

Expression of heart oxytocin receptor and its mRNA in two rat strains with different activity of HPA axis

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Abstract

OBJECTIVES: Oxytocin (OT) is a neuropeptide acting both as a peripheral hormone and in the brain as neurotransmitter and neuromodulator. In addition to its well-known effects on milk-ejection and uterine contraction, OT was shown to exert neuroendocrine regulation of heart functions. The aim of this study was to investigate the expression of mRNA of OT receptors (OTR) in rat hearts by real-time quantitative PCR (qPCR). The study was performed in Sprague-Dawley (SD) and Lewis (LE) rat strains, the latter having lower activity of HPA axis.

METHODS: We used adult male SD and LE rats. OTR mRNA expression was detected in all heart chambers by comparing their threshold cycle values (C_T) to C_T of reference gene β -actin. The relative expression ratios were calculated using the $2^{-\Delta\Delta C_T}$ method. The specificity of reaction of primary antibody with OTRs was tested by Western Blot and localization of OTR in the heart compartments was performed by immunofluorescence with commercial OTR specific antibodies.

RESULTS: We found expression of OTR mRNA in all heart compartments. The expression of OTR mRNA in both atria (LA, RA) was much higher than in the ventricles (RV, LV). By using two-way ANOVA we found no statistical differences between corresponding compartments of SD and LE rats. Immunohistochemical studies showed that OTR staining is not related to neuronal tissue and findings from left atrium indicate that prevalent localization of OTR is on cell membranes of cardiomyocytes.

CONCLUSIONS: The finding of expression of OTR mRNA by real-time qPCR and proof of OTR staining by immunohistochemistry in all heart compartments indicate that OT and its receptors may have function as a cardiovascular hormone. The differences in the HPA axis activity, as is exemplified in Sprague-Dawley and Lewis rat strain, do not project in the expression of OTR mRNA under basal condition. The effect of activity of HPA on OTR expression should be studied under stimulated conditions as it was performed in the behavioral studies.

INTRODUCTION

Oxytocin (OT) is a neuropeptide that is produced in the hypothalamus and is released into to bloodstream and the brain. OT is functioning as peripheral hormone and in the brain as neurotransmitter and neuromodulator (see e.g. Gimpl and Fahrenholz 2001; Brunton & Russell 2008). In addition to its well-known effects on milk-ejection and uterine contraction, OT was shown to exert neuroendocrine regulation through receptor-mediated actions (Zingg & Laporte 2003). All its effects are mediated by only one subtype of OT receptor (OTR), which belongs to G protein-coupled receptors (Cottet *et al.* 2010).

OT acts peripherally at multiple sites including heart and large vessels, and it has been marked as a cardiovascular hormone (Gutkowska *et al.* 2000; Petersson 2002). It was demonstrated that OT exerts negative inotropic and chronotropic effects (Mukaddam-Daher *et al.* 2001), decreases blood pressure, increases natriuresis, reveals others OT cardiovascular effects (Gutkowska & Jankowski 2008), and participates in cardiac ontogeny (Jankowski *et al.* 2004). Nevertheless, the mechanism of its action was not fully elucidated until now. Gutkowska *et al.* (2000) reported that OTRs are localized on atrial cardiomyocytes, and that OT acts through atrial natriuretic peptide (ANP) (Gutkowska & Jankowski 2008). However, there are some controversies as to the localization and amount of OTRs expression in the heart. Cardiovascular actions of OT can be influenced by OT synthesized in the heart as well as by the release of OT from the neurohypophysis to the circulation (Pyner 2009). However, there are several theories of possible indirect actions of OT in the regulation of heart function.

OT may play a regulatory role in the heart under stress conditions (Petersson and Uvnäs-Moberg 2007). The response of the heart to stress is known to be dependent on the activity of hypothalamic-pituitary-adrenal (HPA) axis and possibly on OTRs expression in rat heart. It is known that the effects of stress change in dependence on HPA activity. There are several publications showing differences in HPA axis activity and its impact on some functions, like inflammation, *cfos* expression (Trneckova *et al.* 2006), and several differences in behavior, observed in our laboratory (Kaminsky *et al.* 2001; Klenerova *et al.* 2003; 2007; 2009; 2010). These differences may be attributed to the well documented deficit in HPA axis activity in the LE strain rats (Sternberg *et al.* 1992; Windle *et al.* 1998; Gomez *et al.* 1998).

