# Original Article

# Effect of Stress on the Expression of Galanin Receptors in Rat Heart

(galanin / galanin receptor / rat / heart / stress / immunohistochemistry)

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**Abstract. Neuropeptide galanin, galanin-like peptide and galanin receptors 1, 2 and 3 are a crucial part of the so-called galaninergic system. Our previous studies have shown the possible role of this system in mood modulation, especially regarding stress. So far, the galanin receptors have been found in different tissues including brain and heart. Our study deals with changes in expression of galanin receptor subtypes in the heart of Wistar rats exposed to three different types of stress. Galanin receptor subtypes were determined in fluorescently labelled samples using specific primary antibodies, and all sections were analysed in an immunofluorescent microscope and microphotographs. Image analyses were subsequently performed by software ImageJ, using the threshold method with calculation of the DAPI/galanin receptor signal ratio. We found all three types of receptors in the right and left atria and left and right ventricles. Changes in the density of galanin receptors after application of the stressor depended on the observed heart compartment. We found a significant decrease of galanin receptor 1 in all compartments after all types of stress. For GalR2 and GalR3, the increase/decrease of density was dependent on the tested compartment. These results show that galanin receptors could be involved in the function of heart during the cardiac cycle.**

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Abbreviations: CI – confidence interval, DAPI – 4',6-diamidino-2-phenylindole, Gal – galanin, GalLP – galanin-like peptide, GalR – galanin receptor, IMO – immobilization, LA – left atrium, LV – left ventricle, PBS – phosphate-buffered saline, RA – right atrium, RV – right ventricle, WB – Western blot procedure.

## Introduction

Multitalented neuropeptide galanin is a widely distributed neurotransmitter and it is implicated in numerous central and peripheral physiologic processes as an inhibitory modulator. Galanin (Gal) was discovered in 1983 in porcine intestine (Tatemoto et al., 1983). Newly, galanin-like peptide (GalLP) was discovered in porcine hypothalamus and gastrointestinal tract (Ohtaki et al., 1999), and both neuropeptides were already identified in various tissues of several other species (Cunningham et al., 2002; Lang et al., 2007). So far, three subtypes of galanin receptors (GalR) have been identified, referred to as GalR1, GalR2 and GalR3 (Branchek et al., 2000). All three GalR subtypes are cell surface receptors, which use different G-coupled proteins and different cell signal transduction pathways (see review by Šípková et al., 2017). Their roles in various tissues where galanin receptors have been identified so far (Lang et al., 2007) are also important in the peripheral nervous system and in different peripheral organs. The biological activity of galanin signalling is very complex and plays a role in several important processes including stress-related behaviour and various disorders (Ichikawa and Helke, 1993; Gundlach, 2002; Lang et al., 2007; Klenerova et al., 2011a). The multiple functions of galanin have already been reported to be associated with disturbances in galaninergic system signalling. Therefore, galanin and its receptors present a promising target for pharmacology research and future treatment possibilities.

The literature reports frequent distribution of Gal receptors in various tissues, see a detailed overview by Waters and Krause, but data on GalR 1, 2 3 mRNAs in

the heart are missing (Waters and Krause, 2000). There is evidence that some neuropeptides are involved in the stress responses in the heart (Klenerova et al., 2008, 2009; Slavikova et al., 2016). Other data suggests that Gal may play a role in central cardiovascular control, mainly in the regulation of heart rate and blood pressure (Diaz-Cabiale et al., 2010; Fang et at., 2013), but the exact mechanism of Gal action in the heart is not fully elucidated. Although study of the effect of Gal has made great progress, the presence and function of each GalR subtype in the individual heart sections has not been completely elucidated.

In our laboratory we found that galanin has a modulating effect on restraint stress-induced short- and long-term behaviour changes in rats (Klenerova et al., 2011a), and Gal and GalLP could also be involved in cardiovascular control during stress (Kozlovsky et al., 2009; Skopek et al., 2012; Barnabas et al, 2016). However, so far there are no data on the participation of galanin receptor subtypes in the heart in relation to cardiac function.

