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# Stress-specific changes of galanin and PACAP expression in the rat hypothalamus and adrenal gland

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**Abstract:** Galanin and pituitary adenylate cyclase-activating peptide (PACAP) are involved in the stress response, and both produce multiple effects in the central and peripheral nervous systems. In rodents, galanin brain expression is altered by different stressors, while PACAP has a critical role in the circuits mediating the stress-response. The aim of this study was to investigate whether acute and/or repeated immobilization (IMO) and restraint (R) differently affect galanin and PACAP expression in the rat hypothalamus and adrenal gland. Relative expression of mRNAs was examined by qPCR and relative changes in the protein content were analyzed using Western blotting. The distribution of galanin and PACAP peptides in the hypothalamus was analyzed by immunofluorescence. Relative hypothalamic expression of both galanin and PACAP mRNAs was increased only after repeated restraint. Hypothalamic galanin protein levels were increased after all applied stressors, whereas there were no changes in the PACAP content. In the adrenal glands, galanin and PACAP mRNAs were elevated only after acute IMO, whereas the protein levels were not changed, regardless of the applied stressor or its duration. Immunofluorescence staining demonstrated the presence of galanin in the paraventricular nuclei (PVN) of the hypothalamus in all groups. Galanin immunoreactivity was stronger in the PVN of all stressed groups in comparison to the control. We concluded that the patterns of transcriptional activation and translational control of galanin and PACAP in the examined tissues depended on the type (IMO vs. R) and duration (acute vs. repeated) of the applied stressor.

**Key words**: galanin; hypothalamo-pituitary-adrenal (HPA); immobilization; pituitary adenylate cyclase-activating polypeptide (PACAP); restraint; stress

## INTRODUCTION

Stress is an integral part of life and while organisms have developed mechanisms for coping with stress, prolonged stress can cause psychiatric and cardiovascular disorders. The stress response involves activation of the hypothalamo-pituitary-adrenal (HPA) axis and the sympathoadrenal (SAM) system, although specific stressors may elicit specific responses activating different pathways within the central nervous system. Response patterns depend on type, duration and the severity of stressors, as well as on prior experience and encounters with the same or a different type of stressor [1,2].

Numerous studies have shown that galanin (GAL) and pituitary adenylate cyclase-activating polypeptide (PACAP) and their receptors are expressed in the hypothalamus, pituitary and adrenal glands where they affect the synthesis and secretion of hormones mediating the stress response. They have a stimulatory effect on corticotropin-releasing hormone (CRH) secretion in the hypothalamus [3], on adrenocorticotropic hormone (ACTH) secretion from the pituitary [4], and they directly or indirectly stimulate secretion of corticosterone (CORT) from the adrenal glands [5].

Galanin (GAL) is a neuropeptide widely distributed throughout the central and peripheral nervous systems, where it acts as a neurotransmitter and neuromodulator. It is implicated in the regulation of food intake, memory, the neuroendocrine axis and nociceptive nerve regeneration [6, 7]. GAL is coexpressed with CRH and arginine vasopressin (AVP) in the rat hypothalamic paraventricular nucleus (PVN) [8], and

with AVP and oxytocin in the magnocellular neurons of the supraoptic nucleus (SON) [9]. It is thought that GAL has an essential role in the stress response [10] because it affects the synthesis and secretion of CRH in the hypothalamus. As regards the adrenal gland, GAL increases corticosterone secretion [5], cAMP release from the adrenal cortex cells [11], as well as norepinephrine from the adrenal medulla. Different studies have shown that GAL increases basal and stress-induced sympathetic activity, which is reflected in the increase of plasma catecholamine concentration [12].

