Investigation of the signaling pathways and biological effect of urocortin in human tumor cell lines

Ph.D. thesis

Bálint Balogh

Interdisciplinary Medical Sciences Doctoral School

Chair of the doctoral school: Dr. Ferenc Gallyas

Program leader: Dr. György Sétáló

Tutor: Dr. György Sétáló

University of Pécs

Medical School

Department of Medical Biology and Central Electron Microscopic Laboratory

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INTRODUCTION

Cells consistently interact with their environment and are subject to various extracellular stimuli that influence the communication between individual cells and the coordinated functioning of the multicellular organism. A significant proportion of signaling processes between cells is mediated by chemical substances (e.g., growth factors, cytokines, hormones, neurotransmitters), therefore it is called chemical signaling or signal transduction. During signal transduction, the signal-generating cell builds a ligand that reaches the target cell and binds to its ligand-specific receptor. Following the so-called intercellular signal transduction, intracellular signal transduction begins as the ligand binds to its receptor. The signal often also enters the nucleus, which may result in changes in gene expression. As a result of the signals elicited, the cell gives a biological response to it.

1. Neuropeptides of the CRF family

Members of the corticotropin-releasing factor (CRF) ligand family are CRF, urocortin (UCN), urotensin I, and sauvagine. CRF is a peptide of 41 amino acids that stimulates the secretion of corticotropin from the anterior pituitary. The neuropeptides of the CRF family regulate the hypothalamic-pituitary-adrenal axis, the body's response to stress, and the immune response. The CRF peptides also have positive effects on the cardiovascular system and modulate gastrointestinal functions and inflammatory processes in mammals. CRF family ligands and their receptors are expressed in the central nervous system and are also found in peripheral tissues such as the heart, skin, and skeletal muscle. The broad expression pattern of CRF family members reflects the importance of CRF and its related peptides in the body's reaction to stress stimuli and their role in pathophysiological conditions such as depression, anxiety, and anorexia. The activity of CRF and urocortins is also regulated by a secreted protein, CRF-binding protein (CRF-BP), which coordinates the binding of the peptide ligands to CRF receptors.

2. Urocortins

In 1995, Vaughan and his co-workers reported urocortins, as new members among CRFR (CRF receptor) ligands. Three main types of urocortins are known, urocortin1 (UCN1), UCN2 (or stresscopin-related peptide), and UCN3 (or stresscopin). Their genes are located on different chromosomes, and their expression results in the production of precursor proteins with an N-

terminal end containing a signal peptide that eventually undergoes proteolytic cleavage. The length of the mature proteins varies between 38 and 43 amino acids. The 40 amino acid long UCN1 is predominantly expressed in the cells of the Edinger Westphal nuclei but is also found in peripheral tissues such as the gastrointestinal tract, myocardium, testis, thymus, skin, and spleen. UCN2 is expressed in the hypothalamus, brainstem, and spinal cord. In addition, the peptide is expressed peripherally in blood cells, the heart, and the adrenal gland. UCN3 is produced in the hypothalamus and amygdala, while it is also synthesized peripherally by cells of the gastrointestinal system and pancreas. Urocortin peptides bind to CRF receptors but with different affinities. In vitro, UCN1 can bind to both types of CRF receptors with almost the same affinity but it binds to CRFR1 10 times stronger than CRF itself. UCN2 and UCN3 show binding preferences towards CRFR2, with roughly identical affinities as that of CRF. However, at higher concentrations, UCN2 can also activate CRFR1.

3. CRF receptors

Peptides of CRF family ligands use type B1 G-protein coupled receptors (GPCRs) to transmit their signals. CRF receptors are anchored to the cell membrane by seven transmembrane α-helixes of a single peptide chain. These are the so-called heptahelical receptors. Two main types of receptors can be distinguished, encoded by two different genes, and the proteins are designated CRFR1 and CRFR2. In humans, members of the GPCR family are encoded by 15 genes. Their ligands are polypeptide hormones, which typically act in a paracrine or autocrine manner. They use heterotrimeric G-proteins to transmit their signal. Structurally, the amino acid sequences of CRFR1 and CRFR2 receptors are roughly identical (70%), but they show variations in their N-terminal extracellular domain. This is consistent with the different pharmacological features of the receptor isoforms and their selectivity for agonists. These properties play a pivotal role in the regulation of physiological effects mediated by the receptors. Both receptor types are present in the central nervous system, but CRFR1 is more dominant in the cerebral cortex, cerebellum, hippocampus, amygdala, and pituitary gland. In contrast, CRFR2 is preferentially located in the lateral septum and hypothalamus and is also expressed in peripheral tissues such as the heart, skin, lung, gastrointestinal tract, vasculature, and skeletal muscle. The expression of CRF receptors has already been observed in several human tumor types, including melanoma, small-cell lung cancer, and neuroblastoma.

