THE EFFECTS OF THE UROCORTINS AND THE ENDOMORPHINS ON THE MEDIATORS OF THE STRESS RESPONSE

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CONTENTS

PUBLICATIONS

1. Original publications the present work is based on:

I. Bagosi, Z., Jászberényi, M., Bujdosó, E., Telegdy, G., *The effects of corticoptropin-releasing factor and the urocortins on striatal dopamine release induced by electrical stimulation - an in vitro superfusion study.* (Neurochemical Research, 2006; 31:209-13.) IF: 2,139 C:4

II. Bagosi, Z., Jászberényi, M., Bujdosó, E., Szabó, G., Telegdy, G., *The effects of endomorphins and diprotin A on striatal dopamine release induced by electrical stimulation - an in vitro superfusion study in rats.* (Neurochemistry International, 2006; 49:665-8.) IF: 3,159 C:6

III. Bagosi, Z., Jászberényi, M., Szabó, G., Telegdy, G., *The effects of CRF and the urocortins on [3H]GABA release from the rat amygdala - An in vitro superfusion study.* (Brain Research Bulletin, 2008; 75:15-7.) IF: 1,943 C:2

IV. Bagosi, Z., Jászberényi, M., Telegdy, G., *The effects of endomorphins on striatal [3H]GABA release induced by electrical stimulation - an in vitro superfusion study in rats.* (Neurochemical Research, 2009; 34:905-8.) IF: 2,139 C:1

2. Original publications cited in the present work:

I. Jászberényi, M., Bujdosó, E., **Bagosi, Z.**, Telegdy, G., *Mediation of behavioral, endocrine and thermoregulatory actions of ghrelin.* (Hormones and Behavior, 2006; 50:266-73.) IF: 3,401 C:9

II. Jászberényi, M., **Bagosi, Z.**, Thurzó, B., Földesi, I., Telegdy, G., *Endocrine and behavioral effects of neuromedin S.* (Hormones and Behavior, 2007; 52:631-9.) IF: 3,401 C:6

III. Jászberényi, M., **Bagosi, Z.**, Thurzó, B., Földesi, I., Szabó, G., Telegdy, G., *Endocrine, behavioral and autonomic effects of neuropeptide AF*. (Hormones and Behavior, 2009; 56:24-34.) IF: 3,401 C:0

3. Poster presentations related to the present work:

I. Bagosi, Z., Jászberényi, M., Bujdosó, E., Telegdy, G.: *The effect of endomorphins on ^{3}H-dopamine release from rat striatal slices evoked by electric impulse* (MÉT 2004)

II. Bagosi, Z., Jászberényi, M., Bujdosó, E., Telegdy, G., *The effects of corticotropin-releasing factor and urocortins on striatal dopamine release induced by electric stimulation* (MITT 2005)

III. Bagosi, Z., Jászberényi, M., Bujdosó, E., Telegdy, G., *The actions of selective m opioid receptor antagonists on striatal dopamine release increased by endomorphins* (MÉT 2005)

IV. Bagosi, Z., Jászberényi, M., Telegdy, G., *The actions of selective CRF receptor antagonists on striatal dopamine release increased by CRF and urocortin* (MÉT 2006)

V. Bagosi, Z., Jászberényi, M., Szabó, G., Telegdy, G., *The effects of cortiotropin-releasing factor peptide family on [3H]GABA release from electrically stimulated rat amygdala* (MITT 2007)

VI. Bagosi, Z., Jászberényi, M., Szabó, G., Telegdy, G., *The actions of the selective CRF receptor antagonists on the rat amygdalar transmitter release evoked by electrical stimulation and enhanced by CRF-related peptides* (MÉT 2007)

VII. Bagosi, Z., Jászberényi, M., Telegdy, G., *The effects of the endomorphins on the striatal neurotransmitters* (BKT 2008)

VIII. Bagosi, Z., Jászberényi, M., Szabó, G., *The actions of antalarmin on the consequences of nicotine withdrawal* (MÉT 2008)

IX. Bagosi, Z., Jászberényi, M., Dochnal, R., Szabó, G., *Actions and interactions of ghrelin and nicotine on dopamine released from mouse striatum and amygdala* (MÉT 2009)

X. Bagosi, Z., Simon, B., Jászberényi, M., Szabó, G., *The effects of nicotine withdrawal on the rat limbic system: increase of dopamine release in the striatum and increase of GABA release in the amygdala* (MÉT 2010)

XI. Bagosi, Z., Csabafi, K., Telegdy, G., Szabó, G., *The actions of the urocortins on the mediators of stress response* (MITT 2011)

XII. Bagosi, Z., Csabafi, K., Jászberényi, M., Földesi, I., Gardi, J., Telegdy, G., Szabó, G., *The actions of the endomorphins and the urocortins on the hypothalamic amounts of CRF and AVP in rats* (MÉT 2011)

TOTAL IMPACT FACTOR: **19,583**

TOTAL CITATION: **28 (26)**

ABBREVIATIONS

[^{3}H]DA = tritium-labelled dopamine

[^{3}H]GABA = tritium-labelled gamma-amino-butyric acid

α-MSH = alpha-melanostimulating hormone

ACTH = adrenocorticotropic hormone

AVP = arginine-vasopressin

CNS = central nervous system

CPM = count per minute

CRF = corticotropin releasing factor

CRF-BP = corticotropin releasing factor-binding protein

CRFR = corticotropin releasing factor receptor

DAMGO = Tyr-D-Ala-Gly-MePhe-Gly-Ol

DOR = delta opioid receptor

DPPIV = dypeptidyl-amino-peptidase IV

DV = dorso-ventral

ELISA = enzyme-linked immuno-sorbent assay

EM = endomorphin

GPCRs = G protein-coupled receptors

HPA = hypothalamic-pituitary-adrenal

ICV = intracerebroventricular

IP = intraperitoneal

IR = immunoreactivity

KOR = kappa opioid receptor

ML= medio-lateral

MOR = mu opioid receptor

ORL = opioid receptor-like

POMC = proopiomelanocortin

RC = rostro-caudal

RIA = radio-immuno assay

SCP = stresscopin

SRP = stresscopin-related peptide

UCN = urocortin

1. INTRODUCTION

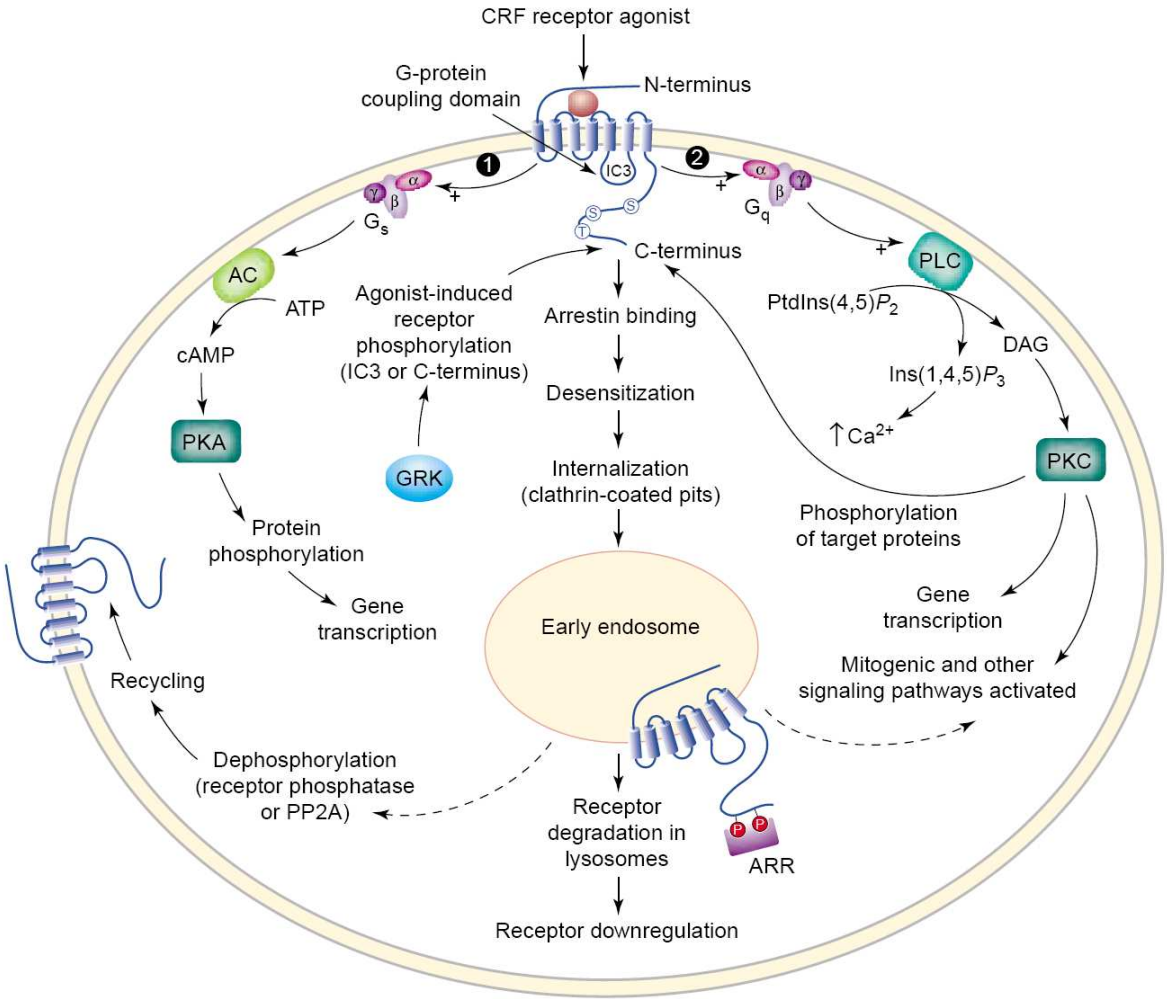
1.1. CRF receptor agonists

Corticotropin-releasing hormone (CRH), originally named corticotropin-releasing factor (CRF) and occasionally called corticoliberin, is a significant neurohormone of the hypothalamic-pituitary-adrenal (HPA) axis and also an important neurotransmitter released from hypothalamic and extrahypothalamic nuclei in mammals. Since CRF was first characterized **[1]**, a growing family of ligands and receptors has been discovered. The mammalian family members include CRF, urocortin I (UCN I) **[2]**, urocortin II (UCN II) **[3]**, also known as stresscopin-related peptide (SRP), and urocortin III (UCN III), also known as stresscopin (SCP) **[4]**, along with two CRF receptors, CRFR1 and CRFR2 **[5]**, and a CRF-binding protein (CRF-BP) **[6]**. These family members share common elements considering their aminoacidic composition and intracellular signalisation **(Figures 1-2)**, but show different aspects regarding their anatomical distribution **(Figures 3-4)** and physiological functions **(Figures 5-6).**

CRF is found mainly in the paraventricular nucleus of the hypothalamus (PVN), the central nucleus of the amygdala and hindbrain regions in the CNS, and in the gut, skin, and adrenal gland in the periphery. UCN I is predominantly expressed in cell bodies of the Edinger-Westphal nucleus in the brain. In the periphery, it has been found in the gastrointestinal tract, testis, cardiac myocytes, thymus, skin, and spleen. However, there is only 40% identity between CRF and UCN I at the aminoacid level. UCN II expression has been described in hypothalamus, brainstem, and spinal cord in the CNS, and in the heart, blood cells, and adrenal gland in the periphery. UCN III expression has been discovered in hypothalamus and amygdala in the CNS, and in the gastrointestinal tract and pancreas in the periphery. Neither UCN II nor UCN III binds the CRF-BP. While CRF has tenfold higher affinity for CRFR1 than for CRFR2, UCN I has equal affinities for both receptors. Though UCN II and UCN III appear to be selective for CRFR2, UCN II may activate CRFR1 at higher concentrations **[7]**.

Previously it has been suggested that CRF-related peptides could play important roles in the regulation of the endocrine, autonomic and behavioral responses to stress **[8]**. Although the role of CRF, activating CRFR1, is stimulatory upon stress responsivity, the role of UCN II and UCN III, both acting on CRFR2, appears to be inhibitory upon stress sensitivity **[9].**

Peptide	Sequence	Length	Identity (%)
hCRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII	41	100
oCRF	SQEPPISLDLTFHLLREVLEMTKADQLABQAHSNRKLLDIA	41	83
URO	NDDPPISIDLTFHLLRNMIEMARIENEREQAGLNRKYLDEV	41	54
hUCN	DNPSLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	40	43
SVG	ZGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI	40	48
hSRP	IVLSLDVPIGLLQILLEQARARAAREQATTNARILARV	38	34
mUCNII	VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV	38	34
hSCP	FTLSLDVPTNIMNLLFNIAKAKNLRAQAAANAHLMAQI	38	32
mUCNIII	FTLSLDVPTNIMNILFNIDKAKNLRAKAAANAQLMAQI	38	26



Figures 1-2

The biochemical composition and intracelullar signalisation of CRFR agonists

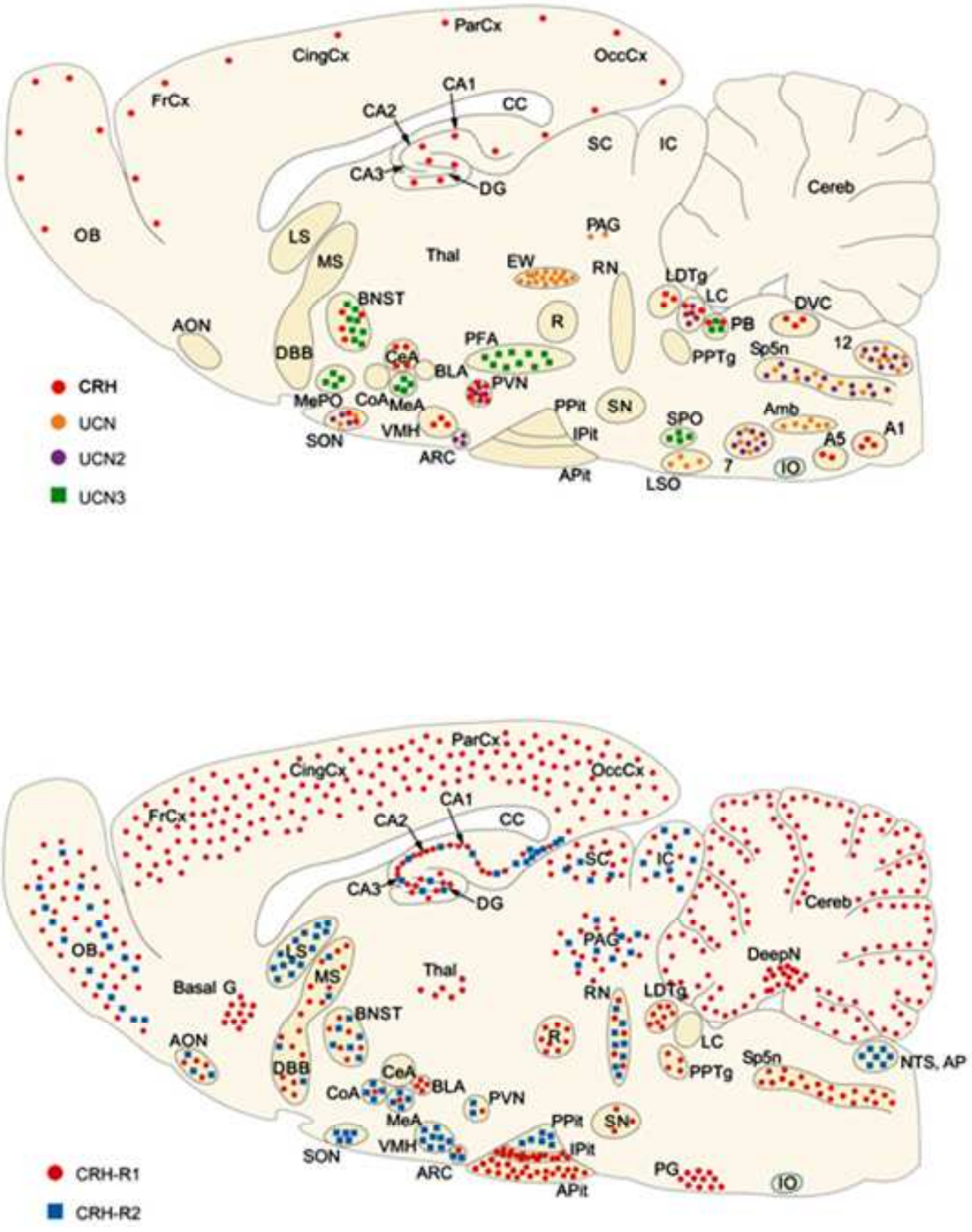
As the only ligand with equally high affinity for both receptors, UCN I's role may be promiscuous **[10-11]**. Lately, it has been proposed that the stress-like actions and reactions to these novel neuropeptides is stressor specific (physical and psychological) and species specific (mice and rats) **[12-13]**.

