IMMUNOHISTOCHEMICAL DETECTION AND GENE EXPRESSION OF TYROSINE HYDROXYLASE AND VESICULAR MONOAMINE TRANSPORTER TYPE 2 IN INTRINSIC CARDIAC GANGLIA OF SOCIALLY ISOLATED RATS

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Abstract - Social isolation induced a significant increase in resting heart rate and reduction in heart rate variability. Dysfunction of the intrinsic cardiac nervous system is implicated in the genesis of cardiovascular diseases. Previous evidence suggests that cardiac ganglia contain noradrenergic neurons. Thus, immunohistochemical expression of catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) and vesicular monoamine transporter 2 (VMAT2) were analyzed, as well as the effects of social isolation stress on mRNA and protein levels of this enzyme and transporter in the intrinsic cardiac nervous system of adult rats. Our results indicate that cardiac ganglion neurons express TH and VMAT2 immunoreactivity. Chronic isolated stress of rats caused a decrease in TH mRNA and VMAT2 mRNA in the neurons of intrinsic cardiac ganglia. No significant alterations in the protein levels of TH and VMAT2 were observed in these neurons. These data indicate that the neurons of intrinsic cardiac ganglia express TH as well as VMAT2 but that social isolation stress does not change their protein levels.

Key words: Social isolation; rats; intrinsic cardiac ganglia; tyrosine hydroxylase; vesicular monoamine transporter 2

INTRODUCTION

Social isolation and lack of social support have deleterious effects on health, and are regarded as important causes of human disease (House, 2001). Grippo et al. (2007) found that social isolation induced a significant increase in resting heart rate and reduction in heart rate variability. These changes in response to social isolation showed predictable interrelations and were mediated by a disruption of autonomic balance. Autonomic fluctuations play an integral role in the modulation of cardiac electrophysiology and could alter heart rate and blood pressure (Kapa et al., 2010). Autonomic regulation of the heart is accomplished through the noradrenergic sympathetic nerves and cholinergic parasympathetic nerves that innervate myocardium (Ardell, 2004). Evidence that augmentation of cardiac function can be evoked by focal activation of the somata of some intrinsic cardiac neurons has led to the suggestion that the somata of adrenergic neurons may be present in the heart (Armour et al., 1993; Huang et al., 1993). The intrinsic cardiac nervous system influences cardiac

rate, atrial ventricular refractoriness, coronary blood flow and appears to be involved in the development of heart failure (Beaulieu and Lambert, 1998; Stevens et al., 1998; Armour, 1999). It has been reported in anatomical studies of the mammalian heart that cardiac ganglia containing the somata of intrinsic cardiac neurons are associated with an intracardiac nerve plexus extending throughout both atria and into the ventricles (Janes et al., 1986; Pardini et al., 1987; Yuan et al., 1994). Several lines of evidence suggest that cardiac ganglion neurons are heterogeneous, including both cholinergic and intrinsic adrenergic neurons (Singh et al., 1999; Slavikova et al., 2003; Weihe et al., 2005). Tyrosine hydroxylase (TH) has been identified within the intrinsic cardiac neurons of adult rats, guinea pigs and humans (Forsgren et al., 1990; Steele et al., 1994; Moravec et al., 1990; Horackova et al., 2000). In addition, there is evidence that many human intrinsic cardiac neurons express all of the enzymes required for the synthesis of catecholamines (Singh et al., 1999; Weihe et al., 2005). Vesicular monoamine transporter type 2 (VMAT2) was also present in a sizable subpopulation of intrinsic cardiac ganglia in humans and rats (Hasan and Smith, 2009). Functional noradrenergic transmission consists of a balance between noradrenaline synthesis, secretion and reuptake. Recently, we demonstrated that chronic isolation stress produced a significant increase in TH mRNA and DBH mRNA levels in stellate ganglia (Gavrilovic et al., 2009). However, there is no literature data with regard to the gene expression of the rate-limiting enzyme in catecholamine synthesis and transport in intrinsic cardiac ganglia during altered homeostasis in stress situations. The catecholamine-synthesizing enzyme TH and VMAT2 immunoreactivity and the effects of social isolation stress on mRNA and protein levels of this enzyme and transporter were investigated in the intrinsic cardiac nervous system of adult rats.

