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Possible Anti-Stressor Effects of Kyotorphin and its Optical Isomer

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ABSTRACT

Background and Objective: Kyotorphin (KTP), an endogenous analgesic neuropeptide in the central nervous system, is considered to be a neurotransmitter or neuromodulator. D-kyotorphin (D-KTP) is a synthetic analogue of KTP. Both peptides bind to a specific receptor and induced Met-enkephalin release. Thus, the effects of both peptides fall into two clearly identifiable groups: the ones, mediated via opioid peptides and the opioid peptide-independent ones. For a long time we have been interested in the neuromodulating properties of KTP and D-KTP in analgesia due to different types of stress. Our previous data showed that both peptides reduced stress-induced analgesia, which suggest that they may act as an anti-opioid peptides counteracting the effects of stress. During acute stress increased secretion of adrenocorticotropic hormone (ACTH) and corticosterone (CORT) plays a key regulatory role on the basal activity of the hypothalamic-pituitary-adrenal axis and on the termination of the stress response. The aim of this study was to compare changes in ACTH and CORT concentration after various stressors, as well as after injection of KTP or D-KTP. **Materials and Methods:** The male Wistar rats were injected with KTP or D-KTP immediately after exposure of acute immobilization, cold and heat stresses. After decapitation plasma ACTH and CORT were assayed by a double antibody radioimmunoassay method. **Results and Conclusion:** The various stressors applied seem to induce a different response of the HPA system as judged by quantitative changes in ACTH and CORT release. In addition, this study points to KTP and its synthesized optical isomer D-KTP as a possible anti-stressor substances with potential clinical importance in the context of stress-related disorders, since they inhibited stress-induced elevations in two investigated hormones helping the organism to reach homeostatic level. Further studies are needed to understand the exact role of KTP in stress response.

Key words: L-and D-kyotorphin, stress, adrenocorticotropic hormone, corticosterone

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INTRODUCTION

Kyotorphin (KTP) represents a naturally occurring dipeptide (L-Tyr-L-Arg, Fig. 1a) with morphine-like characteristic, first described by Takagi *et al*.[1], isolated from bovine brain. It was subsequently isolated from other sources, including the brains of mice and rats[2], guinea pig, rabbits and squirrels[3] and detected in the CSF in humans [4]. KTP pharmacological activity cannot be attributed to its binding to an opioid receptor. The majority of research associated with KTP relates to modulation of pain mechanisms via its ability to excite directly cortical neurons and to exert indirectly μ-and δ-opioid receptors to produce potent naloxone-reversible and long-lasting analgesia by releasing met-enkephalin and β-endorpins[1,5,6,7]. D-Kyotorphin (D-KTP, L-Tyr-D-Arg, Fig. 1b) were synthesized by Dr. H. Yajima and co-workers. D-KTP, which is the most powerful optical isomer of the natural analgesic dipeptide, shows enhanced analgesic activity 5.6-fold higher than that observed with KTP[8]. An action mediated by specific kyotorphin (non-opioid) receptors for KTP and D-KTP has been suggested by several authors[9,10]. Thus, the effects of both peptides fall into two clearly identifiable groups: the ones, mediated via opioid peptides and the opioid peptide-independent ones.

For a long time we have been interested in the neuromodulating properties of KTP and D-KTP in analgesia due to different types of stress. According literature data moderate concentrations of KTP are present in the hypothalamus-the main structure responsible for stress response and its central administration increases plasma levels of oxytocin-the "stress" hormone in rodents[2,11]. Our previous data showed that if KTP or D-KTP are administered in rats

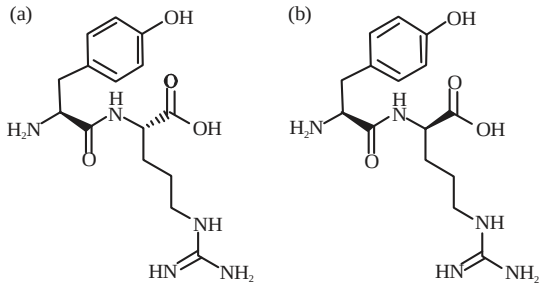

Fig. 1(a-b): Neuroactive dipeptide Kyotorphin (a) and (b) it's optical isomer D-Kyotorphin

subjected to stress procedure, they significantly reduced stress-induced analgesia, which suggest that both peptides may act as an anti-opioid peptides counteracting the effects of stress[12-15].