Recently, new physiological functions have been attributed to CRF-related peptides and receptors, including regulation of food intake and satiety **[14]**, modulation of gastrointestinal motility **[15]**, cardioprotection and vasodilation **[16].**

1.2. CRF receptors

CRFRs belong to the class B subtype of G protein–coupled receptors (GPCRs) **[17]**. CRFR1 and CRFR2 are produced from distinct genes and have several splice variants expressed in various central and peripheral tissues. CRFR1 has α and β isoforms in addition to subtypes designated c-h, which have been detected in human and rodent tissues. Several of these isoforms have been shown to be nonfunctional. CRFR2 is expressed in three functional subtypes, α, β, and γ. These isoforms differ in their N-terminal sequence as well as their distribution in both tissues and species. Both CRFR2α and CRFR2β have been detected in human and rodents. However, to date, CRFR2γ has only been reported in humans. There is nearly 70% identity between CRFR1 and CRFR2 at the aminoacid level. As is consistent with other GPCR family members, the transmembrane and intracellular domains of the CRFRs have the highest homology (over 80% identity). The third intracellular loop is the receptor region thought to interact with the G-proteins for most GPCRs. In the CRFR family, the third intracellular loops are identical between receptors. Specific sites of ligand action on CRFRs have been identified through mutagenesis and chimaeric-receptor studies in which the N terminus, second and third extracellular domains, and the N terminus juxtamembrane region have been shown to be important in determining the ligand binding and receptor specificity **[18].**

The anatomical distribution of these receptors completed the deduction of the physiological functions of their ligands**.** Both receptors are found in the CNS and the periphery, with CRFR1 being more abundant in the CNS and CRFR2 being predominent in the periphery. CRFR1 is distributed throughout the cerebral cortex, cerebellum, olfactory bulb medial septum, hippocampus, amygdala, and pituitary. Central CRFR2 is limited to sites in the lateral septum and hypothalamus, but is widely expressed in peripheral tissues, including the heart, gastro-intestinal tract, lung, skeletal muscle, and vasculature. The choroid plexus is also a major site of CRFR2 expression **[19]**.



Figures 3-4

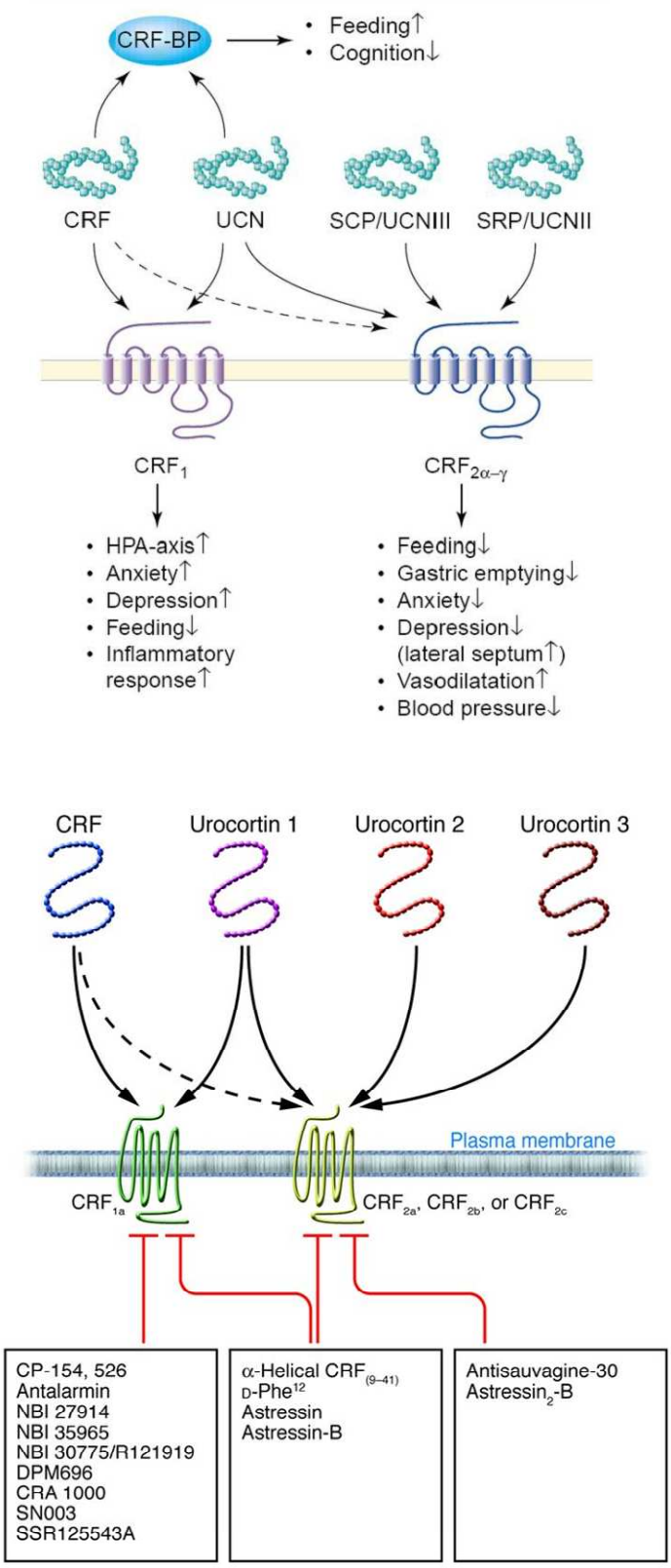
The anatomical distribution of the UCNs and CRFRs

Both CRF and UCN I have high affinities for the CRF-BP that is thought to modulate the endocrine activity of CRF. The CRF-BP is a 37-kDa N-linked glycoprotein expressed in rodent and primate brain and pituitary. In humans, CRF-BP is found in the liver and in the circulation and has been proposed to prevent inappropriate pituitary-adrenal stimulation during pregnancy. Recombinant CRF-BP has been shown to block CRF-induced adrenocorticotropic hormone (ACTH) secretion from rat anterior pituitary cells. CRF-BP has also been detected in brain regions not associated with CRF activity, suggesting that it may also have CRF-independent actions **[18, 20]**.

1.3. CRF receptor antagonists

The first CRFR antagonist synthesised and studied was α-helical CRF 9-41, that efficiently blocked CRF-induced ACTH secretion **[21]** and stress-induced locomotor activation **[22]**, followed by D-Phe CRF 12–41, a more potent antagonist of CRF, than α-helical CRF 9-41 **[23]**. Astressin, a novel CRFR antagonist, was found to be particularly potent at inhibiting the HPA axis. It could reverse the CRF- or stress-induced anxiogenic-like behaviour, but it could not prevent, CRF or stress-induced locomotor hyperactivity **[24]**. Both α-helical CRF 9-41, D-Phe CRF 12-41 and astressin are competitive and nonselective antagonists of CRF, though astressin seem to have a different pharmacologic profile.

CP-154,526 and its structurally related analog antalarmin are selective nonpeptidic CRFR1 antagonists used especially to characterize the central actions of CRFR1s. Both compounds were able to penetrate the blood-brain barrier and antagonize endocrine and behavioural effects of CRF, UCN I or stressors. Though results with CP-154,526 **[25]** may seem confusing, studies with antalarmin **[26-28]** may prove promising for future anxiolytic and antidepressant research **[29].** Antisauvagine 30 and astressin 2B, structurally derived from sauvagine and astressin, respectively, are selective peptidic CRFR2 antagonists **[30]** used preferentially to scrutinize the peripheral functions of CRFR2s **[31-32]**. Selective antagonistic studies suggested that CRF- and stress-induced opposite actions on upper and lower gut transit in mice are mediated by different CRF receptor subtypes: the activation of CRFR1 receptors stimulates colonic propulsive activity, whereas activation of CRFR2 receptors inhibits gastric emptying during stress **[15].** These studies indicate the therapeutical potential of CRF receptor antagonists in disorders of a gut-brain axis, such as inflammatory bowel diseases or irritable bowel syndrome **[33].**



Figures 5-6

The physiological and pharmacological actions of CRFR agonists and antagonists

Previously non-selective CRFR antagonist studies have been performed to demonstrate that classic orexigenic peptides such as, orexin and ghrelin or novel natriuretic peptides, such as BNP and CNP could activate the HPA axis through CRFRs **[34-37]**. Recently selective CRFR antagonists were preferred to investigate if new members of the neuromedin family (neuromedin U and S) or RF-amide family (neuropeptide AF and SF) would stimulate the HPA axis by CRFR1 or CRFR2 **[38-41]**.

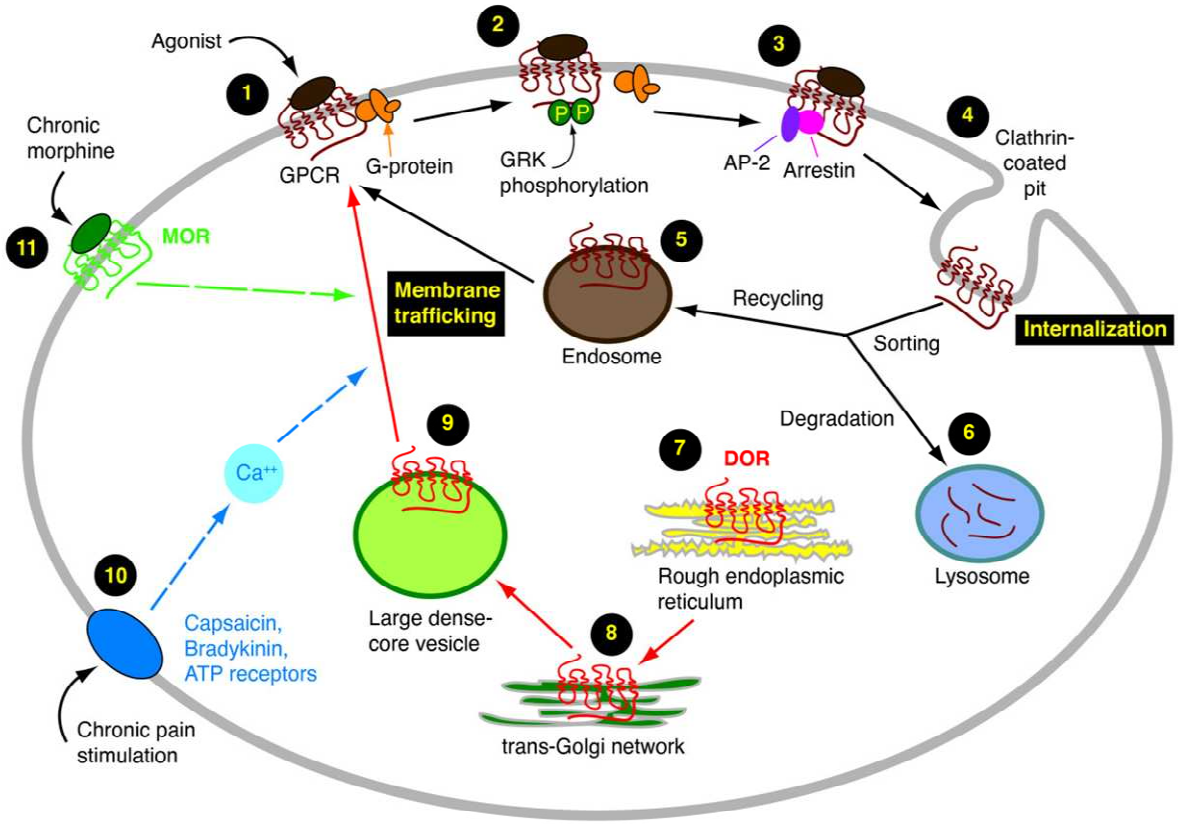
1.4. μ Opioid receptor agonists

There are three classical opioid receptors designated μ (MOR), δ (DOR), and κ (KOR), and one orphan opioid-receptor like receptor (ORL) found in the central and peripheral nervous systems of mammals mediating the biological actions of exogenous and endogenous opioids **[42]**.

β-endorphin, derived from pro-opiomelanocortin (POMC), presenting structural and pharmacological similarities with morphine (highest affinity for the MOR, lower affinity for DOR and lowest affinity for the KOR) was among the first endogenous opioids discovered **[43].** Enkephalins (selective DOR agonists), derived from proenkephalin **[44]**, dynorphins (selective KOR agonists), derived from prodynorphin **[45]** and nociceptin (selective ORL agonist), derived from pronociceptin **[46]** are considered selective endogenous ligands for (non-μ) opioid receptors **[47].**

Though naturally occurring opioid peptides binding preferentially to the MOR were found recently: β-casomorphin (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) from digestion of β-casein **[48-49]**, hemorphin-4 (Tyr-Pro-Trp-Thr) from digestion of hemoglobin **[50]**, Tyr-Pro-Leu-Gly-NH2 (Tyr-MIF-11) and Tyr-Pro-Trp-Gly-NH2 (Tyr-W-MIF-1), both isolated from the brain **[51-52],** no mammalian peptide was proved to be highly selective for MOR until lately. Endomorphin 1 (EM1: Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (EM2: Tyr-Pro-Phe-Phe-NH_2) extracted from bovine brain **[53]** and isolated also in human brain **[54],** were proposed finally as selective endogenous ligands for MOR **(Figures 7-8).** Radioimmunological and immunohistochemical analyses revealed that EM immunoreactivities (IRs) are distributed throughout the human, bovine, and rodent CNS in similar manner than the MORs **(Figures 9-10).** Both EMs are abundant in the areas such as the stria terminalis, the periaqueductal gray, the locus coeruleus, the parabrachial nucleus, and the nucleus of the solitary tract **[55]**.

Receptor	Endogenous Peptide	Amino Acid Sequence
μ (MOR, OP_3)	β-endorphin	Tyr-Gly-Gly-Phe-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu
	β-Casomorphin-5	Tyr-Pro-Phe-Pro-Gly
	β-Casomorphin-7	Tyr-Pro-Phe-Pro-Gly-Pro-Ile
	Morphiceptin	Tyr-Pro-Phe-Pro-NH_2
	β-Casomorphin-5	Tyr-Pro-Phe-Val-Glu
	β-Casomorphin-7	Tyr-Pro-Phe-Val-Glu-Pro-Ile
	Hemorphin-4	Tyr-Pro-Trp-Thr
	Hemorphin-7	Tyr-Pro-Trp-Thr-Gln-Arg-Phe
	Dermorphin	Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2
	Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2
	Endomorphin-2	Tyr-Pro-Phe-Phe-NH_2
	Tyr-MIF-1	Tyr-Pro-Leu-Gly-NH_2
	Tyr-W-MIF-1	Tyr-Pro-Trp-Gly-NH_2
δ (DOR, OP_1)	[Met^5]enkephalin	Tyr-Gly-Gly-Phe-Met
	[Leu^5]enkephalin	Tyr-Gly-Gly-Phe-Leu
		Tyr-Gly-Gly-Phe-Met-Arg-Phe
		Tyr-Gly-Gly-Phe-Met-Arg-Gly-Leu
	Dermenkephalin	Tyr-D-Met-Phe-His-Leu-Met-AspNH_2
	Deltorphin-I	Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2
	Deltorphin-II	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2
κ (KOR, OP_2)	Dynorphin A	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln
		Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile
	Dynorphin A(1-8)	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr
	Dynorphin B	
ORL_1 (NOP_1)	Nociceptin/orphanin FQ	Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln



Figures 7-8.

The biochemical composition and intracelullar signalisation of MOR agonists

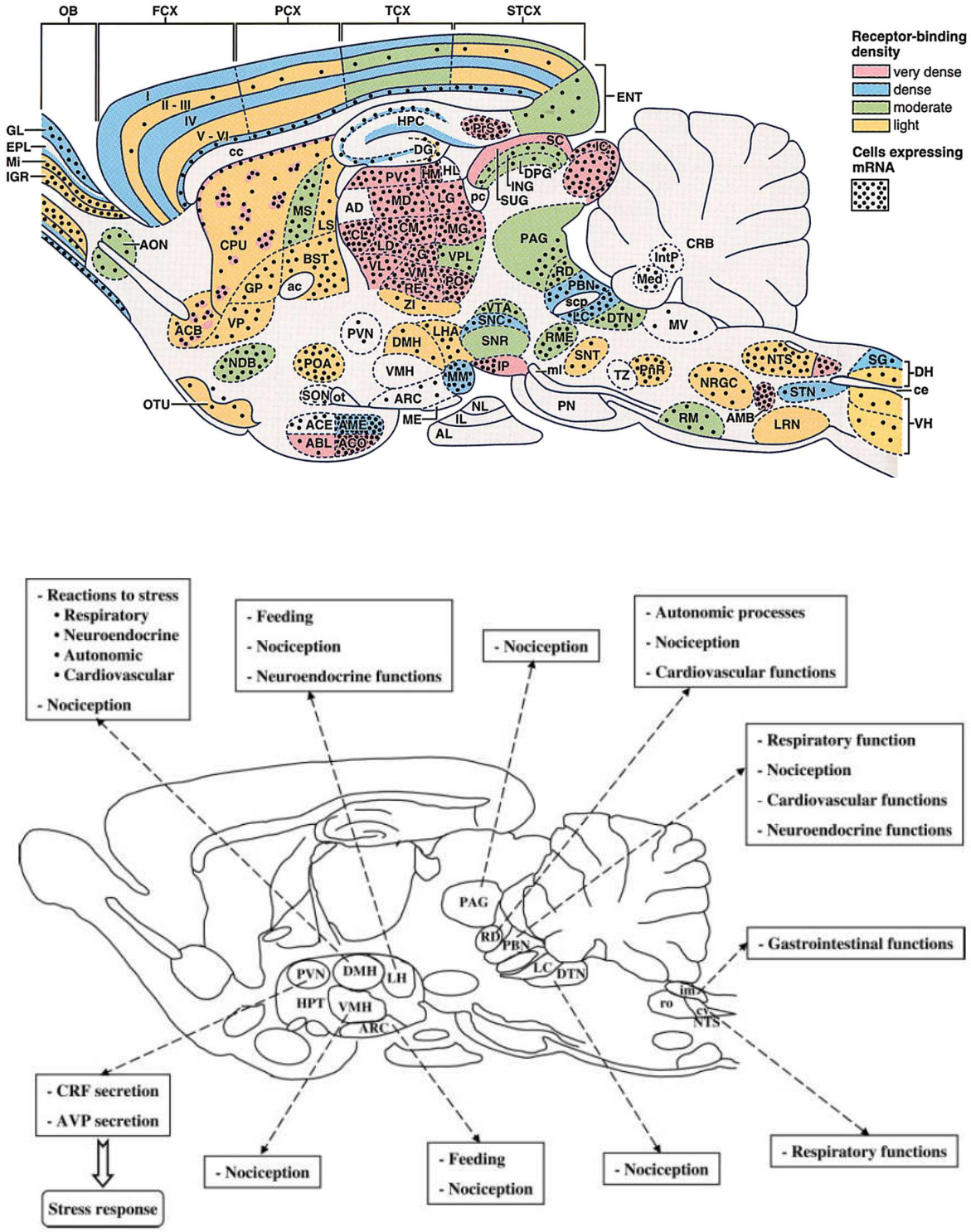
However, there are also important divergencies in the neuroanatomical localization of these peptides. EM1 is predominant the brain and upper brainstem and is most abundant in the nucleus accumbens, the cortex, the amygdala, the thalamus, the hypothalamus, the striatum, and the dorsal root ganglia. In contrast, EM2 is prevalent in the spinal cord and lower brainstem and most prominent in the hypothalamus and the nucleus of the solitary tract. Modest EM2 IRs were seen in the nucleus accumbens, substantia nigra, nucleus raphe magnus, ventral tegmental area, and pontine nuclei and amygdala **[56]**. The differences in the anatomical distribution of EMs presume the existence of two distinct precursors or two different processing pathways of the same precursor, yet to be identified.