The aim of this study was to investigate the expression of mRNA of OTR in rat hearts by real-time qPCR. The study was performed in Sprague-Dawley (SD) and Lewis (LE) rat strains, the latter having lower activity of HPA axis, in order to find a possible role of HPA axis activity also on the expression of OTR receptors in the heart.

MATERIALS AND METHODS

Animals

We used male Sprague-Dawley (SD) and Lewis (LE) rat strains (Charles-River Laboratories, Sulzfeld, Germany) with average starting body weight 216 g and 198 g, respectively. LE rats are known to have deficient activity of HPA axis (Klenerova *et al.* 2007). The 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 ($21 \pm 1^\circ\text{C}$). 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 in Prague.

RNA isolation and gene expression

After decapitation of rats, the hearts were removed, washed in saline and all four compartments were isolated, weighted and quickly frozen in liquid nitrogen and then kept at -80°C until homogenization. Total RNA was isolated from all compartments ($n=6$ per group) using TRI reagent (Sigma, St. Louis, MO, USA) following the protocol of the manufacturer. Contaminating DNA was destroyed with 1 U DNase/ μg of total RNA (Invitrogen, Carlsbad, CA, USA). RNA was reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen) for 50 min at 42°C . Single-strand cDNA was synthesized from 4 μg of total RNA. The primers were designed to amplify the sequence corresponding to nucleotides 823-1106 (forward: ATGACCTTCATCATCGTACTGG, reverse: GTG-GATGAGTTGCTCTTCTTGC) of the published rat OTR cDNA sequence (Genbank Accession No. NM_012871), and to nucleotides 873-969 (forward: TTCCTTCCTGGGTATGGAATC, reverse: GTTG-GCATAGAGGTCTTTACGG) of the published β -actin cDNA (Genbank Accession No. NM_031144). Classical PCR reactions were first conducted to confirm the specificity of primers. As an internal control β -actin was amplified. Real-time qPCR was done in the ICycler (Bio-Rad, Prague, Czech Republic). Reactions were performed in a 25 μl reaction mixture containing 5 μl of diluted cDNA, 1 μl of each primer (20 nmol/l), 5.5 μl of ultrapure water and 12.5 μl iQ SYBR Green Supermix (Bio-Rad, Prague, Czech Republic). The quantitative PCR reactions were performed as follows: denaturation at 95°C for 10 min followed by 45 cycles of amplification (95°C for 30 sec, 60°C for 25 sec and 72°C for 20 sec). After amplification, the samples were slowly heated from 65°C to 95°C with continuous reading of fluorescence to obtain a melting curve. 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. Each pair of primers yielded a single peak in the melting curve and a single band of the expected size in agarose gel. The quantification analysis of the data was performed by using the Optical System Software (Bio-Rad). OTR mRNA was determined in all heart chambers by subtracting their threshold cycle values (C_T) to C_T of reference gene β -actin. The relative expression ratios were calculated using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). The expression level of the β -actin gene was used to normalize for differences in input of cDNA.

Immunohistochemical detection of OTR receptors

Individual heart compartments were covered with kryomount (Bamed, Ceske Budejovice, Czech Republic) and frozen in liquid nitrogen. Sections 7 μ m were prepared on Kryostat Leica CM1850. Samples were exposed at room temperature for two h to the medium blocking the nonspecific binding sites (1% BSA, 0.1% Triton X-100 and 2% normal goat serum). The labeling of OTR receptors was done by primary antibody Rabbit polyclonal to Oxytocin Receptor (diluted 1:500; Abcam, Cambridge, UK). For simultaneous labeling of neuronal tissue (neuronal marker) we used Anti-Neuron specific beta III Tubulin antibody [2G10] – Neuronal Marker (diluted 1:500; Abcam). As secondary antibody we used Alexa Fluor 488 Goat anti rabbit (Invitrogen) diluted 1:400 and Alexa Fluor 546 Goat anti mouse diluted 1:600. The incubation lasted for 2 h at room temperature in the dark. After drying followed coverslip with one drop of Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were observed with fluorescent microscope Leica DM5000 B with color camera Leica DFC420 C.

