Original Article

The Effect of Stress on the Galaninergic System in the Rat Adenohypophysis: mRNA Expression and Immunohistochemistry of Galanin Receptors

(galanin / galanin receptor / rat / hypophysis / stress / expression / RT-PCR / immunohistochemistry)

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Abstract. The neuropeptide galanin is a widely distributed neurotransmitter/neuromodulator that regulates a variety of physiological processes and also participates in the regulation of stress responses. The effect of stress is dependent on the activity of the hypothalamic-adenohypophyseal-adrenal axis. Although the adenohypophysis is a crucial part of this axis, galanin peptides and their receptors have not yet been identified in this part of the pituitary after activation of the stress response. Since there are many controversies about the occurrence of individual galanin receptor subtypes in the adenohypophysis under basal conditions, we decided to verify their presence immunohistochemically, and we clearly demonstrated that the adenohypophysis expresses neuropeptides galanin, galanin-like peptide, and subtypes of galanin receptors GalR1, GalR2 and GalR3. The specificity of the reactions was confirmed by Western blots for galanin receptors. Using real-time qPCR we also demonstrated the presence of three GalR subtypes, with the highest expression of GalR2. In addition, we tested the effect of stress. We found that acute stress did not induce any changes in the GalR2 expression, but increased expression of GalR1 and decreased that of GalR3. We confirmed the involvement of the galanin system in the stress regulation in the adenohypophysis.

Introduction

Environmental factors are very important in human pathologies; however, their precise mechanisms of action are not known. One of these important factors is stress, which can be studied in animal models. We have studied the relationship between acute stress and the regulation by neuropeptides in the brain. The most intensively recently studied neuropeptides are galanin (Gal) and galanin-like peptide (Gal-LP), widely distributed neurotransmitters. Galanin regulates a variety of physiological processes and also participates in some pathological disorders and stress responses. Galanin acts through three receptor subtypes, GalR1, GalR2 and GalR3, which display substantial differences in their functional coupling to G regulatory proteins using different messenger cascades (Šípková et al., 2017a). Subsequent signalling activities are involved in the regulation/modulation of neuroendocrine and behavioural functions.

Our previous results demonstrated that galanin increased the locomotor behaviour and this effect was antagonized by Gal antagonist M40, indicating that galanin penetrates the blood brain barrier (Klenerova et al., 2011). We found that galanin elicits anxiolytic-related and anti-stress effects, which are dependent on the activity of the hypothalamic-adenohypophyseal-adrenal (HPA) axis. Since the hypophysis is a crucial part of this axis and since there are many controversies about the involvement of galanin and individual galanin receptor subtypes in the adenohypophysis (AH), we estimated the expression of this peptide under basal conditions and after stress.

In addition, we investigated the expression of galanin receptor subtypes in the rat adenohypophysis, which plays an important role in the hypothalamic-adenohypophyseal-adrenal axis regulation.

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Abbreviations: AH – adenohypophysis, anterior pituitary, BSA – bovine serum albumin, GAL – galanin, Gal-LP – galanin-like peptide, HPA – hypothalamo-adenohypophyseal-adrenal, IMO – immobilization, PBS – phosphate-buffered saline, qPCR – quantitative polymerase chain reaction, RT qPCR – real-time qPCR.

Material and Methods

Animals

We used male Wistar rats (Velaz, Prague, Czech Republic) with average starting body weight 220 g. Animals had free access to standard pellet food and water. Rats were housed 4–5 per cage and maintained on a standard 12 h light/12 h dark cycle, at a constant temperature (21 ± 1 °C). Treatment of rats was in accordance with the Declaration of Helsinki Guiding Principles on Care and Use of Animals [DHEW Publication, NHI 80-23]. The study was approved by the Ethical Review Committee, First Faculty of Medicine, Charles University.

