Our results are in concordance with literature data about the effects of stressors activating HPA axis [31].

4. Discussion

Experiments about Kyo are partially connected to its` involvement in mechanisms of pain. It`s known that Kyo directly activates cortical neurons and indirectly influences μ- and δ-opioid receptors causing prolonged naloxone-reversible analgesia due to Met-Enk and β-endorphin release [24, 41]. D-Kyo has the same indirect effect on μ- and δ-opioid receptors, but causes prolonged naloxone-reversible analgesia through Met-Enk and β-endorphin release [34, 35, 53]. D-Kyo analgesia in naïve animals results to be 5.6 fold stronger than Kyo`s [41]. In our first experimental series both Kyo and D-Kyo led to decreased nociception which is consistent with literature data. The stronger analgesic effect of D-Kyo is attributed to its enzyme resistance due to replacement of L-arginine with D-arginine [41]. It`s also important that D-Kyo possesses a phenol group participating in interactions of biologically active peptides with cell membrane receptors [29, 30]. Evaluation of nociception after one hour of heat stress showed that heat stress led to higher paw pressure thresholds – results that are fully concordant with literature data about SIA. The underlying mechanism of SIA has two components – an opioid and a non-opioid one [17, 21]. The opioid component is naloxone- and naltrexone-reversible, while the non-opioid one is not sensible to μ-opioid receptor antagonists [27]. In heat-stress-induced analgesia the opioid component of SIA is the more expressed [6, 36]. It`s known that analgesic effects of heat stress are naloxone-reversible [20]. Interestingly, administration of Kyo and D-Kyo did not increase pain thresholds after heat stress. Since in the latter the opioid component is the better expressed and given the mechanisms of Kyo and D-Kyo action (through Met-Enk and β-endorphin release) it was more logic to have a potentiation of heat-stress-induced analgesia. Results showed that Kyo and D-Kyo influenced heat-stress-induced analgesia differently than our expectations. Kyo decreased the pain thresholds and shortened HP-latency for the entire time of the experiment. Pain thresholds observed were comparable with those without heat stress (as if Kyo totally "abolished" the influence of 1h HS on animals). D-Kyo decreased PP-thresholds only on the 15th min, while on the 30th min values were comparable to those after 1h HS, and on the 45th min we had even higher than after-1h HS-values. As to HP-latencies, they were shortened in respect to after-1h HS-values and also in respect to controls on the 45th min. A possible explanation of such results could be that Kyo exerts non-opioid effects, unrelated to enkephalin release [50, 53]. In fact even high Kyo concentrations in the brain steam are tightly connected with sites of opioid analgesia, yet 50% of total Kyo is in brain cortex (where opioid receptors and enkephalin concentrations are low) - suggesting a possible non-opioid pathway in Kyo`s effects. Kyo represents L-tyrosyl-L-arginine – meaning that the semi-essential amino acid L-arg is in the same time a precursor for and a metabolism product of Kyo. A dual effect of L-arg has been described: an analgesic effect through the kyotorphin-Met-enkephalin pathway and a pronociceptive one through the NO-cyclic GMP pathway [24]. In fact a pro-nociceptive effect of the dipeptidic neuropeptide has already been described in the periphery: Ueda and Inoue (2000) demonstrated that intraplantar administration of Kyo (100 fmol) elicited a nociceptive response. The effect was linked to Kyo stimulating its specific receptor, followed by Gai and phospholipase C activation [53]. Another possible explanation of our results could be that Kyo, even having analgesic effect in naïve animals, takes part of the anti-stress system decreasing stress-hormones release and increasing pain perception. Stress activates the hypothalamic-pituitary-adrenal axis causing a specific neuroendocrine pattern of interrelations in order to ensure survival and help the organism to restore homeostasis [47, 49]. Since stress is involved in the etiology and pathophysiology of different pathological condition and diseases (commonly called stress-induced diseases) [48], an anti-stress system also exists aiming at control the stress-response and avoid over-reactivity which could be deleterious to the organism [8]. Our supposition in order to explain the results obtained was that Kyo could be part of the anti-stress system of the body. The D-analog, being "unnatural" to the organism, could differently interfere with stress-answer-mechanisms and could possibly manifest its analgesic effect instead of taking part in the anti-stress system. In order to confirm such hypothesis we evaluated ACTH and corticosterone plasma levels after 1h HS followed by Kyo and D-Kyo administration.