Western Blot

Homogenate of heart compartments and standard Precision Plus Protein Dual Color (BioRad) were loaded at 4–20% Mini-PROTEAN TGX gel (BioRad). 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) on nitrocellulose membrane. Then the membrane was incubated with blocking solution (3% of BSA in TBS buffer). After blocking, the membrane was incubated overnight at 4°C with primary antibody Rabbit polyclonal to Oxytocin Receptor (Abcam) diluted 1:15 000 in blocking solution. Primary antibody was detected with Vectastain ABC kit with horseradish peroxidase and DAB with 3,3'-diaminobenzidine as a substrate (Vector Lab, CA, USA).

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.

Data analysis and statistics

Statistical analyses were performed with two-way ANOVA and one-way ANOVA (GraphPad Prism 5.0; USA). When appropriate the ANOVA was followed by Bonferroni's *post-hoc* test. Differences were considered significant at $p < 0.05$. Data are given as means \pm SEM. The numbers of animals: $n = 6$.

RESULTS

In order to detect the role of oxytocin in the heart, we investigated the expression of OTR mRNA in rat hearts by RT qPCR. In all heart chambers, right and left atria (RA, LA) and ventricles (RV, LV), we detected differences in threshold cycles (ΔC_T) of target and reference genes. Figure 1 shows ΔC_T of OTR mRNA and β -actin. Presented data demonstrate that OTR mRNA was expressed in all heart compartments; however, there are large differences between mRNA OTR expression in atria and ventricles (note that lower values of ΔC_T bars indicate higher expression of OTR mRNA): Two-way ANOVA shows that the main effect of rat strain was not significant: $F(1,40) = 1.42$, $p = 0.24$. The effect of heart chambers is considered extremely significant: $F(3,40) = 100.2$, $p < 0.0001$. One-way ANOVA for SD rats, $F(3,20) = 48.30$: $p < 0.0001$, Bonferroni's Multiple Comparison Test: significant for LA vs LV and RV, and RA vs LV and RV. One-way ANOVA for LE rats, $F(3,20) = 54.88$: $p < 0.0001$, Bonferroni's Multiple Comparison Test: significant for LA vs LV and RV, and RA vs LV and RV.

Table 1 compares relative expression of OTR mRNA in SD and LE rats; it was calculated by the formula $2^{-\Delta\Delta CT}$ and for all other heart compartment the ΔC_T of LA from SD rats was used as calibrator. Two-way ANOVA shows that the main effect of rat strain was not significant: $F(1,40) = 1.44$, $p = 0.237$. The effect of heart chambers is considered extremely significant: $F(3,40) = 73.36$, $p < 0.0001$. One-way ANOVA for SD rats, $F(3,20) = 22.16$, $p < 0.0001$, Bonferroni's Multiple Comparison Test: significant for LA vs LV, RV and RA, and RA vs

Tab. 1. Relative expression of OTR mRNA in two rat strains.

OTR mRNA relative expression in two rat strains normalized to controls in left atria	
Sprague-Dawley strain*	Lewis strain
RE \pm SEM	RE \pm SEM
LA 1.1310 \pm 0.2320 (6) ^x	LA 1.1880 \pm 0.2690 (6) ^x
RA 0.0920 \pm 0.0230 (6) ^{xx}	RA 0.3540 \pm 0.1070 (6) ^{xx}
LV 0.0033 \pm 0.0006 (6)	LV 0.0017 \pm 0.0001 (6)
RV 0.0131 \pm 0.0050 (6)	RV 0.0039 \pm 0.0025 (6)

*Two-way ANOVA did not reveal statistical difference between both strains, however, the effect of heart compartments is very significant (see text). *One-way ANOVA shows very significant effect against all other groups ($p < 0.0001$) and ^{xx} significant effect against LV and RV ($p < 0.01$) in the particular rat strain.

LV and RV. One-way ANOVA for LE rats, $F(3,20) = 30.78$, $p < 0.0001$, Bonferroni's Multiple Comparison Test: significant for LA vs LV, RV and RA. Thus, the overall OTR mRNA expression does not show significant difference between SD and LE rats, which would be dependent on the HPA axis activity.

