THE EFFECTS OF CRF AND THE UROCORTINS ON THE AMYGDALAR-HYPOTHALAMIC-HIPPOCAMPAL SYSTEM

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Ph.D. Thesis
Summary

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LIST OF ABBREVIATIONS

[^{3}H] = tritium
ACH = acetylcholine
ACTH = adrenocorticotropic hormone
AHH = amygdalar-hypothalamic-hippocampal
CNS = central nervous system
CPM = count per minute
CRF = corticotropin-releasing factor
CRF-BP = corticotropin-releasing factor-binding protein
CRFR1 = corticotropin-releasing factor receptor type 1
CRFR2 = corticotropin-releasing factor receptor type 2
CRH = corticotropin-releasing hormone
GABA = gamma-amino-butyric acid
GLU = glutamate
GPCRs = G protein-coupled receptors
HPA = hypothalamic-pituitary-adrenal
ICV = intracerebroventricular
SCP = stresscopin
SRP = stresscopin-related peptide
UCN1= urocortin I
UCN2= urocortin II
UCN3= urocortin III

1. INTRODUCTION

1.1. CRF

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.

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.

1.2. The urocortins

Since CRF was first characterized, a growing family of ligands and receptors has been discovered. The mammalian family members include CRF, urocortin I (UCN1), urocortin II (UCN2), also known as stresscopin-related peptide (SRP), and urocortin III (UCN 3), also known as stresscopin (SCP), along with two CRF receptors, CRFR1 and CRFR2, and a CRF-binding protein (CRF-BP). These family members share common elements considering their aminoacidic composition and intracellular signalization but show different aspects regarding their anatomical distribution and physiological functions.

UCN1 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. UCN2 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. UCN3 expression has been discovered in hypothalamus and amygdala in the CNS, and in the gastrointestinal tract and pancreas in the periphery. While CRF has tenfold higher affinity for CRFR1 than for CRFR2, UCN1 has equal affinities for both receptors. Both CRF and UCN1 can be found attached to CRF-BP. Though UCN2 and UCN3 appear to be selective for CRFR2, they may also activate CRFR1 at higher concentration. UCN2 and UCN3 cannot be bound by CRF-BP.

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. The role of CRF, activating CRFR1, is stimulatory upon stress responsivity, whereas the role of UCN2 and UCN3, both acting on CRFR2, appears to be inhibitory upon stress sensitivity**.**

As the only ligand with equally high affinity for both receptors, UCN1's role may be promiscuous. Lately, it has been proposed that the stress-like actions observed after the

administration of these neuropeptides is stressor dependent (physical and psychological) and species specific (mice and rats).

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

1.3. The CRF receptors

CRFRs belong to the class B subtype of G protein–coupled receptors (GPCRs). 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. 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 with the transmembrane and intracellular domains of the CRFRs presenting 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 chimeric-receptor studies in which the N terminus, second and third extracellular domains, and the N-terminal juxtamembrane region have been shown to be important in determining the ligand binding and receptor specificity**.**

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 predominant 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.

Besides the membrane-bound CRF receptors, there is a soluble one, named CRF-BP that can bind CRF and UCN1 and that is thought to modulate the endocrine activity of these peptides. 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.

In order to investigate the physiological, pharmacological and therapeutical role of CRFR1 and CRFR2 first non-selective, and later selective CRFR antagonists have been developed. The first CRFR antagonist synthesized and studied was α-helical CRF 9-41, that efficiently blocked CRF-induced ACTH secretion and stress-induced locomotor activation, followed by D-Phe CRF 12–41, a more potent antagonist of CRF, than α-helical CRF 9-41. 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 behavior, but it could not prevent, CRF or stress-induced locomotor hyperactivity. 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, UCN1 or stressors. Though results with CP-154,526 may seem confusing, studies with antalarmin may prove promising for future anxiolytic and antidepressant research**.** Antisauvagine 30 and astressin 2B, structurally derived from sauvagine and astressin, respectively, are selective peptidic CRFR2 antagonists used preferentially to scrutinize the peripheral functions of CRFR2s. 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**.** 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**.**