In addition to GAL, PACAP is also involved in the response to stressful stimuli. This peptide was first identified in hypothalamic extracts [13] and named for its ability to stimulate anterior pituitary adenylyl cyclase activity. In rats, PACAP is one of the key components of the neuroendocrine responses to stressful challenges and together with its receptors it is widely distributed throughout the HPA axis, as well as in the hypothalamic PVN and SON, median eminence, hypothalamohypophysial portal system and in the adrenal gland [14,15]. PACAP-positive terminals form synapses with hypothalamic PVN CRH-expressing neurons [16] and can stimulate CRH production and secretion [17]. PACAP can affect ACTH secretion from the anterior pituitary [18] and enhance the synthesis and release of adrenal catecholamines [19], which was shown in experiments on PACAP knockout mice who had impaired catecholamine release during chronic stress exposure.

The detection of a regulatory mechanism for GAL and PACAP in the hypothalamus and adrenal glands in conditions provoked by stressors of different type, duration and severity is very important in stress biology because of the negative impact of stress on health. The aim of this study was to determine whether the type, duration or severity of applied stressors produce specific pattern responses in the hypothalamus and adrenal glands of the rat.

## MATERIALS AND METHODS

### Experimental design

This study was approved by the Ethical Committee of the University of Belgrade, Faculty of Biology, and is in accordance with the Animal Welfare Act and Regulations on Laboratory Animals of the Republic of Serbia. In this study, 9-11-week-old male Wistar rats (*Rattus norvegicus*) weighing 250-320 grams were used. The animals were acclimated to 22±1°C, 12h:12h light-dark cycle, with dark cycle onset at 6 pm. They had *ad libitum* access to standard pelleted food and water. To avoid the impact of day-night rhythm on the tested parameters, all experiments were carried out between 8 am and 12 pm. Experimental animals were divided into five groups as follows: (i) control animals (C); (ii) animals acutely immobilized for three hours (IMO); (iii) animals repeatedly immobilized three hours daily for seven days (R-IMO); (iv) animals acutely restrained for three hours (R), and (v) animals repeatedly restrained three hours daily for seven days (R-R). Each group consisted of six animals. Immobilization was performed according to Kvetnansky and Mikulaj [20]. Animals were placed on the immobilization stands with all four limbs fixed to metal brackets. Restraint was implemented by placing the animal in a transparent cylindrical chamber. At the front of the chamber there was an opening with a few larger holes that allowed the animal to breathe. The other end of the chamber was adjustable to the size of the animal in order to disable movement of the animal. Animals were handled carefully to avoid any kind of injuries. At the end of the treatment the rats were decapitated with a guillotine. The hypothalamus and adrenal glands were quickly excised and stored at -70°C for RNA or protein isolation.

### RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was extracted from the collected tissues using a TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Briefly, tissue samples were homogenized using an IKA T10 basic ultra-turrax homogenizer (IKA-Werke GmbH&Co. KG, Germany) in appropriate volumes of TRIzol reagent, incubated at room temperature and after addition of chloroform, centrifuged (at 12000*xg*, 15 min, +4°C) to separate the homogenate into aqueous (RNA containing), inter- and phenol-chloroform phases (DNA and protein containing). The aqueous phase was transferred to a new tube and RNA was precipitated by the addition of isopropanol; after

several washing steps, the pellet was resuspended in RNase-free diethylpyrocarbonate (DEPC)-treated water. The total RNA concentration was determined by measuring the absorbance at 260 and 280 nm (Biophotometer, Eppendorf AG, Germany) according to the manufacturer's instructions. RNA sample quality was analyzed on a 1.5% agarose gel containing ethidium bromide and visualized by UV transillumination. Reverse-transcription was performed on 1.5 μg of total RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's protocol. Gene expression was analyzed using TaqMan Universal PCR Master Mix and Assay-on-Demand Gene Expression Products with 6-FAM dye-labeled MGB probes (Applied Biosystems, USA). Reactions were performed in a 25-μL reaction mixture containing 1x TaqMan Universal Master Mix with AmpErase UNG, 1x Assay Mix (GAL: Rn00583681_m1; PACAP: Rn00566438_m1; and beta actin as a reference gene: Rn01412977_g1; Applied Biosystems) and the cDNA template (10 ng RNA converted to cDNA). PCR was carried out in the ABI Prism 7000 Sequence Detection System (cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min). Each sample was run in triplicate and the mean value of each Ct triplicate was used for further calculations. Data were analyzed by the comparative $2^{-\Delta\Delta C}$t method according to Livak and Schmittgen [21].

