 200 μl of 26.00 nM of ^{3}H-DAGO (D-Ala^2, Me-Phe^4, Gly(ol)^5 enkephalin for mu receptor), 6.80 nM of ^{3}H-DPDPE (D-Ala^2-,N-Me-Phe^4,Gly-ol for delta receptor subtype) and 59.18 nM of ^{3}H-EKC (ethylketocyclazocine for kappa receptor). Radioligands were purchased from Amersham International (^{3}H-DAGO, ^{3}H-DPDPE) and from New England Nuclear (^{3}H-EKC). The pilot study gave the reason to unify the concentrations of unlabeled ligands used for the nonspecific binding for all three types of opioid receptors (unpublished data). Nonspecific binding was obtained with 10 μM of unlabeled ligands: morphine for mu, Met-enkephalin for delta and Leu-enkephalin-Arg for kappa receptors (Sigma) incubated together with the radioligands. Free ligand was separated from membrane bound radioligand by filtration under reduced pressure through GF/B Whatman glass filters in the room temperature for 1 min. Protein concentration was determined by the BCA method (OLSON & MARKWELL 2007).

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keul's post hoc test.

Results

Mu receptor binding (Fig. 1).

The mu receptor binding of ^{3}H-DAGO in control animals was the lowest in pituitary – 2.30±0.21 fmol/mg protein compared to 5.11±0.31 fmol/mg protein in hypothalamus and 10.02±0.92 fmol/mg protein in adrenal cortex. CRH increased the receptor binding in hypothalamus by 49% (from 5.11±0.31 to 7.60±0.62 fmol/mg protein, P<0.05) and downregulated the binding by 48% in pituitary (decrease from 2.30±0.21 to 1.18±0.10 fmol/mg protein, P<0.05) and by 44% in adrenal cortex (decrease from 10.02±0.92 to 5.60±0.47 fmol/mg protein, P<0.05). Naltrexone potentiated the effect of CRH in hypothalamus what resulted in significant increase of mu receptor binding by 131% compared to control value (increase to the level of 11.80±1.01 fmol/mg protein, P<0.05). Opioid antagonist did not change the effect of CRH in pituitary MOR binding and only slightly increased by 19% the mu receptor binding in the adrenal cortex (P<0.05). Treatment with naltrexone increased the mu receptor binding in hypothalamus by 191%, in anterior pituitary by 136% and in adrenal cortex by 91% (P<0.05). Injection of dexamethasone significantly increased the mu receptor binding in hypothalamus (by 172%), pituitary (by 95%) but decreased the binding by 51% in adrenal cortex (P<0.01).