As already described both dipeptides decreased both the evaluated hormones. ACTH plasma levels decreased to control values (on the 15th and the 30th min) and even below the control levels on the 45th min. The effects of both dipeptides were equivalent. CORT plasma levels were also decreased even without reaching the controls`. Kyo crosses the blood-brain barrier (BBB). Is transported by an H+-coupled peptide transporter PEPT2 with high-affinity and low-capacity [12, 13]. PEPT2 mRNA was reported to be strongly expressed in astrocytes of the cerebral cortex, thalamus and hippocampus [10]. Due to its vast distribution in the brain Kyo influences some neurotransmitter systems [15, 26]. It can be supposed that by modulating opioid and non-opioid neurotransmitter systems Kyo helps the organism to regain homeostasis [4, 37]. According to most of the authors D-Kyo doesn`t cross the BBB since the Kyo-transport system PEPT2 "prefers" L-amino acids [39, 46]. Nevertheless D-Kyo, similarly to Kyo binds Kyo-receptors in the peripheral tissues [7] and due to its enzyme resistance causes β-endorphin release and Met-Enk release 4 times fold compared to basal levels [34, 35, 41]. Our results demonstrated that both the dipeptides exerted the same effect on ACTH and CORT with D-Kyo having a stronger suppressive effect on the 10th min compared to Kyo. It`s known that both the dipeptides induce Met-Enk release and evidence exists that Met-Enk differently modulates other neurotransmitter systems at low and high concentrations [5]. The explanation of the results described should be sought in the involvement of other mediators – opioids, nitric oxide, serotonin, catecholamines [44]. The suppressive effects of both Kyo and D-Kyo were stronger on ACTH plasma levels than on CORT ones. We assume that the anti-stress effect of the dipeptides is better expressed in the beginning of the stress-response and effects strongly the ACTH-release phase than the CORT one. Since Kyo-synthase and Kyo-receptors have been found in rats adrenals, a direct effect of Kyo and D-Kyo on CORT could be involved [22, 54].

5. Conclusion

We assume that by interacting with different neurotransmitter systems after heat stress Kyo and D-Kyo take part in the anti-stress response of the body in the attempt to regain homeostasis. The authors declare no conflict of interests.