Stress procedure

Animals were exposed to an acute restraint (immobilization) stressor (IMO) lasting 60 min, which was followed by 1 h pause after the stress termination (Klenerova et al., 2002). IMO was applied by fixing forelimbs and hind limbs of the rat with adhesive plaster; then the animal was restrained in a snug-fitting plastic mesh. This mesh was bent to conform to the size of an individual animal and a bandage fixed this shape of mesh. After the end of exposure to stress, rats were returned to their home cages. Control animals remained untreated and were used directly after their removal from the home cage. This type of stress was also tested in behavioural studies (Klenerova et al., 2011).

Tissue preparation

Adenohypophyses were promptly removed after rat decapitation and then stored at –80 °C until processed. To determine the expression of mRNAs of galanin peptides with RT qPCR and Western blot procedures, we used AH homogenates. Homogenization was done in a MagNA Lyser homogenizer (Roche Diagnostics GmbH, Mannheim, Germany). Following that, homogenates were exposed to ultrasound and centrifuged (MagNA Lyser, Roche Diagnostic GmBH, Mannheim, Germany) in order to get clear supernatant to assess proteins.

For immunofluorescence determination of galanin peptides we used tissue slides. Frozen AH tissue sections were cut with Cryostat Leica CM1850 (Leica Microsystems, Nussloch, Germany) in thickness of 5 µm. Slides were treated with poly-L-lysine (Sigma-Aldrich, Darmstadt, Germany) and exposed to standard washing phosphate-buffered saline (PBS). Before application of specific primary antibodies, the slices were incubated for 2 h at room temperature with blocking buffer (1% bovine serum albumin (BSA), 0.1% TritonX-100 and 2% normal goat serum) (Bovine Serum Albumin, Sigma-Aldrich, St. Louis, MO; Normal Goat Serum, Gibco, Waltham, MA). Blocking is an important step for minimizing unspecific binding of the primary antibody within the cell, and the permeabilization step with detergent is performed to enable the antibodies to cross the cellular membranes.

mRNA expression of galanin peptides using the real-time PCR technique

The expression of mRNA transcripts of galanin and galanin receptor subtypes was determined by real-time quantitative polymerase chain reaction (RT qPCR) (Kozera and Rapacz, 2013) from AH homogenates. The real-time qPCR reaction was performed in the CFX96 Real-Time System (Bio-Rad, Richmond, CA). Total RNA was isolated from AH of controls and stress-exposed animals using the TRI reagent (Sigma-Aldrich) following the protocol of the manufacturer. RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen, Life Technologies, Prague, Czech Republic) and single-strand cDNA was synthesized from 4 µg of total RNA. The primers were designed to amplify the sequences corresponding to galanin subtype receptors and β-actin was used as a reference housekeeping gene (Genbank Accession No. NM_031144). Classical PCR reactions were first conducted to confirm the specificity of primers (Bio-Rad, Prague, Czech Republic). Reactions for all samples were performed in triplicates, and a reverse transcriptase negative control was tested to exclude any contamination from DNA amplification. Quantification analysis of the data was performed using the Optical System Software (Bio-Rad).

We determined the galanin receptor subtype mRNAs by reference of their C_T value to the C_T value of the reference gene β-actin. The relative expression ratios were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Delta-Delta C_T measured the differences between the values of our reference housekeeping gene and the values of our samples. The expression level of the β-actin gene was used to normalize for differences in the input cDNA. Reactions for all samples were performed in triplicate, and a reverse transcriptase negative control was tested to exclude any contamination from DNA amplification. The specificity of each amplicon was then determined by using the melting curve.

Statistical significance was calculated with one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test; $P < 0.05$ was regarded as significant. As alternative calculation we used the Relative Expression Software Tool (REST-MCS© – version 2) to calculate the relative expression ratios using the pairwise fixed reallocation randomization test (Pfaffl et al., 2002).