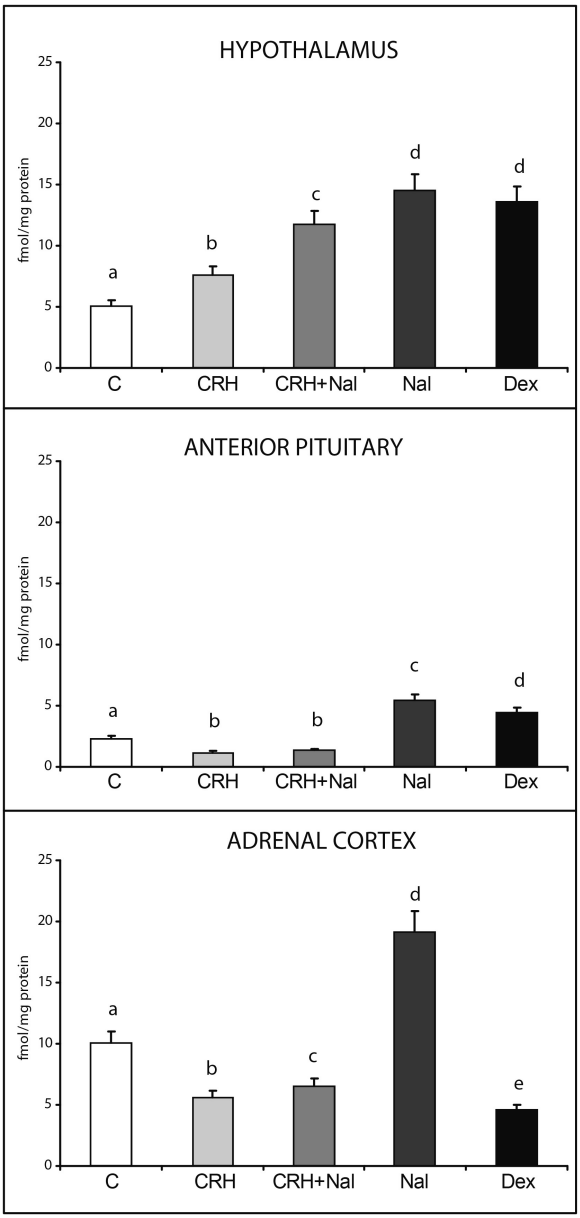


Fig. 1. ^{3}H-DAGO binding to cell membrane preparations from lamb hypothalamus, anterior pituitary and adrenal cortex (fmol/mg of proteins). C-control (saline injected), CRH-(corticotrophin releasing hormone injected), CRH+NAL-(corticotrophin releasing hormone plus naltrexone injected), NAL-(naltrexone injected), DEX-dexamethasone-treated animals. Each bar represents the mean of six animals±SEM. Means with superscripts a,b,c,d,e differ significantly at the P<0.05.

Delta receptor binding (Fig. 2).

The delta receptor binding of ^{3}H-DPDPE in control animals ranged from 55.31±5.18 in hypothalamus to 45.44±4.17 in anterior pituitary and to

44.03±3.91 fmol/mg protein in adrenal cortex. Acute treatment with CRH alone or in combination with naltrexone as well as injection with dexamethasone significantly downregulated the receptor binding (20-90%) in all tested tissues (P<0.05). The strongest effect of CRH on the delta receptor binding was seen in hypothalamus (decrease by 72%) compare to anterior pituitary and adrenal cortex (receptor binding fall by 46%, P<0.05). Naltrexone potentiated that inhibiting effect of CRH on the receptor binding only in the adrenal (by 35%). A single injection of naltrexone upregulated the delta receptor binding in the hypothalamus (increase from 55.31±5.81 to 90.33±10.21 fmol/mg protein, 67%, P<0.05) and caused mild increase in anterior pituitary (by 22%, P<0.05). In contrast, injection of antagonist resulted in significant (by 50%) reduction of delta receptor binding in adrenal cortex from 44.03±3.92 to 22.11±2.32 fmol/mg protein (P<0.05).

Kappa receptor binding (Fig. 3).

The kappa receptor binding of ^{3}H-EKC varied from 18.82±1.53 fmol/mg protein in hypothalamus to 75.04±5.36 in anterior pituitary and to