Radioimmunological assays and reversed phase high-performance liquid chromatography techniques performed in rat and human peripheral tissues revealed that EM-1 IRs and EM-2 IRs are present in significant amounts in human spleen; relatively high levels of EM IRs were also detected in the rat spleen, thymus, and blood. Because very low amounts of EM IR were found in anterior and posterior rat pituitaries, pituitary secretion could not account for the significant plasma concentration of EMs. The differences between central and peripheral concentrations of EMs indicates that EMs are secreted into the systemic circulation from nerve fibers and terminals of the spinal cord **[57].**

The neuroanatomical distribution of EMs reflects their potential participation in many physiological processes, such as perception of pain **(Figures 11-12)** and modulation of stress **(Figures 19-20)**, complex functions such as reward and arousal, as well as regulation of autonomic, cognitive, neuroendocrine and limbic homeostasis **(Figures 9-10)[58].**

1.5. μ Opioid receptors

Numerous in vitro studies clearly demonstrated that the EMs selectively, but not exclusively bind to MORs. In classic binding assays on the rat and mouse brain membrane preparations, both peptides displaced naloxone, Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO), and other MOR-selective ligands in a concentration-dependent manner. The efficacy of EMs in many bioassays is slightly lower than that of DAMGO, but higher than that of morphine **[59]**. Several in vivo studies also demonstrated that EMs are specific ligands and partial agonists of MORs **[58].** The relation between binding efficacy and biological activity of these peptides has yet to be elucidated.



Figures 9-10

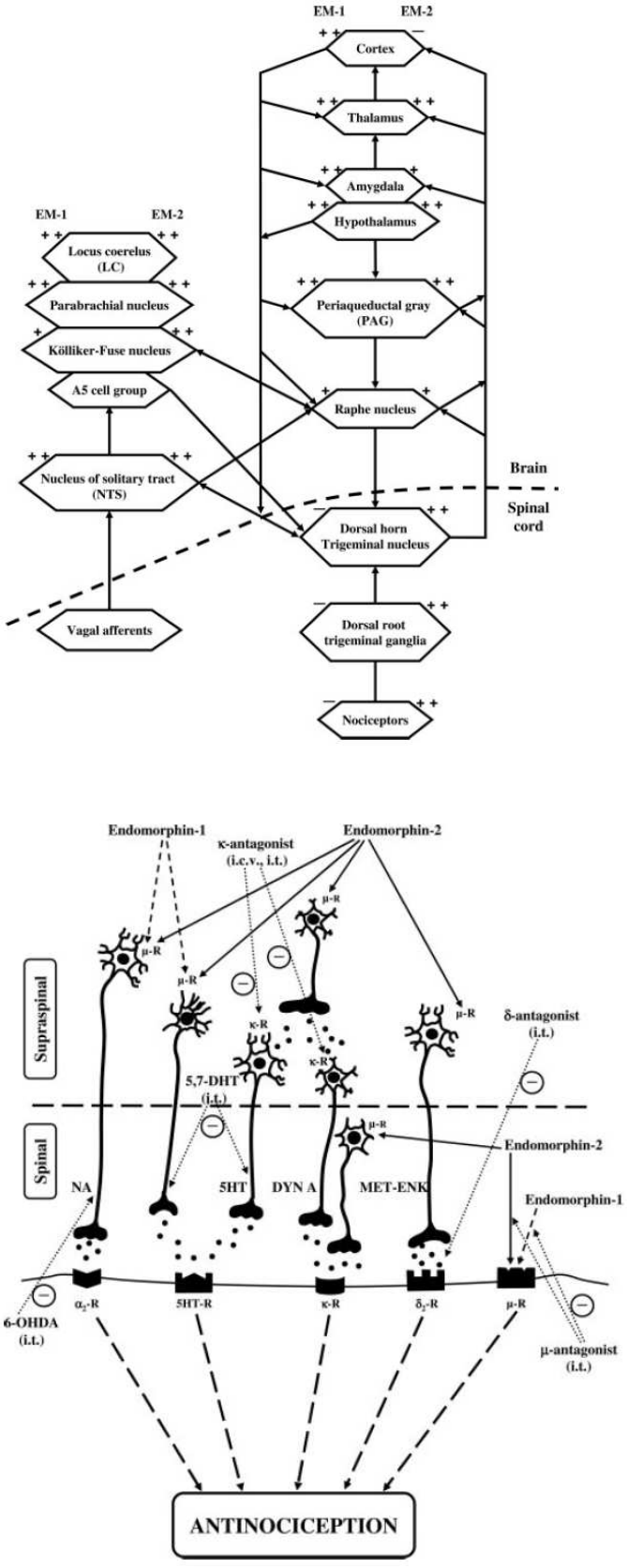
The anatomical distribution of MORs and EMs

The MORs belong to the superfamily of heterotrimeric, guanine-nucleotide binding, G-protein-coupled receptors (GPCRs) **[60]**. In the CNS, EMs, although specifically positioned to activate the MORs, are not selectively associated with the regions expressing these binding sites. In several telencephalic and limbic structures, MORs and EM-IRs are colocalized: the septal nuclei, the bed nucleus of the stria terminalis, the nucleus accumbens, the amygdala, and many hypothalamic nuclei. However, there are brain regions that contain low levels of EMs and high numbers of MORs, namely the amygdala, the thalamus, the hypothalamus, and the periaqueductal gray or the striatum. MORs have also been detected outside the CNS, in the enteric nervous system and throughout the immune tissues, where they were found to be coexpressed with EMs **[57].**

1.6. μ Opioid receptor antagonists

Naloxone and naltrexone are non-selective opioid antagonist drugs commonly used in clinical practice. Naloxone and naltrexone are partial inverse agonists, being used in the treatment of drug abuse (opioids, nicotine, alcohol). Naltrexone is administered to reverse the symptoms of opioid withdrawal and naloxone is preferred to reduce the consequences of opioid intoxication. Some opioid antagonists, like nalorphine and levallorphan, are not pure antagonists, but weak partial agonists, inducing analgesic effects when administered in high doses to opioid-naive individuals **[61]**. Recent advances in pharmacological research led to the development of selective opioid receptor antagonists: cyprodime (selective MOR antagonist), naltrindole (selectivehttp://en.wikipedia.org/wiki/Delta_opioid_receptor DOR antagonist) and norbinaltorphimine (selective KOR antagonist) **[62]**.

Several studies suggested that EM1 and EM2 produce their biological effects by stimulating different subtypes of MORs, MOR1 and MOR2, which might be responsible for their distinct pharmacological activity. Administration of selective MOR1 antagonist naloxonazine **[63-64]** and selective MOR antagonist β-funaltrexamine **[65-66]** differentially attenuated spinal and supraspinal antinociception induced by EM1 and EM2 **[67-68]**. Results revealed that MOR1 was stimulated only by EM2, whereas MOR2 was stimulated by both EM1 and EM2. Antisense oligodeoxynucleotides studies against different exons in the MOR-genes disclosed similar results **[69-70]**. Further studies demonstrated that MOR1 mediate supraspinal analgesia and modulate acetylcholine and prolactin release, whereas MOR2 mediate spinal analgesia, respiratory depression, and inhibition of gastrointestinal transit **[71]**.



Figures 11-12

The EM-ic modulation of spinal and supraspinal pain perception

2. PURPOSES

Though there are relevant reviews demonstrating the function of CRF, arginine-vasopressin (AVP), ACTH, β-endorphin and glucocorticoids in the regulation of stress response **[8, 72]**, there are discrepant results concerning the implication of the UCNs and the EMs in stress-induced mechanisms and stress-related disorders **[9, 58]**. The aim of the present study was to determine the actions of these neuropeptides on hypothalamic neurohormone (CRF and AVP) and extrahypothalamic neurotransmitter (DA and GABA) release in rats for a better understanding of the physiology and pathophysiology of stress response **(Figure 13).**

The activation of the HPA axis is characterized by release of CRF and AVP from hypothalamic nuclei inducing release of ACTH from the anterior pituitary and consequent release of corticosterone from the adrenal gland into the blood **[1, 73]**. Hence, our first interest was to determine the CRF and AVP concentrations by immune assays from homogenised hypothalamic tissues and corticosterone concentrations by chemical assays from plasma samples collected from rats after in vivo administration of UCNs and EMs.

The activation of the HPA axis is associated with release of CRF, DA and GABA from extrahypothalamic sources of CRF **[74-75]**. On one hand, the DA released from the striatum, part of both mesolimbical and nigrostriatal pathways, may mediate the stress-related locomotor hyperactivation **[76]**; on the other hand the GABA release from the amygdala, part of the extended amygdala circle, may transmit the stress-induced stereotype behaviour of the animals **[77]**. Thus, our second purpose was to determine the radio-labelled DA and GABA concentrations from superfused striatal and amygdalar slices isolated from rats after in vitro administration of UCNs and EMs.

The participation of the CRFR subtypes and MOR subtypes in these processes is not fully elucidated; brain slices were pretreated in vitro with selective CRFR antagonists, antalarmin (CRFR1 antagonist) and astressin 2B (CRFR2 antagonist) and selective MOR antagonists, naloxonazine (MOR1 and MOR2 antagonist) and β-funaltrexamine (MOR1 antagonist) to clarify the physiological and therapeutical roles of these receptors and their ligands.

Previous biochemical and behavioural studies reported that both EM1 and EM2, preferentially EM2, were sensitive to the degrading actions of synaptic enzymes, especially dypeptidyl aminopeptidase IV (DPPIV) **[78-80]**. Thus, pretreatment of the brain slices with diprotin A, a potent inhibitor of DPPIV, was justified by both in vitro and in vivo studies **[78]**.

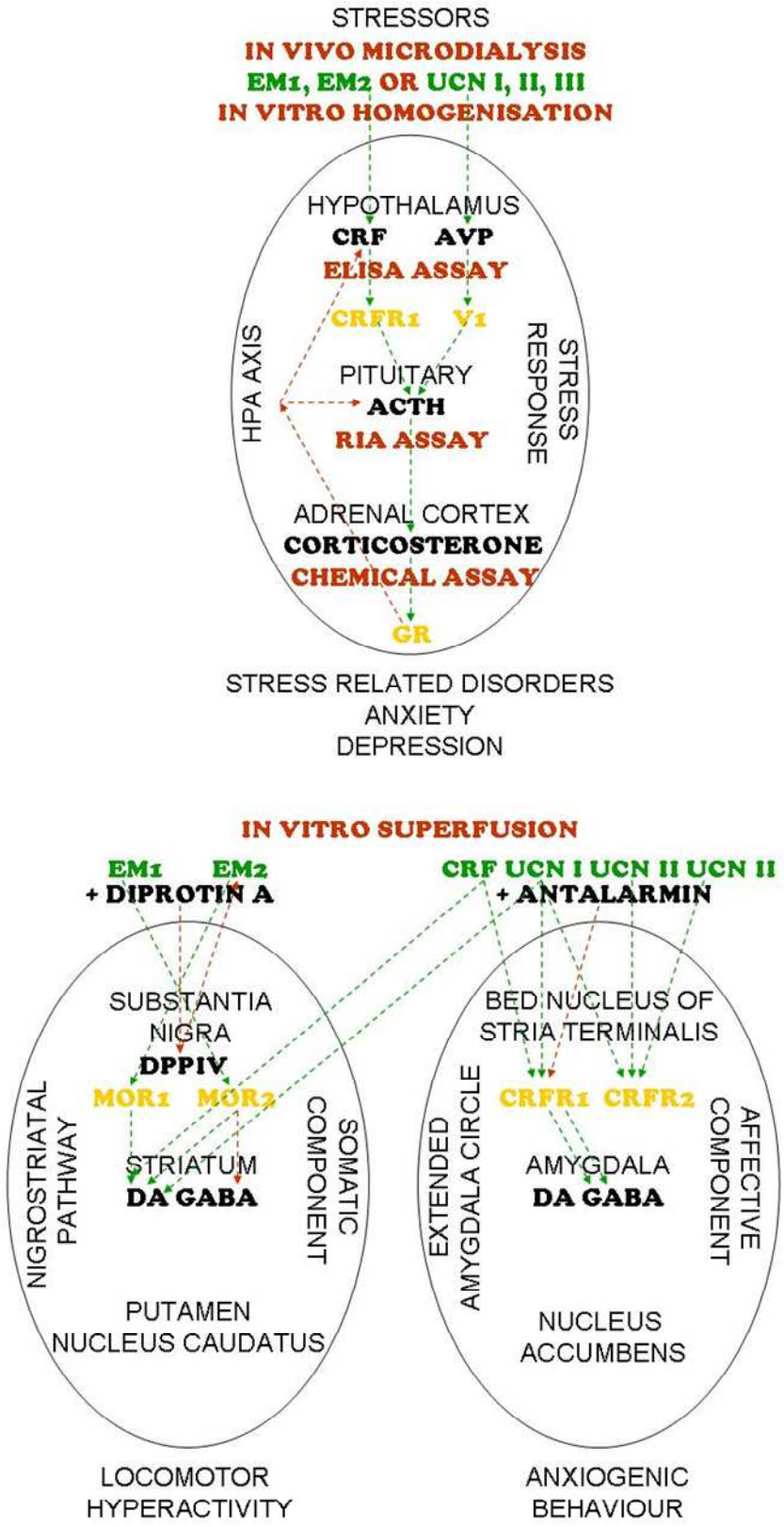


Figure 13

The methods of determination of the hypothalamic and extrahypothalamic mediators of stress

3. MATERIALS AND METHODS

3.1. Materials

The CRFR agonists used in the experiments were:

Corticotropin-releasing hormone, CRH (Bachem, Switzerland);

Urocortin I (Bachem, Switzerland), non-selective CRFR agonist;

Urocortin II (Bachem, Switzerland), selective CRFR2 agonist;

Urocortin III (Bachem, Switzerland), selective CRFR2 agonist.

The CRFR antagonists used in the experiments were:

α-helical CRF 9-41 (Sigma-Aldrich, Austria), non-selective CRFR antagonist;

Antalarmin (Sigma-Aldrich, Austria), selective CRFR1 antagonist;

Asstressin 2B (Sigma-Aldrich, Austria), selective CRFR2 antagonist.

The MOR agonists used in the experiments were:

Endomorphin 1 (Bachem, Switzerland), selective MOR agonist;

Endomorphin 2 (Bachem, Switzerland), selective MOR agonist.

The MOR antagonists used in the experiments were:

β-funaltrexamine (Bachem, Switzerland), selective MOR1 antagonist;

Naloxonazine (Bachem, Switzerland), selective MOR antagonist.

Other substances used in experiments were:

[^{3}H]DA (Amersham, USA), tritium labelled excitatory neurotransmitter;

[^{3}H]GABA (Amersham, USA), tritium labelled inhibitory neurotransmitter;

Diprotin A (Bachem, Switzerland), DPPIV enzyme inhibitor;

Krebs solution: NaCl, KCl, $MgSO_4$, $NaHCO_3$, glucose, KH_2PO_4 and $CaCl_2$ (Reanal, Hungary);

Ringer solution: NaCl, KCl, Na_2HPO_4, $MgCl_2$, glucose, $CaCl_2$ (Reanal, Hungary);

Saline solution (NaCl inj. of 0.9 %, Biogal, Hungary);

Ultima Gold (Perkin Elmer, USA), scintillation fluid;

Mixture of 5 % CO_2 and 95 % O_2 for continuous gassing of the tissues;

Nembutal (CEVA-Phylaxia, Hungary) for general anesthesia of the rats;

Acetic acid of analytical grade (Reanal, Hungary), for CRF and AVP determination;

Ethyl alcohol, methylene chloride and sulfuric acid of analytical grade (Reanal, Hungary), for corticosterone determination.