Immunohistochemical detection of OTR shows that these receptors are expressed in all heart compartments. The Western blot demonstrates the specificity of primary antibody immunoreactions with homogenates containing OTR proteins (Figure 2). After application of comparable amounts of tissue homogenates of LA,

RA, LV and RV, we observe the main band close to the marker of 75 kDa. The second less pronounced band, which may be related to glycosylated receptor, is less pronounced in ventricles compared to atria. Quantitative analysis was not performed yet, however, from simple observation of bands on the Western blot picture it is clear that the quantity difference of detected receptors in atria and ventricles is not as large as in the RT qPCR detection of OTR mRNA.

Figure 3 demonstrates the immunohistochemical images from the left atria, which had the highest OTR mRNA expression from all tested heart chambers. In order to identify the localization of OTRs, we performed simultaneous staining of OTRs, nuclei in cardiomyocytes and staining of the neuronal tissue by neuronal marker beta III tubulin. On the panels A–F there are some merged images: The light green dots on Figure 3A indicate OTR receptors that are mostly localized on the cell plasma membranes. On the panel C, which is merged panel A and B (nuclei, blue staining), there is evident that most OTRs are outside nuclei. In the image shown on panel D we exposed the tissue to the neuronal marker and on the panel E, with merged A and D, it is shown that OTRs are not in positions related to neuronal marker. Finally, panel F is merged figure of OTRs, nuclei and neuronal marker. Thus, the staining of OTRs is on cardiomyocytes, mostly outside nuclei and neuronal tissue.

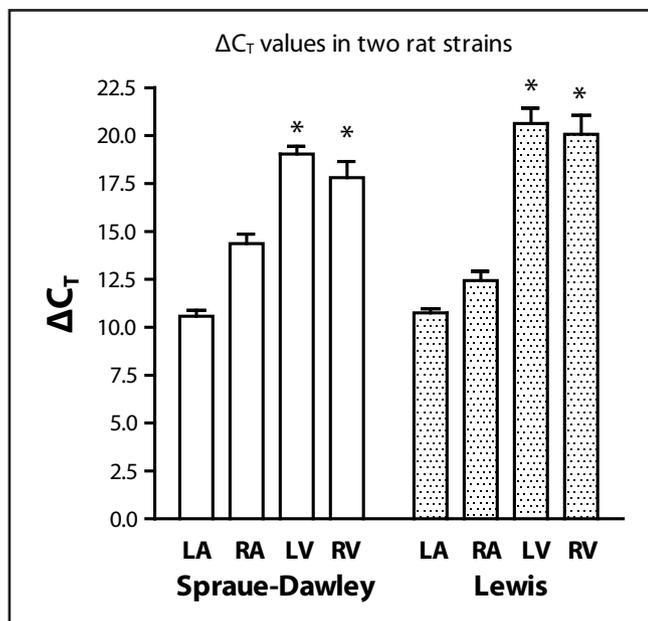


Fig. 1. Comparison of delta C_T values of OTR mRNA in Sprague-Dawley and Lewis rats. Given mean values and SEM. Two way ANOVA values are presented in text. *Significant against both atria for both rat strains.

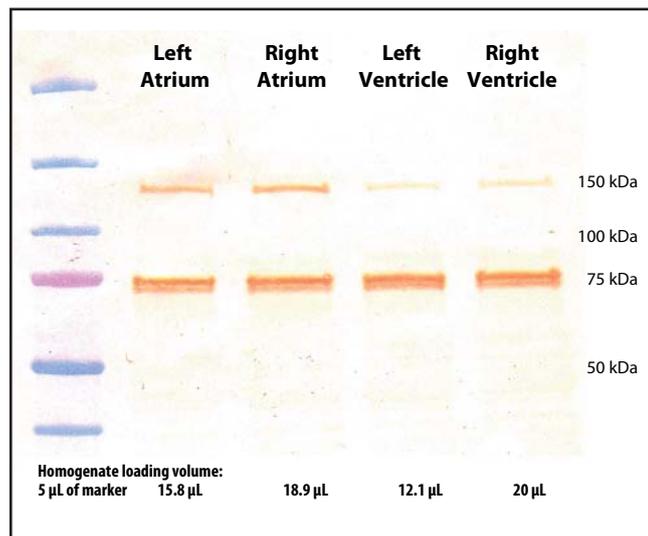


Fig. 2. Western Blot of oxytocin receptors in homogenates of left and right atria and left and right ventricles. See details in Methods.