The aim of this study was to determine the presence of Gal, GalLP and GalR subtypes in rat heart compartments under basal conditions, the possible involvement of these peptides in stress effects, and to evaluate expression of these peptides by using image analysis. We wanted to determine the relative levels of the three Gal receptors in the heart compartments. For this purpose, we had to find optimal conditions for the used immunofluorescence method. A special procedure was applied in order to prepare samples that have been fixed, permeabilized, washed and blocked. Attention was paid to selecting antibodies, their dilution, incubation and determination of antibody specification.

## Material and Methods

### *Animals*

Wistar male rats with starting body weight 250–270 g (VELAZ, Czech Republic) were housed in plexi-glass cages (42 cm × 26 cm × 25 cm) in a breeding room at constant temperature (21 ± 1 °C) with a 12L/12D schedule, the onset of the light phase being at 6:00. The animals were handled daily by the same person and allowed at least 10 days to recover before starting the experimental schedule. Food and water were supplied ad libitum except during the testing. The treatment of animals 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, Prague.

### *Stress application*

The method was assessed as previously described (Klenerová et al., 2003). Animals were randomly assigned to three groups exposed to two types of acute restraint immobilization (IMO) stressors and one control group. Immobilization stress lasting 60 min (IMO60) was applied by fixing the front and hind legs of the rat, and during the stress the animals were kept in vertical position. The second type of stress combined immobilization with immersion of the rats in a water bath (22 °C) lasting 60 min (IMOC60). In the third group, 60 min immobilization was followed by 60 min period without any stress stimuli (adapting period), then the rats were returned to their home cage (IMO60+60). Rats were decapitated directly after the end of the stress and after the adapting period. All groups with stress application were compared with a control group of rats with no stress exposure (CO control).

### *Tissue preparation for immunofluorescence*

Rat hearts were promptly removed and all four heart compartments were isolated, namely, the left atrium (LA), right atrium (RA), left ventricle (LV) and right ventricle (RV). The dissected heart compartments were suffused with Cryomount (Histolab, Gothenburg, Sweden) and frozen in isopentane (Sigma-Aldrich, WGK Germany) prechilled over dry ice gradually to –70 °C and then they were stored at –70 °C until processed.

The frozen blocks were equilibrated in cryostat chamber temperature for approximately 30 min and tissue sections were cut with Cryostat Leica CM1850 (Leica Microsystems, Wetzlar, Germany) in 7 μm thickness at a temperature of –24 °C. Sections were collected after slicing on slides treated with poly-L-lysine (Aldrich, Germany) and exposed to the standard washing phosphate-buffered saline (PBS). Before application of specific primary antibody, slices were incubated for 2 h at room temperature with blocking buffer (1% BSA, 0.1% Triton X-100 and 2% normal goat serum) (Bovine Serum Albumin, Sigma-Aldrich, Delaware Bay, NJ; 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.

### *Tissue preparation for Western blot*

For the Western blot procedure (WB) we used samples of heart compartments immediately after rat decapitation. Homogenization was done in homogenizer MagNA Lyser (Roche Diagnostics GmbH, Mannheim, Germany). The method depended on the type of sample, e.g., for a sample from LV of ca 10–20 mg per 700 μl of PBS we used speed 7000, three cycles every 30 s. Following that, homogenates were exposed for 3 min to ultrasound and centrifuged in order to get clear supernatant for determination of proteins.

### *Immunohistochemical methods*

**Antibodies**

The sections were incubated with primary rabbit polyclonal antibodies (Alomone Labs, Jerusalem, Israel): Anti-Galanin Receptor Type 1 antibody (#AGR-011), a

highly specific antibody directed against the 1st intracellular loop of the mouse galanin receptor type 1 (GALR1); Anti-Galanin Receptor Type 2 (#AGR-012), a highly specific antibody directed against an epitope of mouse galanin receptor type 2 (GALR2) and Anti-Galanin Receptor Type 3 (extracellular) (#AGR-013), a highly specific antibody directed against an extracellular epitope of mouse galanin receptor type 3 (GALR3). The standard quality of all antibodies was checked in Alomone Labs using Western blot analysis and affinity purification on immobilized antigen.