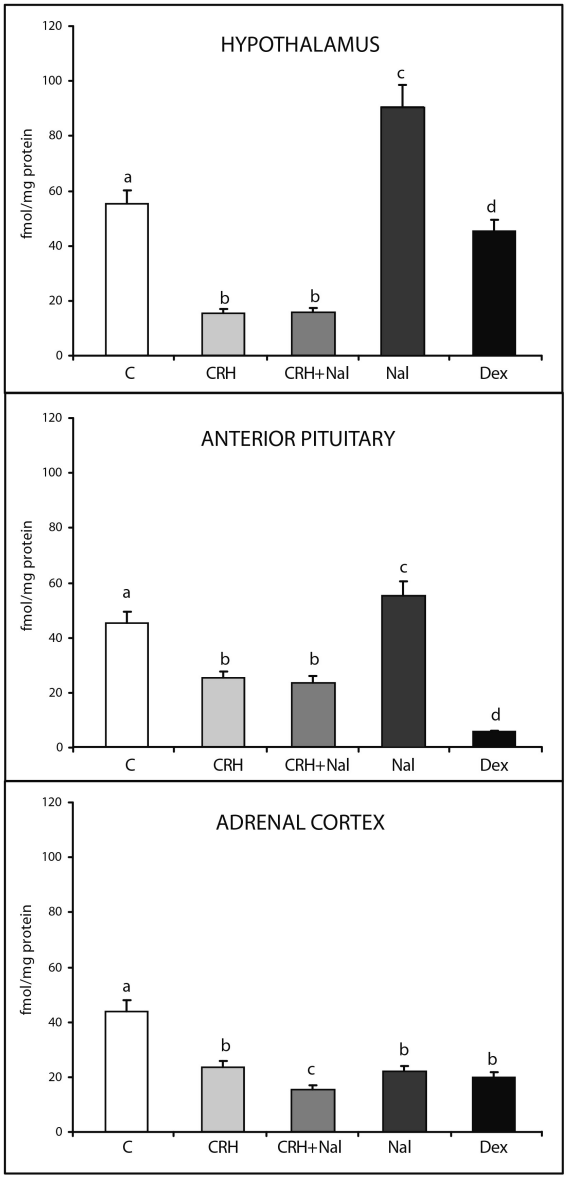


Fig. 2. ^{3}H-DPDPE binding to cell membrane preparations from lamb hypothalamus, anterior pituitary and adrenal cortex (fmol/mg of proteins). C-control (saline injected), CRH-(corticotrophin releasing hormone injected), CRH+NAL-(corticotrophin releasing hormone plus naltrexone injected), NAL-(naltrexone injected), DEX-dexamethasone-treated animals. Each bar represents the mean of six animals±SEM. Means with superscripts a,b,c,d differ significantly at the P<0.05.

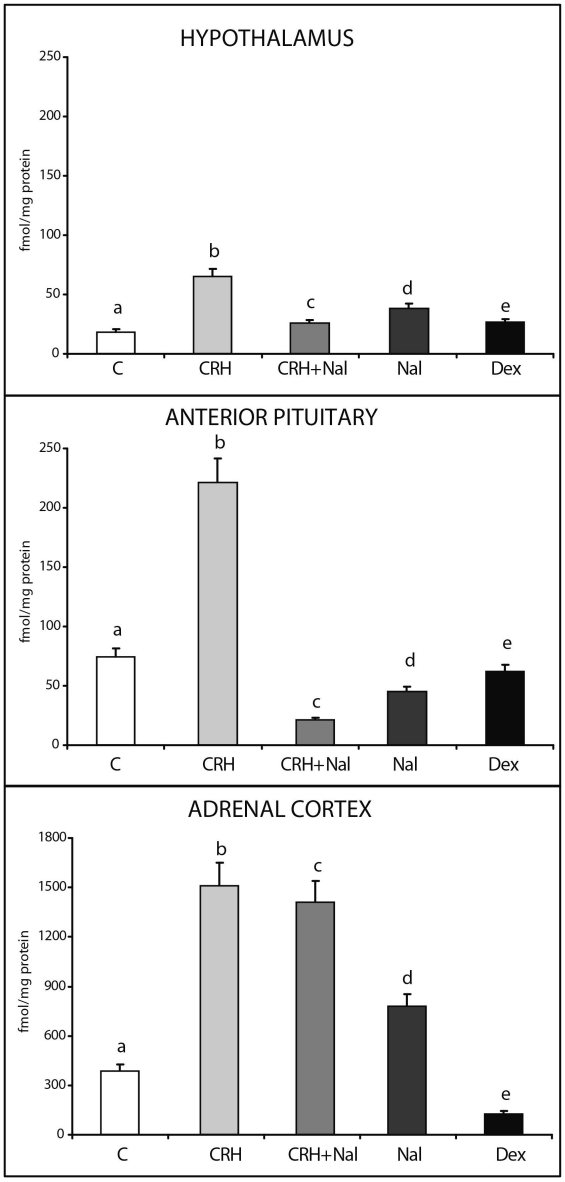


Fig. 3. ^{3}H-EKC binding to cell membrane preparations from lamb hypothalamus, anterior pituitary and adrenal cortex (fmol/mg of proteins). C-control (saline injected), CRH-(corticotrophin releasing hormone injected), CRH+NAL-(corticotrophin releasing hormone plus naltrexone injected), NAL-(naltrexone injected), DEX-dexamethasone-treated animals. Each bar represents the mean of six animals±SEM. Means with superscripts a,b,c,d,e differ significantly at the P<0.05.