**Western blot analysis**

In order to confirm potential differences in GAL and PACAP protein expressions, Western blot analysis was performed. After sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), protein transfer to PVDF membranes and blocking with 5% BSA, the membranes were incubated with GAL primary antibody (1:1000, sc25446, rabbit polyclonal, Santa Cruz Biotechology, CA, USA) or PACAP antibody (1:1000, sc25439, rabbit polyclonal, Santa Cruz Biotechnology, CA, USA). Then the membranes were incubated with goat anti-rabbit IgG; H&L (HRP), Abcam, ab6721-1, 1:5000, and donkey anti-rabbit IgG HRP; sc-2077, Santa Cruz Biotechnology, CA, USA, respectively. Visualization was performed using Clarity Western ECL Substrate (Bio-Rad Laboratories, USA) and film (Kodak Biomax) exposure. The quantification of the signals was determined with the ImageQuant 5.2 software package.

**Immunofluorescence**

For microscopic analysis of the hypothalamus, rats were anesthetized with a mixture of ketamine (30 mg/kg) and xylazine (6 mg/kg). Transcardial perfusion with 0.9% NaCl was performed until the liver was cleared, after which perfusion fixation with 4% paraformaldehyde was applied (Sigma, USA). Brains were removed and post-fixed overnight at +4˚C in the same solution. The tissue was cryoprotected in increasing concentrations of sucrose solution (10%, 20%, 30%). Brains were frozen at -70˚C and cut into 20-μm thick coronal sections on a cryostat (Leica CM1850, Leica Byosystems, Germany). Slices were placed on SuperFrost® slides (Menzel-Glasser, Germany) and stored at +4˚C until further use. Sections were washed in 0.01 M phosphate-buffered saline (PBS) three times, and incubated in 0.1% Triton-X 100 for 15 min. The blocking step was performed for 45 min in a solution containing 2% bovine serum albumin (BSA) in 0.01 M PBS. Both primary and secondary antibodies were diluted in 2% BSA in PBS. Primary antibodies were incubated overnight at +4˚C. The following primary antibodies were used: rabbit anti-GAL (1:100, sc-25446) and rabbit anti-PACAP (1:100, sc-25439). After incubation with primary antibodies, the sections were rinsed in PBS and incubated for 2 h with donkey anti-rabbit Alexa 555 antibody (1:200, A-31572, Molecular Probes). Excess antibodies were removed by washing in PBS, sections were incubated with DAPI (1:4000) for 10 min, washed with PBS several times and mounted with Mowiol. Images were captured with a confocal laser microscope (LSM 510, Carl Zeiss, Germany).

**Statistical analysis**

Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons between groups. All data are presented as mean±SEM values for six animals. Statistical significance was set at $p<0.05$.

## RESULTS

As can be seen in Figs. 1 and 2, hypothalamic GAL and PACAP mRNAs had similar expression patterns. When compared to the controls, the relative expression of GAL and PACAP mRNAs was significantly increased only in the hypothalamus of repeatedly restrained animals (*p<0.05). On the other hand, (Fig. 1B), the GAL protein level significantly increased in all experimental groups (*p<0.05), whereas no changes in PACAP protein level were detected when compared to the control (Fig. 2B).

As far as the adrenal glands are concerned, the relative expression of GAL and PACAP mRNAs exhibited the same patterns of changes. Statistically significant upregulation of mRNA expression for both proteins was observed only after acute immobilization in comparison to the controls (Figs. 3A and 4A). The adrenal glands protein levels of GAL and PACAP did not change relative to the control, regardless of the applied stressor or their duration (Figs. 3B and 4B).