**Fluorescence procedure**

After preparation of sections, the samples were incubated with specific primary antibodies. We diluted antibodies in the blocking solution in a ratio between 1 : 50 – 1 : 1200 to find the concentration with the best fluorescent label and minimal unspecific binding. In our experiment we used primary antibody diluted 1 : 500. The incubation was done overnight at 4 °C. After incubation, the samples were washed three times in standard washing buffer, for 5, 10 and 15 min. Then, the secondary antibody Alexa Fluor 488 goat anti-rabbit (Invitrogen, Carlsbad, CA) (dilution 1 : 600) was applied and antibody incubation lasted for 2 h at room temperature in the dark. After incubation, the samples were washed three times for 5 min in standard washing buffer. After drying, samples were covered with one drop of Vectashield containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA), which intercalates into DNA. Immunofluorescence was observed by fluorescent microscope Leica DM5000 B with digital colour camera Leica DFC420 C with Peltier Cooling System (Leica Microsystems).

**Antibody specificity**

Antibody specificity was determined in two ways: 1) by parallel testing of immunofluorescence obtained after using control antigens, and 2) by Western blot procedure in homogenates of control samples and after using control antigens.

Each antibody was supplied with a control peptide antigen (Alomone Labs – free of charge) to ensure that the primary antibody exclusively labels the desired GalR. The blocking peptide binds GalR and prevents binding of primary antibody, and when the secondary antibody is applied, it cannot specifically bind the primary antibody (see example in Fig. 1). We used control antigens in samples from the heart compartments and we determined blocking of binding of anti-GalR type 1, 2, 3 antibodies to appropriate GalRs. To estimate the antibody specificity **(**experimental validation) we also used Western blots. For confronting problems of antibody specificity and irreproducibility, see Schonbrunn (2014).

**Western blot**

The homogenate of samples and standard marker Precision Plus Protein Dual Color (BioRad, Hercules, CA) were loaded at 4–20 % Mini PROTEAN TGX gel (BioRad). According to the individual bands of a molecular weight size marker we then identified the approximate size of the expected molecule. After electrophoresis (35 min, 200 V), the separated proteins were transferred using Trans-Blot Turbo Transfer System (BioRad) (25 V, 2.5 A, 3 min) and Trans-Blot Turbo Mini Nitrocellulose Transfer Pack (BioRad) onto nitrocellulose membrane. The membrane was then incubated with blocking solution (3% BSA in PBS buffer + 0.1% TritonX-100). After blocking, the membrane was incubated overnight at 4 °C with primary antibody (anti-GalR type 1, 2, 3 antibodies) in blocking solution. The primary antibody was detected using Vectastain ABC kit with horseradish peroxidase and DAB with 3,3´-diaminobenzidine as a substrate (Vector Lab, Burlingame, CA).

**Data collection and analysis**

For the assessment of immunofluorescent studies we evaluated two main parameters: the number of cells reacting with primary antibodies and the density of their immunofluorescence signal.

The data were processed and analysed with software ImageJ (freeware, http://rsb.info.nih.gov) and Nis Elements Viewer (Laboratory Imaging 2015, the Nikon Instruments, Tokyo, Japan) (Rasband, 1997-2014). The final value of the signal was recalculated (sum of all areas marked as signal) as an integer variable with the software built-in function. Thus, two different variables are computed: the area covered by the GalR signal and the area covered by nuclear chromatin (DAPI). The ratio

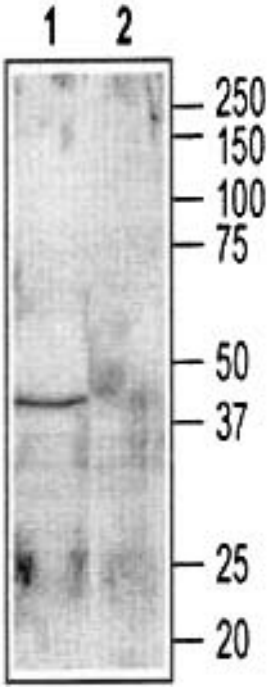


*Fig. 1.* Western blot demonstrating (**1**) a single band of specific binding for GalR1 using anti-galanin receptor type 1, and (**2**) preincubation with control peptide antigen (Alomone Labs).