Western blot procedure

The specificity of primary antibodies was confirmed with the Western blot procedure. The AH homogenate was loaded onto 4–20% Mini-Protean TGX gel (Bio-Rad). After electrophoresis the separated proteins were transferred using the Trans-Blot Turbo Transfer System (Bio-Rad) and the Trans-Blot Turbo Mini Nitrocellulose Transfer Pack (Bio-Rad) to nitrocellulose membranes. The membranes were incubated with primary antibodies detected with the Vectastain ABC kit (Vector Labs, Burlingame, CA).

Immunohistocheminal methods

For immunohistochemical detection of galanin, galanin-like peptide and GalR subtypes we used frozen sections (5 μm) of AH. The detection was performed with the use of rabbit polyclonal antibodies (Alomone Labs, Jerusalem, Israel): Anti-Galanin Receptor Type 1 antibody (#AGR-011), Anti-Galanin Receptor Type 2 (#AGR-012) and Anti-Galanin Receptor Type 3 (extracellular) (#AGR-013) at a dilution of 1 : 500, and the secondary antibody was Alexa Fluor 488 used at a dilution of 1 : 600 (Invitrogen, Carlsbad, CA) (Šípková at al., 2017b). The immunofluorescence was observed by Leica DM5000 B, and the obtained data were analysed with the NIS Elements software (Laboratory Imaging 2015, the Nikon Instruments, Prague, Czech Republic) and/or ImageJ (freeware). We evaluated two main parameters: the number of cells reacting with the primary antibody and the density of their immunofluorescence signal. Statistical evaluation was performed using the *t*-test or one-way ANOVA, and the value of $P < 0.05$ was considered as significant.

Other materials

Materials for RNA isolation and gene expression, antibodies for immunohistochemical studies and Western blot are given above. All other materials were generally available chemicals.

Results

In the first part of our experiments we tested the presence of galanin peptides in the adenohypophysis. We used an immunohistochemical method to determine immunofluorescent labelling of galanin (**A**), galanin-like peptide (**B**) and all three subtypes of galanin receptors, GalR1 (**C**), GalR2 (**D**) and GalR3 (**E**) in AH slices under basal conditions. The magnification is 40× (see Fig. 1). To assess the immunofluorescent studies we evaluated two main parameters: the number of cells reacting with primary antibodies and the density of their immunofluorescence signal. We found the presence of all tested galanin peptides.

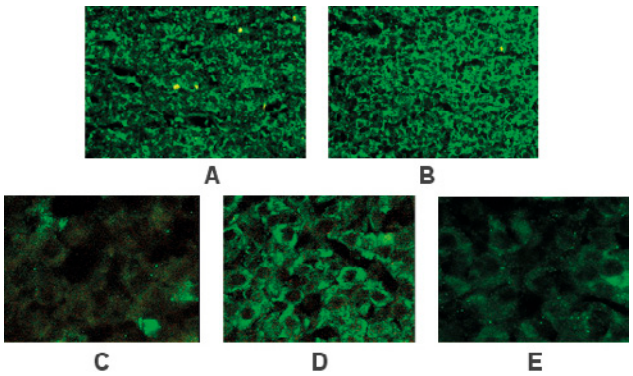

Fig. 1. Control immunofluorescent labelling of galanin peptides

We controlled the specificity of primary antibodies of galanin peptides using the Western blot method. Results in Fig. 2 demonstrate a single band of specific binding for all Gal receptor subtypes GalR1, GalR2 and GalR3 in the rat adenohypophysis.

By the RT qPCR technique we demonstrated the expression of mRNA of GalR1, GalR2 and GalR3 under basal conditions. After subtracting C_T values of the genes from the C_T values of the housekeeping gene β-actin, we found that the expression of GalR2 was much higher than that of the two other genes, GalR 1 and Gal R3, as is shown in Fig. 3.

In the next part of the study we demonstrated the effect of acute stress on the galanin receptor subtypes. Figure 3 shows that acute stress did not induce any changes in GalR2 expression. The relative expression calculation demonstrated increased expression of GalR1 and decreased expression of GalR3; both changes were statistically significant.