CRF ELISA kit (Cosmo Bio Co. Ltd. Japan)

AVP RIA kit (Diagnostics Systems Laboratories Inc., USA)

3.2. Animals

The animals were kept and handled during the experiments in accordance with the instructions of the University of Szeged Ethical Comittee for the Protection of Animals in Research. Male Wistar rats weighing 150-200 g were used. The rats were kept in their home cages at constant room temperature (23°C) on a standard illumination schedule, with 12-h light and 12-h dark periods (lights on from 6:00 a.m.). Commercial food and tap water were available ad libitum. The rats were allowed a minimum of 7 days to acclimatize before surgery, and, to minimize the effects of nonspecific stress the rats were handled daily.

3.3. Surgery

For the intracerebroventricular (ICV) administration of neuropeptides, the rats were implanted with a stainless steel Luer cannula (10 mm long) aimed at the right lateral cerebral ventricle under Nembutal (35 mg/kg, ip) anesthesia. Cannulas were secured to the skull with dental cement and acrylate. The rats were used after a recovery period of at least 5 days. The implantation of the cannula and the isolation of different brain regions were made according to the Stereotaxic Atlas Of The Rat Brain **(Pellegrino et al. 1979),** after the following coordinates: lateral cerebral ventricle: 0.2 mm posterior to the bregma, 1.7 mm lateral to the bregma and 3.7 mm deep from the dural surface; hypothalamus: rostro-caudal, RC +2.6 - -2.6 mm, medio-lateral, ML +1,5 - -1,5 mm, dorso-ventral, DV +7 - +10 mm; striatum RC +4 - -1 mm, ML +1 - +5 mm, DV +3 - +8 mm; amygdala: RC 0 - -2 mm, ML +3 - +6 mm, DV +7 - +10 mm considering the bregma as point of reference **(Figure 14).**

3.4. Administration

The in vivo administration of CRF agonists or MOR agonists was made ICV through the cannula implanted in the right lateral cerebral ventricles 30 min before the animals were sacrificed, either for hypothalamus homogenisation or plasma corticosterone determination. After the interpretation of dose response curves the most effective doses of CRFR agonists and MOR agonists were selected and administered in the combined agonist-antagonist experiments (5 μg EM1 or 0,5 μg EM2 with 1 μg α-helical CRF 9-41 in 2 μl volume). The CRF antagonist was injected ICV 30 min before MOR agonist treatment.

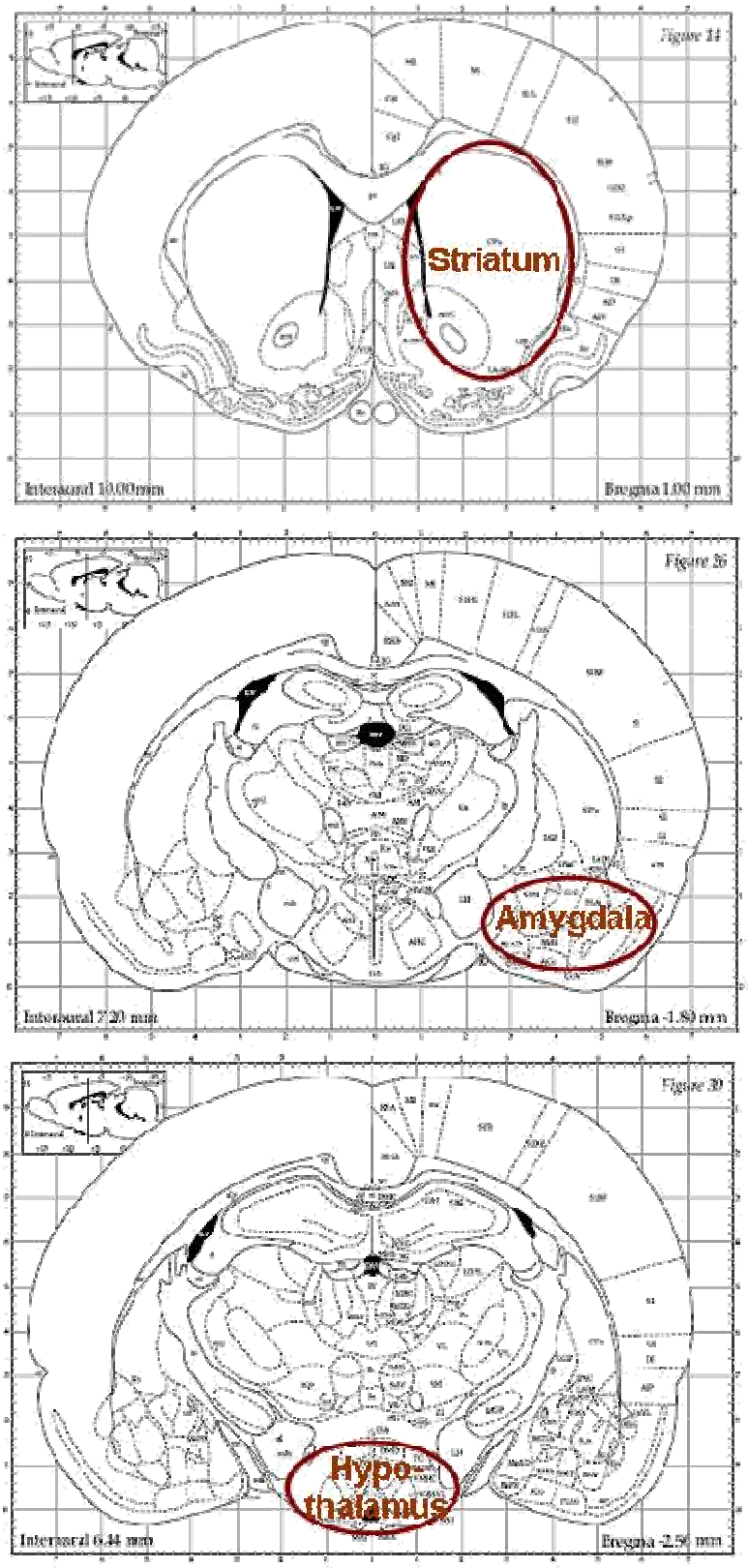


Figure 14

The anatomical regions isolated and dissected for in vitro experiments

The in vitro administration of CRF agonists, 100 nM CRF or 100 nM UCN I, II or III, was made 12 min before the electrical stimulation of the brain slices. When antagonization of a previous effect was intended, the slices were pretreated with selective CRF antagonists, 100 nM antalarmin or 100 nM astressin-2B 22 minutes before the electrical stimulation. The administration of MOR agonists, 10 μM EM1 or 10 μM EM2, and MOR antagonists, 10 μM naloxonazine or 10 μM β-funaltrexamine, was performed in similar way. When DPPIV inhibition was needed, the slices were pretreated with 0.1 mM diprotin A 15 min before the administration of EMs.

3.5. Methods

3.5.1. In vitro homogenisation assay

The hypothalamic neurohormone concentrations were determined by in vitro homogenisation method previously used for CRF determination by Suda and Joanny **[81-82],** and measured by radio-labelled and enzyme-linked immuno-assays. The rats were decapitated and their brains were rapidly removed. The hypothalami were isolated in a Petri dish filled with ice-cold Krebs solution (composition: 113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, pH 7.4). The brain samples were dissolved in 500 μl acetic acid of 2M concentration in Eppendorf tubes and immersed in boiling water for 5 min and then homogenised with ultrasonic homogeniser (Branson Sonifier 250) at 0°C for 30 sec. The homogenisates were centrifuged twice at 10000 rpm at 4°C for 20 min after of which the supernatants were separated and liophylisated for CRF and AVP determination.

3.5.2. Enzyme-Linked Immuno-Sorbent Assay (ELISA)

For the determination of the amount of CRF in the brain homogenisate we used the mouse/rat CRF-high sensitivity ELISA kit provided by CosmoBio Company, Ltd., Japan. This ELISA kit for determination of mouse/rat CRF is based on a sandwich enzyme immunoassay and shows no crossreactivity to UCNs (mouse/rat and human). To the wells of plate coated with highly purified antibody against mouse/rat CRF, standard antigen or sample was added for the 1st step, immunoreaction. After the 1st step incubation and plate washing, biotinylated rabbit anti rat CRF antibody was added as the 2nd step to form CRF antibody-antigen-biotinylated CRF antibody complex on the surface of the wells. After the 2nd step incubation and rinsing out excess biotinylated antibody, horseradish peroxidase labeled streptoavidin was added for binding to biotinylated CRF antibody. Finally, horseradish

peroxidase enzyme activity is determined by 3,3',5,5'-tetramethyl benzidine addition and a typical calibration curve was generated. The CRF concentrations of the extracted samples were corrected for extraction recovery and expressed in ng/ml.

3.5.3. Radio Immuno Assay (RIA)

For the detection of the amount of AVP in the brain homogenisate we used solid-phase extraction and competitive radioimmunoassay used for human determination by the Department of Endocrinology, University of Szeged. This assay uses a rabbit anti-AVP antiserum and a radioiodinated AVP [125I] tracer assured by Diagnostics Systems Laboratories Inc., USA. Bound and free phases were separated by a second antibody bound to solid phase particles, followed by a centrifugation step. The radioactivity in the bound fractions was evaluated and a typical calibration curve was achieved. The AVP concentrations of the extracted samples were corrected for extraction recovery and expressed in ng/ml.

3.5.4. Chemical fluorescence assay

The plasma corticosterone concentration was determined by the fluorescence assay described by Zenker and Bernstein **[83]**, as modified by Purves and Sirett **[84]**. 30 min after the in vivo administration trunk blood was collected from the animals into heparinized tubes and centrifuged for at 3000 rpm 10 min. 200 μl aliquots of the medium were transferred to centrifuge tubes. Meanwhile a reagent blank of 200 μl of distilled water and 2 corticosterone standards of the same volume containing 25 μg or 50 μg, respectively, were prepared. 5 ml of methylene chloride was delivered with an automatic pipette to each tubes and rocked for 30 min to allow complete extraction of corticosterone by the solvent. The extract was centrifuged for 10 min at 3000 rpm. To eliminate any aqueous phase, approximately 3.2 ml of the lower hydrophobic phase was aspired with a glass syringe then transferred into another centrifuge tube. 4 ml of fluorescent reagent (stable mixture of 2.4 volumes of sulfuric acid and 1.0 volume of 50 % v/v aqueous ethyl-alcohol) was added to the extract. The tubes were shaken vigorously for 15 min, centrifuged at 3000 rpm for 10 min and was allowed to stand at room temperature for 2 hours, which permitted the maximum development of fluorescence from corticosterone. Emission intensity was measured from the lower sulfuric acid layer with Hitachi 204-A fluorescent spectrophotometer at 456 nm extinction and 515 nm emission wave-lengths. The concentration of corticosterone of the samples was calculated from the values of the standards and expressed as μg/100 ml.

3.5.5. In vitro superfusion assay

The striatal and amygdalar neurotransmitter releases were determined by the in vitro method described by Gaddum **[85],** as modified by Harsing **[86]**. The rats were decapitated and their brains were rapidly removed. The striata and amygdalae were dissected in a Petri dish filled with ice-cold Krebs solution (composition: 113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, pH 7.4). The dissected tissue was cut with a McIlwain tissue chopper and slices of 300 μM were produced. The slices were incubated for 30 min in 8 ml of Krebs solution, submerged in a water bath at 37 °C and gassed through a single-use needle with a mixture of 5% CO_2 and 95% O_2. During the incubation, the slices were labeled with 0.15 mM of [^{3}H]DA (Amersham Pharmacia Biotech) with a specific activity of 14 Ci/mmol or [^{3}H]GABA (Amersham Pharmacia Biotech) with a specific activity of 87.0 Ci/mM. Two tritiated slices were transferred to each of the four cylindrical perspex chambers of the superfusion system (Experimetria Ltd.). Gold electrodes were attached to both halves of the superfusion chambers and connected to an ST-02 electrical stimulator (Experimetria Ltd.). A multichannel peristaltic pump (Gilson Minipuls 2) was used to maintain a constant superfusion rate of 300 μl/min. The slices were superfused for 30 min to allow tissue equilibrium, and the superfusates were then collected in Eppendorf tubes by a multichannel fraction collector (Gilson FC 203B). 2 min after the sample collecting started, electrical stimulation consisting of square-wave impulses (total duration: 2 min, voltage: 100 V, pulse length: 5 ms, frequency: 10 Hz) was delivered to each of the four chambers. The total collecting time was 32 min (4x16 samples, 2 min each).

3.5.6. Radio scintillation assay

The remnants of superfused brain slices were solubilized in 200 ml of Krebs solution, using an ultrasonic homogenizer (Branson Sonifier 250). The radioactivity in the fractions and the homogenized tissue samples was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard) after the addition of 3 ml of scintillation fluid (Ultima Gold, Packard). The fractional release was calculated as a percentage of the radioactivity (count per minute, CPM) present in the collected sample compared to the total radioactivity of the correspondent tissue.

3.6. Statistics

Values are presented as means ± SEM unless otherwise indicated. Statistical analysis of the results was performed by analysis of variance (ANOVA, Statistica v5.0, StatSoft Inc.). The differences between groups were tested by one-way ANOVA followed by Tukey's post

hoc comparison test in cases of homogenisation study and the differences between samples were determined by two-way ANOVA with repeated measures in cases of superfusion study. A probability level of 0.05 or less was accepted as indicating a statistically significant difference.

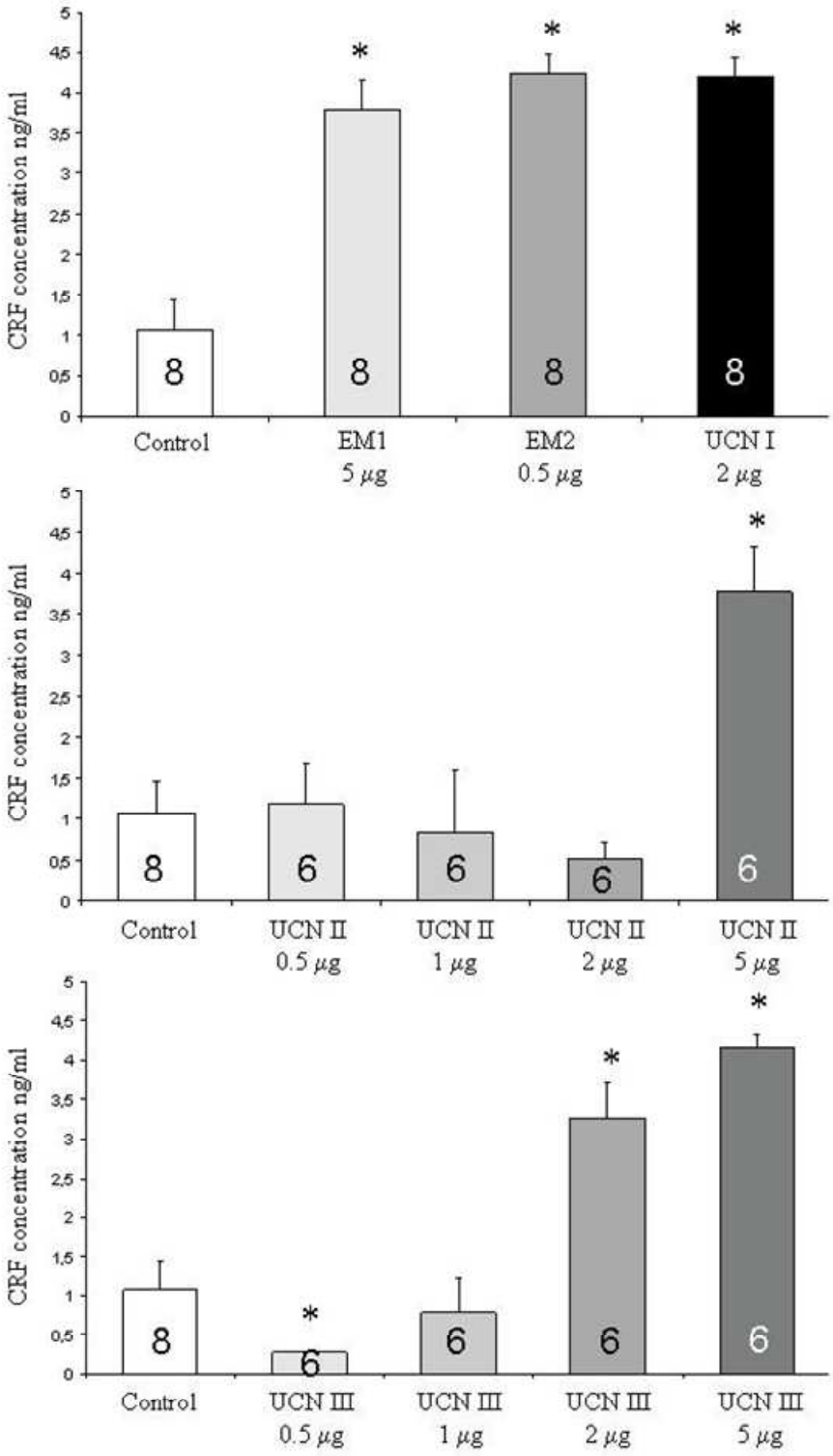
4. RESULTS

4.1. The hypothalamic CRF and AVP release induced by the UCNs

Equimolar doses (2 μg) of UCNs produced important but different effects on hypothalamic hormone contents; UCN I increased the production of CRF [$F(3, 32) = 193{,}55$, $p < 0.05$ for 2 μg UCN I vs. the control] and AVP [$F(3, 24) = 52{,}08$, $p < 0.05$ for 2 μg UCN I vs. the control], UCN II produced a decrease of CRF [$F(4, 32) = 41{,}24$, $p > 0{,}2$ for 2 μg UCN II vs. the control], but not AVP amount, UCN III induced an increase of CRF [$F(4, 32) = 154{,}04$, $p < 0.05$ for 2 μg UCN III vs. the control], but not AVP content **(Diagrams 1-3)**. However, UCN II and UCN III displayed similar bell-shaped dose-response curves, with lower doses decreasing and higher doses increasing the CRF production. Most evident reductions were assessed with 2 μg of UCN II [$F(4, 32) = 41{,}24$, $p > 0{,}2$ for 2 μg UCN II vs. the control] and 0.5 μg of UCN III [$F(4, 32) = 154{,}04$, $p < 0.05$ for 0.5 μg UCN III vs. the control]. Most significant elevations were achieved with 5 μg of both UCN II [$F(4, 32) = 41{,}24$, $p > 0{,}2$ for 5 μg UCN II vs. the control] and UCN III [$F(4, 32) = 154{,}04$, $p < 0.05$ for 5 μg UCN III vs. the control].