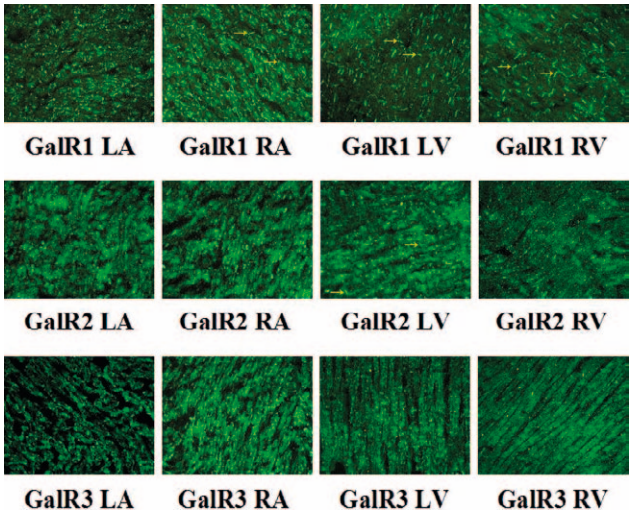


*Fig. 2.* Immunofluorescence with specific antibodies for GalR1, GalR2 and GalR3 in the rat heart compartments. We determined GalR subtypes in all heart compartments with the largest expression for GalR2 in the plasma membranes and nuclei and the lowest for GalR3 in the plasma membranes. GalR1 was expressed most intensively in the nuclei and intercalated discs; in the membranes, the expression was weak (indicated by arrows). Magnification 40×.

of DAPI intensity and particular GalR intensity was determined for each of the slides. The statistical analysis was also performed using the marginal linear model with GalR/DAPI ratio as the target variable and the group type (with categories corresponding to the three stress type exposures and the control group) as the explanatory factor. The parameters in the model were estimated using the generalized least squares method, which allows for considering the dependence between measurements taken within the same subject. The R software (R Core Team, 2014) with the nlme package (Pinheiro et al., 2014) was used for the calculations. The value of 5 % was chosen for the significance level.

## Results

In our previous studies, we investigated the involvement of Gal and GalLP in cardiovascular functions. We have shown the presence of Gal and GalLP in LA and RA and LV and RV. Gal is mostly expressed on the plasma membranes of cardiomyocytes. The frequency of cells expressing galanin is comparable in the atria and ventricles, and the density signal of galanin in the atria seems to be higher compared to ventricles. GalLP is expressed in intercalated discs. To detect the intercalated discs we used immunofluorescent detection of Connexin 40 and Connexin 45 (ThermoFisher Scientific, Waltham, MA). Intercalated discs are complex adhering structures that mediate normal mechanical and electrical coupling between cardiomyocytes. It became clear that the galanin-like peptide may work in conjunction with other transmitters to regulate the cardiac activity. Clarification of this localization will require further experiments.

Since there are many controversies about the occurrence of individual Gal receptor subtypes in the heart, we decided to verify their presence immunohistochemically with specific antibodies. Figure 2 shows detection of GalR1, GalR2 and GalR3 in the rat heart compartments. We determined GalR subtypes in all heart compartments, with the largest level of expression for GalR2 in plasma membranes and nuclei and the lowest for GalR3 in plasma membranes. GalR1 was expressed most intensively in the nuclei and in intercalated discs; the expression in membranes was weak.

In the next phase of the present study we investigated the effect of two types of stressors on Gal receptors in the heart compartments. We characterized and compared the expression of GalRs using immunofluorescent estimation. Our aim was to evaluate the changes in the GalR/DAPI ratio of galanin receptor subtypes (and the overall GalR expression) in the samples from heart compartments representing different types of stress exposure. Table 1 shows the expression of Gal receptor subtypes following application of stressors compared with the number of cardiomyocytes of the four-chambered heart. Using the software ImageJ we calculated approximate P values and borders of 95% confidence interval (CI) for the difference from the values of the control group in comparison to GalR1, GalR2 and GalR3 expression. The stressed groups exhibited lower expression of GalR1 in all heart compartments; in IMO60 and IMOC groups these results were statistically significant ($P < 0.003$). The expression of GalR2 was significantly decreased after IMOC (see example in

*Table 1. Effect of stress on the expression of Gal receptor subtypes in the rat heart*

| | IMO60 | IMOC60 | IMO60+60 |
|---|---|---|---|
| **GALR1/DAPI** | ↓ **P = 0.0009 CI: –0.732 to –0.198** | ↓ **P = 0.0001 CI: –0.801 to –0.272** | ↓ P = 0.1896 CI: –0.440 to 0.089 |
| **GALR2/DAPI** | ↑ P = 0.6649 CI: –0.135 to 0.210 | ↓ **P = 0.0259 CI: 0.024 to 0.369** | ↑ P = 0.9542 CI: –0.167 to 0.177 |
| **GALR3/DAPI** | ↑ P = 0.9817 CI: –0.446 to 0.436 | ↑ P = 0.8965 CI: –0.417 to 0.476 | ↑ P = 0.9367 CI: –0.458 to 0.423 |