Although exposure to stress elicits a wide range of biochemical, physiological and behavioral changes, the two prototypical stress responses in all vertebrates are the activation of the Hypothalamic-Pituitary-Adrenal (HPA) and Sympatho-Medullo-Adrenal (SMA) axis. They both represent the effector limbs, via which the brain influences all body organs during exposure to threatening stimuli[16-19]. During acute stress adrenocorticotropic hormone (ACTH) and corticosterone (CORT) secretion, also known as "stress markers", are increased[20,21]. They play a key regulatory role on the basal activity of the HPA axis and on the termination of the stress response by acting at extrahypothalamic centers, the hypothalamus and the pituitary gland [22].

The aim of this study was to compare changes in ACTH and CORT concentration after various stressors (immobilization, cold and heat), as well as after injection of KTP or D-KTP.

MATERIALS AND METHODS

Animals: Male Wistar rats, 60-90 days old, weighing 180-220 g, were used for the experiments. The animals were acclimated to 22±1°C, kept at a 12:12 h light-dark cycle and given commercial rat food and tap water *ad libitum*.

All experimental procedures were conducted in accordance with the "Guide for Care and Use of Laboratory Animals" published by the National Institutes of Health and ethical guidelines of the International Association for the Study of Pain.

Drugs and treatments: Kyotorphin (5 mg kg^{-1}) was obtained from Tocris. The peptide was dissolved in sterile saline (0.9% NaCl) solution and was injected intraperitoneally (i.p). The rats were divided into groups. The first group represented intact controls. The second, third and fourth group consisted of rats exposed to immobilization (IS), cold (CS) and heat (HS) stress respectively. After stress termination six animals of each group were sacrificed immediately, whereas the rest of the stressed rats were injected with KTP or D-KTP and were decapitated 10 min later.

Stress models:

Acute model of immobilization stress: The animals were placed for 1 h in a plastic tube with adjustable plaster tape on the outside so that the animals were unable to move. There were holes for breathing.

Acute model of cold stress: The animals were placed in a refrigerating chamber at 4°C for 1 h.

Acute heat stress: Rats were continuously exposed to hot environment for 1h in the well-ventilated, thermostatically controlled incubator maintained at 38±1°C (relative humidity 45-50%). The control group was not submitted to 1 h stress procedure.

Blood sampling and hormone analyses: The rats were killed by decapitation without anesthesia. Blood was collected from the trunk and divided into two sets of tubes containing heparin. Plasma was separated by centrifugation at 3000 g for 10 min and frozen at -20°C until assayed for hormone levels. Plasma ACTH and CORT were assayed by a double antibody radioimmunoassay (RIA) method specific for rats using a commercial kits (BRAHMS ACTH, Germany; CORT-CT2, CIS, Bio International, Paris). These assays were performed in the laboratory of Prof. S. Milanov in the Hospital of Ministry of Interior, Sofia and the values expressed as pg ACTH/ml and nmol CORT/l.

Data analysis: One-way ANOVA test was employed for the comparison of the experimental groups followed by Dunnett's multiple comparison test. The values are expressed as Means±S.E.M. and the level of significance was set at $p<0.05$.

RESULTS

Blood ACTH level: Immobilization, cold and heat exposure applied as stressors induced a significant increase in ACTH plasma levels ($p<0.01$) compared to

the controls (Fig. 2a, b and c). Exposure to 38°C produced the largest augmentation in plasma ACTH levels compared to other two stressors (Fig. 2c). The low temperature (4°C) as stressor was less potent in elevating plasma ACTH concentration (Fig. 2b).