4. Activation of cAMP/PKA and ERK1/2 signaling pathways by CRF receptors

The binding of CRF to CRFR usually triggers the activation of a stimulatory G protein (Gs). The G-protein detaches from the receptor and activates its effector, adenylate cyclase, which in turn produces cAMP from ATP. Then, cAMP activates protein kinase A (PKA), which phosphorylates several target proteins, such as the transcription factor CREB (CRE-binding protein). However, other types of G proteins may also be involved in signal transmission between receptor and effector, such as Gq-mediated phospholipase C (PLC) stimulation and inositol phosphate formation in testicular Leydig cells and the placenta.

The activation of the ERK1/2 (extracellular signal-regulated kinase 1/2) proteins can also be mediated by liganded CRF receptors. ERK1 and ERK2 are serine/threonine-specific protein kinases with molecular masses 44 and 42 kDa, respectively. Based on the literature, the activating phosphorylation of EKR1/2 by CRF neuropeptides is a cell-type dependent process, in which MEK1/2 (mitogen-activated protein kinase/extracellular signal-regulated kinase 1/2) and cAMP-dependent PKA can both participate. The cAMP/PKA-dependent activation of ERK1/2 has already been observed in many cellular systems, such as hippocampal neurons, but in some cases, PKA inhibits the activation of ERK1/2. In CRFR1-transfected CHO cells, UCN1-induced CREB phosphorylation but not ERK1/2 activation can be inhibited using the PKA inhibitor H89, suggesting that CRFR1-mediated MAPK signaling is PKA independent in this cell line. Urocortin- and CRF-induced ERK1/2 activation has been described in various cell types, such as rat cardiomyocytes, cortical neurons, human myometrium, or CRFR1-expressing HEK293 cells. It is also known that in CRFR2-expressing CHO cells, CRF ligand-induced ERK1/2 phosphorylation can be inhibited in the presence of the MEK inhibitor PD98059, suggesting that CRFR2-mediated activation of the MAPK cascade is MEK-dependent. Furthermore, the enzyme PI3K (Phosphatidylinositol 3-kinase) may also play a significant role in MAP kinase phosphorylation initiated via CRF receptors, as pretreatment with the PI3K inhibitor Ly294002 reduces sauvagine-induced ERK1/2 phosphorylation.

5. Human cell lines

As already emphasized, CRF family neuropeptides are pivotal regulators of numerous biological processes, such as the development of the stress and immune response, the coordination of inflammatory processes, and the central regulation of food intake. Extreme variations of the listed factors (e.g. chronic stress and/or inflammation, or being overweight) are well-known promoters of tumor development. It is also known that members of the CRF

family and their receptors are expressed in many tumor types – but are also found in normal tissues – and affect cell division, cell survival, metastasis, and apoptosis. Losing control of this fine-tuned balance can lead to the development of tumors. Considering these possible effects, we investigated the biological response and its regulatory signaling induced by urocortin in human tumor cell lines (MCF7 and HeLa).

AIMS

During my Ph.D. studies, I've set the following goals:

- The examination of urocortin's biological effects in human tumor (MCF7 and HeLa cells) cell lines at the cellular and molecular level.
- The mapping of urocortin's possible, so far unrevealed signaling networks in these human tumor cell lines.
- To investigate the expression and localization of CRF receptor isoforms in MCF7 and HeLa cells and to determine the role of the receptors in the signaling of human urocortin.
- Investigation of the possible effect of urocortin on cell proliferation's regulation in the applied cell lines.
- To determine which signaling pathways are parts of the signaling network of human urocortin1 (HU), with an emphasis on the ERK protein. (It is well known from the literature that urocortin treatment induces ERK phosphorylation in many cell types, and this protein is a pivotal regulator of cell division.)
- To decipher the possible connections of signaling pathways induced via the heterotrimer G-protein-binding CRF receptors and that of the Ras/ERK signaling pathway.
- To elucidate the molecular mechanism of the biological response (possible regulation of cell proliferation) induced by human urocortin treatment by investigating proteins (e.g. p53, Rb, and E2F-1) that play a crucial role in cell cycle regulation.

