Amino Acids (2011) 41:937–944
DOI 10.1007/s00726-010-0793-y

ORIGINAL ARTICLE

Effects of D-kyotorphin on nociception and NADPH-d neurons in rat's periaqueductal gray after immobilization stress

Elena B. Dzambazova · Boycho V. Landzhov · Adriana I. Bocheva · Anastasia A. Bozhilova-Pastirova

Received: 2 September 2010 / Accepted: 15 October 2010 / Published online: 3 November 2010

Abstract D-kyotorphin (D-Kyo) is a synthetic analogue of the neuropeptide kyotorphin and produces naloxone reversible analgesia. Stress-induced analgesia (SIA) is an in-built mammalian pain-suppression response that occurs during or following exposure to a stressful stimulus. The periaqueductal gray (PAG) is implicated as a critical site for processing strategies for coping with different types of stress and pain and NO affects its activity. The objectives of the present study were twofold: (1) to examine the effects of D-Kyo (5 mg/kg) on acute immobilization SIA; (2) to investigate the effect of peptide on NO activity in rat PAG after the stress procedure mentioned above. All drugs were injected intraperitoneally in male Wistar rats. The nociception was measured by the paw pressure and hot plate tests. A histochemical procedure for nicotinamide adenine dinucleotide phosphate–diaphorase (NADPH-d)-reactive neurons was used as indirect marker of NO activity. Our results revealed that D-Kyo has modulating effects on acute immobilization stress-induced analgesia in rats may be by opioid and non-opioid systems. Although D-Kyo is incapable of crossing the blood–brain barrier it showed an increased number of NADPH-d reactive neurons in dorsolateral periaqueductal gray (dlPAG) in control but not in stressed groups. We may speculate that the effect of D-Kyo in the brain is due to structural and functional interaction between opioidergic and NO-ergic systems or D-Kyo appears itself as a stressor. Further studies are needed to clarify the exact mechanisms of its action.

Keywords D-Kyotorphin · Immobilization · Stress-induced analgesia · PAG · NADPH-d reactive neurons

E. B. Dzambazova (✉)
Faculty of Medicine, Sofia University St. Kl. Ohridski,
1 Kozyak Str., 1407 Sofia, Bulgaria
e-mail: el_dji@abv.bg

A. I. Bocheva
Department of Pathophysiology, Medical University,
1431 Sofia, Bulgaria

B. V. Landzhov · A. A. Bozhilova-Pastirova
Department of Anatomy and Histology, Medical University,
1431 Sofia, Bulgaria

Introduction

D-Kyotorphin (D-Kyo, L-Tyr-D-Arg) is the most powerful optical isomer (Yajima et al. 1980) of the natural analgesic dipeptide kyotorphin (Kyo, L-Tyr-L-Arg). Kyo was initially isolated from bovine brain (Takagi et al. 1979a) and acts as a neurotransmitter and neuromodulator in nociceptive responses in the central nervous system (Inoue et al. 1999) with a 4.2-fold higher analgesic effect than endogenous opioid peptides such as met-enkephalins (Takagi et al. 1979b; Stone 1983). However, its analogue D-Kyo shows enhanced analgesic activity 5.6-fold higher than that observed with Kyo (Takagi et al. 1982). An action mediated by specific kyotorphin receptors (non-opioid) for Kyo and D-Kyo has been suggested by several authors (Ueda et al. 1989; Ochi et al. 2000). The peptide has shown a non-opioid analgesic effect in the peripheral nervous system, which makes it quite appealing for the treatment of chronic pain (Lopes et al. 2006). Moreover, this peptide inhibits cell proliferation, indicating that peripheral tissue cells also contain D-Kyo-specific receptors (Bronnikov et al. 1997).

Despite the fact that many research articles have been written about stress and stress-related diseases, no scientifically accepted definition of stress exists. Pacák and

Palkovits (2001) define stress as a state of threatened homeostasis. During stress, an adaptive compensatory specific response of the organism is activated to sustain homeostasis. The adaptive response reflects the activation of specific central circuits and is genetically and constitutionally programmed and constantly modulated by environmental factors.

Stress-induced analgesia (SIA) is an in-built mammalian pain-suppression response that occurs during or following exposure to a stressful or fearful stimulus (Butler and Finn 2009). Several factors have been reported to induce analgesia due to stress. One of them is immobilization which evokes extremely variable endocrine, physiological, and behavioral responses by activating motor, autonomic, and hypothalamic-pituitary-adrenal (HPA) systems (Pacák and Palkovits 2001).