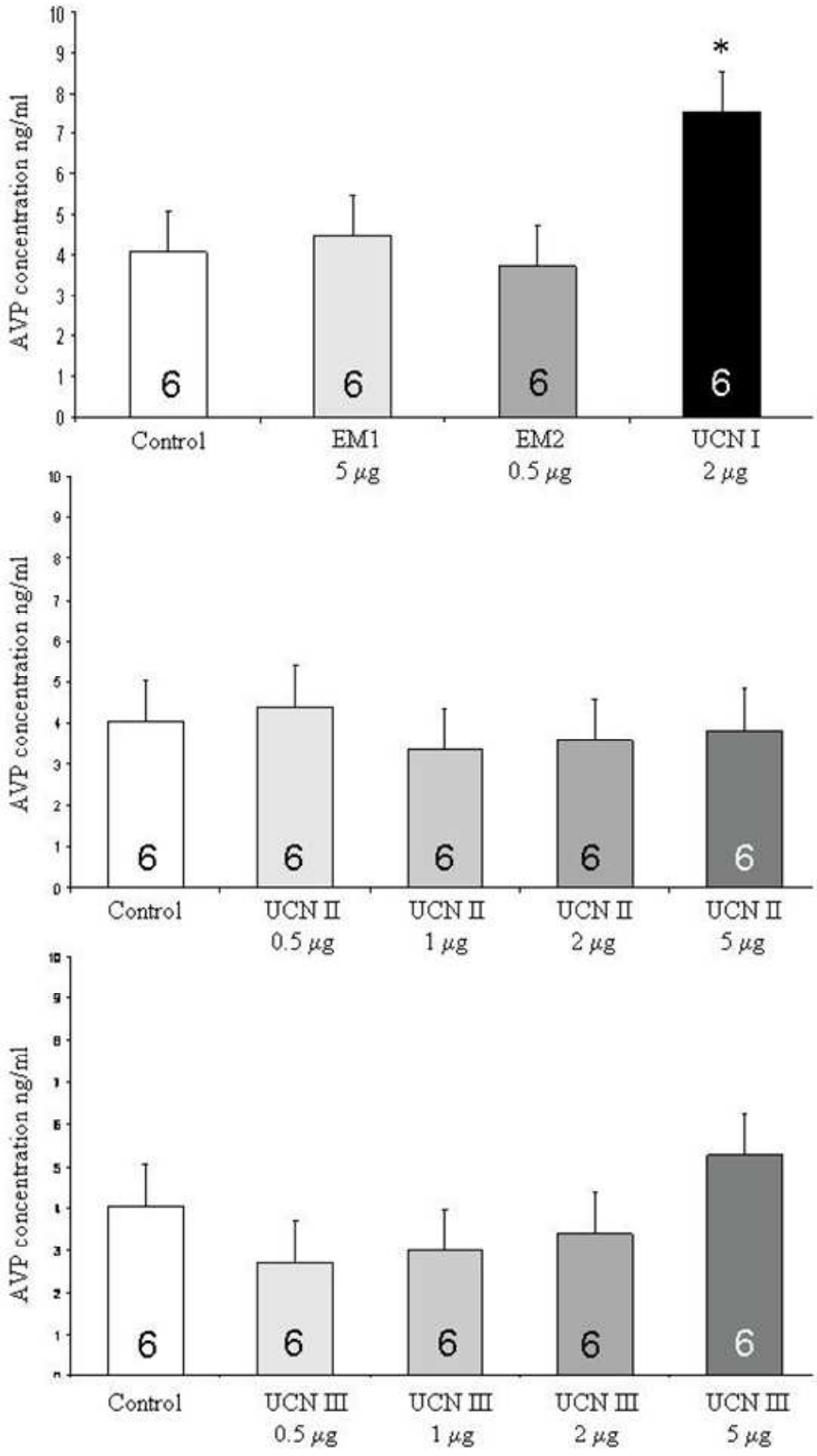
4.2. The hypothalamic CRF and AVP release induced by the EMs

Similarly to UCN I, EM1 and EM2 elicited considerably the CRF response [$F(3, 32) = 193{,}55$, $p < 0.05$ for 5 μg EM1 vs. the control and 5 μg EM2 vs. the control], but contrary to the CRFR ligand, the MOR ligands did not influenced significantly the AVP response **(Diagrams 4-6).** The changes of hypothalamic CRF production were mirrored by the corticosterone secretion, though only the most effective doses of EM1 (5 μg), EM2 (0.5 μg) and UCN I (2 μg) were investigated and represented in the relation of CRF and AVP release.



Diagrams 1-3

The effects of the EMs and the UCNs on the hypothalamic CRF release



Diagrams 4-6

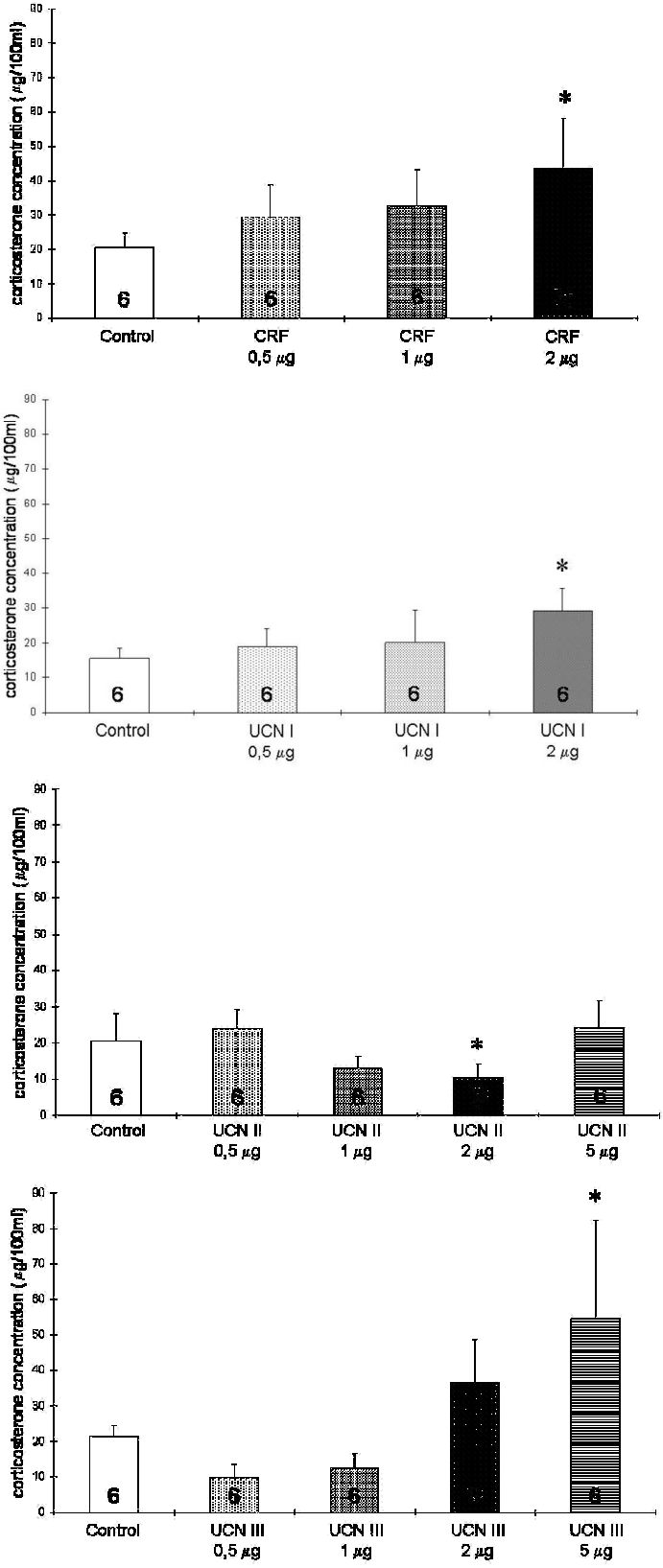
The effects of the EMs and the UCNs on the hypothalamic AVP release

4.3. The adrenal corticosterone release induced by the UCNs

CRF and UCN I induced an important increase of adrenal corticosterone release represented by the plasma corticosterone level elevated considerably by equimolar doses of CRF [$F(3, 12) = 4.95$, $p < 0.05$ for 2 μg CRF vs. the control] and UCN I [$F(3, 12) = 5,06$, $p < 0.05$ for 2 μg UCN I vs. the control]. A stimulating tendency (125-150%) of glucocorticoid release was assesed even at lower doses (0.5-1 μg), but the highest efficacy (200%) was achieved by administration of 2 μg CRFR agonist. UCN II and UCN III had biphasic effects. 2 μg UCN II and 0,5 μg UCN III tent to decrease corticosterone concentration by 100% [$F(4, 12) = 7,56$, $p < 0.05$ for 2 μg UCN II vs. the control]. Lower or higher doses (0.5 or 5 μg) of UCN II seemed ineffective, while higher doses (2 and 5 μg) of UCN III increased the corticosterone concentration by 200-300% as compared to control [$F(4, 12) = 10,75$, $p < 0.05$ for 5 μg UCN III vs. the control] **(Diagrams 7-10).**

4.4. The adrenal corticosterone release induced by the EMs

Both EM1 and EM2 induced a significant increase of the adrenal corticosterone release mirrored by the elevated plasma corticosterone level. EM1 in a dose of 5 μg elevated the corticosterone level by 160% as compared with control [$F(3, 35) = 4.48$, $p < 0.05$ vs. the control]. Lower doses (1-2 μg) of EM1 did not increased efficiently the plasma corticosterone concentration. EM2 in a dose of 0.25 μg elevated the corticosterone level by 140% as compared with control, though this response was not statistically significant. A higher dose (0.5 μg) of EM2 elevated the corticosterone level with 150% more than saline and in a statistically significant manner [$F(3, 26) = 3,42$, $P < 0.05$ vs. the control]. However further doses (1-2-5 μg) did not changed considerably the plasma corticosterone concentration. The adrenal responses induced by either EM1 or EM2 were inhibited by pretreatment with 1 μg α-helical CRF 9-41 [$F(3, 24) = 7.96$, $p < 0.05$ vs EM1] or [$F(3, 47] = 10.88$, $P < 0.01$ vs. EM2) **(Diagrams 11-14).**



Diagrams 7-10.

The effects of the UCNs on the adrenal corticosterone release

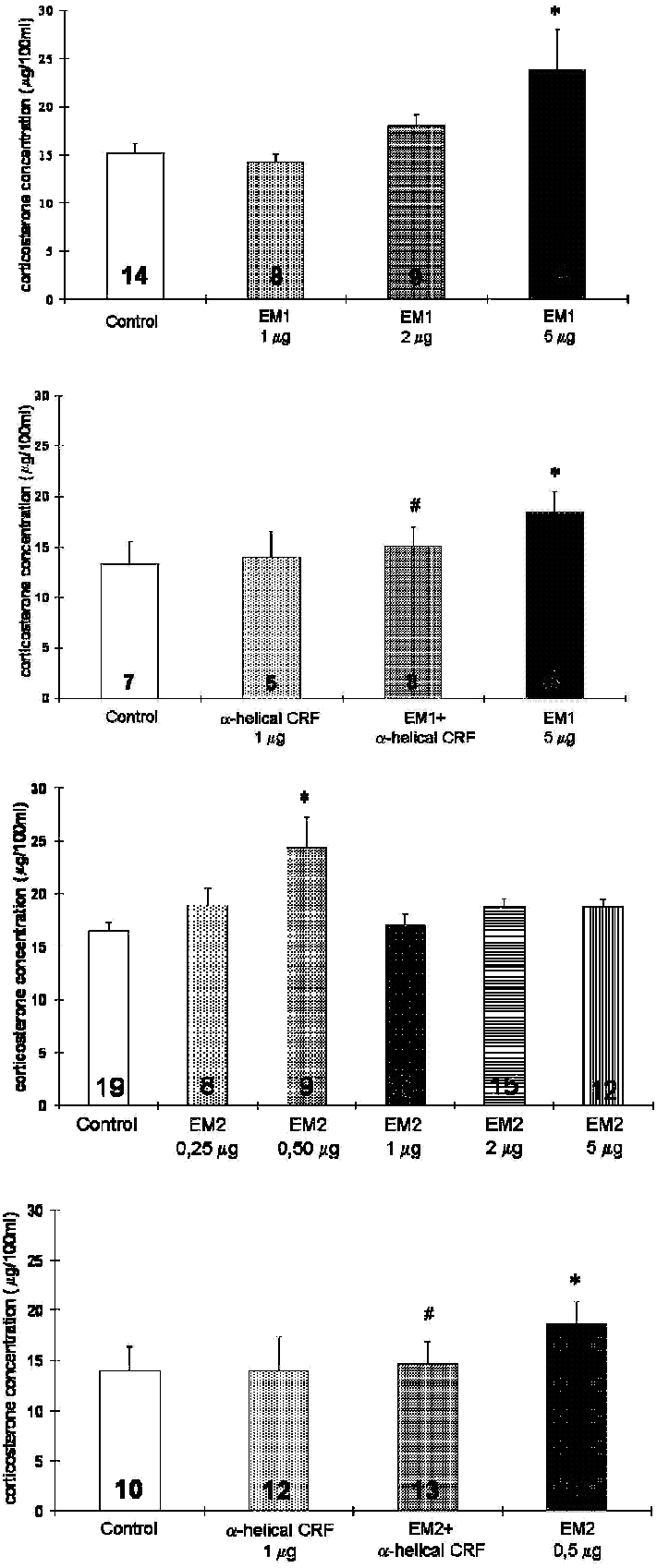


Diagram 11-14.