MATERIALS AND METHODS

For our experiments, we used human MCF7 breast carcinoma and HeLa adenocarcinoma cells of ATCC (American Type Culture Collection) origin, provided by Prof. Dr. József Szeberényi. Most of the chemicals used were purchased from Sigma-Aldrich (part of the Merck Group, Budapest, Hungary) unless otherwise stated. Human urocortin was dissolved in 10% acetic acid according to the manufacturer's instructions. The MEK inhibitor U0126, the PKA inhibitor H89 (Cell Signaling Technology), and the CRFR1-specific antagonist Antalarmin were dissolved in DMSO, while the CRFR2-specific inhibitor Astressin2b in distilled water. Activated proteins were detected by Western blot analysis. Intracellular localization of proteins was examined by laser scanning confocal fluorescence microscopy of relevant immune complexes. Viable cell numbers were determined using CellTiter-Glo 2.0 ATP assay and MTT dye production.

RESULTS AND DISCUSSION

Examination of CRFR expression

We detected the expression of CRFR1 and CRFR2 in lysates of both MCF7 and HeLa cells by Western blotting using antibodies that specifically recognize CRFR isoforms. CRFR1 and CRFR2 mediate a variety of biological effects, but it is mostly a ligand- and cell-type-dependent process. For example, in Cloudman melanoma cells, CRFR1 mediates inhibition of cell division, whereas, in other tumor cells, it stimulates cell proliferation.

Localization of CRFR1 and CRFR2

We detected the intracellular localization of CRFR1 and CRFR2 using laser scanning confocal microscopy and antibodies specifically recognizing these CRF receptors. In MCF7 cells, CRFR1 and 2 immunoreactivities are apparent in the cytoplasm, for CRFR1 with maximal signal intensity towards the periphery and along the plasma membrane. The plasma membrane localization of CRFR1 has been previously described by other research groups too. The CRFR2 immune signal is more granular, has a broader distribution pattern, and appears in deeper layers of the cytoplasm too. In HeLa cells, both CRFR isoforms are present along cell membranes,

exhibiting strong cytoplasmic immunoreactivity. The CRFR1 immune signal is coarser, whereas CRFR2 exhibits finer granules in this cell line. In a previous study, Graziani and colleagues published the expression of CRFR1 at the mRNA level, while Koureta and co-workers detected CRFR2 immunoreactivity in the membrane of MCF7 cells. Although the culture conditions and the steps of immunocytochemistry were identical in our experiments with the two cell lines, there were differences in the signal distribution of CRF receptor isoforms. These variations may be due to cell type-dependent specific properties.

Time kinetics of HU-induced ERK1/2 phosphorylation by Western blotting

Human urocortin treatment of MCF7 cells at a final concentration of 10 nM (HU10) induced robust ERK1/2 phosphorylation 5 minutes after addition of the peptide, then the signal intensity fell back to around that of the untreated control. In HeLa cells, 15 minutes of HU10 treatment induced maximal ERK1/2 phosphorylation, followed by the rapid decrease in signal intensity to around baseline. Urocortin-induced ERK1/2 activation protects the isolated rat heart against ischemic and reperfusion injury, but its role in stress adaptation, neuroprotection, and regulation of apoptosis has also been described. Furthermore, it is also known that transient ERK1/2 activation stimulates cell proliferation in PC12 cells.

Dose-dependence of HU-induced ERK1/2 phosphorylation by Western blotting

Our results showed that 10 nM and 100 nM final concentrations of HU treatment induced marked ERK1/2 phosphorylation in MCF7 cells. Since there was no significant difference between the effects induced by the two treatment concentrations, we decided to use the lower concentration throughout our experiments. In the case of HeLa cells, the treatment concentration of 10 nM was found to be the most effective again, which is also the ideal dose based on literature data.

Inhibition of MEK in HU-treated human cell lines

Pretreatment of MCF7 and HeLa cells with the MEK inhibitor U0126 completely abolished the ERK1/2-phosphorylating effect of HU. The inhibitor alone could reduce even basal ERK1/2 phosphorylation to below detectable. Based on our Western blot results, MEK appears to play a pivotal role in HU-induced ERK1/2 activation in MCF7 and HeLa cells too. A similar inhibitory effect of this chemical on ERK1/2 phosphorylation induced by urocortin

was also observed by Karteris and colleagues in human uterine myometrial cells cultured in the presence of U0126.