SIA is mediated by activation of descending inhibitory pain pathway. Pharmacological and neurochemical studies have demonstrated involvement of large number of endogenous opioid peptides which are produced in the body and take part in various functions of hormones or neuromodulators. It is known that stimulation of opioid receptors within the midbrain periaqueductal gray (PAG) activates descending inhibitory pathways and plays a pivotal role in the modulation of nociception (Xing and Li 2007; Butler and Finn 2009). In addition, the PAG receives direct spinal nociceptive projections and is reciprocally connected with all levels of the central nervous system, including diencephalic and forebrain structures involved in pain transmission. Therefore, besides descending systems of pain inhibition, the PAG is strategically located to allow integration of brain areas that subserve the motivational-affective components of pain, and has been implicated as a critical site for processing strategies for coping with different types of stress, threat, and pain (Vaccarino et al. 1997).

Literature data revealed that stress caused the activation of nitric oxide (NO)-producing neurons and NO played an important role in regulating the response of the HPA axis to various stress models (Uribe et al. 1999). It was reported that the NO system fulfils the main criteria of a stress-limiting system and nitric oxide is involved in NO-molecular ways, which affect, through auto regulation, different signaling molecules such as opioids, endocannabinoids and others (Gilinsky et al. 2005). Also NO affects neuronal activity of the PAG (Xing et al. 2008).

The objectives of the present study were twofold: (1) to examine the effects of D-Kyo (5 mg/kg, i.p.) on acute immobilization stress-induced analgesia (ISIA) in rats. Naloxone (1 mg/kg, i.p.) was used to determine whether opioidergic system is involved; (2) to investigate the effect of Kyo analogue on nitric oxide activity in rat's periaqueductal gray after stress procedure mentioned above. A histochemical procedure for NADPH-d-reactive neurons was used as indirect marker of NO activity.

Materials and methods

Animals

The experiments were carried out on male Wistar rats (180–200 g), housed at 12 h light/dark cycle. Food and water were available ad libitum. All experiments were carried out between 09.00 a.m. and 12.00 p.m. Each group included eight rats for nociceptive tests and five rats for histochemistry.

Drugs and treatment

D-Kyo (5 mg/kg, i.p.) and naloxone (Nal, 1 mg/kg, i.p.), a non-specific opioid receptor antagonist, were obtained from Sigma. All drugs were dissolved in a sterile saline (0.9% NaCl) solution and injected intraperitoneally (i.p.). The immunohistochemical reagents were the products of Santa Co.

The experimental procedures were carried out in accordance with the institutional guidance and general recommendations on the use of animals for scientific purposes.

Acute model of immobilization stress

The animals were placed in a plastic tube with adjustable plaster tape on the outside, which immobilizes them. Holes were made to allow breathing. The control group was not submitted to restraint. The immobilization procedure was carried out for 1 h.

Nociceptive tests

Paw-pressure test (Randall-Selitto test)

The changes in the mechanical nociceptive threshold of the rats were measured using an analgesimeter (Ugo Basile). The pressure was applied to the hind-paw and the pressure (g) required eliciting nociceptive responses, such as squeak, and struggle was taken as the mechanical nociceptive threshold. A cut-off value of 500 g was used to prevent damage of the paw.

Hot plate test

The latency of response to pain was measured from the moment of placing an animal on a metal plate (heated to

55 ± 0.5°C) to the first signs of pain (paw licking, jump). The cut-off time was 30 s.

Histochemistry

The animals were anesthetized with thiopental (40 mg/kg b.w.). Transcardial perfusion was done with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Post fixation of the obtained material was conducted in 4% buffered solution (0,1 M phosphate buffer, pH 7,4) of paraformaldehyde overnight at 4°C. Coronal sections were cut on a freezing microtome (Reichert-Jung) at 25 μm and washed repeatedly in 0.01 M PBS (phosphate buffer, pH 7,4). First, every fifth section was processed for double staining in NADPH-d. The slices were stained with NADPH-d-technique using: 0.2 mg/ml NBT (nitrobluetetrazoliumchlorid), 1 mg/ml NADPH-tetranatriumsalt, 0.5% Triton X-100 diluted in 0.1 M Tris–HCl, pH 7.6 for 5 h at 37°C. Afterwards, they were rinsed with 0.1 M Tris–HCl, pH 7.6 and thrice with 0.01 M PBS for 5 min. They were mounted on gelatin-coated glass, dried for 24 h and coverslipped with Entellan. Ten coronal sections were utilized for calculation of the neuronal packing density in dorsolateral periaqueductal gray (dlPAG) of rats. NADPH-d activity was visualized as blue color in perikarya, dendrites, and axons. The intensity of the staining was evaluated visually and number of NADPH-d reactive neurons was counted. We used Paxinos and Watson's atlas (1986) in anterior–posterior localization from bregma-7.64 mm for an analysis of the sites.

Data analysis

Results from the nociceptive tests were statistically assessed by analysis of variance (ANOVA) followed by the Newman–Keuls multiple comparison test.

Morphometric analysis was performed using a micro-analysis system (primary magnification 20× objective). Data of the entire drawings were entered in computer programme (Olympus CUE-2), recorded automatically, calculated and compared by Student's t test.