The effects of the EMs on the adrenal corticosterone release

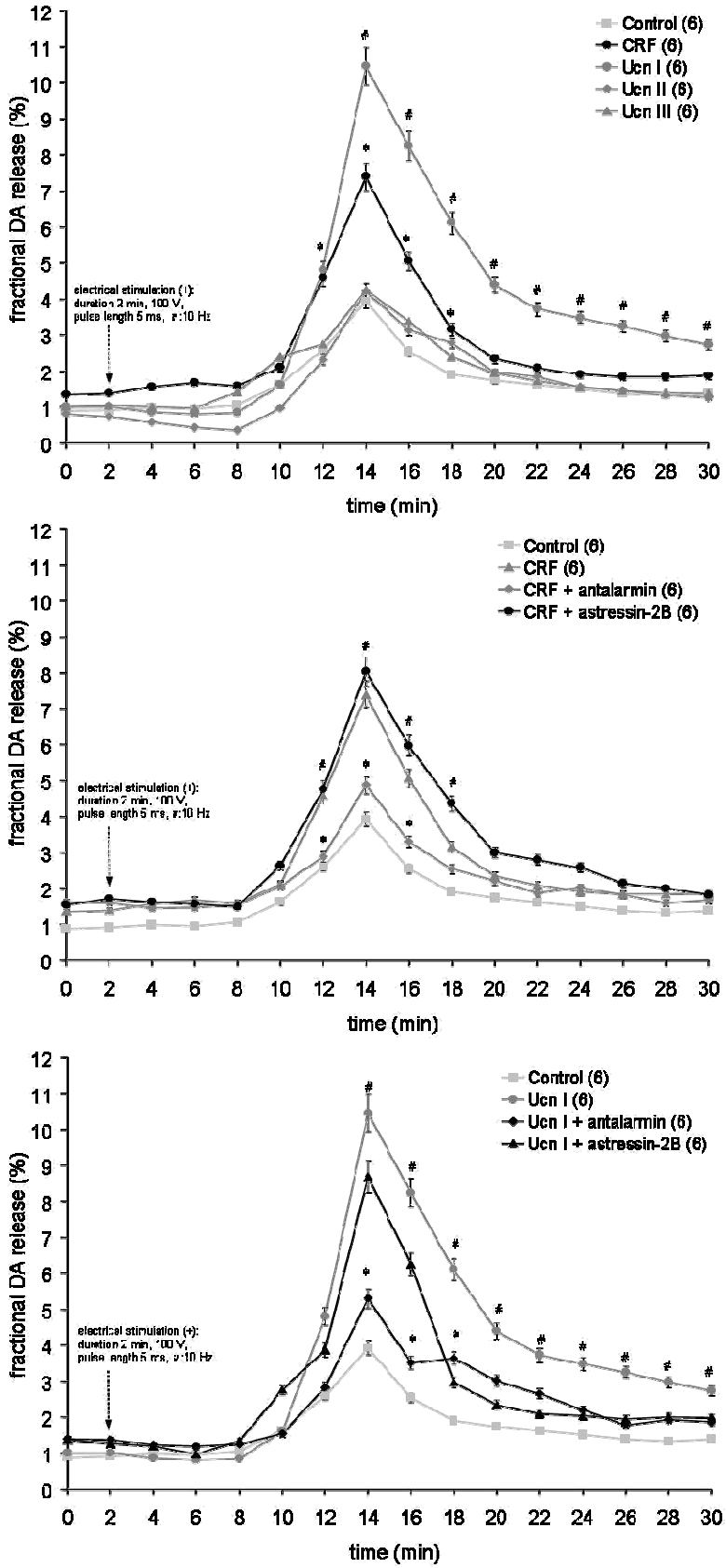
4.5. The extrahypothalamic DA and GABA release induced by the UCNs

Both CRF and UCN I increased significantly the striatal [^{3}H]DA release evoked by the electrical stimulation [$F_{14min}(1,10)=57.92$; $p<0.001$ and $F_{14min}(1,10)=197.15$; $p<0.001$, respectively], though a difference between the stimulatory effects of CRF (175%) and UCN I (275%) was clearly visible. [$F_{14min}(1,10)=25.63$; $p<0.001$]. However both effects were inhibited by antalarmin [$F_{14\ min}(1,8)=17.82$; $p=0.003$ for CRF + antalarmin vs. CRF alone and $F_{14min}(1,8)=70.25$; $p<0.001$ for UCN I + antalarmin vs. UCN I alone] and unaffected by astressin 2B. UCN II and UCN III appeared to be ineffective **(Diagrams 15-17).**

CRF and UCN I also increased significantly the amygdalar [^{3}H]GABA release elicited by electrical stimulation [$F_{16min}(1,8)=10.56$; $p=0.006$ for CRF vs. the control and $F_{16min}(1,8)=22.02$; $p<0.001$ for UCN1 vs. the control. The stimulatory effects of CRF and UCN I were blocked by the selective CRFR1 receptor antagonist antalarmin [$F_{14min}(1,8)=35.36$; $p<0.001$ for CRF + antalarmin vs. CRF alone and $F_{14min}(1,8)=79.59$; $p<0.001$ for UCN I + antalarmin vs. UCN I alone], but not by the selective CRFR2 antagonist astressin 2B. The effects of the selective CRFR2 agonists, UCN II and UCN III, proved to be insignificant as well **(Diagrams 18-20).**

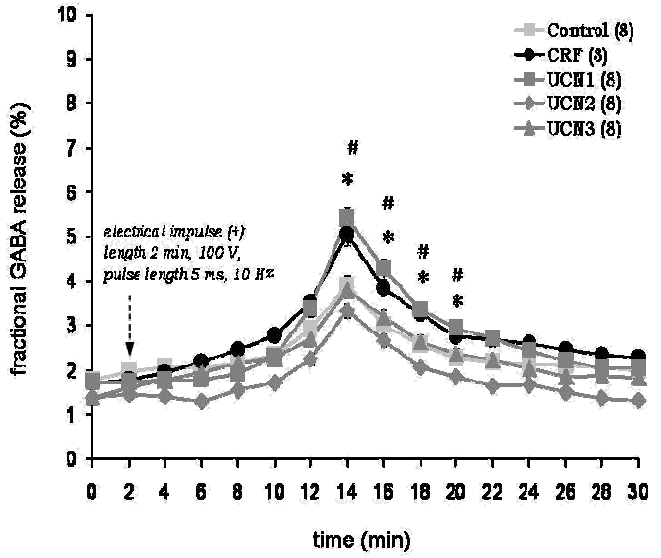
4.6. The extrahypothalamic DA and GABA release induced by the EMs

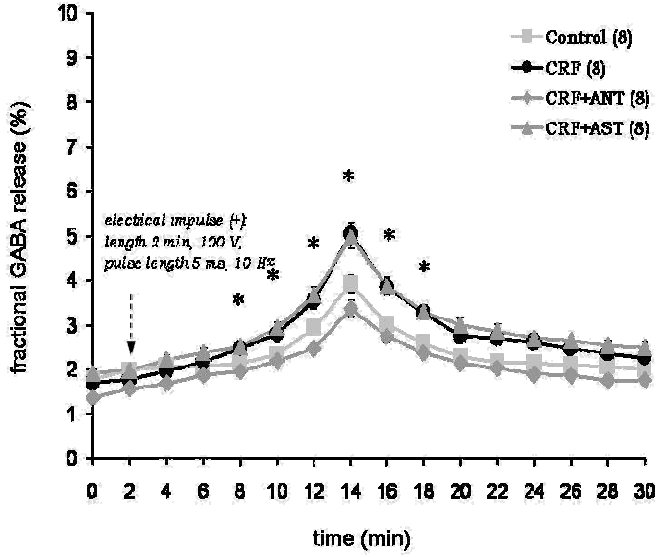
EM1 increased significantly the striatal [^{3}H]DA release induced by electrical stimulation [$F_{14min}(1,12)=76.42$; $p<0.001$]. The DPPIV inhibitor, diprotin A did not potentiate this effect. The stimulatory effect of EM1 was antagonized only by β-funaltrexamine [$F_{14min}(1,12)=16,56$; $p=0.001$], but not by naloxonazine. EM1 inhibited significantly the striatal [3H]GABA release increased by electrical stimulation [$F_{14min}(1,12)=16.90$; $p<0.005$]; the inhibitory effect of EM1 was antagonized by β-funaltrexamine [$F_{14min}(1,12)=88,75$; $p<0.001$], but not by naloxonazine **(Diagrams 21-23).** In contrast, the increasing effect of EM2 on striatal DA release was significant only when striatal slices were pretreated with diprotin A [$F_{14min}(1,14)=28.82$; $p<0.001$]. This effect of EM2 was antagonized by both β-funaltrexamine [$F_{14min}(1,12)=24.99$; $p<0.001$] and naloxonazine [$F_{14min}(1,14)=30.21$; $p<0.001$], at least when the slices were pretreated with diprotin A. EM2, whether administered alone or with diprotin A, did not influence considerably the striatal [3H]GABA release **(Diagrams 24-26).** The effects of the EMs on amygdalar neurotransmitter release were insignificant, so they were not interpreted in the present work.