Administration of KTP immediately after IS significantly reduced the increased levels of ACTH on 10th min ($p<0.01$) (Fig. 2), while D-KTP showed the same effect on 20th min (not shown on the Fig.). Both investigated peptides abolished the effect of CS and HS reversing increased ACTH concentration back to control level (Fig. 2b and 2c).

Blood CORT level: The exposure to extreme environmental temperatures (4 and 38°C for 1 h) and immobilization produced a higher increment of CORT secretion compared to the controls ($p<0.01$) (Fig. 3a, b and c). In comparison, again the heat exposure produced the largest increment (Fig. 3c) while the cold exposure showed the lower increase in plasma CORT levels (Fig. 3b).

Intraperitoneal injections of both peptides immediately after stress procedure significantly reduced the increased levels of CORT ($p<0.01$) (Fig. 3). The effect was more pronounced for D-KTP and also only

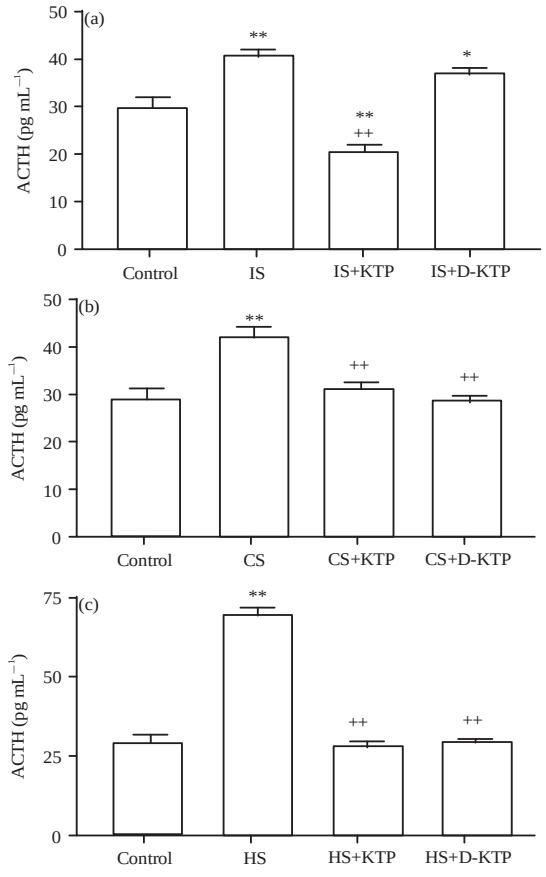

Fig. 2(a-c): Influence of KTP and D-KTP (both at a dose 5 mg kg^{-1}, i.p.) on adrenocorticotropic hormone (ACTH) plasma levels in Wistar rats after exposure to (a) 1 h Immobilization Stress (IS), (b) 1 h cold stress (CS), (c) 1 h heat stress (HS). The data are presented as Means±S.E.M. of six animals (n = 6) in pg ACTH mL^{-1}. Differences between the groups: *$p<0.05$, **$p<0.01$ vs. control, $^{++p}<0.01$ vs. relevant stress