DISCUSSION

The regulatory role of oxytocin (OT) in CNS was discovered later than its role in the reproductive system. It deals with the role of OT in stress, anxiety, learning and memory, social behavior, long-lasting effects in pair bonding, influence on motherhood etc. Then, the description of other peripheral effects of OT followed. Already in 1998, the group of Jankowski and Gutkowska (Jankowski *et al.* 1998) described that OT is produced in rat heart and acts on cardiac OTRs. These authors described that the highest concentration of OT was found in the right atrium, which was about the same range as found in the hypothalamus (Gutkowska *et al.* 2000). In spite of all this information there are still some controversies concerning production of OT in the heart and the role of OT in cardiovascular function. It is not clear yet, what the role of HPA axis activity is in the expression of OT receptors, whether stress affects expression of OT receptors, and where the expressed receptors are located. Thus, the aim of this study was to investigate the expression of mRNA of OTRs in rat hearts by real-time qPCR. The study was performed in SD and LE rat strains, the latter having lower activity of HPA axis.

For the expression of OTR mRNA, we used the real-time qPCR procedure, which is one of the most sensible, accurate and reliable methods to quantify gene expressed in controls as well as under pathological con-

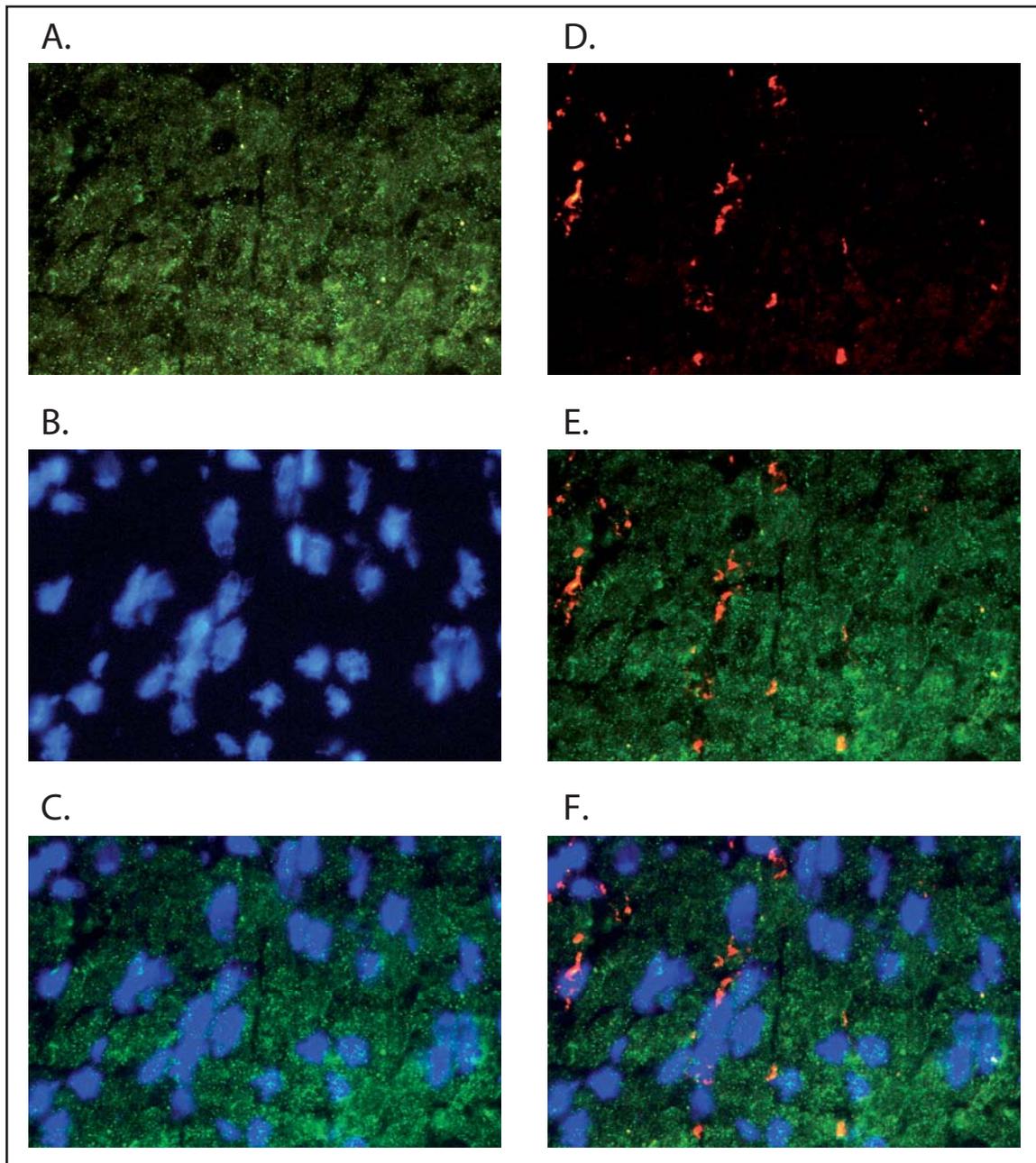


Fig. 3. Immunohistochemical detection of OTR in rat left atria. Specific labeling of OTR; B) nuclei; C) Merged image of OTR and nuclei; D) neuronal marker; E) merged OTR and neuronal marker; F) Merged OTR, nuclei and neuronal marker. Magnification 40x.

ditions, including after stress. The calculation was performed as ΔC_T values of reference and target genes, and in the following step, we calculated relative expression; as calibrator, we used data from left atria, which in our experiments produced the highest OTR mRNA from all heart compartments. It is evident that the expression of OTR mRNA is significantly much higher in atria than in ventricles. Statistical analysis by two-way ANOVA revealed that there are no statistical differences between SD and LE rat strains. Since in behavioral studies the effect of changed activity of HPA axis manifested itself under stress conditions (Klenerova *et al.* 2007), similar

approach should be used in studies testing the expression of OTR mRNA in the heart. Only this approach can provide a more reliable information about the role of HPA activity in OTR regulation in the heart.