References

[1] Aloisi Ceccarelli I, Lupo C. Behavioural and hormonal effects of restraint stress and formalin test in male and female rats. Brain Res Bull. 1998; 47(1):57-62.
[2] Amir S, Amit Z. Endogenous opioid ligands may mediate stress-induced changes in the affective properties of pain related behavior in rats. Life Sci. 1978; 23:1143–1151.
[3] Appelbaum BD, Holtzman SG. Restraint stress enhances morphine-induced analgesia in the rat without changing apparent affinity of receptor. Life Sci. 1985; 36: 1069–1074.
[4] Armario A. The hypothalamic-pituitary-adrenal axis: what can it tell us about stressors? CNS Neurol. Disord. Drug Targets. 2006; 5(5):485-501.
[5] Beani L, Bianchi C, Siniscalchi A. The effect of naloxone on opioid-induced inhibition and facilitation of acetylcholine release in brain slices. Br. J. Pharmac.1982; 76: 393-401.
[6] Bocheva A, Dzambazova E. Involvement of endogenous nitric oxide in the effects of Tyr- W-MIF-1 and Tyr-K-MIF-1 after three models of stress. Compt Rend Acad Bulg Sci. 2008; 61(2):261-268.
[7] Bronnikov GE, Kolaeva SG, Dolgacheva LP, Kramarova LI. Kyotorphin suppresses proliferation and Ca2+ signaling in brown preadipocytes. Bull Exp Biol Med. 2006; 141(2):223-225.
[8] Cullinan WE, Herman JP, Helmreich DL, Watson SJ. The neuroanatomy of stress. In: Neurobiologicaland Clinical Consequences of Stress: From Normal Adaptation to PTSD. 1995.
[9] Dallman MF. Stress update: adaptations of the hypothalamic-pituitary-adrenal axis to chronic stress. Trends Endocrinol. Metab. 1993; 4:62-69.
[10] Dieck ST, Heuer H, Ehrchen J, Otto C, Bauer K. The peptide transporter PEPT2 is expressed in rat brain and mediates the accumulation of the fluorescent dipeptide derivative h-Ala-Lys-Nq-AMCA in astrocytes. Glia. 1999; 25:10–20.
[11] Djordjevic J, Vuckovic T, Jasnic N, Cvijic G. Effect of various stressors on the blood ACTH and corticosterone concentration in normotensive Wistar and spontaneously hypertensive Wistar-Kyoto rats. Gen Comp Endocrinol. 2007; 153(1-3):217-220.
[12] Fujita T, Kishida T, Okada N, Ganapathy V, Leibach FH, Yamamoto A. Interaction of kyotorphin and brain peptide transporter in synaptosomes prepared from rat cerebellum: implication of high affinity type H+/peptide transporter PEPT2 mediated transport system. Neurosci Lett. 1999; 271(2):117-120.
[13] Fujita T, Kishida T, Wada M, Okada N, Yamamoto A, Leibach FH, Ganapathy V. Functional characterization of brain peptide transporter in rat cerebral cortex: identification of the high-affinity type H+/peptide transporter PEPT2. Brain Res. 2004; 997(1):52-61.
[14] Galina ZH, Kastin AJ. Tyr-MIF-1 attenuates antinociceptive responses induced by three models of stress-analgesia. Br J Pharmacol., 1987, 90(4):669-74.
[15] Ganapathy V, Miyauchi S. Transport systems for opioid peptides in mammalian tissues. AAPS J. 2005; 7(4):E852-856.
[16] Ignat'ev DA, Vorob'ev VV, Ziganshin RKh. The effect of short peptides isolated from the brain of a hibernating suslik on the rat EEG and behavior. Neurosci Behav Physiol. 1998; 28(2):158-166.
[17] Inoue M, Rashid MH, Kawashima T, Matsumoto M, Maeda T, Kishioka S, Ueda H. The algogenic-induced nociceptive flexion test in mice: studies on sensitivity of the test and stress on animals. Brain Res Bull. 2003; 60:275-281.
[18] Kavaliers M. Responsiveness of deer mice to a predator, the short-tailed weasel: population differences and neuromodulatory mechanisms. Physiol Zool. 1990; 63:338–407.
[19] Kavaliers M, Colwell DD. Sex differences in opioid and non-opioid mediated predator-induced analgesia in mice. Brain Res. 1991; 568:173–177.
[20] Kavaliers M, Innes D. Stress-induced opioid analgesia and activity in deer mice: sex and population differences. Brain Res. 1987; 425:49–56.
[21] Kavaliers M. Evidence for opioid and non-opioid forms of stress-induced analgesia in the snail, Cepaea nemoralis. Brain Res. 1987; 410(1):111-115.
[22] Kawabata A, Muguruma H, Tanaka M, Takagi H. Kyotorphin synthetase activity in rat adrenal glands and spinal cord. Peptides. 1996; 17(3):407-411.
[23] Kawabata A, Nishimura Y, Takagi H. L-leucyl-L-arginine, naltrindole and D-arginine block antinociception elicited by L-arginine in mice with carrageenin-induced hyperalgesia. Br J Pharmacol. 1992, 107(4):1096-1101.
[24] Kawabata A, Umeda N, Takagi H. L-Arginine exerts a dual role in nociceptive processing in the brain: involvement of the kyotorphin-Met-enkephalin pathway and NO-cyclic GMP pathway. Br J Pharmacol. 1993; 109:73-79.
[25] Koko V, Djordjeviae J, Cvijiae G, Davidoviae V. Effect of acute heat stress on rat adrenal glands: a morphological and stereological study. J Exp Biol. 2004; 207(Pt 24):4225-4230.
[26] Kolaeva SG, Semenova TP, Santalova IM, Moshkov DA, Anoshkina IA, Golozubova V. Effects of L-thyrosyl - L-arginine (kyotorphin) on the behavior of rats and goldfish. Peptides. 2000; 21(9):1331-1336.
[27] Lapo IB, Konarzewski M, Sadowski B. Effect of cold acclimation and repeated swimming on opioid and nonopioid swim stress-induced analgesia in selectively bred mice. Physiol Behav. 2003; 78(3):345-350.
[28] Lester LS, Fanselow MS. Exposure to a cat produces opioid analgesia in rats. Behav Neurosci. 1985; 99:756–759.
[29] Lopes SC, Fedorov A, Castanho MA. Chiral recognition of D-kyotorphin by lipidic membranes: relevance toward improved analgesic efficiency. Chem Med Chem. 2006; 1(7):723-728.
[30] Lopes SC, Soares CM, Baptista AM, Goormaghtigh E,