1.4. The hypothalamic-pituitary-adrenal (HPA) system

CRF a hypothalamic neurohormone and an extrahypothalamic neurotransmitter that mediates the endocrine, autonomic and behavioral responses to stress. As a hypothalamic neurohormone, CRF activates the hypothalamic-pituitary-adrenal (HPA) axis. CRF is secreted

from the paraventricular nucleus (PVN) of the hypothalamus and released into circulation at the level of median eminence; reaching the anterior pituitary it stimulates the secretion of adrenocorticotropic hormone (ACTH), which on its turn stimulates the production of glucocorticoids in the adrenal cortex. The increase of plasma glucocorticoid concentration not only reflects the activation of the HPA axis, but it exerts negative feedback effects on the hypothalamus, the anterior pituitary and the hippocampus and positive feedback effect on the amygdala.

1.5. The amygdalar-hypothalamic-hippocampal (AHH) system

As an extrahypothalamic neurotransmitter, CRF may also modulate the HPA axis. CRF is also synthesized in the central nucleus of the amygdala (CeA) found in the vicinity of the medial nucleus of the amygdala (MeA) and the ventral subiculum (vSub) region of the hippocampus. Neurons from these regions (especially MeA and vSub) send GABAergic or glutamatergic projections to the GABAergic neurons of the bed nucleus of the stria terminalis (BNST) and the peri-paraventricular nuclei (peri-PVN), which exert a tonic GABAergic inhibition upon the paraventricular CRF synthesis/release. Thus, the amygdala through GABAergic-GABAergic disinhibition increases, whereas the hippocampus through glutamatergic-GABAergic inhibition decreases the activity of the HPA axis, respectively.

1.6. CRF, urocortins, CRF receptors and the hippocampus

Besides the regulation of the stress responses, CRF and the urocortins could play an important role in the modulation of cognitive processes along with other neuropeptides. This second role is supported by the following lines of evidence: CRF-positive neurons and their projections are also found in various brain regions linked to cognition, including the hippocampus and the prefrontal and cingulate cortex. UCN1 expression is represented predominantly in the Edinger-Westphal nucleus, but it was also reported in one of the cholinergic brainstem nuclei, the laterodorsal tegmental nucleus, that may represent a major site of interaction of the CRF/urocortin system with the cholinergic system. UCN2 is expressed, amongst other sites, in the noradrenergic locus coeruleus, that, together with the cholinergic laterodorsal tegmental nucleus, is implicated in mediation of arousal. UCN3 expression was not reported in any regions implicated in cognitive processes, but projections from regions rich in UCN3 expression, such as the perifornical area of the hypothalamus, the medial nucleus of the amygdala, the bed nucleus of the stria terminalis to sites involved in learning and memory, such as the hippocampus, cannot be excluded. In addition, both CRFR1

and CRFR2 receptors are moderately to strongly expressed in the olfactory bulbs, the hippocampus, the entorhinal cortex, the bed nucleus of the stria terminalis and the periaqueductal grey, many of these brain regions being involved in the mediation of arousal, attention, learning and memory. These observations raise the possibility that activation or inhibition of the CRF receptors can affect these types of behavior.

2. PURPOSES

Our previous *in vitro* studies have demonstrated that CRF and UCN1 stimulate the hypothalamic and amygdalar GABA release in rats and that this stimulatory effect is mediated *via* CRFR1, and not CRFR2. The aim of the present studies was to investigate the effects of CRF and the urocortins on the hippocampal glutamate and acetylcholine release in rats in similar *in vitro* conditions.

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:

Antalarmin (Sigma-Aldrich, Austria), selective CRFR1 antagonist; Asstressin2B (Sigma-Aldrich, Austria), selective CRFR2 antagonist.

Other substances used in experiments were:

[^{3}H]ACH (Amersham, USA), tritium labelled acetylcholine; [^{3}H]GLU (Amersham, USA), tritium labelled glutamate; Krebs solution: NaCl, KCl, $MgSO_4$, $NaHCO_3$, glucose, KH_2PO_4 and $CaCl_2$ (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;

3.2. Animals

Male Wistar rats (Animal Husbandry Services, Domaszék, Hungary) weighing 150-250 g were used. During the experiments they were kept and handled in accordance with the instructions of the University of Szeged Ethical Comittee for the Protection of Animals in

Research which are concordant with the European Communities Council Directive of 24 November 1986 (86/609/EEC).

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**,** 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.