As can be seen in Fig. 5, GAL-immunofluorescence staining demonstrated its presence in the PVN of the hypothalamus in all groups. However, GAL immunoreactivity was stronger in the PVN of stressed groups than in that of the control. Among different types of stressors, the strongest GAL immunopositivity was detected in the PVN of repeatedly restrained animals. Besides the increase in the number of GAL-positive cells, particularly long and straight neuron extensions could be detected in all stressed groups as compared to the control. In addition, strong GAL immunopositivity was observed in the ependymal

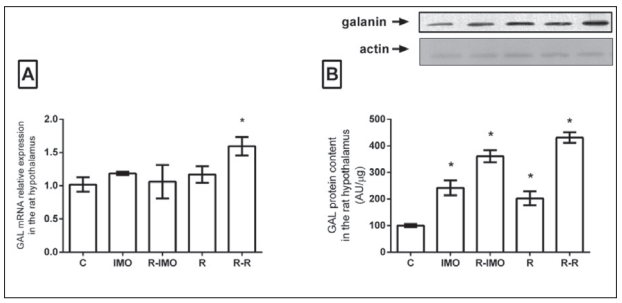


**Fig. 1.** GAL mRNA and protein levels in the hypothalamus of rats acutely or repeatedly exposed to immobilization (IMO, R-IMO) and restraint (R, R-R). **A** – RT-qPCR quantification of GAL transcript levels. **B** – Relative levels of GAL protein normalized to β-actin.

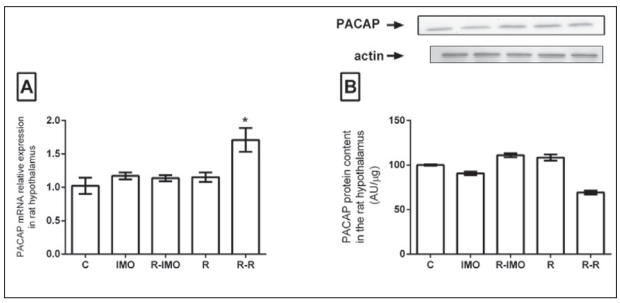


**Fig. 2.** PACAP mRNA and protein levels in the hypothalamus of rats acutely or repeatedly exposed to immobilization (IMO, R-IMO) and restraint (R, R-R). **A** – RT-qPCR quantification of PACAP transcript levels. **B** – Relative levels of PACAP protein normalized to β-actin.

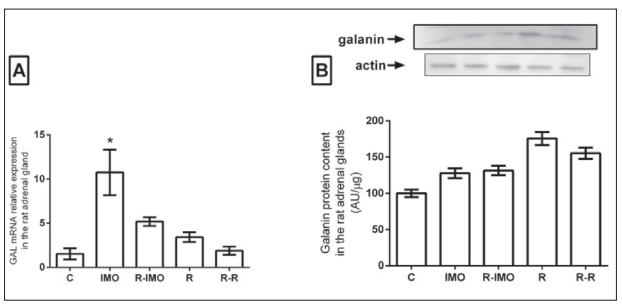


**Fig. 3.** GAL mRNA and protein levels in the adrenal glands of rats acutely or repeatedly exposed to immobilization (IMO, R-IMO) and restraint (R, R-R). **A** – RT-qPCR quantification of GAL transcript levels. **B** – Relative levels of GAL protein normalized to β-actin.