Cabral BJ, Castanho MA. Conformational and orientational guidance of the analgesic dipeptide kyotorphin induced by lipidic membranes: putative correlation toward receptor docking. J Phys Chem B Condens Matter Mater Surf Interfaces Biophys. 2006; 110(7):3385-3394.
[31] Martí O, Gavaldà A, Gómez F, Armario A. Direct evidence for chronic stress-induced facilitation of the adrenocorticotropin response to a novel stressor. Neuroendocrinology. 1994; 60:1-7.
[32] Miczek KA, Thompson ML, Shuster L. Opioid-like analgesia in defeated mice. Science. 1982; 215:1520–1522.
[33] Nishimura K, Kaya K, Hazato T, Ueda H, Satoh M, Takagi H. Kyotorphin like substance in human cerebrospinal fluid of patients with persistent pain. Masui. 1991; 40(11):1686-1690.
[34] Ochi T, Ohkubo Y, Mutoh S. Blockade of the antinociceptive effect of spinally administered kyotorphin by naltrindole in mice. Neurosci Lett. 2002; 322(2):95-98.
[35] Ochi T, Ohkubo Y, Mutoh S. The spinal antinociceptive effect of kyotorphin in mice: involvement of the descending noradrenergic and serotonergic systems. Neurosci Lett. 2002; 329(2):193-196.
[36] Pacák K, Palkovits M. Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr Rev. 2001; 22:502-548.
[37] Pan W, Kastin AJ. From MIF-1 to endomorphin: The Tyr-MIF-1 family of peptides. Peptides. 2007, 28(12):2411-2434.
[38] Randall LO, Selitto J. A method for measurement of analgesic activity on inflamed tissue. Arch Int Pharmacodyn Ther. 1957; 61:409-419.
[39] Sakaeda T, Tada Y, Sugawara T, Ryu T, Hirose F, Yoshikawa T, Hirano K, Kupczyk-Subotkowska L, Siahaan TJ, Audus KL, Stella VJ. Conjugation with L-Glutamate for in vivo brain drug delivery. J Drug Target. 2001; 9(1):23-37.
[40] Sinha RK. Study of changes in some pathophysiological stress markers in different age groups of an animal model of acute and chronic heat stress. Iran Biomed J. 2007; 11(2):101-111.
[41] Takagi H, Shiomi H, Kuraishi Y, Ueda H. Analgesic dipeptide, L-Tyr-D-Arg (D-kyotorphin) induces Met-enkephalin release from guinea pig striatal slices. Experientia. 1982; 38:1344-1345.
[42] Takagi H, Shiomi H, Ueda H, Amano H. A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. Nature. 1979; 282(5737):410-412.
[43] Takagi H, Shiomi H, Ueda H, Amano H. Morphine-like analgesia by a new dipeptide, L-tyrosyl-L-arginine (Kyotorphin) and its analogue. Eur J Pharmacol. 1979; 55(1):109-111.
[44] Tanaka M, Tsuda A, Ida Y, Ushijima I, Tsujimatu S, Nagasaki N. Methionine- enkephalin inhibits stres-induced increases in noradrenaline turnover in brain regions of rats. Jpn J Pharmacol. 1985; 37(1):17-19.
[45] Teskey GC, Kavaliers M, Hirst M. Social conflict activates opioid analgesic and ingestive behaviors in male mice. Life Sci. 1984; 35:303–315.
[46] Teuscher NS, Keep RF, Smith DE. PEPT2-mediated uptake of neuropeptides in rat choroid plexus. Pharm Res. 2001; 18(6):807-813.
[47] Tsigos C, Chrousos GP. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J Psychosom Res. 2002; 53(4):865-871.
[48] Tsigos C, Kyrou I, Chrousos G. Stress, Endocrine Physiology and Pathophysiology. In: Adrenal physiology and diseases, Ed. George Chrousos, 2004.
[49] Tsigos C., Chrousos GP. Physiology of the hypothalamic– pituitary–adrenal axis in health and dysregulation in psychiatric and autoimmune disorders. Endocrinol. Metab Clin North Am. 1994; 23:451-466.
[50] Ueda H, Inoue M. In Vivo Signal Transduction of Nociceptive Response by Kyotorphin (Tyrosine-Arginine) through Gai- and Inositol Trisphosphate-Mediated Ca2+ Influx. Molecular Pharmacology. 2000; 57:108–115.
[51] Ueda H, Matsumoto S, Yoshihara Y, Fukushima N, Takagi H. Uptake and release of kyotorphin in rat brain synaptosomes. Life Sci. 1986; 38(26):2405-2411.
[52] Ueda H, Shiomi H, Takagi H. Regional distribution of a novel analgesic dipeptide kyotorphin (Tyr-Arg) in the rat brain and spinal cord. Brain Res., 1980; 198(2):460-4.
[53] Ueda H, Yoshihara Y, Misawa H, Fukushima N, Katada T, Ui M, Takagi H and Satoh M. The kyotorphin (tyrosine-arginine) receptor and a selective reconstitution with purified Gi, measured with GTPase and phospholipase C. J Biol Chem. 1989; 264:3732–3741.
[54] Ueda H, Yoshihara Y, Fukushima N, Shiomi H, Nakamura A, Takagi H. Kyotorphin (tyrosine-arginine) synthetase in rat brain synaptosomes. J Biol Chem. 1987; 262(17):8165-8173.
[55] Yajima H, Ogawa H, Ueda H, Takagi H. Studies on peptides. Synthesis and activity of kyotorphin and its analogues. Chem Pharm Bull. 1980; 28(6):1935-1938.