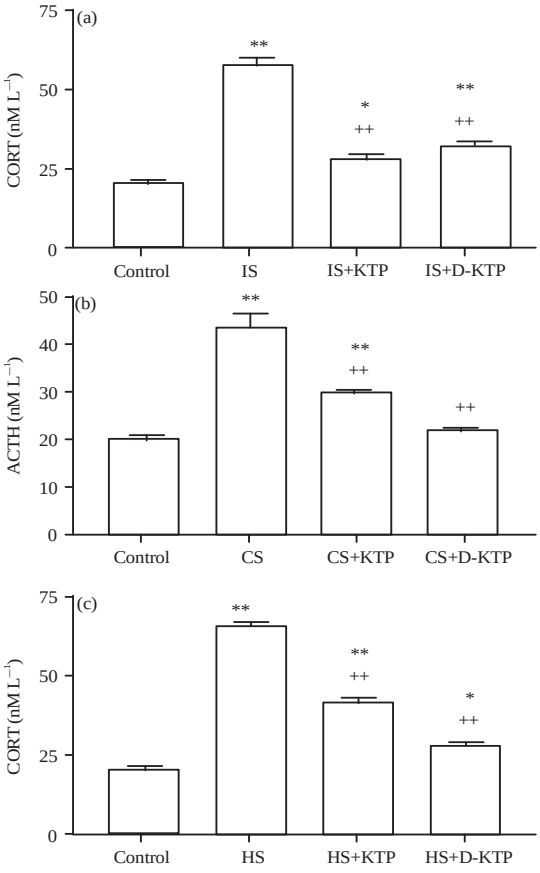

Fig. 3(a-c): Influence of KTP and D-KTP (both at a dose 5 mg kg^{-1}, i.p.) on corticosterone (CORT) plasma levels in Wistar rats after exposure to (a) 1 h immobilization stress (IS), (b) 1 h cold stress (CS), (c) 1 h heat stress (HS). The data are presented as Means±S.E.M. of six animals (n = 6) in nmol CORT/l. Differences between the groups: *$p<0.05$, **$p<0.01$ vs. control, $^{++}p<0.01$ vs. relevant stress

D-KTP completely abolished increased CORT concentration after CS and reversed it to the control level ($p<0.01$) (Fig. 3b). Although CORT was significantly decreased in most of the cases by KTP compared to the stressed group ($p<0.01$), its concentration still remains increased compared to the control ($p<0.05$ or 0.01) (Fig. 3a, b and c).

DISCUSSION

The present results support previous findings that stressors activate the pituitary and adrenal cortex, which are functional parts of the HPA axis[16-19]. They showed that plasma ACTH and CORT concentrations were elevated under the influence of all types of stressors applied but quantitatively differently. The most intense ACTH and CORT increase was provoked by heat exposure, as well as by IS, being more than 2 folds greater than the controls, respectively. The CS was weaker stressor, as compared to both other stressors, as it produced a 1.2 increment of CORT concentration. This is in agreement with findings of some authors, reported that the increment in plasma ACTH was larger under the influence of HS and IS in respect to CS and the concept suggests that each type of stressor has its own central neurochemical and peripheral neuroendocrine "signature", with quantitatively and qualitatively distinct mechanisms[23-26].

The stress response is characterized by a synchronized set of endocrine, immunological, autonomic, behavioral and cognitive responses to perceived threats that is necessary for survival and has been conserved throughout evolution. The prevalence of stressors in the dynamic environment of an animal, make it essential to have mechanisms that limit activity of stress response systems and promote rapid recovery to pre-stress levels. For example, activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis by stress is under tight feedback regulation that serves to restrain and terminate the response[19,27,28].

The orchestrated interplay of several neurotransmitter systems in the brain underlies the characteristic phenomenology of behavioral, endocrine, autonomic and immune responses to stress[29-31]. These transmitters include Corticotrophin-Releasing Hormone (CRH), vasopressin (AVP), dopamine, serotonin, norepinephrine and opioid peptides.

The obtained results showed that both investigated opioid-like peptides KTP and D-KTP significantly reduced increased levels of ACTH and CORT after IS, CS and HS. Also different potency of peptides was observed. In most of the cases the optical isomer D-KTP showed more potent reducing effect on increased levels of ACTH and CORT measured 10 min after peptide injection (Fig. 2 and 3). The same effect was observed for IS on 20 min after D-KTP administration which is not shown on the figures. Thus, the observed more potent and prolonged effect of D-KTP may be due to their peptide structure, specific binding sites and different interaction with opioid or non-opioid components of three stress models. Literature and our data showed that opioid and non-opioid components are differently involved in each of stress models mentioned above. The non-opioid system is mostly involved in CS, opioid-in HS, while both systems are equally presented in IS[23,32]. On the other hand stressors are a potent modulator of opioid activities. For instance, KTP and D-KTP have anti-opioid properties, since they decreased some forms of stress-induced analgesia[13,14,15,31,33]. The anti-opioid peptides are able to reduce some of the acute opioid effects and permanently mask the effects of exogenous and endogenous opioids. As anti-opioid peptides, KTP and D-KTP participate in a homeostatic system, which acts to dampen the effects of opioids. Kyotorphin receptors mediate an activation of phospholipase C in synaptosomal membranes trough G_{i1}, followed by an opening of inositol 1, 4, 5-triphosphate (IP_3)-gated calcium channels, located in the plasma membrane of nerve terminals, thereby directly leading to a production of action potential[34-36]. The KTP is actively taken up into nerve terminals by relatively specific mechanism. The evidence that KTP receptor is identified in the membrane preparations of the brain and it is taken up into nerve terminals and released by depolaraizing stimuli supports the view that this peptide plays a role as a neurotransmitter or neuromodulator. Besides their anti-opioid properties, we may suggest that KTP and D-KTP also exert anti-stressor properties due to their ability to reduce stress-induced rising in ACTH and CORT levels. Although the exact mechanisms of these anti-stressor properties are unclear and future investigations are needed for identification of KTP signaling pathways, we may suggest some possibilities regarding HPA neuromodulation. Probably, the anti-stressor properties are related to both opioid and non-opioid KTP action. It is known that opioid