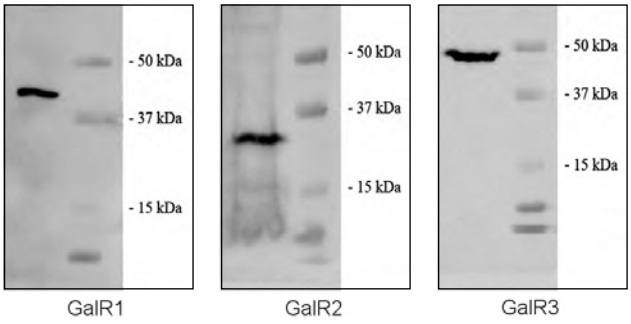

Fig. 2. The results of Western blots of GalRs in the rat adenohypophysis. There are single bands with specific binding for all galanin receptor subtypes. The molecular weights of receptor subtype proteins differ.

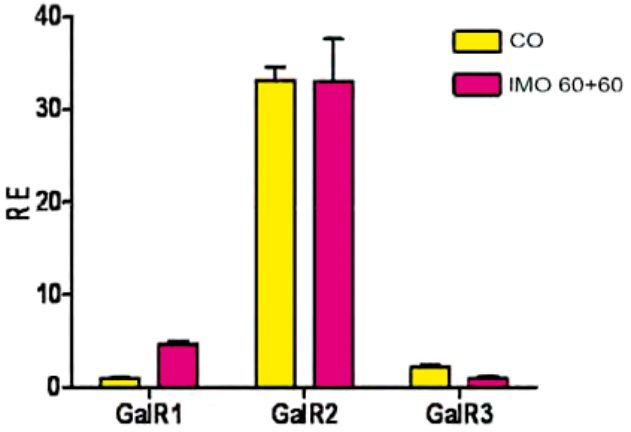

Fig. 3. Comparison of mRNA expression of galanin receptor subtypes GalR1, GalR2 and GalR3 in the rat adenohypophysis. Results from the control group (yellow colour) and under application of acute stress one hour after termination of the stress (red colour). Given $100/\Delta C_T$ mean values ± SE.

We also tested the effect of systemic application of galanin immediately after the stress. The results from behavioural studies for total movement distance and speed of movement (data not shown) demonstrate that this systemic application of galanin prevented the decrease of locomotion induced after stress (Klenerova et al., 2011). This finding indicates that the galanin peptide penetrated the blood brain barrier (Begley, 1994) and Gal displayed anxiolytic-like effects and anti-stress actions (Klenerova et al., 2009**).** Our results demonstrated that galanin is involved in the stress regulation.

Discussion

For our study of the galaninergic system we have chosen the anterior pituitary (AH), which is the main part of the hypothalamic-pituitary-adrenal (HPA) axis, and thus plays a substantial role in stress responses. Tortorella et al. (2007) have shown in their survey that galanin has an important role in the autocrine/paracrine functional regulation of the central and peripheral branches of the HPA axis. The mechanism of regulation of the AH is rather complex, and it has been generally accepted that AH is regulated by the hypothalamus via a portal system (Raisman, 1997), but the exact mechanism of galanin action has not been fully elucidated (Liu and Ju, 1998). Extensive evidence indicates that galanin stimulates the activity of the central branch of the HPA axis, i.e., the release of the corticotropin-releasing hormone and ACTH, thereby enhancing glucocorticoid secretion from the adrenal cortex (Klenerova et al., 2017). There is also evidence that galanin plays a role in the modulation of the HPA axis response to stress (Kozlovsky et al., 2009), as well as in the pathogenesis of pituitary adenomas and perhaps pheochromocytomas (Rauch and Kofler, 2010). Although galanin and its receptors are expressed in all anatomical components of the HPA axis, data on the role of the galanin system in the physiology and pathophysiology of the HPA axis remain unexplained (Packard et al., 2016).