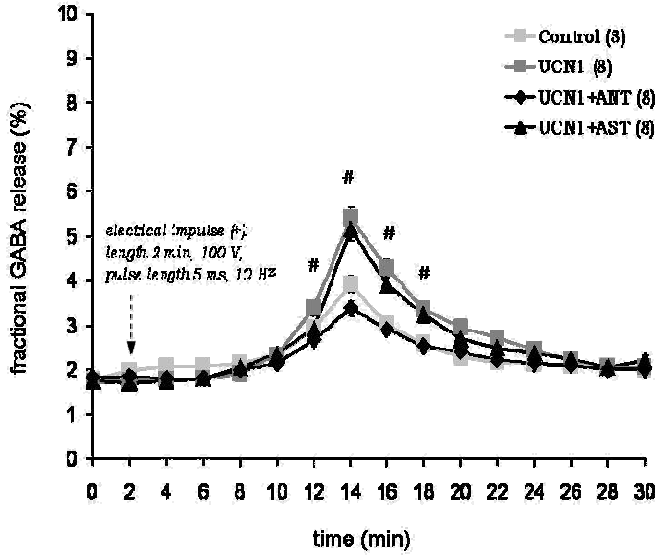


Diagrams 15-17

The effects of the UCNs on the striatal DA release

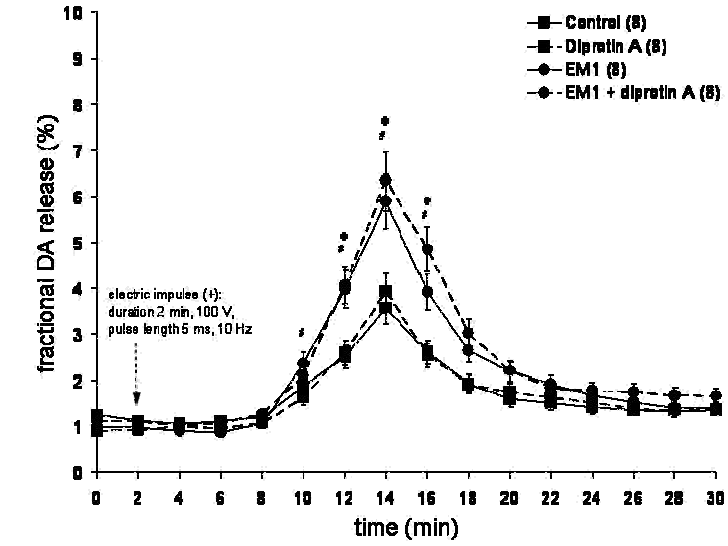


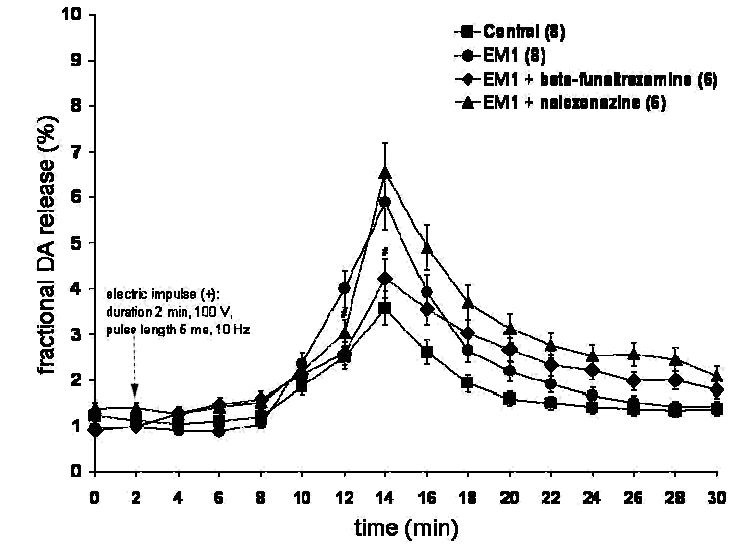


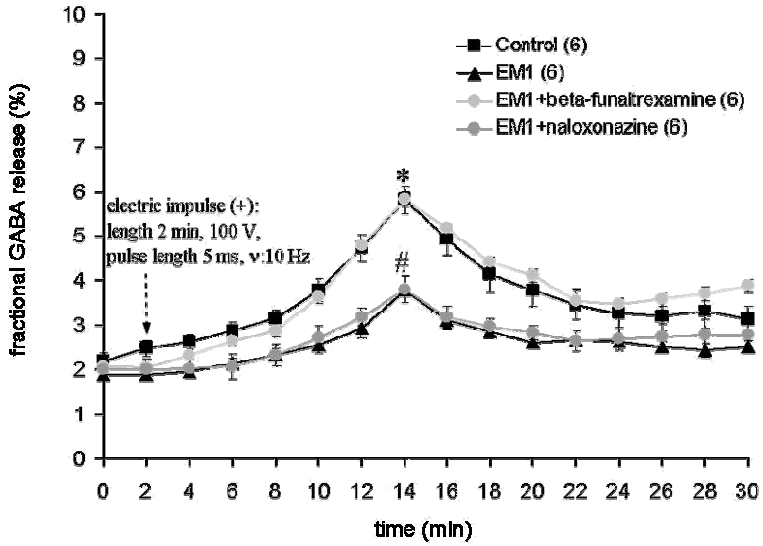


Diagrams 18-20

The effects of the UCNs on the amygdalar GABA release

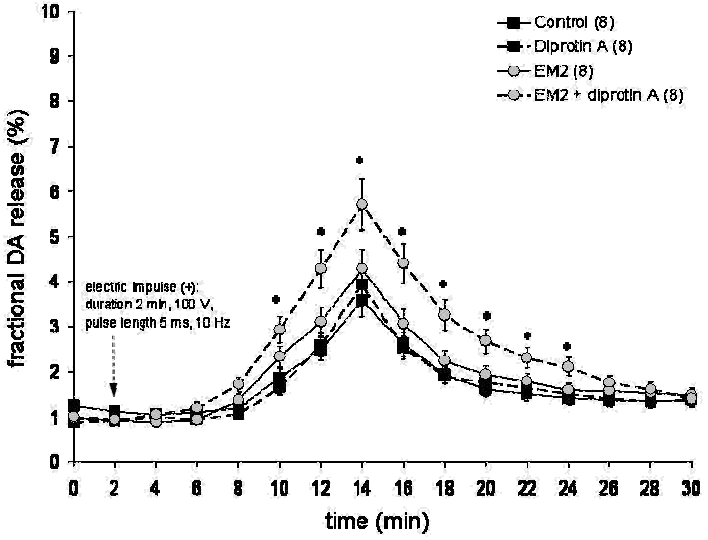


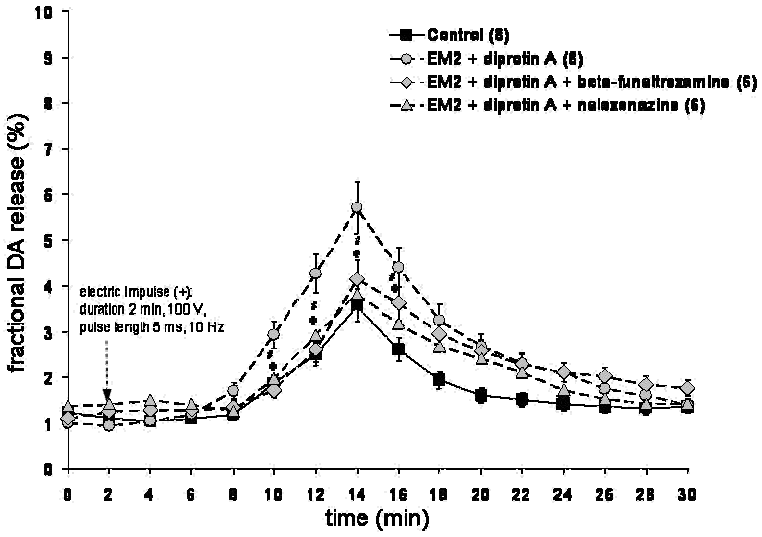


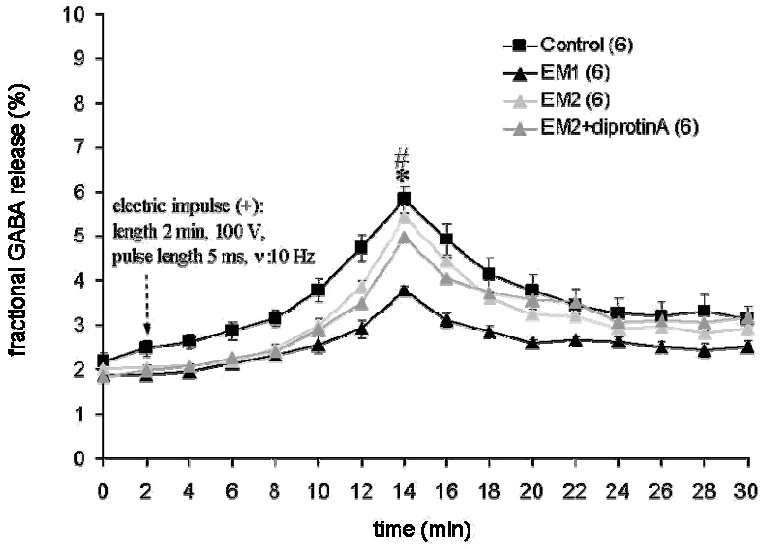


Diagrams 21-23

The effects of the EM1 on the striatal DA and GABA release







Diagrams 24-26

The effects of the EM2 on the striatal DA and GABA release

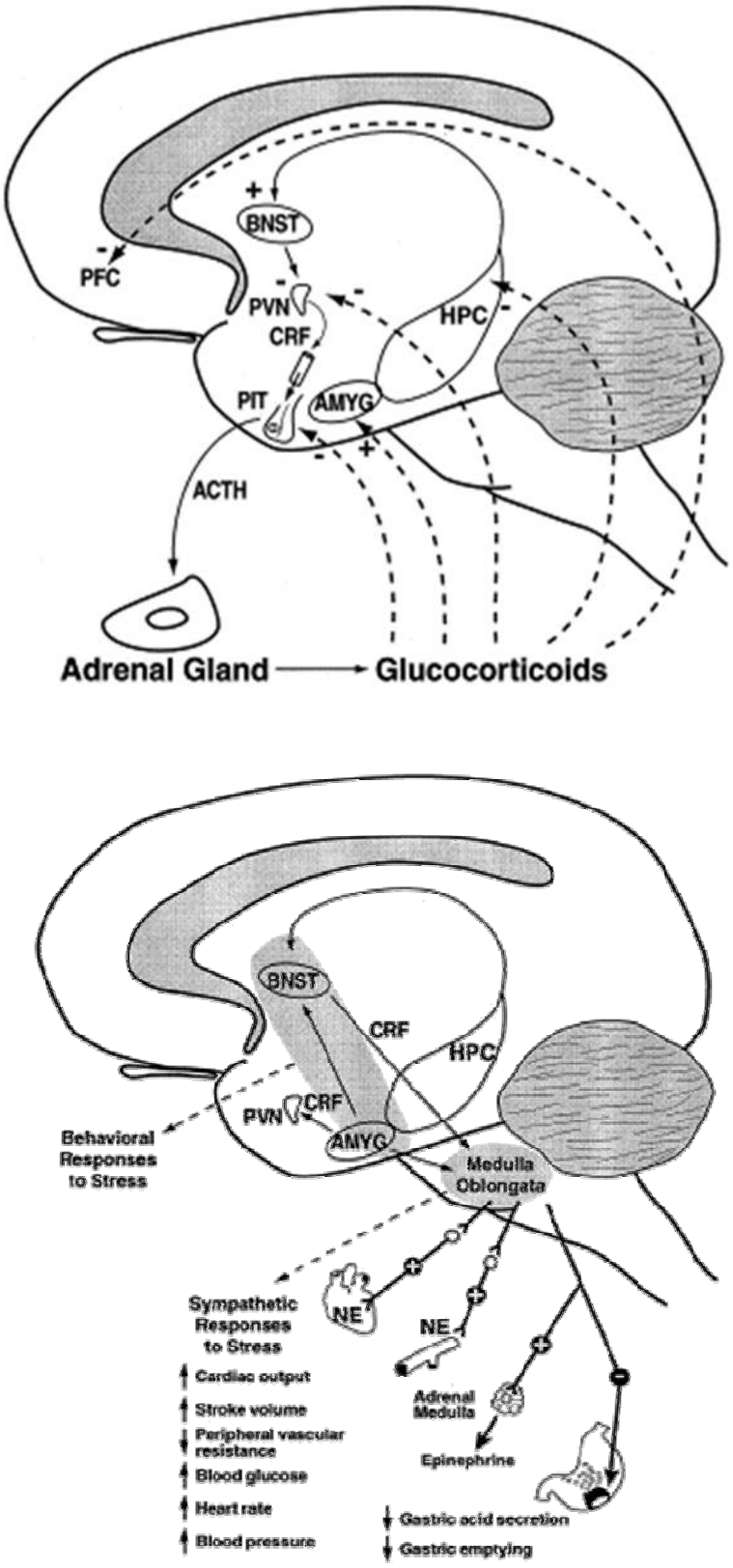
5. DISCUSSION

5.1. The effects of the UCNs on the HPA neurohormones

Since the isolation of CRF, it has been postulated that CRF is the principal regulator of HPA axis in mammals, stimulating ACTH release from the pituitary and glucocorticoid release from the adrenal gland **[1]** inducing a series of endocrine, autonomic and behavioural responses **(Figures 15-16).** Since the description of the UCNs, it has been speculated that UCNs are putative fine-tuners of the stress response, stimulating anxiogenic behaviour through CRFR1 or anxiolytic behaviour through CRFR2 **[12, 87-93].** It was shown that CRF evokes ACTH and glucocorticoid production through CRFR1, a specific G protein-coupled receptor that activates the adenylate cyclase - protein kinase A pathway. AVP is also known to provoke ACTH and glucocorticoid secretion through V3 receptors and adenylate cyclase - protein kinase C pathway **[94].**

Our results are in agreement with previous studies which demonstrated that both CRF and UCN I induce increase of corticosterone production in rats in dose-dependent manner. Previous experiments indicated that CRF and UCN I share common receptors and pathways but show different potencies in their abilities to stimulate ACTH and/or glucocorticoid production and/or secretion **[90, 95]**. However in the present experiments the receptors and second messengers were not investigated and differences between the two effects were not observed. Indeed, the higher affinity of UCN I for CRFR1 than that of CRF could explain a higher potency of UCN I than that of CRF, but an equal affinity of UCN I for both CRFR1 and CRFR2, with putative antagonistic actions on the HPA axis, could invalidate these discrepancies.

Though there is no doubt about the role of CRFR1 and its principal agonists in the activation of HPA axis, there is still a debate about the role of CRFR2 and their preferential agonists in the modulation of stress response. Our studies are clearly discordant with studies in mice suggesting that central administration of UCN II or UCN III does not evoke HPA responses **[96-97]**. They are partly concordant with agonist studies in rats, demonstrating that activation of CRFR2s provokes activation of HPA axis **[89, 91],** and partly concordant with antisense oligonuclotide studies in rats, suggesting that the antagonization of CRFR1 and CRFR2 provokes antagonistic changes of ACTH and corticosterone release **[87, 93]**.



Figures 15-16

The regulation of hypothalamic and extrahypothalamic CRF pathways

As a possible explanation for the biphasic effects of the UCNs should be mentioned that physiological and pharmacological concentrations could activate different receptor subtypes (CRFR1 vs. CRFR2α or CRFR2β) and stimulate different brain circuits (hypothalamic vs. extrahypothalamic). However the differences in the potency of UCN II and UCN III may not be explained by their binding affinities, but the differencies in their solubilizing and signalling properties **[3-4]**. The administration of the CRFR2 agonists and antagonists into different species (mice vs. rats) or different strains (Wistar vs. Sprague Dawley), and the determination of neurohormone concentrations after various periods of time (minutes vs. hours) and at various levels (central vs. peripheral) should be also considered for the integration of these results **[89, 91, 96-97].**

It must be admitted as well that surgery and injection itself, in addition to the solitary housing and chronic cannulation, could stimulate or sensitize the HPA axis. Though this nonspecific stress could possibly increase the basal hormone level even after saline injection, it could not influence the effect of peptide administration as it could be easily differentiated from that of the vehicle injection. Despite these observations, we suppose that in basal conditions or physiological doses CRF and UCN I, acting mainly through CRFR1 receptors on the anterior pituitary stimulate the release of ACTH and corticosterone, while UCN II and UCN III acting probably through CRFR2 on the hypothalamus inhibit the HPA axis.

5.2. The effects of the UCNs on striatal and amygdalar neurotransmitters

There are various studies suggesting a strong correlation of CRF with DA and GABA in brain regions like the paraventricular and arcuate nuclei of hypothalamus, the ventral tegmental area, the basal ganglia, the basolateral and central nuclei of amygdala **[75-77, 82, 98-112].** The anatomical and functional connections between these brain regions are reviewed in the works of Meloni, Gerety et al. 2006 **[107]** and Cullinan, Ziegler et al. 2008 **[100] (Figures 15-16)**. Our studies are in accord with these studies, demonstrating that both CRF and UCN I increase DA release from the striatum and GABA release from the amygdala following electrical stimulation, through the activation of CRFR1. The differences between the effects of equimolar doses of CRF and UCN I could be explained by the higher affinity or lipid solubility of UCN I, as compared to CRF.

The CRFR2 agonists, UCN II and UCN III, did not influence the neurotransmitter release. This is not surprising since distribution of CRFR2 in the basal ganglia and in the central amygdala is very low. However these results do not rule out the possibility that activation of CRFR2 in other regions of the brain may affect DA or GABA function in the

basal ganglia or in the extended amygdala, respectively. Furthermore, some would speculate that not CRFR1 or CRFR2, but CRF-independent mechanisms might participate in the nonspecific stress represented by electrical stimulation.

These neurochemical changes may explain the behavioral consequences of CRF and UCN I administration in rats: locomotor activation in a familiar environment and suppression of exploration in an unfamiliar one. Indeed, the locomotor and the anxiogenic actions of CRF in rats might be mediated by CRFR1s and extrahypothalamic sites as they were inhibited by α-helical CRF 9-41 and antalarmin, but not by hypophysectomy or glucocorticoids **[113-116]**. UCN II and UCN III might inhibit these processes through activation of hypothalamic and extrahypothalamic CRFR2s, though our studies are not conclusive in this direction **[96-97, 117-118]**.

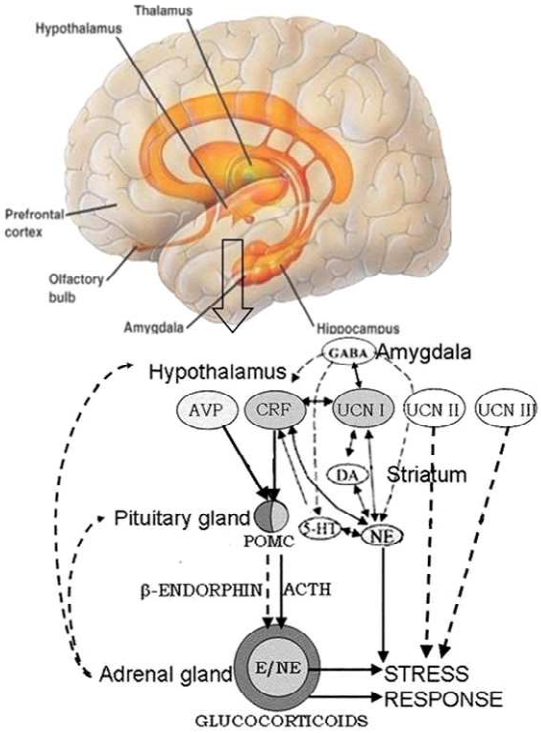
5.3. The significance of antalarmin

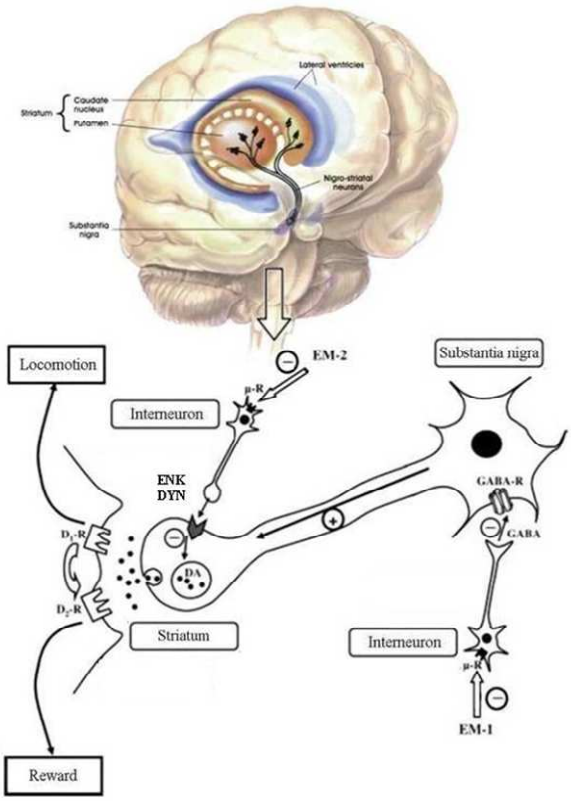
Antalarmin is a selective and competitive antagonist of CRFR1 with nonpeptidic structure that efficiently antagonized the neuroendocrine and autonomic consequences of HPA activation **[26].** It also exhibited antidepressant and anxiolytic properties in rodents and inhibited the stress-induced responses in primates **[27, 119-121].**

Previous studies demonstrated that acute administration of CRF and UCN I induce locomotor hyperactivation and anxiogenic behaviour through the activation of CRFR1 receptors. The present study claims that these behavioural changes might be mediated or associated by biochemical processes, such as, the DA release from the striatum and GABA release from the amygdala **[75, 95, 97, 113, 122-128] (Figures 17-18).**

Chronic stress would induce a sensitisation of the HPA axis and increase of ACTH and glucocorticoids and through this, desensitisation of the glucocorticoid receptors and decrease of the negative feedback effects exerted by glucocorticoids upon CRF and ACTH. Chronic stress would induce a stimulation of the amygdala with the release of CRF and UCN I and GABA that would overactivate the HPA axis directly (through CRFR1 activation) or indirectly (through GABA-GABA interaction) leading to the development of stress.related disorders **[129-130].** The putative pathophysiological and potential therapeutical role of neurotransmitters and neuropeptides in stress were reviewed by Carrasco and updated by Rotzinger **[8, 131] (Figures 17-18).**

In this order of thoughts, antalarmin, or any other selective CRFR1 antagonist with similar behavioural and biochemical properties, could represent future therapy of psychiatric diseases, such as major depression and general anxiety disorder **[132-135].**





Figures 17-18

The regulation of hypothalamic and extrahypothalamic DA/GABA pathways

5.4. The effects of the EMs on the HPA neurohormones

Though a close relationship between the levels of endogenous opioids and corticosteroids has been demonstrated, a precise role of opioid peptides and opioid receptors in the stress response has not been fully elucidated **[136-137].** As some authors claim that β-endorphin inhibits the HPA axis through MORs in the hypothalamus **[138-139]**, and other authors sustain that morphine stimulates ACTH and corticosterone release through the same receptors **[140-141]**, the role of the MORs and their ligands in the control of the HPA axis remains ambiguous **[142-143].**

Results with the most specific endogenous ligands for MORs, EMs are also dichotomous. Centrally administered EMs did not stimulate the HPA system and had no effect on corticosterone release, at doses which were sufficient to activate other physiological systems **[144]**. Furthermore, activation of the HPA axis did not influence the plasma level of EMs in rats exposed to chronic inflammatory, immunological and psychological stress, although plasma corticosterone, ACTH, and β-endorphin had been elevated. These results suggest that EMs do not play an important role in mediating the stress response or regulating the HPA axis.

However parallel studies described EMs having high affinity for the HPA axis **[145-146]**. Thus, our colleagues demonstrated that both EM1 and EM2 elicited corticosterone release exhibiting two dose-response curves with similar inverted U-shape but different dose-ranges. This EM-evoked endocrinological response could be completely blocked by α-helical CRF 9-41 preatreatment, as well as the EM-induced behavioural responses associated to stress, such as anxiogenic behaviour and locomotor hyperactivity. Consequently, these effects are likely to be mediated through CRF, however the participation of other ACTH secretagogues (with the exception of AVP) cannot be fully excluded. The concentration of EM2 accomplishing similar action to that of EM1 was 10-fold lower and the concentration of α-helical CRF 9-41 abolishing this action was 2-fold higher than that in the case of EM1. These discrepancies in dose-effect could be explained more likely by the differences in the receptor activation and signal transduction, than the differences in the diffusion and degradation of EMs. Apparently, EM1 and EM2 activate different subtypes of the MORs and G-proteins: EM1 activates both MOR1 and MOR2 subtypes and Gi1a and Gi3a proteins, while EM2 stimulates just MOR1 subtype and Gi2a protein **[69, 147].**

Our experiments validated this second opinion, not the first observation. Indeed, if activation of the HPA axis induced by morhine is mediated by MORs, the inefficiencies of its

endogenous analogues might be due to the differences in diffusion and degradation of these peptides, compared with morphine **[79, 148] (Figures 19-20).**

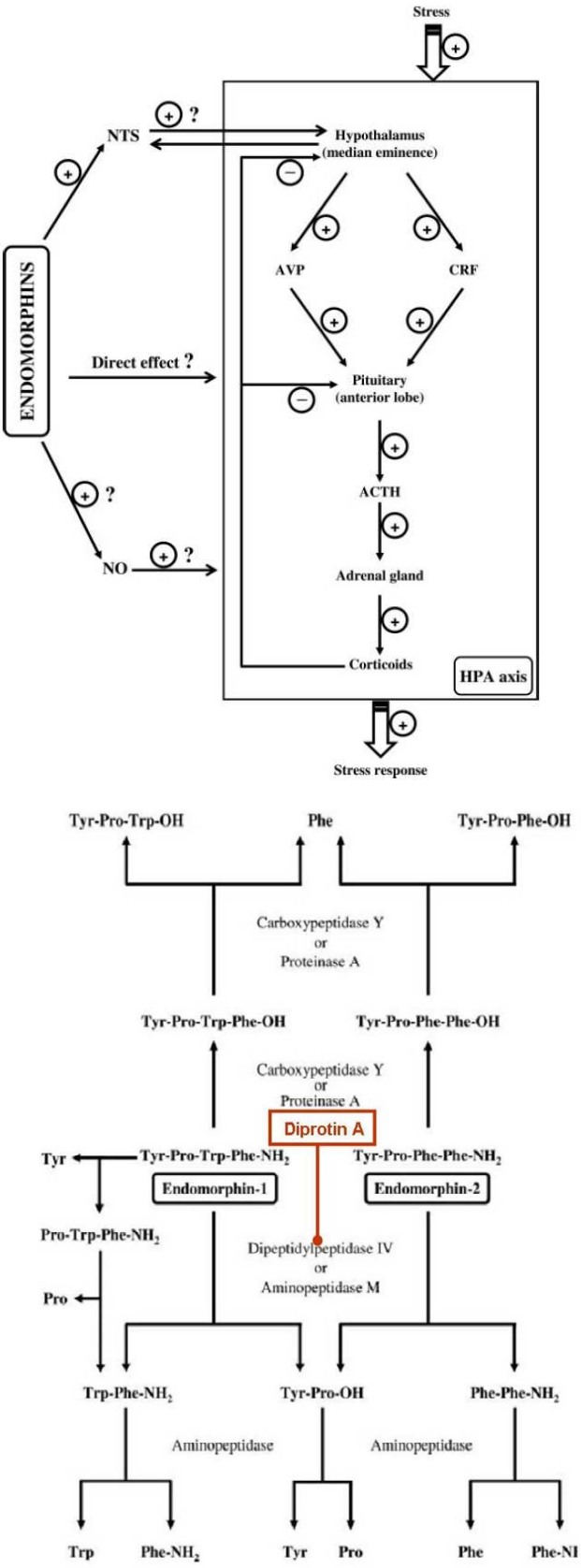
Some studies depicted reciprocal connections between hypothalamic sites of CRF production and extrahypothalamic sites of EM expression, such as nucleus tractus solitarius and periaqueductal gray, inevitable brain regions in the modulation of the stress response **[149-152].** These observations suggest that EMs might be involved in nucleus tractus solitarius-mediated activation of the HPA axis and periaqueductal gray-mediated stress-induced analgesia.

