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# Effects of Immobilization Stress on Nitric Oxide Active Neurons in Rat's dlPAG; Histochemical Study

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## ABSTRACT

**Background:** Exposure to stress-factors caused an array of biochemical, physiological and behavioral changes. According to literature data, specific stressors may elicit specific responses, and different stressors may activate different brain systems by using specific pathways within the central nervous system. Several brain structures, including the periaqueductal gray (PAG), have been implicated in the functional neuroanatomy of stress response. The dorsolateral column of the periaqueductal gray (dlPAG) integrates aversive emotional experiences and represents an important site responding to life threatening situations. It was reported that nitric oxide (NO) affects the neuronal activity of the PAG. The goal of the present study was to investigate the changes of NO activity in the dlPAG of immobilized rats using a histochemical examination of the distribution of NADPH-d reactivity neurons. Our results showed that NO activity in rat's dlPAG was significantly increased by acute immobilization stress. This suggests a pivotal role of this part of the brain and NO-ergic system in stress response which main role is to attenuate the effect of stress and to restore the homeostasis. **Methods:** The experiments were carried out on male Wistar rats (180-200g), divided into two groups. The first group represented intact controls. The second group was subjected to acute immobilization stress. **Results:** The acute stressor – 1 hour immobilization, showed statistically significant increase in the number of the NADPH-d positive neurons compared to the control group ($p < 0.01$). **Conclusion:** NO activity in rat's dlPAG was significantly increased by acute immobilization stress.

***Keywords:*** *Acute immobilization stress; Periaqueductal gray; Nitric oxide; Histochemistry; Rat.*

## INTRODUCTION

Stress is associated with activation of the hypothalamic–pituitary–adrenocortical (HPA) axis. Exposure to stress-factors caused an array of biochemical, physiological and behavioral changes.[1-3] For instance, animal faced with artificial or natural threatening stimuli manifest a decrease in pain sensitivity, called stress-induced analgesia.[4]

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This phenomenon has been demonstrated in a wide range of species and can be elicited by various emergencies, such as restraint, cold or heat exposure, predator-prey interactions. Also, changes in body temperature have been used as important criterion for stress reactions in animals.[5,6] So called stress-induced hyperthermia, is thought to result from a "regulated" thermoregulatory response since it occurs when animals are studied in stressful environment[7,8] and is accompanied by activation of heat-producing and heat-conserving mechanisms.[9,10] It is known that in the mechanisms that initiate response due to stress, activation of endogenous opioids system appears to play important roles, and glucocorticoids, as well as nitric oxide, appear to play an important role in

modulating this response.[11,12]

Several brain structures that organize defensive reactions and represent the neural substrate of fear and anxiety have been implicated in the functional neuroanatomy of stress response. Among those are prefrontal regions, amygdala, hippocampus, and parahippocampal area, hypothalamus, thalamus, and the periaqueductal gray (PAG). Opioid receptors in the PAG contribute to a wide range of behaviors. These include nociceptive modulation, cardiovascular regulation, thermoregulation, and locomotor activity.[13-19] The dorsolateral column of the periaqueductal gray (dlPAG) integrates aversive emotional experiences and represents an important site responding to life threatening situations.[20-21] It was reported that nitric oxide system fulfils the main criteria of a stress-limiting system and nitric oxide (NO) affects the neuronal activity of the PAG.[22-24] Nitric oxide acts as an intracellular signaling molecule – neurotransmitter itself and/or as a neuromodulator and influences the plastic properties of the neurons.[25-26] It is also involved in NO-molecular ways, which affect through auto-regulation different signaling molecules–like opioids, endocannabinoids and others.[27] In addition, it is well known that immobilization stress activates neuronal NOS in the HPA axis.[28,29] While there are reports to support a role for NO in dlPAG function,[30-34] exactly how NO influences dlPAG function remains to be clearly understood.

The goal of the present study was to investigate the changes of NO activity in the dlPAG of rats subjected to acute immobilization stress using a histochemical examination of the distribution of NADPH-d reactivity neurons.

## MATERIALS ANDMETHODS

**Animals**

The experiments were carried out on male Wistar rats (180-200g), divided into two groups. The first group represented intact controls. The second group was subjected to acute immobilization stress.

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 hour.

**Histochemistry**

The animals were anesthetized with thiopental (40mg/kg b.w.). Transcardial perfusion was done with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. Postfixation of the obtain material was conducted in 4 % buffered solution (0,1 M phosphate buffer, pH 7,4) of para-formaldehyde for overnight at 4°C. Coronal sections were cut on a freezing microtome (Reichert-Jung) at 25 µm and washed repeatedly in 0,01M PBS (phosphate buffer, pH 7,4). First, every fifth section was processed for double staining for NADPH-d. The slices were stained with NADPH-d-technique using: 0,2 mg/ml NBT (nitrobluetetra-zoliumchloride), 1 mg/ml NADPH-tetranatriumsalt, 0,5 % Triton X – 100 diluted in 0,1M Tris HCl, pH 7,6-for 5 hours at 37° C. Afterwards, they were rinsed with 0,1 M Tris HCl, pH 7,6 and 3 time with 0,01 M PBS for 5 min. They were mounted on gelatin-coated glass, dried for 24 hours and cover slipped with Entellan. Ten coronal sections were utilized for calculation of the neuronal packing density in dlPAG of rats. The intensity of the staining was evaluated visually and number of NADPH-d reactive neurons was counted. We used Paxinos and Watson's atlas in anterior-posterior localization from bregma -7,64 mm for an analysis of the sites.

**Data analysis**

Morphometric analysis was performed using a microanalysis system (primary magnification 20 x 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 (S.E.M.). Statistical significance was accepted when $P < 0.05$.