390.12±41.21fmol/mg protein in adrenal cortex of control lambs (P<0.05). Corticotrophin releasing hormone significantly increased the kappa receptor binding in every level of HPA axis by 2-4 folds (P<0.05). Naltrexone in combination with CRH reversed the stimulating effect of CRH in anterior pituitary (completely) and adrenal cortex (slightly). A single injection of naltrexone significantly increased the kappa receptor binding in hypothalamus and adrenal, and downregulated in pituitary (P<0.05). Dexamethasone increased the kappa receptor binding in hypothalamus (by 43%, P<0.05) but decreased in pituitary (by 20%, P<0.05) and in adrenal cortex (by 67%, P<0.05). Interestingly, kappa receptor binding was much higher in the adrenal cortex than in hypothalamus and anterior pituitary, and varied from 390.12±41.21 in control lambs to 1514.33±99.23 fmol/mg protein in adrenal from CRH treated animals, and to 780.45±101.27 fmol/mg protein in adrenal taken from naltrexone injected lambs.

Discussion

The distribution of opioid binding sites in the brain was analyzed by different methods in rats (UNDERWALD *et al.* 1998; WAND *et al.* 2012), mice (BRADY *et al.* 1999), guinea pig (LEWIS *et al.* 1985; FOOTE & MAURER 1986). In our study, the coexistence of specific binding sites for mu, delta and kappa opioid receptors was demonstrated in each level of hypothalamic-pituitary-adrenal axis in control lambs, however their distribution was uneven. The ^{3}H-DPDPE binding to DOR was about 20% higher in hypothalamus than in pituitary or adrenals. Unexpectedly, ^{3}H-EKC binding to kappa sites in the adrenal cortex was 18 and 5 fold higher than in hypothalamus and in pituitary, respectively. An unequal distribution of the opioid receptors and their mRNA's was also found in the rat study (GEORGE *et al.* 1994; MANSOUR *et al.* 1994; GERRA *et al.* 2006). They showed the highest expression of mu receptor mRNA in several thalamic nuclei together with increased binding properties of that receptor compared to pituitary. In contrast, expression of delta receptor mRNA and the agonist binding were much lower in the hypothalamus. Distribution of kappa receptor mRNA in hypothalamus was quite similar to that seen for mu receptor, however the agonist binding was found only in several hypothalamic nuclei.

Similarly to the varied radioligand receptors binding in control lambs, we found inconsistent reaction to the i.v. of drug injection. Generally, corticotrophin releasing hormone increased the kappa agonist binding and decreased delta agonist binding in all tested tissues. CRH also increased the MOR binding in hypothalamus but caused significant fall of the binding in anterior pituitary and adrenal cortex. It seems probable that CRH stimulated endogenous enkephalins release which were bound to delta receptors not only in hypothalamus but also in the pituitary and adrenal cortex, where the ^{3}H-DPDPE binding *in vitro* was much lower than in control animals. Agonist activation of the delta opioid receptor is able to initiate G-protein-dependent and independent signaling pathway. It was found that agonist induced activation of the delta opioid receptor leads to receptor desensitization and finally to internalization (PRADHAN *et al.* 2009).

The presence of kappa opioid receptor in the lamb hypothalamus was proved in several experiments what may suggest an important role of dynorphin in the modulation of hypothalamic neuropeptides secretion (DEPAOLI *et al.* 1994; FUNK *et al*. 2014). In the present experiment, kappa receptor binding in the hypothalamus was much lower than in pituitary and adrenal but every treatment evoked significant increase of KOR in this structure. Similar results were showed by BOYADJIEVA *et al.* (2004) in experiment testing the opioid effects on immune system at the brain level. In contrast, the stimulating effect of acute CRH injection on the ^{3}H-EKC binding was much more potentiated in the anterior pituitary and adrenal cortex compared to the reaction seen in the hypothalamus. It seems probable, that increased level of CRH caused upregulation of kappa receptors and/or inhibition of dynorphin release from the nerves endings. This effect was similar to that seen after naltrexone, an opioid receptors antagonist, treatment. Previously, it was found that exercise stress (long walking of sheep) and restraint stress in hens which were accompanied by increased level of CRH significantly lowered the plasma level of dynorphin-like peptide (PIERZCHALA & PRZEWLOCKI 1989; PIERZCHALA-KOZIEC *et al.* 1999).