All values are presented as mean ± standard error of the mean (SEM). Statistical significance was accepted when $P < 0.05$.

Results

Rats were immobilized by placement in plexiglas restrainers for 1 h. The investigations started immediately after stress procedure or 15 min after i.p. injection of peptide.

Antinociceptive effects were evaluated using the paw pressure (PP) and hot plate (HP) tests. Our results showed that D-Kyo (5 mg/kg, i.p.) administered alone in control animals has significant well pronounced and time dependent analgesic effect versus control group during the whole investigated period in PP test ($p < 0.01$). Administration of non-selective opioid blocker naloxone (Nal, 1 mg/kg, i.p.) before D-Kyo significantly reduced its analgesic effect to values compared to control ($p < 0.01$) (Fig. 1).

In HP test D-Kyo (5 mg/kg, i.p.) administered alone in intact animals has significant analgesic effect compared to the control ($p < 0.05$) only on 15th min, and Nal administered before peptide significantly decreased it ($p < 0.01$) (Fig. 2).

In PP test immobilization stress (IS) showed significant analgesic effects on 15th ($p < 0.01$) and 30th min ($p < 0.01$), which have been reduced by Nal $p < 0.05$ and $p < 0.01$, respectively. Administration of D-Kyo, immediately after IS decreased significantly the pain threshold 15 min later ($p < 0.01$), but increased it on 30th min ($p < 0.05$) from the beginning of the experiment.

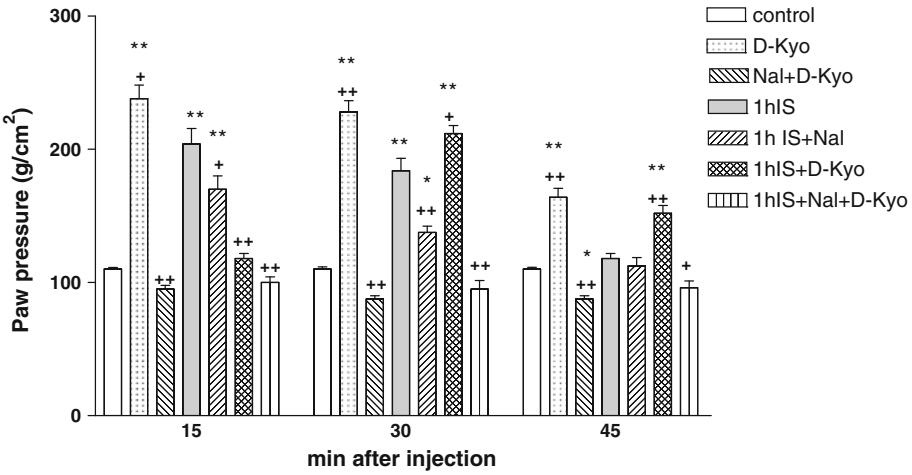

Fig. 1 Effects of D-Kyo (5 mg/kg), and its co-administration with Nal (1 mg/kg, i.p) on nociception before and after 1hIS estimated by PP test. Data are presented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ versus control; $^{+}p < 0.05$, $^{++}p < 0.01$ versus 1hIS

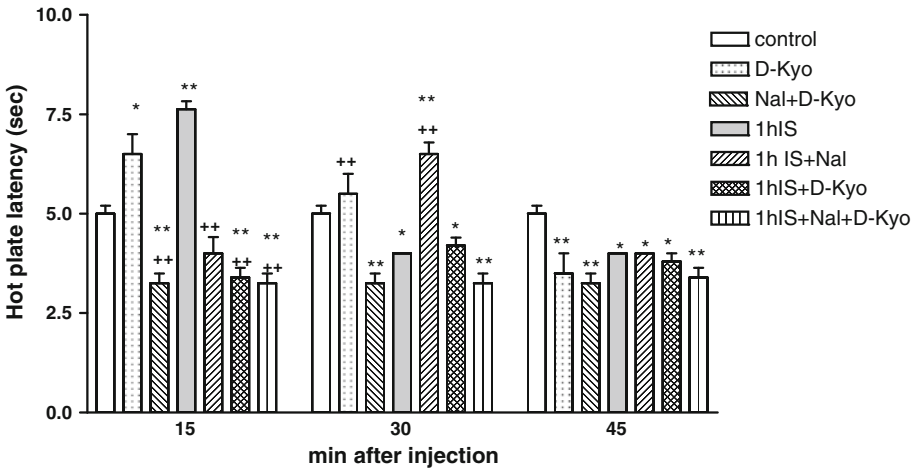

Fig. 2 Effects of D-Kyo (5 mg/kg), and its co-administration with Nal (1 mg/kg, i.p) on nociception before and after 1hIS estimated by HP test. Data are presented as mean ± SEM; $^{*}p < 0.05$, $^{**}p < 0.01$ versus control; $^{+}p < 0.05$, $^{++}p < 0.01$ versus 1hIS