## RESULTS & DISCUSSION

It's known that stress activates the HPA axis by stimulating neuronal activity within the paraventricular nucleus of the hypothalamus. 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 [1]. According to literature data, specific stressors may elicit specific responses, and different stressors may activate different brain systems by using specific pathways within the central nervous system.[35,36] Several brain structures, including PAG, organize defensive reactions and represent the neural substrate implicated in the functional neuroanatomy of stress response. PAG activates descending inhibitory pathways to the medulla, which is connected with the spinal cord and suppresses nociception.[37,38]The 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.[38] Besides, dlPAG is a midbrain region surrounding the aqueduct and controlling different reactions such as defensive behaviors, analgesia and autonomic changes.[39] NO is produced from L-arginine through calcium dependent pathways by the nitric oxide synthase enzyme (NOS).[40] 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.[41] So, in spite of some limitations,[42] 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.[42,43]

In our experiments a histochemical procedure for NADPH-d-reactive neurons in rat's PAG was used as marker of NO activity. Increased NO stimulates guanylate cyclase and increases the levels of cyclic guanosine 3′5′-monophosphate in the cells. Thus, the NADPH-d histochemical method allows the direct visualization of the neurons, which use NO. The control animals that were not immobilized showed a cluster of intensely stained NADPH-d positive neurons with varicose fibers in the PAG. The acute stressor – 1 hour immobilization, showed statistically significant increase in the number of the NADPH-d positive neurons compared to the control group [Figure 1-3] ($p < 0.01$). These results support some author's data about stress-induced increasing of NO activity in PAG[2,22,44,45], PVN[46-48], cerebral cortex [3], caudate putamen[49], striatum[50], claustrum[51,52], in the thalamic reticular nucleus.[53,54]

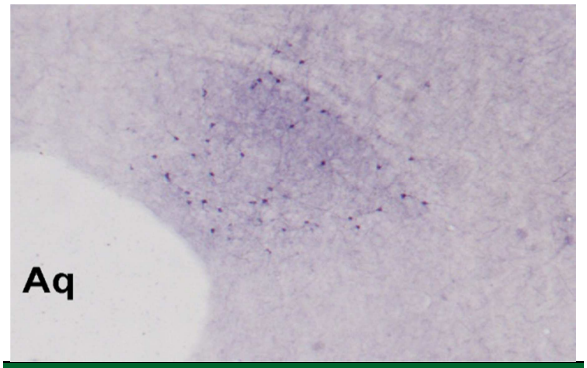


**Figure 1:** Photomicrograph showing NADPH-d-reactive neurons in intact male Wistar rat's dlPAG (x100); Aq - aqueduct.

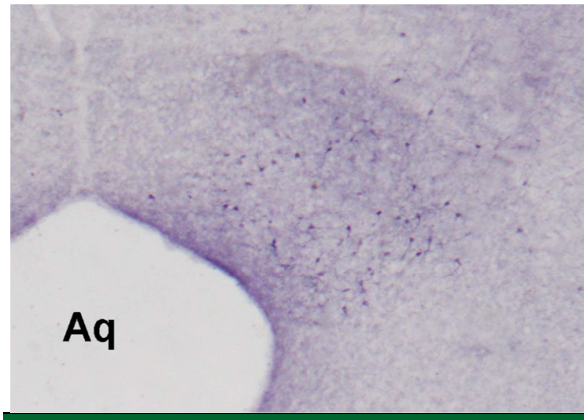


**Figure 2:** Photomicrograph showing NADPH-d-reactive neurons in male Wistar rat's dlPAG, sacrificed immediately after 1h immobilization stress (IS) – increased number of neurons (x100); Aq - aqueduct.

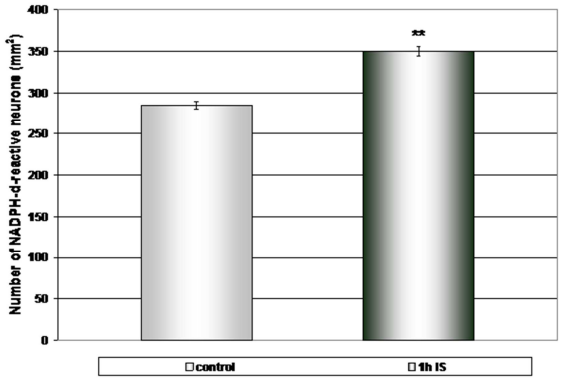


**Figure 3:** Effect of 1h immobilization stress (IS) on NADPH-d-reactive neurons in male Wistar rat's dlPAG. Mean values ± S.E.M. are presented **P < 0.01 vs. control.

Although a large number of neurotransmitters[55-67], neuropeptides, and neuromodulators [68] 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.[1] The orchestrated interplay of several neurotransmitter systems in the brain underlies the characteristic phenomenology of behavioral, endocrine, autonomic and immune responses to stress.[69] These transmitters include CRH, AVP, opioid peptides, dopamine and norepinephrine. Also newly synthesized analogues of neuropeptides which neuromodulatory effect on nitric oxideergic system was even stronger have been studied.[70]

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 stress response. Previous works have demonstrated that NO causes a neuronal release of β-endorphin[71] and that NO antinociception is suppressed by pretreatment with various NOS inhibitors.[72] 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.[73] Since a group of PAG cells has been reported to be enkephalin immunoreactive [74], 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.

## CONCLUSION

In conclusion our results showed that NO activity in rat's dlPAG was significantly increased by acute immobilization stress. This suggests a pivotal role of this part of the brain and NO-ergic system in stress response which main role is to attenuate the effect of stress and to restore the homeostasis.

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