inhibition of the HPA axis is most likely exerted at the hypothalamic level and may be mediated through AVP rather than CRH[37]. As we know, moderate concentrations of KTP are present in the hypothalamus when it may excite directly magnocellular neurons or may inhibit HPA axis indirectly by releasing the opioids met-enkephalin and β-endorpin. Opioid peptides expressed in AVP neurons[38,39] are known to attenuate release of neurohypophysial hormones and would be a potential mechanism by which KTP could indirectly affect the magnocellular system[11]. There is evidence that opioids are capable of inhibiting the release of CRH from rat hypothalami in vitro[40,41,42,43].

The other possible mechanism for HPA modulation is production of Nitric Oxide (NO). This unique gas acts as a neurotransmitter and neuromodulator in the CNS. According to literature data that NO plays an important role in regulating the response of the HPA axis to various stressors. Some morphological studies have provided evidence for the existence of a signaling pathway between an opioid-and the nitric oxideergic systems in the hypothalamus of rat brain[44,45,46]. On the other hand, KTP is rapidly metabolized to L-Arg, which ia possible substrate for inducible and neuronal nitric oxide synthase NOS[47].

Concerning the penetration of Blood-Brain Barrier (BBB) of KTP, the literature data are contradictory. The majority of research associated with KTP claimed that it has limited capacity to cross the BBB[48]. Some literature data showed that KTP was transported by H^+-coupled peptide transporter PEPT2[49-52]. The D-KTP is incapable of crossing the BBB, because PEPT2 prefers L-amino acids[51,53]. Therefore, it is surprising that in our experiments D-KTP injected intraperitoneally showed more potent anti-stressor effect. These results maybe due to following suggestion that D-KTP strongly stimulates opioidergic system, which has structural and functional relations with NO-ergic system in the brain[54].

In conclusion, the various stressors applied seem to induce a different response of the HPA system as judged by quantitative changes in ACTH and CORT release. In addition, this study points to KTP and its synthesized optical isomer D-KTP as a possible anti-stressor substances with potential clinical importance in the context of stress-related disorders, since they inhibited stress-induced elevations in two investigated hormones helping the organism to reach homeostatic level. Further studies are needed to understand the exact role of KTP in stress response.