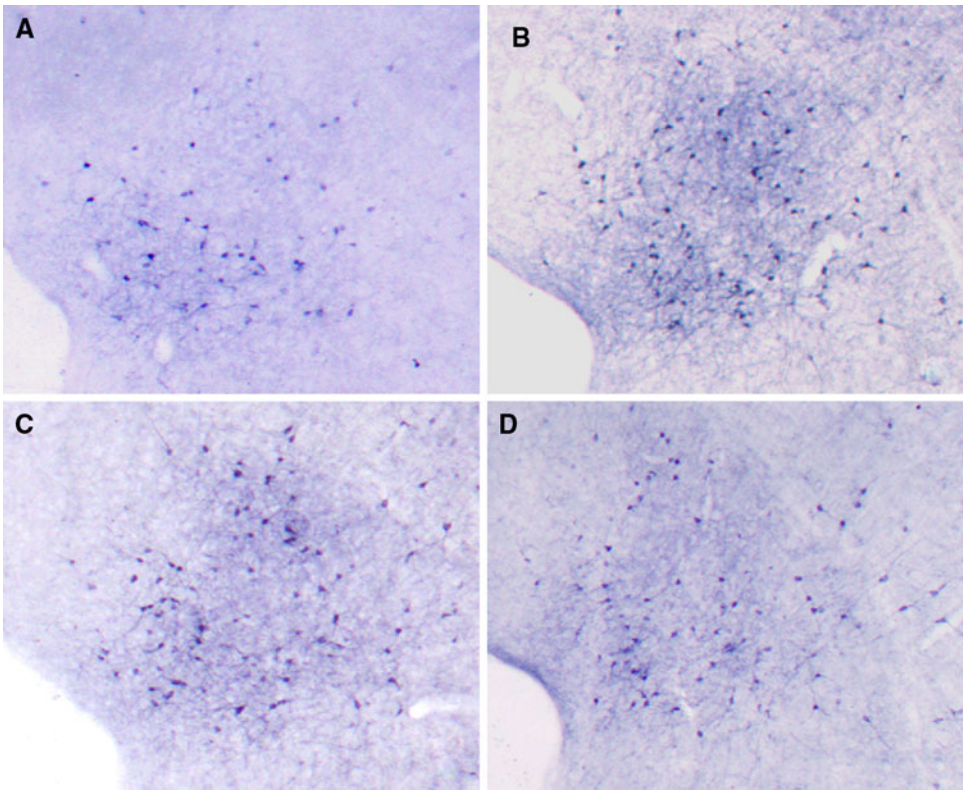

Fig. 3 Photomicrographs showing NADPH-d-reactivity neurons density in the dlPAG: **a** control, **b** immobilization stress (IS), **c** D-Kyo (5 mg/kg), **d** IS+D-Kyo (×100)

Administration of Nal after IS and before the peptide significantly decreased the pain threshold compared to IS on 15th and 30th min ($p < 0.01$) (Fig. 1).

In HP test IS significantly increased HP latency only on 15th min ($p < 0.01$). Injected immediately after 1 h IS, D-Kyo significantly inhibited ISIA on 15th min ($p < 0.01$). Combination of Nal+D-Kyo after 1 h IS decreased significantly the HP latency compared to IS ($p < 0.01$) (Fig. 2).

A histochemical procedure for NADPH-d-reactive neurons in rat's PAG was used as marker of NO activity. The animals injected with saline i.p. showed a cluster of intensely stained NADPH-d positive neurons in the dlPAG (Fig. 3a) and a few scattered NADPH-d positive neurons in other PAG areas. In the animals subjected to IS (Fig. 3b) or injected with D-Kyo (Fig. 3c), stress procedure or dipeptide injection induced an evident and statistically significant increase in the number of the NADPH-d positive neurons in the PAG area mentioned above compared to the control group ($p < 0.05$) (Figs. 3a, 4). Administration of D-Kyo after 1 h IS (Figs. 3d, 4) also showed an increased number of NADPH-d reactive neurons but they were comparable to immobilized group which suggests that D-Kyo injected after stress had no effect on NO activity (Fig. 4).

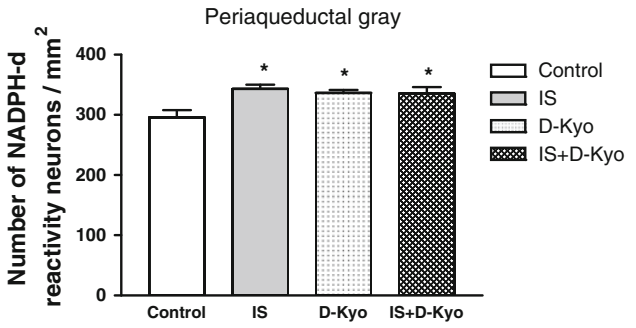

Fig. 4 Effects of 1 h immobilization stress (IS), D-Kyo (5 mg/kg) and their combination on NADPH-d-reactive neurons in dlPAG in male Wistar rats. Mean values ± SEM are presented; $*p < 0.05$ versus control