Inhibition of PKA in HU-treated cells

In the presence of the PKA inhibitor compound H89, HU-induced maximal ERK1/2 phosphorylation (5 minutes) could be significantly inhibited in MCF7 cells, examined by Western blotting. No similar inhibition was observed in HeLa cells in the presence of the inhibitor. These results suggest that HU-induced ERK1/2 phosphorylation is a PKA-dependent process in MCF7 cells. Based on the literature, the PKA dependence of ERK1/2 activation induced via CRF receptor isoforms varies with the cell type. In neuronal cells, ERK1/2 phosphorylation is mediated by the cAMP/PKA signaling pathway, whereas in mouse cardiac myocytes, MAPK activation is a PKA-independent process.

Selective inhibition of CRFR1 and CRFR2

Pretreatment of MCF7 and HeLa cells with the CRFR1 antagonist Antalarmin could significantly reduce the HU-induced activating ERK1/2 phosphorylation in both human cell lines, examined by Western blotting. By contrast, in the presence of the CRFR2-specific inhibitor Astressin2b, we couldn't detect a similar phosphorylation inhibitory effect in either of the human cell lines used. Our results highlight the importance of CRFR1 in the HU-stimulated ERK1/2 activating phosphorylation in both MCF7 and HeLa cells. In HL-1 myocardial cells expressing CRF receptors, urocortin-stimulated ERK1/2 phosphorylation can be blocked by the CRFR1-specific chemical inhibitor Antalarmin and partially inhibited by the CRFR2 antagonist antisauvagine-30. In rat esophageal cells, the CRFR2-specific inhibitor Astressin2b blocks the ERK1/2 phosphorylating effect of urocortin.

Intracellular localization of phosphorylated ERK1/2 by confocal microscopy

A weak pERK1/2 signal could be detected in the cytoplasm of untreated MCF7 cells by laser scanning confocal fluorescence microscopy. Five minutes after the start of HU treatment the cytoplasmic signal intensity became stronger and it appeared in the nuclei of MCF7 cells as well. By the end of the treatment day, the level of ERK1/2 phosphorylation fell back to around baseline in this cell line. In HeLa cells, a more robust cytoplasmic pERK1/2 immune signal was observed after 15 minutes in the presence of HU compared to untreated control cells. Then a

weaker cytoplasmic ERK1/2 signal appeared in HeLa cells by the end of the treatment day (24 hours). The literature suggests that cytoplasmic-localized ERK1/2 increases the proliferation of PC12 cells, for example, whereas nuclear-localized ERK1/2 slows the cell cycle down and induces differentiation in the same cell type.

Examination of cell proliferation by MTT-assay

Regarding the cell proliferation regulating effect of urocortins and ERK1/2, we examined the biological response in MCF7 and HeLa cells induced by HU10-treatment using MTT-assay. The MTT dye is a tetrazolium salt that is converted to formazan by mitochondrial oxidoreductase enzymes, that are only active in healthy cells. Consequently, the amount of formazan converted is proportional to the number of viable cells. Culturing MCF7 and HeLa cells in the presence of HU10 significantly increased the measurable amount of MTT dye converted to formazan crystals in both cell lines, suggesting that HU10 treatment boosts the ability of these cells to divide. This effect of HU could be inhibited in the presence of the CRFR1 inhibitor Antalarmin or the CRFR2 antagonist Astressin2b in MCF7 cells, respectively. On the contrary, in HeLa cells, the cell proliferation-stimulating effect of HU could be inhibited in the presence of the CRFR1-specific inhibitor Antalarmin, whereas the CRFR2 antagonist Astressin2b did not prevent urocortin from exerting this biological effect. These differences again point to specific, cell-type-dependent features of urocortin signaling.

Examination of HU-induced Rb phosphorylation by Western blotting

We could also detect increased phosphorylation of the key cell cycle regulatory protein Rb induced by HU10 using Western blot analysis in MCF7 cells. This effect could be inhibited in the presence of the MEK inhibitor U0126 and the PKA inhibitor H89, suggesting that both enzymes are central elements of the cell-proliferation-regulating signaling of urocortin in MCF7 cells. In contrast, HU treatment did not induce a significant increase in Rb phosphorylation in HeLa cells.

Examination of E2F-1 protein expression by Western blotting

The E2F-1 protein is a transcription factor that stimulates the expression of S-phase genes, thus promoting cell division. HU10 treatment could significantly increase the level of E2F-1 protein expression in both human cell lines, and this effect could be blocked in the

presence of the MEK inhibitor U0126, suggesting that MEK plays an essential role in HU signaling in MCF7 and HeLa cells. Furthermore, in MCF7 cells, the urocortin-induced increase in E2F-1 protein expression could also be inhibited in the presence of the PKA inhibitor H89, suggesting that this enzyme is also part of the signaling pathway of HU in this cell type.