We are the first to describe the presence of galanin, galanin-like peptide and galanin receptor subtypes in AH under physiological conditions. Numerous authors have reported the distribution of galanin receptors in various tissues (see a detailed overview by Waters and Krause (2000)), but data on messenger RNAs of the galanin receptor subtypes in AH are missing. There is evidence that some neuropeptides are involved in the stress responses (Klenerova et al., 2006, 2017; Slavikova et al., 2016); however, so far there were no data on the participation of galanin receptor subtypes during stress. We found that galanin has a modulating effect on restraint stress-induced short- and long-term behaviour changes in rats (Klenerova et al., 2011) and Gal could also be involved in the cardiovascular control during stress (Skopek et al., 2012; Šípková et al., 2017b). Although investigation of the effect of galanin has made great progress, we still do not fully understand the presence and function of each GalR subtype in AH. We demonstrated the expression of mRNA of all subtypes of galanin receptors (GalR1, GalR2 and GalR3) under physiological conditions and we found that the expression of GalR2 was much higher than that of the other two (GalR1 and GalR3) receptors. In our experiments with stress application, we showed that acute stress did not produce any changes in the GalR2 expression, but increased expression of GalR1 and decreased expression of GalR3.

To explain these results we have to recall that the link between all three galanin receptors in the cell signalling cascades is different (Šípková et al., 2017a). All three GalR are members of the family of G-protein-coupled receptors, but GalR1 stimulation activates the adenylate cyclase pathway with Gal_{α} inhibitory protein, GalR2 stimulation activates phospholipase C and finally, the stimulation of GalR3 activates the $G_{i/0}$ protein pathway (Lang et al., 2007). A number of pituitary hormones are synthesized in AH, which may have a functional relationship to galanin, in particular the relationship between ACTH and galanin. Estimation of the co-localization of galanin and galanin receptor subtypes with ACTH will help determine the role of galanin in various types of stress. We have made preliminary experiments to study the co-localization of ACTH with the galanin system. We found co-localization of ACTH with the GalR2 receptor subtype, GalR1 co-localized only rarely, and GalR3 co-localized with ACTH in only some cells. These results indicate the involvement of galanin receptors in ACTH secretion in the rat AH, and this research warrants continuation.

In summary, the results of our study show that the biological activity of galanin signalling is very complex and plays a role in several important processes including acute stress. Galanin receptor subtypes GalR1, GalR2 and GalR3 probably mediate many of galanin actions, and therefore we intended to determine the relative levels of these receptors in galanin-responsive tissues, including the adenohypophysis. The multiple functions of galanin have already been reported to be associated with disturbances in the galaninergic system signalling, which contributes not only to the diversity of possible physiological effects, but also to the diversity of after-stress effects. Therefore, galanin and its receptors present a promising target for pharmacology research and future treatment possibilities.

Conclusions

In our study with the immunohistochemistry procedure, we clearly demonstrated that the adenohypophysis expresses neuropeptides galanin, galanin-like peptide and subtypes of galanin receptors GalR1, GalR2 and GalR3. The specificity of the reaction was confirmed by Western blots. The effect of restraint stress revealed a statistically significant increase in the GalR2 receptor subtype expression, with the increase of both the number of positive cells and the specific signal density. Using real-time qPCR we have also demonstrated the presence of three GalR subtypes, with the highest expression of

GalR2. This might be due to the messenger cascade that is utilized by GalR2. The dynamics of the acute restraint stress effects seems to be similar as the expression of galanin receptors under basal conditions. We confirmed the involvement of the galaninergic system in the stress regulation in the adenohypophysis.

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Disclosure of conflict of interest

The authors declare no conflict of interest.

References

Begley, D., J. (1994) Peptides and the blood-brain barrier: the status of our understanding. *Ann. N. Y. Acad. Sci.* **739**, 89-100.