Approximate P values and borders of 95% confidence interval (CI) for the difference from the values of the control group, for subtypes of Gal receptor expression following application of three stressors in all heart compartments. Statistically significant results ($P < 0.003$) are printed in bold, the elevation of expression is marked by an arrow pointing upwards, lowering of expression is marked with an arrow pointing down.

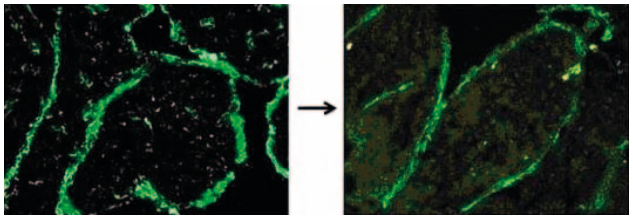

*Fig. 3.* Immunofluorescence detection of GalR1 in the left atrium of rat heart after exposure to stress
The left panel shows GalR1 in the left atrium in a control heart, i.e., without stress application. The right panel shows the expression of GalR1 in a heart from the group exposed to IMO60 stress. The density of the signal in the control heart is stronger than in the heart from the animal exposed to stress. The results are described in Table 2 and indicate that the stress exposure leads to a decrease of signal intensity.

Fig. 3), while expression was higher in both IMO60 and one hour after the end of IMO60+60, but this trend was not statistically significant. Finally, for GalR3 we observed a slight elevation of the expression in all three types of the study groups, yet this elevation was not statistically significant.

The immunofluorescence was evaluated by determining signal density. Table 2 shows the GalR/DAPI fluorescence signal ratio in individual heart compartments after application of three types of stress: IMO60, IMOC60 and IMO60+60. Table 2 presents the varying density of Gal receptor subtypes in the atria and ventricles, and also the difference between the left and right heart for GalR2 and GalR3, in the controls and following application of stressors. The decrease of GalR1 density seems to be very sensitive to stressors in the heart ventricles. The compared ratios of GalR1/DAPI in the right and left ventricles after IMO and IMOC revealed a return of decreasing expression of GalR1 to control values after one hour of adapted period (IMO60+60).

By using immunohistochemistry procedures we clearly demonstrated different distribution and density of Gal, GalLP and their receptors GalR1, 2 and 3 in all rat heart compartments. These findings suggest that the efficacy of Gal and GalLP to induce an effective coupling of its receptors to G proteins could be different depending on the heart localization. It has been sufficiently proved that Gal receptor subtypes are involved in the stress response.

## Discussion

The cardiovascular system is regulated by a diverse array of hormones, neurotransmitters and neuropeptides. In our previous study of hormones and neuropeptides in the heart and the effect of stress, we demonstrated the involvement of oxytocin receptors in all heart compartments (Klenerova et al., 2011b), the involvement of atrial natriuretic peptide (Slavíková et al., 2016), acetylcholintransferase and muscarinic $M_2$ receptors (Chottová-Dvořáková et al., 2010) and newly the possible role of Gal (Klenerova et al., 2011a). Various stressful stimuli are known to activate the sympathetic nervous system to release catecholamines and the hypothalamic–pituitary–adrenal axis to release glucocorticoids in the circulation to influence the function of the cardiovascular system. So far, however, there have been no reports on the participation of Gal receptor subtypes in the regulation/modulation of the heart function or in the individual heart sections.

In the literature, there are only scarce reports regarding the possible role of GalRs in the heart muscle cells. Since there are many controversies about the occurrence of individual Gal receptor subtypes in the heart, we decided to verify their presence immunohistochemically with highly specific antibodies. The purpose of the present study was to determine whether the exposure of rats to an acute stress could change the expression of GalRs. Our previous studies have shown the possible role of Gal as a stress modulator (Klenerova et al., 2011a). Potter and Smith-White (2005) described modulation of cholinergic neurotransmission in the heart muscle in GalR1 knockout mice. Herring et al. (2012) demonstrated that exogenous Gal inhibits the heart rate response to peripheral vagal nerve stimulation thanks to a presynaptic mechanism and GalR1 stimulation in guinea pigs. However, the mechanism of stimulation of all GalRs in the cardiac muscle cells following stress is not yet fully understood and further research is needed to explain this phenomenon.