Discussion

In this study D-Kyo injected alone in control rats showed analgesic effect more pronounced in paw-pressure test. Some authors (Takagi et al. 1979b) suggested that the enhanced analgesic effect of the dipeptide is the result of protease resistance conferred by the substitution of L-arginine with a D-arginine residue, but this might not be the only factor behind the enhanced analgesic effect. Literature data suggested that Kyo and D-Kyo action is mediated by specific non-opioid receptors (Ueda et al. 1989; Ochi et al. 2000). Moreover, it is known that D-Kyo has a phenolic group, which is common and essential for the interaction of biologically active peptides with cell-surface receptors. It has already been demonstrated for related peptides that different membrane properties can modulate the exposure and orientation of this critical group, which is most likely involved in receptor interaction (Lopes et al. 2006). A study of D-Kyo based on modifications in the lipid bilayer–water interface is important, as Sargent and Schwyzer (1986) proposed that peptides interact with membrane lipids prior to receptor binding, and this interaction allows them to adopt an appropriate conformation for docking cell receptors. This hypothesis has gained experimental support, which highlights the fact that critical groups, such as the phenolic ring of tyrosine, may indeed be exposed and oriented by lipid membranes. Lopes et al. (2006) suggested that interaction and recognition of D-Kyo by membranes is potentially important to its increased analgesic effect. In compliance with literature data our results showed that the analgesia produced by D-Kyo is Nal-reversible in control animals (Takagi et al. 1982).

Exposure to a stressful stimulus is perceived as a threat to the organism's homeostasis and elicits a variety of physiological adaptations, encompassing endocrine, autonomic, and behavioral aspects (Yamada and Nabeshima 1995; Carrasco and Van de Kar 2003). At the behavioral level, response to stress includes a transient decrease of pain sensitivity (Rodgers and Randall 1988; Yamada and Nabeshima 1995). Stress-induced analgesia plays an adaptive role to threat and is a component of the defensive behavioral response (Amit and Galina 1988). It appeared early that stress-induced analgesia could be mediated by at least two distinct neuronal mechanisms: opioid (Nal-reversible) versus non-opioid (Nal-irreversible) (Tsuda et al. 1989; Watkins and Mayer 1986; Lapo et al. 2003).

It was demonstrated that the type of SIA was partly determined by the severity of stress or type of the stressful stimulus (Bodnar 1990; Contet et al. 2006). Interestingly, a collateral inhibition between opioid and non-opioid mechanisms was demonstrated, with both pathways being mutually antagonistic (Bodnar 1990). Although a large number of neurotransmitters, neuropeptides, and neuromodulators are activated in various brain regions during exposure to stress, one can suppose that specific neuronal circuits exist to optimize effective, rapid, and efficient responses to restore disturbed homeostasis and ensure minimal damage to the organism (Pacák and Palkovits 2001). Stress caused by immobilization should be viewed as a mixture of physical and psychological stressors, including decreased body temperature and pain stress as important components. In our studies, 1-h immobilization increases significantly the pain threshold, measured by the paw-pressure test, which is in accordance with data in the literature, showing that immobilization produces antinociception increases in the tail-flick (Aloisi et al. 1998), hot-plate (Amir and Amit 1978), thermal pain models, and formalin tests (Appelbaum and Holtzman 1985). Naloxone significantly reduced the anti-nociception, induced by IS, which also correlated with literature data that opioidergic system appears to play an important role in provoked antinociception (Choi et al. 2003).

Our results showed that in both tests injection of D-Kyo immediately after IS leads to decreased ISIA on the 15th min from the beginning of the experiment back to the control value. Nal pretreatment also showed the analgesic effect compared to D-Kyo. This suggests that D-Kyo might be involved in analgesia, induced by IS via non-opioid pathway. It is interesting that on the 30th min of PP test, D-Kyo after IS slightly potentiated ISIA, and the effect is Nal-reversible.

Taking into consideration that D-Kyo showed different effects on ISIA and since our and literature data (Pacák and Palkovits 2001; Bocheva and Dzambazova 2009) indicated that opioid and non-opioid components are equally presented in immobilization stress, we may propose that D-Kyo has modulating effects on nociception, induced by IS acting via non-opioid and opioid pathways.