3.4. Treatment

In vivo administration of CRF agonists or antagonists was performed ICV through the cannula implanted in the right lateral cerebral ventricles 30 min before the animals were sacrificed. *In vitro* administration of the substances will be described as part of the in vitro superfusion studies.

3.5. Methods

3.5.1. *In vitro* superfusion studies

The hippocampal glutamate and acetylcholine release were measured by means of an *in vitro* superfusion system. First, the rats were decapitated, and their brains were removed and dissected in a Petri dish filled with ice-cold Krebs solution. The hippocampus was isolated from each rat according to the following stereotaxic coordinates: rostro-caudal - 4.0 to - 6.0 mm, medio-lateral + 2.0 to + 5.0 mm, dorso-ventral + 3.0 to + 8.0 mm, using bregma as a point of reference. The brain tissue was cut into 3 μm slices with a tissue chopper (McIlwain Inc., USA). The brain slices were incubated for 30 min in 8 ml of Krebs solution (Reanal Ltd., Hungary), 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, 15 mM of [^{3}H] glutamate (PerkinElmer Inc., USA) or [^{3}H] acetylcholine (PerkinElmer Inc., USA) was added

to the incubation medium. After incubation, the tritium-labelled brain slices were transferred to each of the four cylindrical perspex chambers of a superfusion system (Experimetria Ltd., Hungary). Golden electrodes were attached to both halves of the chambers and connected to an ST-02 electrical stimulator (Experimetria Ltd., Hungary). A multichannel peristaltic pump (Gilson Minipuls 2) was used to perfuse the brain slices at a constant rate of 300 μl/min. The slices were superfused for 30 min to allow tissue equilibrium, and then the superfusates were collected in Eppendorf tubes by a multichannel fraction collector (Gilson FC 203B).

The hippocampal slices were pretreated with 0.1 nM of the selective CRFR1 antagonist antalarmin (Sigma-Aldrich Inc., USA) or 1 nM of the selective CRFR2 antagonist astressin$_2$B (Sigma-Aldrich Inc., USA), and then treated with 100 nM of non-selective CRF agonists, such as CRF (Bachem Ltd., Switzerland) and UCN1 (Bachem Ltd., Switzerland) or 100 nM of selective CRFR2 agonists, such as UCN2 (Bachem Ltd., Switzerland) and UCN3 (Bachem Ltd., Switzerland). CRF and the urocortins did not affect the baseline acetylcholine release and therefore, the hippocampus was stimulated electrically that may mimic the stress-induced hippocampal acetylcholine release. Hence, after 2 min, electrical stimulation consisting of square-wave impulses (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) and the peak of the fractional release was observed at 14 minutes.

The brain tissue was removed from each chamber and solubilized in 200 ml of Krebs solution, using an ultrasonic homogenizer (Branson Sonifier 250). After the addition of 3 ml of Ultima Gold scintillation fluid (PerkinElmer Inc., USA) to the samples and the remaining brain tissue, the radioactivity was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard Inc., USA) and expressed in count per minute (CPM). The fractional release was calculated as the ratio between the radioactivity of the samples and that of the remaining brain tissue.

3.5.2. *In vivo* behavioral studies

3.5.2.1. Elevated plus-maze test

Thirty minutes after the ICV treatment, the rats were evaluated in an elevated plus-maze test, validated by Lister and Rodgers to investigate anxiety-like behavior. The apparatus consists of a plus-shaped wooden platform elevated at 50 cm from the floor, made-up by four opposing arms of 50 cm × 10 cm. Two of the opposing arms are enclosed by 40 cm-high side and end walls (closed arms), whereas the other two arms have no walls (open arms). The

principle of the test is that open arms are more fear-provoking, and the ratio of the times spent in open vs. closed arms, or the ratio of the entries into open vs. closed arms, reflects the relative safety of closed arms, as compared with the relative danger of open arms. Each rat was placed in the central area of 10 cm × 10 cm of the maze, facing one of the open arms. For a 5-minute period the following parameters were recorded by an observer sitting at 100 cm distance from the center of the plus-maze: a. the percentage of the number of entries into the open arms relative to the total number of entries, b. the percentage of the time spent in the open arms relative to the total time and c. the total number of entries into the open and the closed arms. Entry into an arm was defined as the entry of all four feet of the animal into that arm. The apparatus was cleaned up with sodium hypochlorite solution (HIP-TOM Ltd., Hungary) between the subjects.