MATERIALS AND METHODS

Animals

Male Wistar 11-week-old rats were acclimated to 22±1°C, synchronized to a 12 h light/dark regime and kept in groups of four per cage. The animals had free access to commercial rat food and tap water. Care was taken to minimize the pain and discomfort of the animals according to the recommendations of the Ethical Committee for the use of laboratory animals of the "Vinča" Institute based on Directive 2010/63EU, and all procedures with animals were approved by the Ethical Committee.

In the experiment, we used 18 animals that were randomly divided into two groups. One group was subjected to social isolation with a single animal per cage, while the second group consisted of naive, group-housed controls. After 12 weeks, the rats were decapitated and the heart was removed and dissected on ice. The base of the heart including atria, which includes the cardiac ganglia, was removed, frozen and stored at -70°C. Three hearts from each group were fixated in 10% buffered formalin, and processed for paraffin sections.

Histology and immunohistochemistry

For histological examinations, 5-μm-thick sections from the atria level were stained with Masson's trichrome technique.

For immunohistochemistry, 5-μm-thick sections were placed on positively charged slides, deparaffinized and rehydrated. After microwave treatment in citrate buffer (pH 6.0), sections were rinsed in 0.1 M PBS and endogenous peroxidase activity was blocked with 3% H_2O_2. Slices were then washed, treated with protein block reagens to reduce non-specific binding of primary and secondary antibodies and incubated in primary antibodies: polyclonal anti-VMAT 2, rabbit (1:1500; Abcam, Cambridge, UK) and monoclonal anti-TH, mouse (1:1000; DiaSorin, Stillwater, USA) for 24 h at room temperature. Labeling was performed using biotin-conjugated secondary antibody, followed by streptavidin-HRP; for visualization, 3,3'-diaminobenzidine (DAB) served as a chromogen (Peroxidase Detection System RE 7120-K, Novocastra, UK) was used. Mayer's hematoxylin was used for counterstaining. Sections were dehydrated and coverslipped with DPX Mountant (Fluka, Swit-

zerland). Photomicrographs were taken using an Olympus BX41 microscope with an Olympus C5060-ADU wide zoom digital camera.

RNA isolation and real time polymerase chain reaction (qPCR)

Total RNAs were isolated using TRIZOL reagent (Invitrogen, CA, U.S.A.). Reverse transcription was performed using Ready-To-Go You-Prime First-Strand Bead (AP, 90 Biotech) and pd (N)6 primer according to manufacturer's protocol. The qPCR assay was performed exactly as previously described (Gavrilovic et al., 2013). Reactions were performed in the ABI Prism 7000 Sequence Detection Systemat. TaqMan PCR reactions were carried out using Assay-on-Demand Gene Expression Products (Applied Biosystems, United States) for TH (ID:Rn00562500_m1), and for VMAT2 (ID:Rn00565488_m1). A reference, endogenous control, was included in each analysis to correct the differences in the inter-assay amplification efficiency and all transcripts were normalized to cyclophylineA (ID:Rn 00690933) expression.

Protein isolation and Western blotting

Samples were homogenized in 0.05 M sodium phosphate buffer pH 6.65 and the homogenates were clarified by centrifugation (20 min, 4°C). The protein content in the supernatant was determined by the method of Lowry et al. (1951). Thirty µg of heart tissue protein extract separated by 12% SDS-polyacrylamide gel electrophoresis were transferred to a supported PVDF membrane (Hybond™ P, Amersham Bioscience, GE Healthcare, Buckinghamshire, UK). The membrane was blocked in 5% non-fat dry milk in Tris-buffered saline-Tween (TBST). All subsequent washes and antibody incubations were also performed in TBST at ambient temperature on a shaker. For measuring the TH and NET protein levels, a polyclonal anti-TH antibody (rabbit, ab51191, dilution 1:1000, Abcam, Cambridge, UK), and a polyclonal anti-VMAT2 primary antibody (rabbit, ab81855, dilution 1:5000, Abcam, Cambridge, UK), respectively, were used. The washed membrane was further incubated with the horseradish peroxidase

conjugated secondary anti-rabbit antibody for luminol based detection (ab6721, dilution 1:5000, Abcam, Cambridge, UK). The secondary antibody was then visualized by Immobilion Western Chemiluminescent HPR Substrate (Millipore Corporation, Billerica, USA). Western blot analysis was performed as previously described (Gavrilovic et al., 2013).