One major module in the circuitry mediating stress-induced analgesia (Basbaum and Fields 1984) is the PAG as it sends descending inhibitory fibers to the medulla, which

in turn modulates incoming noxious signals in the spinal cord (Sandkühler 1996; Behbehani 1995). Stimulation of opioid receptors within the PAG activates these descending inhibitory pathways and suppresses nociception. It was reported that the NO system fulfils the main criteria of a stress-limiting system and nitric oxide is involved in NO-molecular ways, which affect through auto regulation different signaling molecules such as opioids, endocannabinoids and others (Gilinsky et al. 2005). According to Uribe et al. (1999) stress causes the activation of nitric oxide producing neurons and NO plays an important role in regulating the response of the HPA axis to various stress models. Also literature data revealed that NO affects neuronal activity of the PAG (Xing et al. 2008). NO is produced from L-arginine through calcium dependent pathways by the nitric oxide synthase enzyme (NOS) (Ignarro 1990). At least two different types of constitutive NOS, called neuronal and endothelial NOS, have been identified in the brain. Neuronal NOS has been co-purified with reduced nicotinamide adenine dinucleotide phosphate–diaphorase (NADPH-d) and, in the nervous system, NOS immunoreactivity has been consistently co-localized with NADPH-d activity reflecting the two functions carried out by the same molecule (Hope et al. 1991). So, in spite of some limitations (Matsumoto et al. 1993), it is generally accepted that the histochemical detection of the NADPH-d is one of the most useful ways of identifying the putative NOS containing neurons (Matsumoto et al. 1993; Saxon and Beitz 1996). The contribution of spinal NO to the processing of sustained nociceptive inputs has been clearly illustrated by our previous studies using L-nitro-arginine-methyl ester (L-NAME) as NOS inhibitor (Dzambazova et al. 2009).

According to our discovery using histochemistry and literature review about immunicytochemistry studies, the results confirmed that NADPH-d positive neurons are localized mainly in the dlPAG (Onstott et al. 1993; Rodella et al. 1998). Generally, the NADPH-d neurons appeared highly positive, although some cells exhibiting a weak positivity were observed.

In the animals subjected to IS or injected with D-Kyo, stress procedure or dipeptide induced an evident and statistically significant increase in the number of the NADPH-d positive neurons in the dlPAG (Fig. 4). The NADPH-d data we reported correlate with literature data that neuronal NOS is increased in response to stress (Costa et al. 1993; Karanth et al. 1993) and the dlPAG represented a region that is specifically activated by different types of stress, threat, and pain (Vaccarino et al. 1997).

The marked increase in the number of NADPH-d positive cells we found after stress procedure suggested that in the PAG, like in the spinal cord, NO may play a role in the central mechanisms of stress response or that it was involved in modulation of SIA. Previous works have demonstrated that NO causes a neuronal release of β-endorphin (Hodges et al. 1994) and that NO antinociception is suppressed by pretreatment with various NOS inhibitors (McDonald et al. 1994). This indicates that the mechanism of NO antinociception in rats might involve both NO and β-endorphin release. It has also been suggested, that NO may mediate the β-endorphin induced release of Met-enkephalin in the rat spinal cord (Hara et al. 1995). Since a group of PAG cells has been reported to be enkephalin immunoreactive (Gioia and Bianchi 1988), it is possible to suppose that NO generated by NADPH-d positive neurons in the PAG can influence the activity of the enkephalin positive neurons. Further knowledge of NO's role in these mechanisms in dlPAG may have potential implications in the development of novel anxiety and analgesic strategies.

According to the literature that optical isomer of Kyo is incapable of crossing the blood–brain barrier (BBB), because the peptide transporter PEPT2 responsible for the transport of Kyo across the BBB prefers L-amino acids (Fujita et al. 1999; Teuscher et al. 2001). Therefore, it is surprising that in our experiments D-Kyo injected intraperitoneally in control rats showed an increased number of NADPH-d reactive neurons. These results may be due to following suggestions: first, D-Kyo strongly stimulates opioidergic system, which has structural and functional relations with NO-ergic system in the brain (Hori et al. 2005) or second, maybe exogenous D-Kyo interrupting homeostasis between opioid and anti-opioid systems appears itself as a stressor because regarding Pacák and Palkovits (2001) stress is a state of threatened homeostasis. During stress, an adaptive compensatory specific response of the organism is activated to sustain homeostasis. One of them is activation of large number of opioid peptides and NO-ergic system which in turn leads to anti-nociception and increased number of NADPH-d reactive neurons.

Conclusions

Our results revealed that D-Kyo has modulating effects on acute immobilization stress-induced analgesia in rats maybe by opioid and non-opioid systems. Although D-Kyo is incapable of crossing the blood–brain barrier it showed an increased number of NADPH-d reactive neurons in dlPAG. We may speculate that this effect depends on indirect mechanism, where the strong stimulation of opioidergic system, according to literature, is structurally and functionally related to NO-ergic system in the brain. On the other hand, D-Kyo itself may appear as a stressor interrupting homeostasis. The injection of the peptide alone showed increased number of NADPH-d reactive neurons, which are comparable to the effect of IS. However the

injection of dipeptide after stress did not show any changes. This confirms the second hypothesis. Further studies are needed to clarify the exact mechanisms of its action.

Acknowledgments This work was supported by Grant 3-D/2007 of the Medical University of Sofia, Bulgaria.