3.5.2.2. Porsolt's forced swim test

In parallel, the rats were evaluated for 5 minutes in a forced swim test, invented by Porsolt et al. to investigate depression-like behavior. The apparatus consists of a plexiglass cylinder of 50 cm height and 20 cm diameter positioned on a table. The cylinder was half-filled with water maintained at 25±1 °C. The principle of the test is that in such a situation, from which they cannot escape, animals rapidly became immobile, that is, floating in an upright position and making only small movements to keep their heads above water. Meanwhile their attempt to escape the cylinder by climbing or swimming may decrease or cease eventually. Each rat was dropped individually into the water. For a 5 minutes period the following parameters were recorded by an observer sitting at 100 cm distance from the table: a. the climbing activity (the time that rats spent with climbing the walls, in their attempt to escape the cylinder), b. the swimming activity (the time that rats spent with swimming in the water, in their attempt to remain at the surface) and c. the time of immobility (the time that rats spent in an upright position on the surface with its front paws together). A 3 second period was considered a time unit, thus the climbing and the swimming activities and the time of immobility were expressed in time units. The water from the apparatus was changed between the subjects.

3.5.2.3. Morris' water-maze test

To assess the effects of CRFR1 antagonists and CRFR2 agonists on acquisition of spatial reference memory, subjects were tested in the Morris water maze, as described previously. Briefly, subjects were trained to reach a hidden platform in a 1.8 m open-field

water (25±1 °C) maze after receiving ICV pretreatment (−30 min) with antalrmin, UCN2 or UCN3. The submerged platform (size of platform 10 cm×10 cm, 1 cm below surface of water) was maintained in the northeast quadrant. The room was dimly lit by diffuse light reflected from four floodlights situated beneath the rim of the maze and was equipped with prominent extra-pool cues. The rats' performance was monitored by an observer situated at 1 m distance. The following three dependent measures: latency to reach the platform, path length (distance traveled) to the platform, and cumulative distance from the platform (an index of search error or proximity to the platform during the trial). Swim speed was calculated as the total path length divided by the total trial duration. The animals were trained for 3 days, before the test. On each day, subjects started each trial facing the exterior at a different, pseudorandomly determined compass point (N, E, S, W), except for the first trial of the first day when they were restricted to a starting point distant from the platform (S, W). Trials ended when the platform was located, after which the subjects spent 30 s on the platform or, for subjects that did not escape, 90 s had elapsed.

3.6. Statistics

For the statistical analysis of our in vitro studies, the fractional release of glutamate and acetylcholine was calculated and analyzed 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 homogenized tissues and the differences between samples were determined by two-way ANOVA with repeated measures in cases of superfused tissues. A probability level of 0.05 or less was accepted as indicating a statistically significant difference.

Statistical analysis of the results of our in vivo studies was performed by analysis of variance (GraphPad Prism, GraphPad Software Inc., USA). The differences between groups were determined by one-way ANOVA followed by Holm-Sidak's post hoc test for pair-wise comparisons. The probability level of 0.05 or less was accepted as indicating a statistically significant difference.

4. RESULTS

4.1. The effects of CRF and urocortins on the hippocampal glutamate release

CRF and UCN1 (both of 100 nM concentration) decreased significantly the hippocampal [^{3}H]glutamate release elicited by electrical stimulation. In contrast, UCN2 and UCN3 (both of 100 nM concentration) did not affect significantly the hippocampal

[^{3}H]glutamate release enhanced by electrical stimulation. The effect of CRF was reversed remarkably by antalarmin, but not by astressin 2B, both being administered in equimolar doses (100 nM). Also, the effect of UCN1 was reversed completely by antalarmin, but not by astressin 2B, both being administered in equimolar doses (100 nM). CRF, UCN1, UCN2 or UCN3 did not change the basal release of [^{3}H]glutamate. Nevertheless, antalarmin and astressin 2B alone, did not change the stimulated release of [^{3}H]glutamate.