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REFERENCES

1. Takagi, H., H. Shiomi, H. Ueda and H. Amano, 1979. A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. Nature, 282: 410-412.
2. Ueda, H., H. Shiomi and H. Takagi, 1980. Regional distribution of a novel analgesic dipeptide kyotorphin (Tyr-Arg) in the rat brain and spinal cord. Brain Res., 198: 460-464.
3. Ignat'ev, D.A., V.V. Vorob'ev and R.K. Ziganshin, 1998. Effects of a number of short peptides isolated from the brain of the hibernating ground squirrel on the EEG and behavior in rats. Neurosci. Behav. Physiol., 28: 158-166.
4. Nishimura, K., K. Kaya, T. Hazato, H. Ueda, M. Satoh and H. Takagi, 1991. [Kyotorphin like substance in human cerebrospinal fluid of patients with persistent pain]. Masui, 40: 1686-1690, (In Japanese).
5. Takagi, H., H. Shiomi, H. Ueda and H. Amano, 1979. Morphine-like analgesia by a new dipeptide, L-tyrosyl-L-arginine (Kyotorphin) and its analogue. Eur. J. Pharmacol., 55: 109-111.
6. Vlaskovska, M., 1989. Opioid Peptides. In: Neurotransmitters and Neuromodulators, Mitzov, V., M. Vlaskovska and L. Kazakov (Eds.). Medicina I Fizkultura, Sofia, Bulgaria, pp: 128-162.
7. Kawabata, A., Y. Nishimura and H. Takagi, 1992. L-leucyl-L-arginine, naltrindole and D-arginine block antinociception elicited by L-arginine in mice with carrageenin-induced hyperalgesia. Br. J. Pharmacol., 107: 1096-1101.
8. Yajima, H., H. Ogawa, H. Ueda and H. Takagi, 1980. Studies on peptides. XCIV. Synthesis and activity of kyotorphin and its analogs. Chem. Pharmaceut. Bull., 28: 1935-1938.

9. Ueda, H., Y. Yoshihara, H. Misawa, N. Fukushima and T. Katada *et al*., 1989. The kyotorphin (tyrosine-arginine) receptor and a selective reconstitution with purified Gi, measured with GTPase and phospholipase C assays. J. Biol. Chem., 264: 3732-3741.
10. Ochi, T., Y. Motoyama and T. Goto, 2000. The spinal antinociceptive effect of FR140423 is mediated through kyotorphin receptors. Life Sci., 66: 2239-2245.
11. Summy-Long, J.Y., V. Bui, S. Gestl, E. Koehler-Stec, H. Liu, M.L. Terrell and M. Kadekaro, 1998. Effects of central injection of kyotorphin and L-arginine on oxytocin and vasopressin release and blood pressure in conscious rats. Brain Res. Bull., 45: 395-403.
12. Dzambazova-Maximova, E.B., H.H. Nocheva and A.I. Bocheva, 2006. Is kyotorphin an antiopioid peptide? Bulgarian Chem. Commun., 38: 36-40.
13. Dzhambazova, E.B., A.I. Bocheva and V.P. Nikolova, 2009. Involvement of endogenous nitric oxide in the effects of kyotorphin and its synthetic analogue on immobilization and cold stress-induced analgesia. Bulgarian Chem. Commun., 41: 116-121.
14. Dzhambazova, E.B., B.V. Landzhov, A.I. Bocheva and A.A. Bozhilova-Pastirova, 2011. Effects of D-kyotorphin on nociception and NADPH-d neurons in rat's periaqueductal gray after immobilization stress. Amino Acids, 41: 937-944.