ATP measurement of MCF7 cells

We examined the effect of HU on cell proliferation by CellTiter-Glo 2.0 ATP-assay. The luminescent signal that is proportional to the amount of ATP present was detected using an ELISA microplate reader. In the presence of the peptide, a significant reduction in ATP production was measured in MCF7 cells, which tightly corresponds to their metabolic activity. However, the markedly increased E2F-1 expression and the significant increase in Rb phosphorylation in the presence of HU confirm the biological effect detected by the MTT-assay. The observed decrease in ATP production in the presence of HU was thus attributed to other metabolic mechanisms that we have not investigated.

Examination of PKB/Akt phosphorylation by Western blotting

The enzyme PKB/Akt is a central regulator of cell survival. Urocortin-induced activation of the protein can be mediated either by CRFR1 or 2, but there are also cases in which UCN inhibits Akt phosphorylation. Phosphorylation of the cell survival-promoting protein PKB/Akt increased in MCF7 cells in the presence of HU10 and reached its maximum 3 hours after the start of urocortin treatment. Phosphorylation of PKB/Akt was also increased in HeLa cells, reaching peak activation 1 hour after the addition of HU. HU-stimulated PKB/Akt phosphorylation may contribute to enhanced survival of MCF7 and HeLa cells.

Decreased p53 level in HU-treated HeLa cells

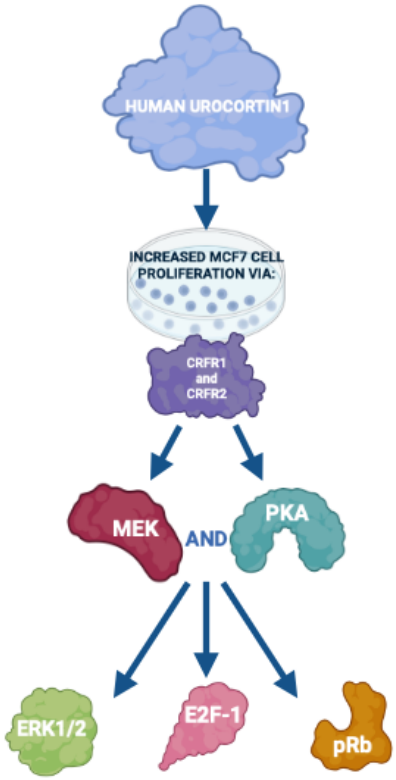
Apoptosis, or programmed cell death, is another biological phenomenon often mediated by CRF receptors. We examined the expression of the cell survival- and proliferation-regulating transcription factor p53 by Western blotting. In HeLa cells, HU10 treatment resulted in a prolonged, statistically significant decrease in p53 protein levels, which returned to around baseline by the end of the treatment day. Reduced p53 levels may contribute to the enhanced survival of HeLa cells.

SUMMARY

In our experiments, we examined the expression of CRFR isoforms in human tumor cell lines by Western blotting and laser scanning confocal fluorescence microscopy. In MCF7 cells, the CRFR1 immune signal was localized along the plasma membrane, whereas in the HeLa cell line, the immune signal also penetrated deeper into the cytoplasm, as did CRFR2 immunoreactivity in both cell types. HU10 treatment could induce transient ERK1/2 phosphorylation in both MCF7 and HeLa cells. The ERK1/2 phosphorylating effect of HU could be blocked by the CRFR1-specific inhibitor Antalarmin, while pretreatment of the cells with the CRFR2 antagonist Astressin2b did not inhibit this effect of HU. HU-stimulated ERK1/2 phosphorylation could be inhibited in both cell types in the presence of the MEK inhibitor U0126, suggesting that MEK plays a central role in urocortin-induced ERK1/2 phosphorylation in both MCF7 and HeLa cells. Similarly, HU-induced ERK1/2 phosphorylation inhibition was also detected in MCF7 cells upon administration of the PKA inhibitor H89, meaning that this enzyme also plays an important role in HU-induced ERK1/2 phosphorylation, but only in MCF7 cells. Urocortin treatment increased the expression of the transcription factor E2F-1 in both human cell lines, which could be inhibited in the presence of the MEK inhibitor U0126, again underlining the role of MEK in HU-stimulated signaling pathways in these cell types. Moreover, in MCF7 cells, the phosphorylation of Rb protein was enhanced in the presence of the neuropeptide, an effect that could be blocked by the MEK inhibitor U0126, as well as in the presence of the PKA inhibitor H89. These inhibitors had the same effect onto increased E2F-1 protein levels in the presence of HU. Our results suggest that urocortin activates cell proliferation-promoting proteins in both MCF7 and HeLa cells, and MEK is an essential regulator of this effect. Furthermore, HU10 treatment also increased the amount of MTT dye converted to formazan in both cell types, which means that the ability of the cells to divide was increased in the presence of urocortin. In MCF7 cells, this effect could be inhibited in the presence of the CRFR1 inhibitor Antalarmin or the CRFR2 antagonist Astressin2b, while in the HeLa cell line, only Antalarmin prevented urocortin from exerting this biological effect. This is one of the cell type-dependent differences that we could identify during our work. The elevation in E2F-1 protein level and the significant increase in formazan dye production in the presence of urocortin in both cell lines, – as well as the increased phosphorylation of Rb protein in MCF7 cells – is suggestive of the enhanced ability of MCF7 and HeLa cells to divide in the presence of HU. Furthermore, phosphorylation of the essential