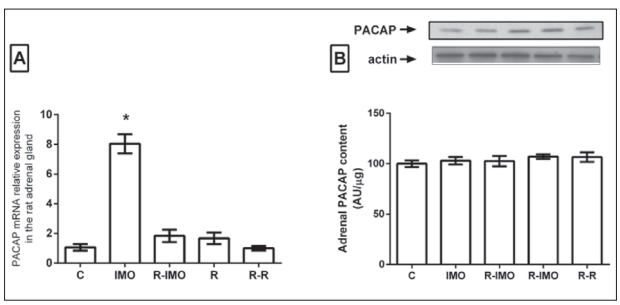


**Fig. 4.** PACAP mRNA and protein levels in the adrenal glands of rats acutely or repeatedly exposed to immobilization (IMO, R-IMO) and restraint (R, R-R). **A** – RT-qPCR quantification of PACAP transcript levels. **B** – Relative levels of PACAP protein normalized to β-actin. **C** – control, non-stressed rats; IMO – acute immobilization (3 h); R-IMO – repeated immobilization (7 days, 3 h daily); R – acute restraint (3 h); R-R – repeated restraint (7 days, 3 h daily). The values are means of six animals±SEM. Statistical significance: p<0.05.

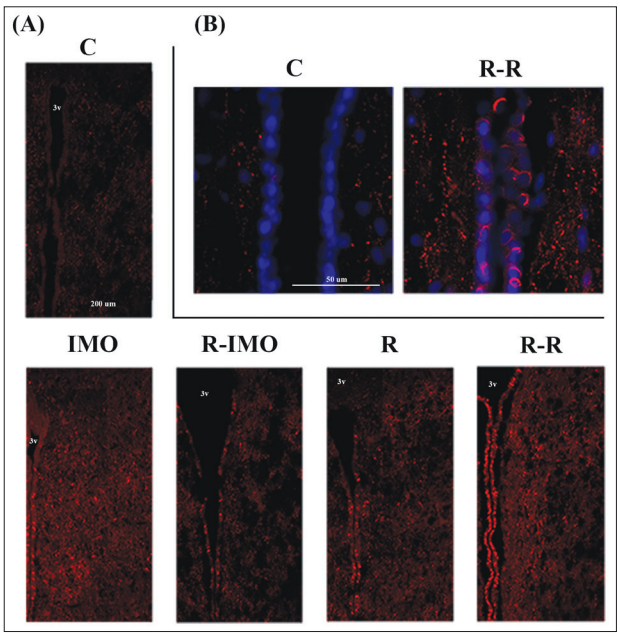


**Fig. 5.** The effect of different type of stressors on GAL expression in the paraventricular nucleus (PVN) of the hypothalamus and in the ependymal cells surrounding the third ventricle (3v). **A** – Immunofluorescence staining demonstrating GAL expression (red) in the PVN of the hypothalamus in all groups of animals (C, R, IMO, R-R and R-IMO). In all stressed groups GAL immunoreactivity in PVN was stronger than in the control. Among different types of stressors, repeated restraint showed the strongest immunopositivity (R-R). Original magnification: x40. In addition, particularly strong GAL (red) immunopositivity was observed in the ependymal cells surrounding the third ventricle in stressed animals (white arrow). **B** – Representative confocal image showing GAL immunoreactivity in ependymal cells of control (C zoom) and repeated restraint (R-R zoom). Note the strong GAL immunoreactivity in the cytoplasm of ependymal cells. DAPI was used to stain all nuclei (blue). Orig. magnification: x80. C – control, non-stressed rats; IMO – acute immobilization (3 h); R-IMO – repeated immobilization (7 days, 3 h daily); R – acute restraint (3 h); R-R – repeated

cells lining the lumen of the third ventricle of stressed animals. Some GAL-positive cells and cell projections were also detected in other hypothalamic regions, such as the periventricular, ventromedial and supraoptic, although there were no significant differences between control and treated groups.

As far as PACAP-immunofluorescence results are concerned, there were no differences between control and stressed animals, and the signal was too weak (data not shown).