4.2 The effects of the selective CRFR1 antagonist and selective CRFR2 agonists on anxiety and depression

CRFR1 antagonist antalarmin (100 nM concentration) significantly increased the time spent in open arm and entries into the open arm. In contrast, UCN2 and UCN3 (both of 100 nM concentration) did not affect significantly neither the time spent in open arm, nor the entries into the open arm compared to the control group.

CRFR1 antagonist antalarmin (100 nM concentration) significantly increased the time spent with swiming and climbing while significantly decreased the time spent with inmobility. In contrast, UCN2 and UCN3 (both of 100 nM concentration) did not affect significantly neither the time spent with swiming and cimbing, nor the time spent with inmobility compared to the control group.

4.3. The effects of CRF and urocortins on the hippocampal acetylcholine release

CRF and UCN1 significantly increased, while UCN2 and UCN3 significantly decreased the stimulated hippocampal acetlycholine release. The increasing effect of CRF and UCN1 was significantly reduced by antalarmin, but not astressin$_2$B. In contrast, the decreasing effect of UCN2 and UCN3 was significantly reversed by the selective CRFR2, but not the selective CRFR1 antagonist.

4.4. The effects of the selective CRFR1 antagonist and selective CRFR2 agonists on cognitive functions

On the 2nd day CRFR1 antagonist antalarmin (100 nM concentration) and CRFR2 agonists (UCN2 and UCN3) decreased the latency time significantly, compared to the control group. Meanwhile, ont he 3rd day only Antalarmin significantly reduced the latency time, compared to the control group.

On the 4th day, CRFR1 antagonist antalarmin (100 nM concentration) significantly increased the time spent in the N-E quadrant. In contrast, UCN2 and UCN3 (both of 100 nM concentration) did not affect significantly the time spent in the N-E quadrant.

On the 4th day, CRFR1 antagonist antalarmin (100 nM concentration) significantly increased the number of crossovers. In contrast, UCN2 and UCN3 (both of 100 nM concentration) did not significantly affect the number of crossovers.

5. DISCUSSION

5.1. The impacts of CRF and urocortins on the hippocampal glutamate release

The presnt study demonstrates that of CRF and UCN1 inhibit the glutamate release in the hippocampus *via* CRFR1, as the selective CRFR1 antagonist was able to increase the hippocampal glutamate release decreased previously by the non-selective CRFR1 agonists. CRFR2 is not involved in this process, since the selective CRFR2 antagonist did not reverse the effects of CRF or UCN1 and the selective CRFR2 agonists UCN2 and UCN3 did not affect the hippocampal glutamate release either.

Our previous *in vitro* superfusion study reported that CRF and UCN1 stimulate the amygdalar GABA release *via* CRFR1, but not CRFR. We speculated that this amygdalar GABA is released from both the MeA and the CeA, which respond to distinct stressors and are thought to have divergent roles in HPA regulation. Neurons from the MeA are activated following exposure to emotional stressors including predator, social interaction, forced swimming and restraint stress paradigms and send mainly GABAergic projections to GABAergic neurons of BNST and the peri-PVN which directly innervate the PVN leading to activation – actually to disinhibition – of the HPA axis. In contrast, the CeA is activated following exposure to homeostatic stressors, including hemorrhage and immune challenge and exerts its feed-forward effect on the HPA axis through interneurons localized in the brain stem.

The present study completes our previous report with the observation that CRF and UCN1 inhibit the hippocampal glutamate release *via* CRFR1, and not CRFR2. We presume that a similar process may occur under different stressors in the vSub region of the hippocampus, which has been implicated in the regulation of the HPA axis. Hippocampal lesions involving the vSub were shown to produce exaggerated HPA responses to restraint and open field exposure, but not to hypoxia or ether exposure, suggesting that hippocampal neurons respond to distinct stress modalities. Neurons from this region send mostly

glutamatergic projections to GABAergic neurons of BNST and the peri-PVN which directly innervate the PVN, resulting ultimately in inhibition of the HPA axis.