References

Aloisi AM, Ceccarelli I, Lupo C (1998) Behavioural and hormonal effects of restraint stress and formalin test in male and female rats. Brain Res Bull 47:57–62

Amir S, Amit Z (1978) Endogenous opioid ligands may mediate stress-induced changes in the affective properties of pain related behavior in rats. Life Sci 23:1143–1151

Amit Z, Galina ZH (1988) Stress induced analgesia plays an adaptive role in the organization of behavioral responding. Brain Res Bull 21:955–958

Appelbaum BD, Holtzman SG (1985) Restraint stress enhances morphine-induced analgesia in the rat without changing apparent affinity of receptor. Life Sci 36:1069–1074

Basbaum AI, Fields HL (1984) Endogenous pain control systems: brainstem spinal pathways and endorphin circuitry. Annu Rev Neurosci 7:309–338

Behbehani MM (1995) Functional characteristics of the midbrain periaqueductal gray. Prog Neurobiol 46:575–605

Bocheva AI, Dzambazova EB (2009) Opioidergic system and second messengers affected the nociceptive effects of Tyr-MIF-1's after three models of stress. Bulg Chem Commun 41:153–159

Bodnar RJ (1990) Effects of opioid peptides on peripheral stimulation and 'stress'-induced analgesia in animals. Crit Rev Neurobiol 6:39–49

Bronnikov G, Dolgacheva L, Zhang SJ, Galitovskaya E, Kramarova L, Zinchenko V (1997) The effect of neuropeptides kyotorphin and neokyotorphin on proliferation of cultured brown preadipocytes. FEBS Lett 407:73–77

Butler RK, Finn DP (2009) Stress-induced analgesia. Prog Neurobiol 88(3):184–202

Carrasco GA, Van de Kar LD (2003) Neuroendocrine pharmacology of stress. Eur J Pharmacol 463:235–272

Choi S, Lee J, Suh H (2003) Effect of ginsenosides administered intrathecally on the antinociception induced by cold water swimming stress in the mouse. Biol Pharm Bull 26:858–861

Contet C, Gavériaux-Ruff C, Matifas A, Caradec C, Champy MF, Kieffer BL (2006) Dissociation of analgesic and hormonal responses to forced swim stress using opioid receptor knockout mice. Neuropsychopharmacol 31:1733–1744

Costa A, Trainer P, Besser M, Grossman A (1993) Nitric oxide modulates the release of corticotropin-releasing hormone from the rat hypothalamus in vitro. Brain Res 605:187–192

Dzambazova EB, Bocheva AI, Nikolova VP (2009) Involvement of endogenous nitric oxide in the effects of kyotorphin and its synthetic analogue on immobilization and cold stress-induced analgesia. Bulg Chem Commun 41:116–121

Fujita T, Kishida T, Okada N, Ganapathy V, Leibach FH, Yamamoto A (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

Gilinsky MA, Petrakova GM, Amstislavskaya TG, Maslova LN, Bulygina VV (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

Gioia M, Bianchi R (1988) The distribution of substance P and Met-enkephalin in the periaqueductal gray matter of the rat. Basic Appl Histochem 32:103–108

Hara S, Kuhns ER, Ellenberger EA, Mueller JL, Shibuya T, Endo T, Quock RM (1995) Involvement of nitric oxide in intracerebroventricular β-endorphin-induced neuronal release of methionine enkephalin. Brain Res 675:190–194

Hodges BL, Gagnon MJ, Gillespie TR, Breneisen JR, O'Lealy DF, Hara S, Quock RM (1994) Antagonism of nitrous oxide antinociception in the rat hot plate test by site-specific mu and epsilon opioid receptors blockade. J Pharmacol Exp Ther 269:596–600

Hope BT, Michael GJ, Knigge KM, Vincent SR (1991) Neuronal NADPH–diaphorase is a nitric oxide synthase. Proc Natl Acad Sci USA 88:2811–2820

Hori N, Lee MC, Sasaguri K, Ishii H, Kamei M, Kimoto K, Toyoda M, Sato S (2005) Suppression of stress-induced nNOS expression in the rat hypothalamus by biting. J Dent Res 84:624–628

Ignarro LJ (1990) Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol 30:535–560

Inoue M, Yamada T, Ueda H (1999) Low dose of kyotorphin (tyrosine-arginine) induces nociceptive responses through a substance P release from nociceptor endings. Mol Brain Res 69:302–305

Karanth S, Lyson K, McCann SM (1993) Role of nitric oxide in interleukin 2-induced corticotropin-releasing factor release from incubated hypothalamus. Proc Natl Acad Sci USA 90:3383–3387

Lapo IB, Konarzewski M, Sadowski B (2003) Effect of cold acclimation and repeated swimming on opioid and nonopioid swim stress-induced analgesia in selectively bred mice. Physiol Behav 78:345–350