15. Dzhambazova, E.B. and A.I. Bocheva, 2010. The unique brain dipeptide kyotorphin-from discovery to nowadays. J. Biomed. Clin. Res., 3: 3-11.
16. Gold, P.W., F. Goodwin and G.P. Chrousos, 1988a. Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (1). N. Engl. J. Med., 319: 348-353.
17. Gold, P.W., F.K. Goodwin and G.P. Chrousos, 1988b. Clinical and biochemical manifestations of depression. Relation to the neurobiology of stress (2). New Engl. J. Med., 319: 413-420.
18. Marti, O., A. Gavalda, F. Gomez and A. Armario, 1994. Direct evidence for chronic stress-induced facilitation of the adrenocorticotropin response to a novel acute stressor. Neuroendocrinology, 60: 1-7.
19. Armario, A., 2006. The hypothalamic-pituitary-adrenal axis: What can it tell us about stressors? CNS Neurol. Disord. Drug Targets, 5: 485-501.
20. Tsigos, C. and G.P. Chrousos, 1994. Physiology of the hypothalamic-pituitary-adrenal axis in health and dysregulation in psychiatric and autoimmune disorders. Endocrinol. Metab. Clin. North Am., 23: 451-466.
21. Sinha, R.K., 2007. 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., 11: 101-111.
22. De Kloet, E.R., 1991. Brain corticosteroid receptor balance and homeostatic control. Front. Neuroendocrinol., 12: 95-164.
23. Pacak, K. and M. Palkovits, 2001. Stressor specificity of central neuroendocrine responses: Implications for stress-related disorders. Endocrine Rev., 22: 502-548.
24. Goldstein, D.S., K. Pacak and I.J. Kopin, 1996. Nonspecificity versus Primitive Specificity of Stress Response. In: Stress: Molecular Genetic and Neurobiological Advances, Mccarthy, R., G. Aguilera, E. Sabban and R. Kvetnansky (Eds.). Gordon and Breach, New York, USA., pp: 3-20.
25. Djordjevic, J., G. Cvijic and V. Davidovic, 2003. Different activation of ACTH and corticosterone release in response to various stressors in rats. Physiol. Res., 52: 67-72.
26. Bocheva, A., E. Dzambazova, R. Hadjiolova, L. Traikov, R. Mincheva and I. Bivolarski, 2008. Effect of Tyr-MIF-1 peptides on blood ACTH and corticosterone concentration induced by three experimental models of stress. Autonomic Autacoid Pharmacol., 28: 117-123.
27. Dallman, M.F., M.T. Jones, J. Vernikos-Danellis and W.F. Ganong, 1972. Corticosteroid feedback control of ACTH secretion: Rapid effects of bilateral adrenalectomy on plasma ACTH in the rat. Endocrinology, 91: 961-968.
28. Valentino, R.J. and E. Van Bockstaele, 2015. Endogenous opioids: The downside of opposing stress. Neurobiol. Stress, 1: 23-32.
29. Chrousos, G.P., 1995. The hypothalamic-pituitary-adrenal axis and immune-mediated inflammation. N. Engl. J. Med., 332: 1351-1363.
30. Tsigos, C. and G.P. Chrousos, 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res., 53: 865-871.
31. Pan, W. and A.J. Kastin, 2007. From MIF-1 to endomorphin: The Tyr-MIF-1 family of peptides. Peptides, 28: 2411-2434.