## DISCUSSION

Organisms have developed mechanisms for responding to stress, and gene and protein expression changes are major factors in the response. In the present study, to examine the possible effects on GAL and PACAP mRNA expression and their protein levels in the rat hypothalamus and adrenal glands, adult male rats were subjected to acute and/or repeated immobilization and restraint stress. To confirm activation of the HPA axis, we measured plasma ACTH after IMO and R stress. In comparison to the control, the concentration of ACTH in the plasma was increased 4- and 3.5-fold after acute and repeated immobilization, respectively [22]. Similarly, we observed that acute and repeated restraint led to 3- and 2-fold increases, respectively (unpublished data).

Relative expression of both GAL and PACAP mRNAs was increased only after repeated restraint, and not after acute or repeated IMO or acute restraint. This result suggested for the first time that two stressors, which slightly differ in nature, elicited a very specific hypothalamic response. It also demonstrated a fine regulation of the hypothalamic response that depended on the type and duration of stressor. The magnitude of the stress response varies depending on the previous encounter with the same stressor. If the stress response is not altered after repeated exposure, adaptation or habituation is observed. Habituation only develops following exposure to the same stressor [23] and is likely to result from behavioral familiarization to this particular stressor. The difference between the effects of R-IMO and R-R on GAL mRNA expression, along with the fact that IMO is thought to be the strongest stressor, could be considered to be the effect of habituation within the hypothalamus. According to our results, habituation was stress-specific since it was shown only after repeated IMO, but not after R-R. The elevation in GAL mRNA that was noted only after R-R is in agreement with other studies. Sweerts et al. [7] showed that the increase of preproGAL mRNA depended only on restraint stress and that the same pattern of preproGAL mRNA expression could not be observed for other types of stress. They also showed that the expression of preproGAL mRNA increased as the number of restraint sessions increased.

Hypothalamic PACAP mRNA exhibited the same pattern of expression as GAL mRNA expres-

sion, where the most prominent effect of IMO and R on hypothalamic PACAP mRNA expression was observed after R-R. The absence of change in PACAP mRNA after acute restraint was in accordance with other studies. Hannibal et al. [14] showed that an hour of restraint stress had no effect on PACAP mRNA increase. They also suggested that different stressors could alter PACAP mRNA in a different time range [24]. Therefore, considering the effects of acute and repeated IMO on PACAP mRNA expression where no significant alterations were observed, we assume that three hours of acute treatment may not be of sufficient duration to alter PACAP mRNA expression in the hypothalamus. In conclusion, PACAP mRNA in the hypothalamus is influenced not only by the duration of stress but the type of stressor.

Considering that GAL agonists are used to treat behavioral signs of stress-related emotional disorders [25], it was expected that the amount of GAL in the hypothalamus was significantly elevated after all four stressors were applied. Five out of six mRNA-processing genes showed a negative correlation between mRNA and protein levels [26]. There are numerous reasons for such cases. Many processes are involved besides transcription and translation and protein stability is an important factor. For example, the half-life of different proteins can vary from minutes to days, whereas mRNA degradation occurs more rapidly: 2-7 h for mammalian mRNAs as compared to 48 h for proteins [27]. Since there was rise in hypothalamic GAL levels regardless of the type of stressors or their duration, we presumed that there was pre-stored GAL in cell bodies, which was not released during increased activity of the HPA axis; this is similar to what happens to tyrosine hydroxylase with which GAL is co-localized in the hypothalamus [28]. Based on the immunofluorescence results, although a positive signal was present in the periventricular, ventromedial and supraoptic hypothalamic nuclei, the most significant effect was noted in the PVN. According to Levin [29], GAL-containing terminals are in the PVN of the rat, and they preferentially innervate oxytocin-containing cells. Furthermore, there is evidence that GAL coexists with vasopressin and that they are transported together to the posterior pituitary [30]. Thus, we surmise that GAL is highly involved in the stress response and that it modulates the activity of the neuroendocrine axis by assuming an important neuroregulatory role.