Statistical analysis

The results are reported as means±S.E.M. The significance of the differences in mRNA and protein levels of TH and VMAT2 in the heart tissue of rats subjected to social isolation was estimated by one-way ANOVA test. The Tukey post-hoc test was used to evaluate the differences between the groups. Statistical significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

In both experimental groups (housed control and socially isolated animals), the presence of cardiac neurons between cardiomyocytes was evident in slices from the atria, stained with Masson's trichrome technique. These cells differ from cardiomyocytes; they do not contain contractile elements, they are oval in shape and contain one mainly centrally located nucleus. Immunohistochemical analysis of serial sections shows that these cells express TH and VMAT2 immunoreactivity (Fig.1).

Post-hoc analysis of obtained results showed that the level of TH mRNA significantly decreased in the socially isolated rats (by 65%, $p < 0.05$) compared with the group-housed control. There was no change in the TH protein level between these two groups (Fig.2). The level of VMAT2 mRNA was also significantly decreased in the socially isolated rats (38%, $p < 0.05$) but the VMAT2 protein level did not significantly differ between the socially isolated rats and group-housed control (Fig.3).
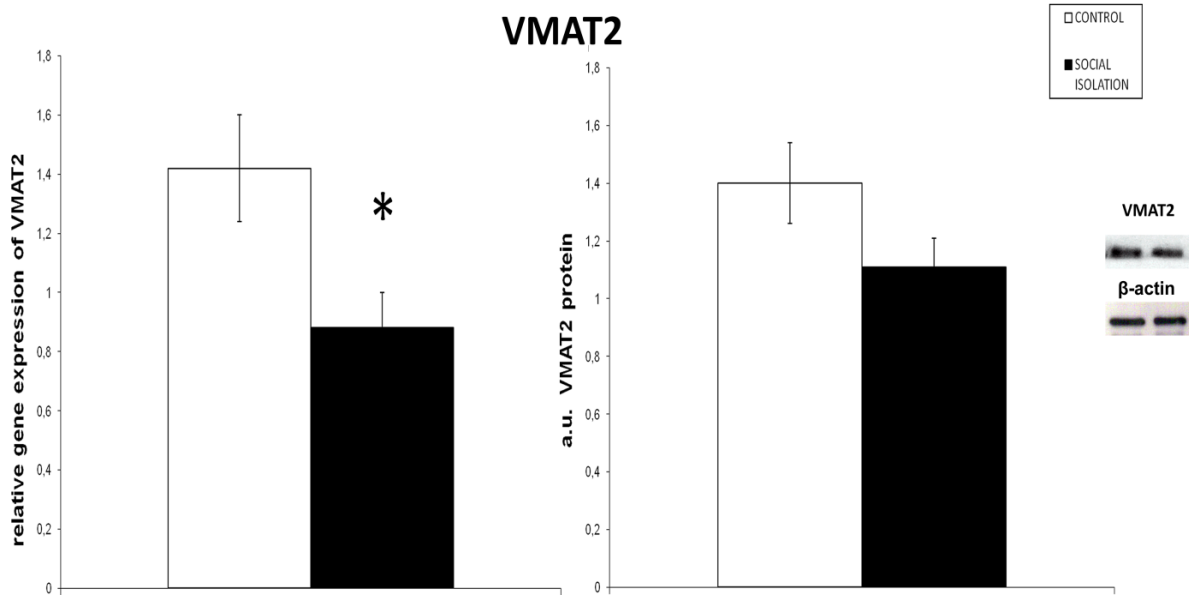
Neural control of heart rate, particularly its sympathetic component, is generally thought to reside primarily in the central nervous system, though accumulating evidence suggests that intrathoracic
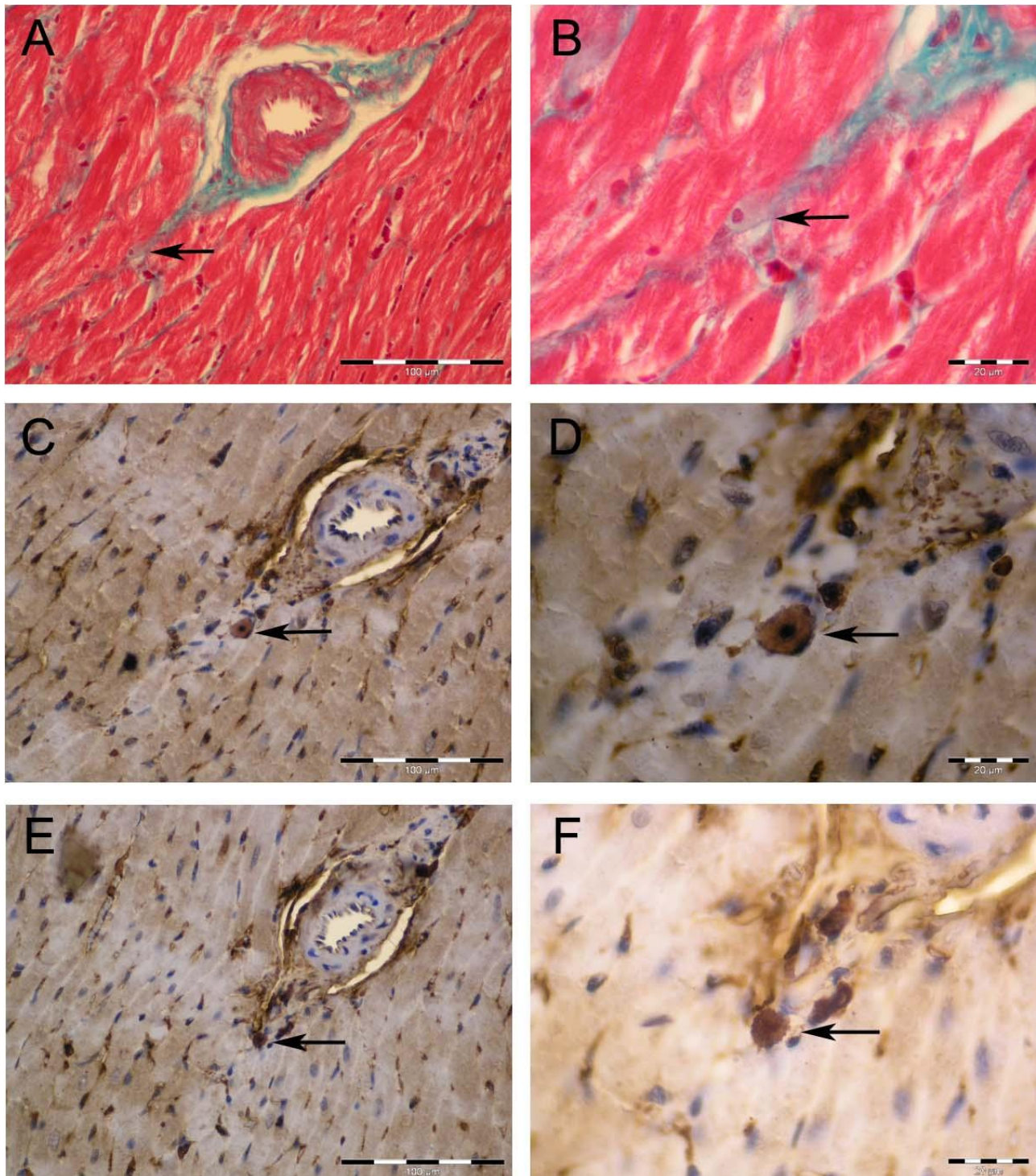
Fig. 1. Digital photomicrographs of cardiac ganglion neuron on serial cross sections through the interatrial septum stained with Masson's trichrome technique (A, B), immunolabeled with tyrosine hydroxylase (TH) (C, D) and vesicular monoamine transporter type 2 (VMAT2) (E, F). Arrows indicate position of the cardiac ganglion neuron.
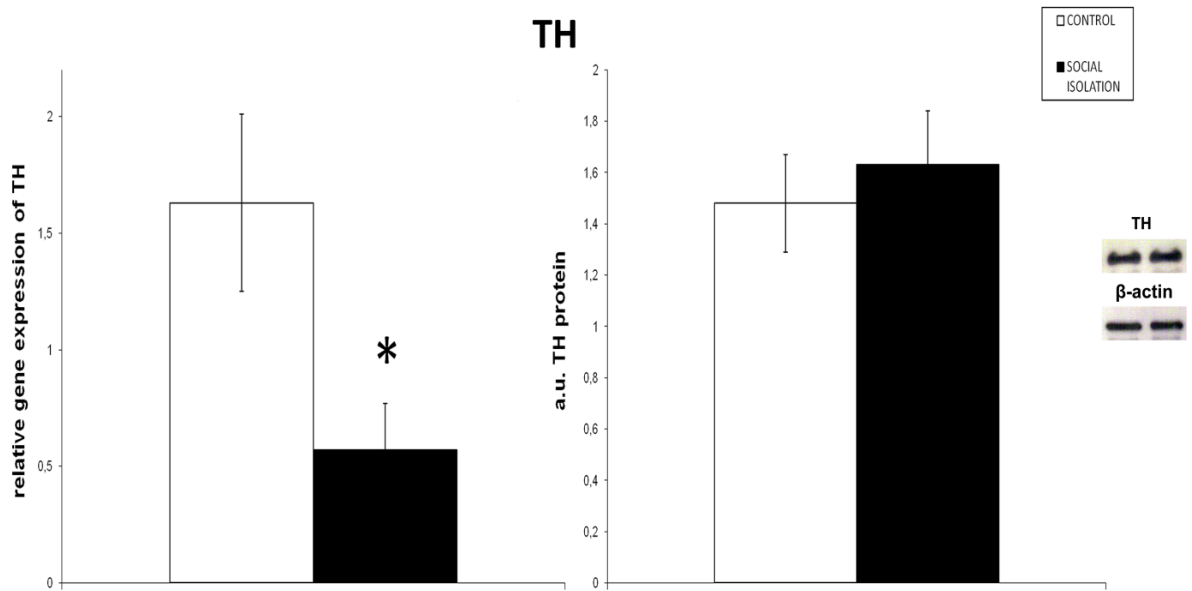
Fig. 2. Social isolation stress-related changes in tyrosine hydroxylase (TH) mRNA and protein level in intrinsic cardiac ganglia of adult male rats. The values are means ± S.E.M. of 7 rats. Statistical significance: *p <0.05 group housed control vs. socially isolated rats (Tukey test). The results are expressed as fold change to the calibrator and normalized to cyclophyline A. Protein levels are express d in arbitrary units normalized in relation to β-actin.