The interaction of CRF and urocortins with glutamate have been investigated in other *in vitro* settings also. An earlier study indicated that CRF and UCN1 modulate differently the excitatory glutamatergic synaptic transmission in the CeA and the lateral septum, which are reciprocally innervated. Another study concluded that UCN1, but not UCN2, protects cultured hippocampal neurons from oxidative stress and glutamatergic excitotoxicity *via* CRFR1, even more potently than CRF does. We suggest that besides having role in neurotransmission and neuroprotection, CRF-glutamate and UCN1-glutamate interactions may also take part in the regulation of the HPA axis. Based on the previous and the present results, we propose that CRFR1 agonists can activate the HPA axis not only directly by stimulating the pituitary ACTH and consequently the adrenal glucocorticoid secretion, but also indirectly by increasing the amygdalar GABA release and decreasing the hippocampal glutamate release.

5.2. Therapeutical implications in anxiety and depression

The principal role of CRF and the urocortins is to regulate the neuroendocrine, autonomic, and behavioral stress responses, including the regulation of the hypothalamic-pituitary-adrenal (HPA) axis, which consists of the paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary and the adrenal cortex. Intracerebroventricular (ICV) administration of CRF and UCN1 induces activation of the HPA axis reflected by elevation of the plasma corticosterone concentration, anxiety- and depression-like behavior in mice and rats. In contrast, ICV administration of UCN2 and UCN3 produces anxiolytic and anti-depressant actions in rodents. It was hereby hypothesized that in physiological conditions, CRF and UCN1 would initiate the responses to stress activating CRFR1 in the anterior pituitary, whereas UCN2 and UCN3 would terminate these responses activating CRFR2 in the PVN. Consequently, CRFR1 and CRFR2 are presumed to mediate antagonistic effects, as regards stress, anxiety, and depression. However, our previous *in vivo* studies suggested that the role of CRFR2 in the regulation of the HPA axis can be inhibitory or stimulatory, depending on the actual concentration of their agonists. In addition, CRF- and UCN1-induced stimulation of the amygdalar GABA release and inhibition of the hippocampal glutamate release *via* CRFR1, but not CRFR2, may also modulate the activity of the HPA axis. This notion is supported by the observation that, next to the PVN, CRF is also expressed in the central nucleus of the amygdala (CeA) and the ventral subiculum (vSub) of the hippocampus. Overwhelming stress may induce a pathological stimulation of

CRF/CRFR1 system in the cerebral cortex and the amygdala over the urocortin/CRFR2 system in the lateral septal nucleus and the hippocampus that may result in hyperactivity of the HPA axis, anxiety and depression. In this order of thoughts, administration of selective CRFR1 antagonists, such antalarmin and/or selective CRFR2 agonists, such as UCN2 and UCN3 are promising therapy in stress-related disorders, such as anxiety and depression.

Our recent in vivo behavioral studies support these observations. In the elevated plus-maze test, administration of the CRFR1 antagonist induced anxiolysis, since the rats treated with antalarmin spent significantly more time in the open arm of the maze. In addition, in the forced swim test, the rats treated with CRFR2 agonists expressed an increased swimming and climbing time and decreased immobility time, indicative of anti-depressant effects. In contrast, administration of UCN2 and UCN3 did not affect the parameters observed either in the elevated plus-maze test or the forced swim test. This is in concert with our in vitro results, according to which CRFR1, but not CRFR2, inhibits the release of hippocampal GLU, that may induce anxiety and depression through GLU-ergic-GABA-ergic stimulation of the hypothalamic CRF release and consequent activation of the HPA axis.

Nevertheless, a recent hypothesis suggested that the action of CRFR1 and CRFR2 is not a matter of simple dualism, but it depends on the brain regions and neuron populations that are activated, and despite the promising pre-clinical results, the clinical application of CRFR1 antagonists produced no therapeutical effects, but some side effects, such as liver toxicity.

5.3. The impacts of CRF and urocortins on the hippocampal acetylcholine release

The present study also demonstrates that CRF and UCN1 increase the hippocampal acetylcholine release through CRFR1, while UCN2 and UCN3 decrease the hippocampal acetylcholine release through CRFR2. Previous results have already indicated that icv administration of CRF stimulates the hippocampal acetylcholine release, and that this process is mediated *via* CRFR1. Nevertheless, the present study is the first to demonstrate that UCN1 stimulates the hippocampal acetylcholine release through activation of CRFR1, whereas UCN2 and UCN3 inhibit the hippocampal acetylcholine release through activation of CRFR2.