Although there was very significant GAL protein elevation, we did not observe a change in the hypothalamic PACAP protein content. This may be a consequence of the fact that PACAP was already released from its depots; it is known that it acts at the level of the hypothalamus to control induction of early genes as well as CRH mRNA in the hypothalamus [31]. Results obtained from immunofluorescence confirmed the Western blot results as there were no significant differences in PACAP protein levels among treated groups as compared to the control (data not shown).

The pattern of the adrenal gland response to IMO and R was completely different from that of the hypothalamus. Namely, for both GAL and PACAP, upregulation of mRNAs was detected only after acute IMO, whereas there were no changes at the protein level.

Following acute immobilization stress there was a significant change in GAL mRNA level in the adrenal gland, but very little or none after application of repeated immobilization and acute and/or repeated restraint. Anouar et al. [32] showed that adrenal GAL mRNA levels were maximally increased at 4 h, and that they remained maximally elevated for at least 48 h after insulin administration. Based on our results, the immobilization was strong enough to elicit a change in GAL mRNA within 3 h, so we suspect that there is a time course of mRNA level change that depends on the type of applied stressor. As far as GAL protein is concerned, there were no changes in protein levels regardless of the stressor type or duration. In other words, increases in transcriptional activities were not accompanied by increases in proteins levels. GAL is implicated in the activities of chromaffin cells on glucocorticoid and mineralocorticoid production and participates in stress responses [29]. We assume that during 3 h, all pre-stored GAL is released in the blood, so that it was not detected in the adrenal gland.

When it comes to PACAP mRNA change in the adrenal gland, our results indicate that only acute IMO was strong enough to activate transcription of the PACAP gene during that time. It is known that PACAP is co-stored with acetylcholine in the splanchnic nerve terminals innervating the adrenal medulla, and that the release of different neurotransmitters from the same nerve terminal at low and high frequencies will have different postsynaptic effects. PACAP is re-

leased only at high frequencies of stimulation and has a highly specialized role in epinephrine secretion [33]. According to our results, neither IMO nor R affected the PACAP protein level in the adrenal gland, regardless of whether they were applied acutely or repeatedly. The possible explanation for the absence of changes in PACAP protein level in the adrenal gland in response to the applied stressors could be due to depletion of all internal storages through exocytotic PACAP release immediately after the application of stressors.

## CONCLUSIONS

Although IMO and R are thought to be very similar stressors, for the first time we showed that there were different responses in the hypothalamus and adrenal glands as far as the expression of two neuropeptides, GAL and PACAP, are concerned. Based on our results, the hypothalamic and adrenal GAL and PACAP mRNA patterns of change are specific adaptive responses that are the consequence of a specific type of stress, since different responses were elicited depending on the type of stress and its duration. At the hypothalamic level, the strongest response was after R-R, whereas in the adrenal gland it was after acute IMO. This study suggested that there was a very well-defined tissue-specific response to a particular stress. On the other hand, considering the results obtained from protein expression, the stronger response, at least at the hypothalamic level, was for GAL. Taken together these results suggest the presence of fine regulation of the hypothalamo-adrenal responses, and the ability of the hypothalamus to detect and react differently, even when the animals were subjected to two strong and similar stressors. The physiological significance and molecular mechanisms underlying these differences remain to be determined.

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**Author contributions:** Iva Lakić participated in study design, performed the Western blots, handled the animals. Tanja Jevđović performed the qPCR experiments and analysis. Predrag Vujović conducted Western blotting and interpreted the results. Nebojsa Jasnić performed the statistical analysis. Tamara Dakić worked on the confocal microscope. Jelena Đorđević was responsible for the overall supervision. All authors edited and approved the final version of this paper and approved the final draft.

**Conflict of interest disclosure:** All authors read and approved the final manuscript and declare no conflict of interests. The authors whose names are listed on the cover page certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria, educational grants, participation in speakers' bureaus, membership, employment, consultancies, stock ownership, or other equity interest, and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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