*Table 2. Galanin receptor/DAPI fluorescence signal ratio in heart compartments after stress*

| | GAL R 1 | | | | GAL R 2 | | | | GAL R 3 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | IMO 60 | IMO C60 | IMO 60+60 | Control | IMO 60 | IMO C60 | IMO 60+60 | Control | IMO 60 | IMO C60 | IMO 60+60 | Control |
| **Left Atrium** | 0.378 | 0.315 | 0.422 | 0.564 | 0.304 | 0.217 | 0.107 | 0.304 | 0.918 | 1.039 | 0.449 | 0.388 |
| **Right Atrium** | 0.377 | 0.120 | 0.446 | 0.931 | 0.131 | 0.479 | 0.093 | 0.277 | 0.258 | 0.213 | 0.378 | 0.456 |
| **Left Ventricle** | 0.334 | 0.129 | 0.619 | 0.566 | 0.383 | 0.539 | 0.346 | 0.138 | 0.422 | 0.399 | 0.597 | 0.308 |
| **Right Ventricle** | 0.201 | 0.404 | 0.881 | 0.892 | 0.253 | 0.473 | 0.305 | 0.199 | 0.300 | 0.330 | 0.547 | 0.791 |

Expression of GALR1, GALR2 and GALR3 in rat heart compartments (N = 12) after application of three types of stress (IMO60, IMOC60 and IMO60+60).
The galanin receptor/DAPI fluorescence signal ratio (mean values) presents the varying density of Gal receptor subtypes in the atria and ventricles. The compared values of the ratio GALR1/DAPI in the right and left ventricles after IMO60 and IMOC60 revealed the return of decreasing expression of GalR1 to control values after one hour of adapted period (IMO60+60).

Our results have proved that all three subtypes of GalR are expressed in the rat heart, both under physiological conditions and after the induced stress exposure. The quantity of this expression is modified by the stress application. The response of the particular receptor types, however, differed. For GalR1, the expression was very significantly lowered in answer to both IMO and IMOC, for GalR2 the response was different depending on the type of stress, for IMO with emotional preference, a nonsignificant elevation was observed, while for IMOC with physical preference, there was a very significant decrease. For GalR3, the expression was not significantly elevated in answer to IMO and IMOC. To explain these results, we have to recall that the connection of all three GalRs into the cell signalling cascades is different (see further Šípková et al., 2017). All three GalRs are members of the large family of G-protein-coupled receptors, but GalR1 stimulation activates the adenylate cyclase pathway with $Gal_{\alpha}$ inhibitory protein, GalR2 stimulation activates phospholipase C and finally, the stimulation of GalR3 activates the $G_{i/0}$ protein pathway (Lang et al., 2007).

It is known that GalR subtypes may have a major role in modulating the emotional networks of the brain through heteromerization with 5-HT1A (5-hydroxytryptamine), NPYY1 (neuropeptide Y) (see review Fuxe et al. 2012). GalR subtypes may form heteromers with each other and other types of GPCRs in the CNS, which implies the possibility of this mechanism also in the heart.

In summary, our findings show that GalR subtypes have substantial differences in their functional coupling and subsequent signalling activities in the rat heart cardiac cycle, which contributes not only to the diversity of possible physiological effects, but also to the diversity of after stress effects. The results also support the view that the galaninergic system is very prospective in possible therapeutic use.

## *Conclusions*

We have shown the expression of Gal and GalLP in atria and ventricles in the rat heart. Gal is mostly expressed on the plasma membranes of cardiomyocytes, while GalLP is mainly expressed at intercalated discs. We found the presence of all subtypes of GalRs with ratios GalR2 > GalR1 > GalR3. Their expression had changed upon different stressor application, while the effect of the physical and/or emotional stress component influenced this change. Namely, the decreased expression of GalR1 following stress exposure was very significant. This study contributes additional evidence that galanin is worthy of further study, possibly as a novel therapeutic target for the treatment of stress-related disorders.

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## *Disclosure of conflict of interest*

The authors declare no conflict of interest.

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