Stress affects learning and memory functions that are mediated mainly by hippocampal acetylcholine**.** This effect of stress could be modulated by CRF/CRFR1 system, the presence of which was indicated within the pyramidal cell layers of hippocampus. Furthermore, the effect of systemic, distantly released CRF on hippocampal neurons has been already demonstrated by previous studies. However, the role of the native, locally released

CRF and that of urocortin/CRFR2 system in stress-evoked enhancement of hippocampal synaptic function has not been clarified yet**.**

Previous studies indicated that CRF is stored in GABAergic terminals, while CRFR1 is found in glutamatergic synapses of the hippocampus. This is quite interesting, considering that endogenous CRF has been localized to inhibitory interneurons, yet exogenous CRF acts as an excitatory neurotransmitter. However, the way in which CRF reaches and activates the receptor is not clear. The finding that stress increases CRF-immunoreactivity in extracellular spaces adjacent to the pyramidal cells suggests the possibility that local CRF is released by electrical stimulation from hippocampal interneurons, and then it diffuses slowly to the pyramidal cell dendritic spines. Although under physiological conditions, an extrahippocampal source and a rapid transport of CRF from distant brain sites that is shared by the urocortins cannot be excluded. Moreover, previous studies also indicated that the CRF-induced hippocampal acetylcholine release might be the result of the increased release of acetylcholine from the terminals of neurons projecting from the medial septal nucleus or a decreased uptake of high-affinity choline in the hippocampus.

5.4. Therapeutical implications in cognitive deficits

Hippocampal acetylcholine plays a critical role in cognitive functions, including attention, learning and memory, however studies investigating this role led to contradictory results, due to the complexity of acetylcholine's actions in the brain. Comprehending the complexity of neuropeptide-acetylcholine interactions might allow us to better understand the physiological roles of acetylcholine in cognitive functions and help us to design better treatment options for dementia that is observed in neurodegenerative disorders, including Alzheimer's, Parkinson's, and Huntington disease.

Overall, these studies suggest the existence of two apparently opposing CRF systems in the hippocampus, through which CRF and the urocortins might modulate cholinergic activity and thereby cognitive functions. We further speculate that CRF and UCN1 would facilitate processing and storing of affectively negative information, by activating CRFR1 expressed abundantly in the medial septal nucleus and hippocampus, whereas UCN2 and UCN3 would help to neglect this information, by activating CRFR2 expressed in the lateral septal nucleus and the hippocampus. In physiological conditions, a delicate balance between CRF/CRFR1 and urocortin/CRFR2 systems must exist to secure a normal HPA axis activity and cognitive functioning. However, in pathological states, such as anxiety, depression, and post-traumatic stress disorder an overstimulation of CRF/CRFR1 system may occur that

results in hyperactivity of the HPA axis and deterioration of cognitive functions. In this order of thoughts, administration of selective CRFR1 antagonists, such as antalarmin and/or selective CRFR2 agonists, such as UCN2 and UCN3, might be able to attenuate the hyperactivity of the HPA axis, and the disturbance of attention, learning and memory associated with anxiety, depression, and other stress-related disorders.

Our recent in vivo behavioral studies also support these observations. In the Morris' water-maze test, CRFR1 antagonist and CRFR2 agonists affected similarly the cognitive parameters, as both processes of learning/memory. Namely, antalarmin, UCN2 and UCN3 decreased the latency time on the 2nd day, whereas on the 3rd day only antalarmin reduced the latency time, suggestive of facilitated learning. Also, on the 4th day, only antalarmin (100 nM) increased significantly the time spent in and the number of crossovers through the the N-E quadrant, indicative of enhanced memory. These findings partly coincide, but certainly do not contradict, with our in vitro results, according to which CRFR1 antagonists increased, whereas CRFR2 antagonists decreased the release of the hippocampal ACH, indicative of the dualistic effects of the CRF/CRFR1 and urocortin/CRFR2 systems.

Despite of the fact that the animal experiments are very promising, most clinical trials concluded that CRF antagonists are not effective at treating the affective symptoms of anxiety and depression disorder. Nevertheless, the cognitive actions of CRFR1 antagonists administered alone or together with CRFR2 agonists were not explicitly investigated, hence more animal and human studies are needed to demonstrate the efficacy of these drugs in the therapy of these disorders.