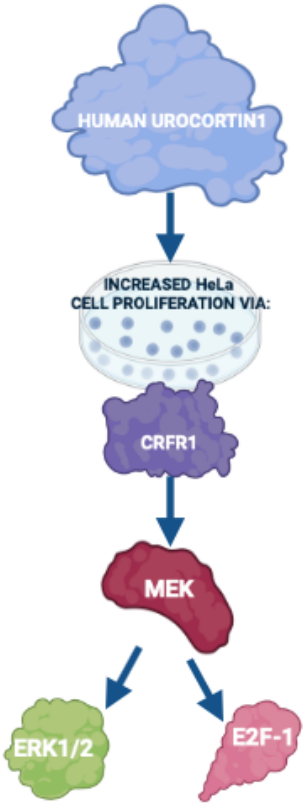
cell survival-regulating protein PKB/Akt was also increased in the presence of HU10 in both examined cell types. HU-induced activating PKB/Akt phosphorylation promotes the survival of the examined cell lines, which is also supported by the reduced p53 protein expression, measured in HeLa cells.

Summarizing our results, we can conclude that in MCF7 cells HU mediates its effects via CRFR1, PKA, and MEK towards ERK1/2, but with respect to the effect of Astressin2b on MTT measurement, we cannot exclude the involvement of other signaling pathways activated via CRFR2. MEK and PKA also play key roles in urocortin-stimulated Rb protein phosphorylation and increased expression of the transcription factor E2F-1, the latter two proteins both promote cell proliferation.



Schematic model of HU-induced signaling in MCF7 cells

Based on our findings, we can further conclude that in HeLa cells, HU mediates its effects via CRFR1 and MEK towards the MAPK ERK1/2. MEK also plays a pivotal role in mediating the increased E2F-1 protein expression induced by HU, which is supportive of HeLa cells' enhanced proliferation during urocortin treatment.



Schematic model of HU-mediated signal transduction in HeLa cells

We believe that our results can help to better understand cell type-dependent differences in urocortin's signal transduction and they further illuminate the role of this neuropeptide in the regulation of cell proliferation, and thus in tumor formation. This could also contribute to the success of developing more selective therapeutic approaches for the targeted treatment of various malignancies in the future.

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PUBLICATIONS

Aggregate impact factor: 13,711
Impact factor of peer-reviewed articles on the topic of the thesis: 6,7

The thesis is based on the following publications:

Balogh B, Vecsernyés M, Stayer-Harci A, Berta G, Tarjányi O, Sétáló G Jr. Urocortin stimulates the ERK1/2 signaling pathway and the proliferation of HeLa cells via CRF receptor 1. FEBS Open Bio. 2023 Mar 27. doi: 10.1002/2211-5463.13602. Epub ahead of print. PMID: 36971048. *IF: 2,6*

Balogh B, Vecsernyés M, Veres-Székely A, Berta G, Stayer-Harci A, Tarjányi O, Sétáló G Jr. Urocortin stimulates ERK1/2 phosphorylation and proliferation but reduces ATP production of MCF7 breast cancer cells. Mol Cell Endocrinol. 2022 May 1;547:111610. doi: 10.1016/j.mce.2022.111610. Epub 2022 Feb 24. PMID: 35219718. *IF: 4,1*

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