5.5. The effects of the EMs on striatal and amygdalar neurotransmitters

There are numerous studies demonstrating a wide colocalization of MORs and a strong interaction of morhine with the DA- and GABA-ergic neurons of the mesolimbical pathway (from the ventral tegmental area to the nucleus accumbens) and the nigrostriatal pathway (from the substantia nigra to the putamen and nucleus caudatus) **[153-160].** Since the discovery of the selective agonists of MORs, several of the actions of morphine have been attributed to its endogenous analogues **[102, 145-146, 161-163].** The physiological functions and pharmacological actions of EMs are reviewed in the works of Horvath and Fichna **[57-58]**.

Our results are in concert with these studies, evidencing that EM1 and EM2 have stimulating impact on striatal DA release, and emphasizing that EM1, but not EM2, have inibiting effect on striatal GABA release, at least in the presence of enzyme inhibitor and electrical stimulation. These actions of the EMs seem to be mediated by different subtypes of MORs (MOR1 for EM2, and MOR2 for EM1) in the basal ganglia, yet the participation of non-opioid mechanisms can not be fully excluded. Further interactions with other neurotransmitter systems were detailed in previous in vitro superfusion studies which led to the conclusion that the basal neurotransmitter releases are not influenced significantly by EMs and the degradation of these tetrapeptides is not inhibited importantly by enzyme inhibitors **[162, 164]**.

We suppose that the striatal neurotransmitter changes induced by in vitro administration of EMs, might mediate the locomotor hyperactivity produced by in vivo injection of morphine and EMs, and antagonized by naloxone and naloxonazine **[145-146, 153-154, 156-157, 165-166].** We propose an EM-ergic model for the activation of nigrostriatal system and locomotor activity based upon that of mesolimbical system and drug reward **[102, 161, 163, 167-168].**



Figures 19-20

The EM-ic modulation of the central and peripheral stress response

EM1, acting on MOR2 situated on GABAergic neurons may reduce the GABA release from the recurrent axon terminals and induce indirectly DA release from striatal DA-ergic axon terminals. EM2, activating MOR1 located on DAergic neurons may stimulate directly the DA release or eventually with the participation of dynorphynic, enkephalinergic or non-opioidergic interneurons.

The interaction of morphine with DA- and GABA-receptors in the central and the basolateral nuclei of amygdala were investigated recently but the actions of EMs on the DA and GABA releases from these centers of fear and reward were not significant in our studies **[169-172].**

5.6. The significance of diprotin A

Diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) are apparently competitive inhibitors but actually substrates for DPPIV that is present in brain synapses and hydrolizes peptides having Pro as a penultimate aminoacid in their composition **[173-174]**. The enzymatic degradation of EMs was studied in vitro and in vivo and detailed in earlier publications. The integration of behavioural and biochemical results led to the observation that EM1 is more resistant to DPPIV due to its Pro(2)-Trp(3) protected link and EM2 is more sensitive to DPPIV because of its Pro(2)-Phe(3) cleavage site **[78-79].** However the half-life of EMs in membrane preparations were appreciated to be around 15-20 minutes as both tetrapeptides were degraded by synaptic enzymes; carboxipeptidases catabolized preferentially EM1, while EM2 was metabolized by aminopeptidases **(Figures 19-20)**.

The present in vitro superfusion study confirmed previous results, as the effects of EM1 on striatal neurotransmitters were not really influenced by DPPIV inhibition, but the effects of EM2 were significant only with the addition of diprotin A. However a similar in vitro superfusion study investigating the effects of EM1 and EM2 on norepinephrine release from the tractus solitarii–dorsal motor vagal nucleus complex denied the importance of enzymatic degradation in this process **[164]**. Though the EMs and diprotin A were administered in similar conditions, the divergent conclusion may result from the different MOR expression and DPPIV concentration in this region.

From our point of view diprotin A, reducing the affinity of DPPIV for the EM2, may increase the efficacy of the EM2, especially in the striatal synapses. Furtherly, EM2 analogues resistant to DPPIV, with different chemical structure but similar pharmacological profile, could be as potent antinociceptive or anti-panic drugs as EM1 **[175-176].**

6. CONCLUSIONS

We believe that our studies provide an intimate view of the hypothalamic and extrahypothalamic actions of these novel neuropeptides and their interactions with the classic neurohormones and neurotransmitters mediating the stress-induced responses, through the following findings:

6.1. The central administration of UCN I, equipotent agonist of CRFR1 and CRFR2, is associated with CRF and AVP production in the hypothalamus, that ends with the secretion of corticosterone from the pituitary after 30 minutes, proving an activating role for CRFR1 agonists on the HPA axis

6.2. The central administration of UCN II and UCN III, selective agonists of CRFR2, is associated with a biphasic release of CRF and consequently biphasic release of corticosterone 30 minutes following its administration, showing a dose-dependent or time-dependent tuning role of CRFR2 agonists on the HPA axis.

6.3. The local administration of UCN I, activating CRFR1, but not CRFR2, provokes DA release from the striatum and GABA release from the amygdala, mediating the anxiogenic behaviour observed in CRFR1 agonist administration or stressful conditions.

6.4. The in vitro administration of UCN II and UCN III, both selectively activating CRFR2, does not influence DA release from the striatum or GABA release from the amygdala; the anxiolytic behaviour elicited by UCN II or UCN III in vivo might be explained by stimulation of other extrahypothalamic sites or mediators.

6.5. The central administration of EM1 and EM2, endogenous agonists of MORs, increases CRF, but not AVP production in the hypothalamus, inducing corticosterone secretion from the pituitary that peaks after 30 minutes, suggesting a modulating role for the EM on the HPA axis.

6.6. The local administration of EM1 and EM2, activating different subtypes of MORs, evokes DA release from the striatum, through GABA-ergic and non-GABA-ergic mechanisms, mediating the locomotor hyperactivity observed in MOR agonist administration.

We hope that our results bring strong evidence for the pathophysiological significance of the UCNs and the EMs and awakens new interest for the therapeutical potential of CRFR1 antagonists, such as antalarmin, or DPPIV inhibitors, such as diprotin A, in stress-related disorders.

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SUMMARY

INTRODUCTION: The urocortins (UCNs) are recently discovered members of the mammalian CRF peptide family, including CRF (1981), UCN I (1997), UCN II (2001), UCN III (2001), presenting similar amino acid sequences but different binding capacities for CRF receptors (CRFR1 and CRFR2) and CRF-binding protein (CRF-BP). The endomorphins (EMs) are two endogenous opioid tetrapeptides, EM1 and EM2, lately isolated from mammalian brain (1997), with strong affinity and high selectivity for the μ-opioid receptors (MOR1 and MOR2). Though the stimulating action of CRF on ACTH and corticosterone release is unquestionable, there is still a dispute concerning the role of the UCNs and the CRFRs in the regulation of the hypothalamic-pituitary-adrenal (HPA) axis. The principal functions of the endogenous opoids, such as antinociception, has been revealed as well, but the role of the EMs and the MORs in the modulation of the HPA axis is still under debate.

PURPOSES: Our first interest was to determine the interactions of these novel neuropeptides, with the classic neurohormones regulating pituitary ACTH release, such as hypothalamic CRF and AVP, and their actions on consequent adrenal corticosterone release. Our second purpose was to determine the participation of the different CRFR and MOR subtypes and their ligands in the release of extrahypothalamic neurotransmitters, such as striatal DA and amygdalar GABA, mediating various behavioural responses to stress.

METHODS: Male Wistar rats were decapitated, their trunk blood collected and their brains removed 30 minutes after intracerebroventricular administration of the peptides. In vitro homogenisation and immunosorbent assays (ELISA for CRH, RIA for AVP, respectively) were brought about to evaluate the hypothalamic neurohormone levels. The plasma concentration of corticosterone was measured by chemical assay and fuorescent photometry. The striata and amygdalae were isolated and dissected for in vitro superfusion experiments. The brain tissues were incubated (for 30 minutes) with tritium labelled DA and GABA, treated with CRFR or MOR antagonists (for 20 minutes) and agonists (for 10 minutes), and then electrically stimulated. Ultrasonic homogenisation and scintillometry were carried out to appreciate the extrahypothalamic neurotransmitter levels.

RESULTS: CRF, UCN I, EM1 and EM2 elevated the plasma corticosterone levels dose-dependently; in addition, the most effective doses of UCN I (2 μg), EM1 (5 μg) and EM2 (0.5 μg) elicited the hypothalamic CRF production and corticosterone secretion significantly. The administration of UCN I (2 μg) elevated the AVP production also. UCN II and UCN III

produced similar biphasic dose-response curves, both decreasing the hypothalamic CRF levels in physiological ranges (≤ 2 μg), but increasing it in pharmacological doses (≥ 5 μg). These changes were mirrored by the plasma corticosterone levels, but not accompanied by hypothalamic AVP release. CRF and UCN I increased the amygdalar GABA and striatal DA release; their stimulatory effects were blocked by CRFR1 antagonist antalarmin, but not by CRFR2 antagonist astressin 2B. UCN I and UCN II were ineffective. EM1 increased the striatal DA and decreased the striatal GABA release; both effects were inhibited by specific but non-selective MOR1 and MOR2 antagonist naloxonazine, but not by selective MOR1 antagonist β-funaltrexamine. The effect of EM2 was significant on striatal DA release (at least in the presence of 0,1 mM DPPIV inhibitor diprotin A), but insignificant on striatal GABA release and was inhibited by both (seletive MOR1 and non-selective MOR1 and MOR2) antagonists. We used equimolar and the most effective doses (100 nM in case of UCNs and 10 μM in case of EMs) of both agonists and antagonists.

CONCLUSIONS: The results of the homogenisation experiments demonstrate that the UCNs are fine-tuners of the HPA axis determining antagonistic changes of the corticosterone secretion, and that these dose-dependent or time-dependent responses are initiated or associated with changes of CRF production. We also claim, what our colleagues has been already suggested, that the EMs activate the hypothalamic-pituitary-adrenal (HPA) axis and that CRF, but not AVP, participates in this process. Based upon the superfusion experiments, it can be speculated that the anxiogenic behaviour provoked by CRF and UCN I and the locomotor hyperactivity evoked by EM1 and EM2 could be mediated by amygdalar GABA release and striatal DA release, respectively. We also sustain that the anxiolytic behaviour and the locomotor hypoactivity elicited by UCN II and UCN III might be explained by stimulation of other extrahypothalamic sites or mediators.

ÖSSZEFOGLALÁS

BEVEZETÉS: Az urokortinok, UCN I (1997), UCN II (2001) és UCN III (2001), a CRF peptid-család újonnan felfedezett tagjai, melyek hasonló aminosav-szekvenciával, de különböző kötődési affinitással rendelkeznek, ami a CRF receptorait (CRFR1 és CRFR2) és a CRF-kötő fehérjét (CRF-BP) illeti. Bár a CRF stimuláló hatása az ACTH és a kortikoszteron termelésre már felfedezése korában bizonyítást nyert, az UCN-ok és a CRFR-ok szerepe a hypothalamus-hypophysis-mellékvesekéreg (HPA) tengely szabályozásában még mindig tisztázásra szorul. Az endomorfinok, EM1 (1997) és EM2 (1997), nemrégiben kimutatott endogén opioid tetrapeptidek, melyek erős affinitást és jelentős szelektivitást mutatnak a mu opioid receptorok (MOR1 and MOR2) iránt. Míg az EM-ok és MOR-ok főbb funkciói, mint a fájdalomérzet csökkentése, időközben egyértelművé váltak, egyéb hatásai, mint a HPA tengely befolyásolása, mai napig vita tárgyát képezik.

CÉLOK: Dolgozatom célja, elsősorban, ezen újszerű neuropeptidek és a klasszikus hypothalamikus stressz-regulátorok (pl. CRF és AVP) kölcsönhatásainak tisztázása. Másodsorban, dolgozatom tárgya különböző CRFR és MOR altípusok és ligandjainak hatásai az extrahypothalamikus stimuláló- és gátló transzmitterekre (pl. striatális DA és amygdaláris GABA).

MÓDSZEREK: Kísérleteinkben hím Wistar patkányokat használtunk; 30 perccel a peptid intracerebroventrikuláris beadását követően az állatok törzsvérét összegyűjtöttük és az agyát elkülönítettük. A hypothalamikus neurohormonok szintjeinek (CRF, illetve AVP) megállapításához in vitro homogenizációt és immunoszorbens vizgálatokat (ELISA, illetve RIA) végeztünk, az extrahypothalamikus neurotranszmitterek (pl. striatális DA és amygdaláris GABA) szintjeinek kimutatásához in vitro szuperfúziót és szcintillometriás viszgálatokat alkalmaztunk. A plazma kortikoszteron koncentrációt kémiai és fluoreszcens módszerrel állapítottuk meg.

EREDMÉNYEK: A CRF, az UCN I, az EM1 és az EM2 dózis-függő módon emelte a plazma kortikoszteron szintet, valamint, a leghatékonyabb dózisokban (2 μg UCN I, 5 μg EM1 és 0,5 μg EM2), a hypothalamikus CRF szintet is. Ezek mellett az UCN I adása az AVP termelését is fokozta, míg az UCN II és az UCN III, valamint az EM1 és az EM2 nem volt hatással rá. Az UCN II és az UCN III hasonló bifázisos dózis-görbét produkált, fiziológiás dózisban (≤ 2 μg) emelve, farmakológiás dózisban (≥ 5 μg) csökkentve a hypothalamus CRF és a plazma kortikoszteron tartalmát. A CRF és az UCN I equimoláris dózisban (100 nM)

fokozta az amygdalából való GABA és a striátumból való DA felszabadulást; a CRFR1 antagonista antalarmin, szemben a CRFR2 antagonista astressin 2B-vel, hatékonyan gátolta ezeket a hatásokat. Ezzel szemben, az UCN II és az UCN III ugyanazokban a dózisokban, hatástalanok voltak. Az EM1 fokozta a DA és csökkentette a GABA felszabadulását a striátumból, miközben az amygdalában nem váltott ki semmilyen hatást; a specifikus de nem-szelektív MOR1 és MOR2 antagonista naloxonazin, megakadályozta, míg a szelektív MOR1 antagonista β-funaltrexamin nem befolyásolta az EM1 striatális hatásait. Az EM2 jelentős stimuláló hatást mutatott a striatális DA szintre (legalábbis 0,1 mM DPPIV inhibitor diprotin A jelenlétében), de jelentéktelen hatással volt a striatális GABA szint esetében; az EM2 striatális DA-ra kifejtett hatását mindkét specifikus (szelektív és nem-szelektív) MOR antagonista blokkolta. Mind a MOR agonistákból, mind a MOR antagonistákból equimoláris dózisokat (10 μM) használtunk.

KÖVETKEZTETÉS: In vitro homogenizációs kísérleteink bizonyítják, hogy az UCN-ok élettani körülmények között, dózis-függő és ellentétes hatásokkal vannak a HPA tengelyre, melyeket a hypothalamikus CRF termelődés és az adrenális kortikoszteron felszabadulás egyaránt tükröz. Azt is kijelenthetjük, amire már kollegáim is utaltak, hogy az EM-ok stimuláló hatással vannak a HPA tengelyre, és hogy ezt a hatást valószínűleg a CRF és nem az AVP közvetíti. In vitro szuperfúziós kísérleteink alapján feltételezzük, hogy a CRF és az UCN I anxiogén hatásait CRFR1-on keresztül az amygdaláris GABA és a striatális DA felszabadulás közvetíti, valamint hogy az EM1 és az EM2 a lokomotoros aktivitást fokozó hatásukat, különböző MOR-okon hatva, a striatális dopaminerg rendszer aktiválásán keresztül fejthetik ki. Hozzátesszük, hogy az UCN II és az UCN III centrális adásakor megfigyelt anxiolytikus viselkedés és lokomotoros hypoaktivitás más agyrégiók vagy jelátvivők stimulálását feltételezi.

APPENDIX