Previous studies in our laboratory and elsewhere have established that the stress-related corticotrophin releasing hormone is a critical mediator of responses to different stressors including isolation, restraint, footshock and yohimbine (PIERZCHALA-KOZIEC *et al.* 1999; LE *et al.* 2000; MARINELLI *et al.* 2007). Recent work has begun to determine how CRH may interact with other neurotransmitters in stress-related behaviors such as anxiety and place aversion. Results from MCLAUGHLIN *et al.* (2006) showed that one of the neurotransmitter, the endogenous opioid dynorphin and its receptor (kappa opioid receptor) are involved in responses to stress. LAND *et al.* (2008) prooved that dynorphin is involved in motivation to seek alcohol and other drugs (WALKER *et al.* 2011; SCHANK *et al.* 2012). Although KOR and CRH receptors have been shown to interact in stress-related behaviors (LAND *et al.* 2008), little is known about how they

may interact in stress-induced reinstatement of mood and emotional disturbances.

The mu receptor binding performed with ^{3}H-DAGO in *in vitro* conditions was increased in hypothalamus and anterior pituitary of lambs treated by naltrexone what might indicated that this antagonist caused MOR desensitization. LAM *et al.* (2011) suggested that constitutive activity at the mu receptor can be reversed by naltrexone which is also proposed as an inverse agonist. However, in the present study, in hypothalamus and in adrenal cortex the join effect of CRH and naltrexone caused an increase of the mu receptor binding compared to CRH-alone treated lambs. In both tissues the level of agonist binding was significantly higher than in CRH-treated group.

Endogenous opioidergic systems, especially pro-opiomelanocortin (POMC)-derived beta-endorphin, exert inhibitory effects on the hypothalamic-pituitary-adrenal (HPA) axis. Beta-endorphin immunoreactive fibers and corticotrophin-releasing hormone perikarya are colocalized in the paraventricular nucleus (PVN) of the hypothalamus.To exert a tonic inhibition on CRH neuronal activity, it is suggested that beta-endorphin acts primarily at the mu opioid receptor. It might be also postulated that CRH inhibited the mu receptor activity through paracrine mode.

Glucocorticoid treatment and stress have been reported to augment the reinforcing and locomotor-activating properties of opiates, cocaine and amphetamine (KALIVAS & STEWART 1991). Opiate like peptides such as endorphins, enkephalins and dynorphins were changed concomitantly with glucocorticoids during stress in rats (PIERZCHALA & VAN LOON 1990) and lambs (PIERZCHALA-KOZIEC *et al*. 2006). Intravenous injection of dexamethasone increased the mu receptor binding in hypothalamus and pituitary, what might be the effect of proopiomelanocortin (precursor of ACTH and beta-endorphin) synthesis and/or inhibition of opioid secretion. On the other hand, injection of dexamethasone resulted in lower mu, delta and kappa tritiated agonists receptor binding in lamb adrenal cortex. Thus, it appears that in peripheral level of HPA axis, dexamethasone caused down-regulation of all three classes of opioid receptors.

The present data confirm the previous findings (PIERZCHALA-KOZIEC *et al.* 2000) that three major classes of receptors and their activity are distinct from each other. Thus, it seems probable that during stress reaction, when CRH, ACTH and glucocorticoids are elevated, endogenous opioid peptides modulated the regulatory mechanism by different separable binding to specific receptors at the central and peripheral levels. Our results observed after i.v. injection of CRH and DEX are in agreement with those of PRADHAN *et al.* (2012) and also indicated that there is independent modulation of the hypothalamic-pituitary-adrenal axis by endogenous opioid peptides at μ-, δ- and κ-opioid receptors.

In contrast, recent studies on mice demonstrated the potential heteromeric interaction between delta and mu receptors *in vivo* through the use of monoclonal antibodies, where chronic morphine exposure upregulated their expression (GUPTA *et al.* 2010; HE *et al.* 2011). However, in light of these findings, mu and delta heteromers might be promising and desirable targets mainly for the pharmacological management of chronic or neuropathic pain (ROZENFELD & DEVI 2010) and treatment of addiction disorders (STOCKTON & DEVI 2012).

In conclusion, corticotrophin releasing hormone effects on mu, delta and kappa opioid receptors binding appeared to be dependent on the level of HPA axis and were modulated by naltrexone. Dexamethasone, a potent cortisol analog decreased the mu, delta and kappa opioid receptor binding in adrenal cortex. It seems probably that opioid receptors play differential roles in modulating neuronal activity across different brain and peripheral regions.

In particular, the evaluation of opioid interaction with the hormones of HPA axis may provide new elements for the development of mu, delta or kappa receptors-based therapeutic strategies.

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