32. Bocheva, A.I. and E.B. Dzhambazova, 2009. Opioidergic system and second messengers affected the nociceptive effects of Tyr-MIF-1's after three models of stress. Bulgarian Chem. Commun., 41: 153-159.
33. Cesselin, F., 1995. Opioid and anti-opioid peptides. Fundam. Clin. Pharmacol., 9: 409-433.
34. Ueda, H., Y. Yoshihara and H. Takagi, 1986. A putative met-enkephalin releaser, kyotorphin enhances intracellular Ca^{2+} in the synaptosomes. Biochem. Biophys. Res. Commun., 137: 897-902.
35. Ueda, H., T. Miyamae, C. Hayashi, S. Watanabe, N. Fukushima, Y. Sasaki, T. Iwamura and Y. Misu, 1995. Protein kinase C involvement in homologous desensitization of delta-opioid receptor coupled to Gi1-phospholipase C activation in *Xenopus* oocytes. J. Neurosci., 15: 7485-7499.
36. Ueda, H., T. Miyamae, N. Fukushima, S. Watanabe and Y. Misu, 1995. Evidence for a metabostatic opioid κ-receptor inhibiting pertussis toxin-sensitive metabotropic glutamate receptor-currents in *Xenopus* oocytes. FEBS Lett., 375: 201-205.
37. Delitala, G., P.J. Trainer, O. Oliva, G. Fanciulli and A.B. Grossman, 1994. Opioid peptide and α-adrenoceptor pathways in the regulation of the pituitary-adrenal axis in man. J. Endocrinol., 141: 163-168.
38. Martin, R. and K.H. Voigt, 1981. Enkephalins co-exist with oxytocin and vasopressin in nerve terminals of rat neurohypophysis. Nature, 289: 502-504.
39. Watson, S.J., H. Akil, W. Fischli, A. Goldstein, E. Zimmerman, G. Nilaver and T.B. van Wimersma Griedanus, 1982. Dynorphin and vasopressin: Common localization in magnocellular neurons. Science, 216: 85-87.
40. Calogero, A.E., W.T. Gallucci, P.W. Gold and G.P. Chrousos, 1988. Multiple feedback regulatory loops upon rat hypothalamic corticotropin-releasing hormone secretion. Potential clinical implications. J. Clin. Invest., 82: 767-774.
41. Tsagarakis, S., P. Navarra, L.H. Rees, M. Besser and A. Grossman, 1989. Morphine directly modulates the release of stimulated corticotrophin-releasing factor-41 from rat hypothalamus *in vitro*. Endocrinology, 124: 2330-2335.
42. Tsagarakis, S., L.H. Rees, M. Besser and A. Grossman, 1990. Opiate receptor subtype regulation of CRF-41 release from rat hypothalamus *in vitro*. Neuroendocrinology, 51: 599-605.
43. Jessop, D.S., 1999. Central non-glucocorticoid inhibitors of the hypothalamo-pituitary-adrenal axis. J. Endocrinol., 160: 169-180.
44. Uribe, R.M., S. Lee and C. Rivier, 1999. Endotoxin stimulates nitric oxide production in the paraventricular nucleus of the hypothalamus through nitric oxide synthase I: Correlation with hypothalamic-pituitary-adrenal axis activation. Endocrinology, 140: 5971-5981.
45. Gilinskii, M.A., G.M. Petrakova, T.G. Amstislavskaya, L.N. Maslova and V.V. Bulygina, 2005. Hypothalamic monoamines in cold stress on the background of changes in the activity of the nitric oxide system. Neurosci. Behav. Physiol., 35: 171-175.
46. Gupta, V., A. Gupta, S. Saggu, H.M. Divekar, S.K. Grover and R. Kumar, 2005. Anti-stress and adaptogenic activity of *l*-arginine supplementation. Evidence-Based Complement. Altern. Med., 2: 93-97.
47. Arima, T., Kitamura, Y., T. Nishiya, H. Takagi and Y. Nomura, 1996. Kyotorphin (L-tyrosyl-L-arginine) as a possible substrate for inducible nitric oxide synthase in rat glial cells. Neurosci. Lett., 212: 1-4.
48. Santos, S.M., L. Garcia-Nimo, S. Sa Santos, I. Tavares, J.A. Cocho and M.A. Castanho, 2013. Neuropeptide kyotorphin (tyrosyl-arginine) has decreased levels in the cerebro-spinal fluid of Alzheimer's disease patients: Potential diagnostic and pharmacological implications. Front. Aging Neurosci., Vol. 5. 10.3389/fnagi.2013.00068
49. Berger, U.V. and M.A. Hediger, 1999. Distribution of peptide transporter PEPT2 mRNA in the rat nervous system. Anat. Embryol., 199: 439-449.
50. Dieck, S.T., H. Heuer, J. Ehrchen, C. Otto and K. Bauer, 1999. The peptide transporter PepT2 is expressed in rat brain and mediates the accumulation of the fluorescent dipeptide derivative β-Ala-Lys-N_{ε}-AMCA in astrocytes. Glia, 25: 10-20.
51. Fujita, T., T. Kishida, N. Okada, V. Ganapathy, F.H. Leibach and A. Yamamoto, 1999. 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., 271: 117-120.

52. Fujita, T., T. Kishida, M. Wada, N. Okada, A. Yamamoto, F.H. Leibach and V. Ganapathy, 2004. Functional characterization of brain peptide transporter in rat cerebral cortex: Identification of the high-affinity type H^+/peptide transporter PEPT2. Brain Res., 997: 52-61.
53. Teuscher, N.S., R.F. Keep and D.E. Smith, 2001. PEPT2-mediated uptake of neuropeptides in rat choroid plexus. Pharmaceut. Res., 18: 807-813.
54. Hori, N., M.C. Lee, Sasaguri, K., H. Ishii and M. Kamei *et al*., 2005. Suppression of stress-induced nNOS expression in the rat hypothalamus by biting. J. Dent. Res., 84: 624-628.