6. CONCLUSIONS

6.1. Our in vitro studies demonstrate that CRF and UCN1 inhibit the hippocampal glutamate release via CRFR1 and that UCN2 and UCN3 and CRFR2 do not participate to this process. Based on the previous and the present results we conclude that CRFR1 agonists can modulate the HPA axis, anxiety and depression not only directly by stimulating the pituitary ACTH release, but also indirectly by increasing the amygdalar GABA release and decreasing the hippocampal glutamate release.

6.2. Our recent in vivo behavioral studies support these observations. In the elevated plus-maze test, administration of the CRFR1 antagonist induced anxiolysis, since the rats treated with antalarmin spent significantly more time in the open arm of the maze. In addition, in the forced swim test, the rats treated with CRFR2 agonists expressed an increased swimming and climbing time and decreased immobility time, indicative of anti-depressant

effects. In contrast, administration of UCN2 and UCN3 did not affect the parameters observed either in the elevated plus-maze test or the forced swim test.

6.3. Our in vitro studies also demonstrate that CRF and UCN1 stimulate the hippocampal acetylcholine release via CRFR1, whereas UCN2 and UCN3 inhibit the hippocampal acetylcholine release via CRFR2. Based on the present results we suggest the existence of two apparently opposing CRF systems in the hippocampus, through which CRF and the urocortins might modulate the cognitive functions, such as attention, learning and memory.

6.4. Our recent in vivo behavioral studies also support these observations. In the Morris' water-maze test, both the CRFR1 antagonist antalarmin, and the CRFR2 agonists UCN2 and UCN3 decreased the latency time on the 2nd day, whereas on the 3rd day only antalarmin reduced the latency time, suggestive of facilitated learning. Also, on the 4th day, only antalarmin increased significantly the time spent in and the number of crossovers through the N-E quadrant, indicative of enhanced memory.

LIST OF PUBLICATIONS

1. Original publications the present work is based on:

I. Bagosi Z, Balangó B, **Pintér D**, Csabafi K, Jászberényi M, Szabó G, Telegdy G: The effects of CRF and urocortins on the hippocampal glutamate release (Neurochemistry International, 2015 Nov; 90:67-71.) **IF: 3.385**

II. **Pintér D,** Balangó B, Simon B, Palotai M, Csabafi K, Dobó É, Ibos KE, Bagosi Z: The effects of CRF and the urocortins on the hippocampal acetylcholine release in rats Neuropeptides, 2021 Aug; 88: 102147) **IF: 2.411**

2. Other publications cited in the present work:

I. Bagosi Z**,** Palotai M, Simon B, Bokor P, Buzás A, Balangó B, **Pintér D**, Jászberényi M, Csabafi K, Szabó G: Selective CRF2 receptor agonists ameliorate the anxiety- and depression-like state developed during chronic nicotine treatment and consequent acute withdrawal in mice (Brain Research, 2016 Dec; 1652:21-29.) **IF: 3.033**

II. Buzás A, Bokor P, Balangó B, **Pintér D**, Palotai M, Simon B, Csabafi K, Telegdy G, Szabó G, Bagosi Z: Changes in striatal dopamine release and locomotor activity following acute withdrawal from chroni*c* nicotine are mediated by CRFR1, but not CRFR2, receptors (Brain Research, 2019 Mar; 1706: 41-47) **IF: 3.370**

III. Bagosi Z, Csabafi K, Balangó B, **Pintér D**, Szolomájer-Csikós O, Bozsó Z, Tóth G, Telegdy G, Szabó G. Anxiolytic- and antidepressant-like actions of Urocortin 2 and its fragments in mice (Brain Research, 2018 Feb 1; 1680:62-68.) **IF: 3.103**

3. Poster presentations related to the present work:

I. Bagosi Z, Bokor P, Buzás A, Balangó B, **Pintér D**, Csabafi K, Szabó G. The effects of the selective CRF2 receptor agonists in mice exposed to chronic nicotine treatment and consequent acute withdrawal (FAMÉ, Pécs, Hungary, 2016)

II. Bagosi Z, Balangó B, **Pintér D**, Bokor P, Buzás A, Csabafi K, Szabó G. The effects of selective CRF receptor antagonists in rats exposed to chronic nicotine treatment and consequent acute withdrawal (FENS, Pécs, Hungary, 2017)

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