Lopes SC, Fedorov A, Castanho MA (2006) Chiral recognition of D-kyotorphin by lipidic membranes: relevance toward improved analgesic efficiency. Chem Med Chem 1:723–728

Matsumoto T, Nakane M, Pollock JS, Kuk JE, Forstermann U (1993) A correlation between soluble brain nitric oxide synthase and NADPH-d activity is only seen after exposure of the tissue to fixative. Neurosci Lett 155:61–64

McDonald CE, Gagnon MJ, Ellenberger EA, Hodges BL, Ream JK, Tousman SA, Quock RM (1994) Inhibitors of nitric oxide synthesis antagonize nitrous oxide antinociception in mice and rats. J Pharmacol Exp Ther 269:601–608

Ochi T, Motoyama Y, Goto T (2000) The spinal antinociceptive effect of FR140423 is mediated through kyotorphin receptors. Life Sci 66:2239–2245

Onstott D, Mayer B, Beitz AJ (1993) Nitric oxide synthase immunoreac-tive neurons anatomically define a longitudinal dorsolateral column within the midbrain periaqueductal gray of the rat: analysis using laser confocal microscopy. Brain Res 610:317–324

Pacák K, Palkovits M (2001) Stressor specificity of central neuroendocrine responses: implications for stress-related disorders. Endocr Rev 22:502–548

Paxinos G, Watson C (1986) The rat brain in stereotaxic coordinates. Academic Press, Orlando

Rodella L, Rezzani R, Agostini C, Bianchi R (1998) Induction of NADPH–diaphorase activity in the rat periaqueductal gray matter after nociceptive visceral stimulation. Brain Res 793:333–336

Rodgers RJ, Randall JI (1988) Environmentally induced analgesia: situational factors, mechanisms and significance. In: Rodgers RJ, Cooper SJ (eds) Endorphins, opiates and behavioural processes. John Wiley & Sons, New York, pp 107–142

Sandkühler J (1996) The organization and function of endogenous antinociceptive systems. Prog Neurobiol 50:49–81

Sargent DF, Schwyzer R (1986) Membrane lipid phase as catalyst for peptide-receptor interactions. Proc Natl Acad Sci USA 83:5774–5778

Saxon DW, Beitz A (1996) Induction of NADPH–diaphorase/nitric oxide synthase in the rat brainstem trigeminal system resulting from cerebellar lesions. J Comp Neurol 371:41–71

Stone TW (1983) A comparison of the effects of morphine, enkephalin, kyotorphin and D-phenylalanine on rat central neurones. Br J Pharmacol 79:305–312

Takagi H, Shiomi H, Ueda H, Amano H (1979a) A novel analgesic dipeptide from bovine brain is a possible Met-enkephalin releaser. Nature 282:410–412

Takagi H, Shiomi H, Ueda H, Amano H (1979b) Morphine-like analgesia by a new dipeptide, L-tyrosyl-L-arginine (Kyotorphin) and its analogue. Eur J Pharmacol 55:109–111

Takagi H, Shiomi H, Kuraishi Y, Ueda H (1982) Analgesic dipeptide, L-Tyr-D-Arg (D-kyotorphin) induces Met-enkephalin release from guinea pig striatal slices. Experientia 38:1344–1345

Teuscher NS, Keep RF, Smith DE (2001) PEPT2-mediated uptake of neuropeptides in rat choroid plexus. Pharm Res 18:807–813

Tsuda A, Ida Y, Satoh H, Tsujimaru S, Tanaka M (1989) Stressor predictability and rat brain noradrenaline metabolism. Pharmacol Biochem Behav 32:569–572

Ueda H, Yoshihara Y, Misawa H, Fukushima N, Katada T, Ui M, Takagi H, Satoh M (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

Uribe RM, Lee S, Rivier C (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

Vaccarino AL, Clemmons HR, Mader GJ Jr, Magnusson JE (1997) A role of periaqueductal grey NMDA receptors in mediating formalin-induced pain in the rat. Neurosci Lett 236:117–119

Watkins LR, Mayer DJ (1986) Multiple endogenous opiate and non-opiate analgesia systems: evidence of their existence and clinical implications. Ann NY Acad Sci 467:273–299

Xing J, Li J (2007) TRPV1 receptor mediates glutamatergic synaptic input to dorsolateral periaqueductal gray (dl-PAG) neurons. J Neurophysiol 97:503–511

Xing J, Li DP, Li J (2008) Role of GABA receptors in nitric oxide inhibition of dorsolateral periaqueductal gray neurons. Neuropharmacol 54:734–744

Yajima H, Ogawa H, Ueda H, Takagi H (1980) Studies on peptides. XCIV. Synthesis and activity of kyotorphin and its analogues. Chem Pharm Bull 28:1935–1938

Yamada K, Nabeshima T (1995) Stress-induced responses and multiple opioid systems in the brain. Behav Brain Res 67:133–145