In the parallel study, we investigated the expression of mRNA of muscarinic M2 receptors and cholinacetyltransferase. Obtained results also did not show different expression of their mRNA in the tested SD and LE rats. In progress there are experiments studying the effects of various immobilization stresses on mRNA expression of OTR, M2 receptors and atrial natriuretic peptide in rat heart compartments. Preliminary

experiments showed that the effects of immobilization stresses differed according to the heart compartment investigated. There are also interesting differences in the effects of two types of stressors having the prevalence of psychogenic or physical components of their actions; these stresses are known to produce different behavioral effects (Klenerova *et al.* 2007).

If we accept the idea that OT functions as a cardiovascular hormone (Gutkowska *et al.* 2000; Gutkowska & Jankowski 2008), further studies should investigate, how much of this effect is done by OT system in the heart and by OT released into the circulation, and what are the indirect effects with the participation of the sympatho-adrenal axis (Wsol *et al.* 2009).

Immunohistochemical data determine the expression of the oxytocin receptor protein while detected OTR mRNAs are carriers of the message for the synthesis of protein. There does not need to be direct relationship between these two parameters. It is of interest that bands in Western blot do not show such high differences between atria and ventricles as is shown in results calculated as RE.

Our immunohistochemical studies with specific OTR antibodies are also of great interest. In the study with left atria, we demonstrated the prevalent localization of OTRs on cell membranes of cardiomyocytes and not their connection with neuronal tissue studied with the neuronal marker. Under physiological conditions the expression of OTRs in left atria is not related to cell nuclei. Further studies are needed to show what are the effects of stress on OTR localization and how it is changed due to receptor translocation due to their internalization under various regulatory conditions.

In conclusion: the finding of expression of OTR mRNA by real-time qPCR and proof of OTR staining by immunohistochemistry in all heart compartments indicates that OT and its receptors may have function as a cardiovascular hormone. The differences in the HPA axis activity, as is exemplified in Sprague-Dawley and Lewis rat strains, do not project in the expression of OTR mRNA under basal condition. The effect of activity of HPA on OTR expression should be studied under stimulated conditions as it was performed in the behavioral studies.

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