Klenerova, V., Kaminsky, O., Sida, P., Krejci, I., Hlinak, Z., Hynie, S. (2002) Impaired passive avoidance acquisition in Spraque-Dawley and Lewis rats after restraint and cold stress. *Behav. Brain Res.* **36**, 21-29.

Klenerova V., Krejci I., Sida P., Hlinak Z., Hynie S. (2006) Timing of stress and testing influence the long-lasting behavioral performance in rats. *Neurosci. Lett.* **410**, 100-104.

Klenerova, V., Krejci, I., Sida, P., Hlinak, Z., Hynie, S. (2009) Modulary effects of oxytocin and carbetocin on stress-induced changes in rat behavior in the open-field. *J. Physiol. Pharmacol.* **60**, 57-62.

Klenerova, V., Flegel, M., Skopek, P., Sida, P., Hynie, S. (2011) Galanin modulating effect on restraint stress-induced short- and long-term behavioral changes in Wistar rats. *Neurosci. Lett.* **502**, 147-151.

Klenerova, V., Kvetnansky, R., Hynie, S. (2018) The effect of acute and repeated stress on CRH-R1 and CRH-R2 mRNA expression in pituitaries of wild type and CRH knock-out mice. *Cell Mol. Neurobiol.* **38**, 163-169.

Kozera, B., Rapacz, M. (2013) Reference genes in real-time PCR. *J. Appl. Genet*. **54**, 391-406.

Kozlovsky N., Matar M., A., Kaplan Z., Zohar J., Cohen H. (2009) The role of the galaninergic system in modulating stress-related responses in an animal model of posttraumatic stress disorder. *Biol. Psychiatry* **65**, 383-391.

Lang, R., Gundlach, A., L., Kofler, B. (2007) The galanin peptide family: receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacol. Ther.* **115**, 177-207.

Livak, K., J., Schmittgen, T., D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods* **25**, 402-408.

Raisman, G. (1997) An urge to explain the incomprehensible: Geoffrey Harris and the discovery of the neural control of the pituitary gland. *Ann. Rev. Neurosci.* **20,** 533-566.

Rauch, I., Kofler, B. (2010) The galanin system in cancer. *EXS* **102**, 223-241.

Packard, A., E., Egan, A., E., Ulrich-Lai, Y., M. (2016) HPA axis interactions with behavioral systems. *Compr. Physiol.* **6,** 1897-1934.

Pfaffl, M., W., Horgan, G., W, Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.

Slavikova, J., Mistrova, E., Klenerova, V., Kruzliak, P., Caprnda, M., Hynie, S., Sida, P., Dvorakova, M. (2016) Effects of immobilizations stress with or without water immersion on the expression of atrial natriuretic peptide in the hearts of two rat strains. *Am. J. Transl. Res.* **8**, 3148-3158.

Skopek, P., Hynie, S., Chottova-Dvorakova, M., Sida, P., Slavikova, J., Mistrova, E., Klenerova, V. (2012) Effects of acute stressors on the expression of oxytocin receptor mRNA in hearts of rats with different activity of HPA axis. *Neuroendocrinol. Lett.* **33**, 124–132.

Šípková, J., Kramáriková, I., Hynie, S., Klenerová, V. (2017a) The galanin and galanin receptor subtypes, its regulatory role in the biological and pathological functions. *Physiol. Res.* **66**, 729-740.

Šípková, J., Šída, P., Kaspříková, N., Kramáriková, I., Hynie, S., Klenerová, V. (2017b) Effect of stress on the expression of galanin receptors in rat heart. *Folia Biol. (Praha)* **63**, 98-104.

Tortorella, C., Neri, G., Nussdorfer, G. G. (2007) Galanin in the regulation of the hypothalamic-pituitary-adrenal axis. *Int. J. Mol. Med.* **19**, 639-647.

Waters, S. M., Krause, J. E. (2000) Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. *Neuroscience* **95**, 265-271.