Fig. 3. Social isolation stress-related changes in vesicular monoamine transporter 2 (VMAT2) mRNA and protein level in intrinsic cardiac ganglia of adult male rats. The values are means ± S.E.M. of 7 rats. Statistical significance: *p <0.05 group housed control vs. socially isolated rats (Tukey test). The results are expressed as fold change to the calibrator and normalized to cyclophyline A. Protein levels are expressed in arbitrary units normalized in relation to β-actin.

extracardiac and intrinsic cardiac ganglia are also involved (Kember et al., 2011). Our study demonstrates that the neurons of intrinsic cardiac ganglia express the catecholaminergic synthetic enzyme TH as well as a VMAT2 transporter. Based on the present observations, it is evident that TH and VMAT2 are localized in neuronal soma. The presence of fibers within the cardiac ganglia suggests that the release of biogenic amines may have powerful modulating effects on ganglionic neurotransmission. Noradrenergic regulation of the heart rate and force of contraction might similarly be under the control of the intrinsic innervation of the heart, in concert with the heart's noradrenergic extrinsic sympathetic innervation. Therefore, we examined the influence of socially isolated stress on the gene expression of TH and VMAT2 in rat intrinsic cardiac ganglia. TH catalyzes the initial rate-limiting step in the synthesis of catecholamines whereas VMAT2 facilitates the transport of catecholamines into storage vesicles. As we have already mentioned, chronic isolation stress produced a significant increase in TH mRNA (Gavrilovic et al., 2009) and did not change VMAT2 protein levels (Jovanovic et al., 2014) in stellate ganglia, which represents an extrinsic noradrenergic innervation of heart. Our results show that chronic isolated stress of rats caused a decrease in TH mRNA and VMAT2 mRNA in the intrinsic cardiac ganglia. On the other hand, no significant alterations in the protein levels of TH and VMAT2 were observed in these neurons. The present results show that mRNA reduction did not parallel the protein expression level. One explanation for this finding could be attributed to the decreased stability and half-life of mRNA. The lower levels of TH mRNA and VMAT2 mRNA and unchanged protein levels in the observed intrinsic cardiac ganglia after exposure to chronic isolation stress suggest that this stress did not lead to the activation of these neurons. Dysfunction of the intrinsic cardiac nervous system is implicated in the genesis of atrial and ventricular arrhythmias (Hoover et al., 2009; Gibbons et al., 2012). Saygili et al. (2011) reported that intrinsic cardiac adrenergic cells respond to irregular electrical activation with an increase in catecholamine synthesizing enzymes. Based on these results, it could be hypothesized that excessive activation of intrinsic

cardiac ganglion neurons occurs in cardiovascular disease, while under mild stress, such as social isolation, these neurons are not activated.

The data presented here suggest that while both extrinsic and intrinsic catecholaminergic systems contribute to cardiac properties, extrinsic innervation appears to predominantly influence stress caused by social isolation.

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