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The influence of exercise on morphological and neurochemical properties of neurons in rat nodose ganglia

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ABSTRACT

Physical exercise can induce immunohistochemical changes and cell proliferation in the hippocampus. One of the main effects of prolonged exercise is resting bradycardia, most probably caused by enhanced vagal activity. To investigate whether physical exercise can cause neurochemical and morphological changes in vagal afferent neurons, we performed immunohistochemical studies of nodose neurons using isolectin B4 (IB4), 200-kDa neurofilament protein (N52) and calretinin in adult female rats. To distinguish subpopulations of neurons projecting to the left ventricle, we applied a Fast Blue patch to the epicardial surface of the left ventricle. Treadmill running for 8 weeks significantly increased the size of N52-positive cardiac projecting neurons. Furthermore, the proportion of IB4-positive neurons among all nodose ganglia neurons was significantly higher in trained animals. These data indicate that exercise leads to plastic changes in nodose ganglia neurons that may initiate changes of vagal activity caused by prolonged exercise.

The levels of expression of neuropeptides, neurotransmitters and/or related enzymes in mature nodose ganglia neurons are not static and their modifications can be related to changes in their target tissues [38]. The heart, being an important target for visceral afferents, could influence those modifications, since the largest number of primary heart-projecting somata originates from nodose ganglia [16]. It is well known that intensive exercise leads to the functional and structural changes of cardiac contractile elements [1,3,24,25,30,37]. Also, regular exercise in animals and humans results in adaptation of the autonomic nervous system, which is most commonly observed as reduced heart rate at rest [36]. The precise mechanism(s) by which exercise produces changes in autonomic control are still unknown. It is very likely that the mechanism(s) are mediated by alterations beginning in cardiac vagal afferents since the heart provides the primary source of afferent input for the control of sympathetic outflow by the vagal cardiopulmonary reflex during changes in thoracic blood volumes and pressures [22]. Also, enhanced stimulation of cardiac sensory receptors contributes importantly to the control of sympathetic drive and vascular resistance during exercise [28]. It seems that vagal afferent input from the left ventricle has the most important role in regulating sympathetic outflow mediated through cardiopulmonary reflexes [22]. Are all those inputs strong enough to provoke neuronal plasticity among nodose ganglia neurons? Several studies have shown that physical exercise may induce neuronal plasticity (detected by immunostaining) [2,6,10], and even increase neurogenesis [34], but mostly among hippocampal neurons. Based on these observations, our study was designed to determine whether physical activity is able to provoke morphological and neurochemical changes of nodose ganglia neurons with a special focus on neurons projecting to the left ventricle of the heart. In order to detect those changes we performed double-labeling of nodose ganglia neurons with isolectin B4 (IB4) that was combined with immunoreactivity either to 200-kDa neurofilament protein (N52) or to calretinin (CALR). IB4 and N52 are previously described as markers for a subset of nonmyelinated nonpeptidergic and myelinated neurons, respectively [21,35] Positivity for each of these markers has already been confirmed in cardiac nodose ganglia neurons [7,20]. CALR has been detected in a significant number of nodose ganglia neurons [17,38]. Since it has been proven that mechanical stimulation increases Ca^{2+} in a subpopulation of nodose ganglia neurons [32], it is likely that CALR is a marker of mechanosensitive neurons. For detecting neurons projecting to the left ventricle of the heart we applied a retrograde neuronal tracer – Fast Blue (FB) to the epicardial surface of the left ventricle as previously described by Guić et al. [15].

The experimental protocol was approved by the Ethics Committee of the School of Medicine in Split, and laboratory animal guidelines (European Communities Council Directive of 24 November 1986) were followed and applied in all experiments. Twelve female Sprague–Dawley rats (160–180 g) were randomly divided into 2 groups of six animals: control group and exercise group.

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The exercise protocol consisted of one week of adjustment, during which the speed and the inclination of the treadmill (Exer 3/6 Treadmill, Columbus Instruments, OH, USA), as well as, the duration of running was increased gradually in order to reach 28 m/min at a 10° inclination. Following a week of adaptation, animals were running 60 min/day 5 days/week over a period of 8 weeks. After the exercise training period ended, animals from both groups were anaesthetized with an intramuscular injection of a mixture containing ketamine (Ketaminol, Intervet, EU, 60 mg/ml) and xylazine (Xylapan, Vetoquinol, Bern, Switzerland, 50 mg/ml) and underwent surgery for application of FB, which was left in place for one week. Animals were then re-anaesthetized and perfused through the ascending aorta via the left ventricle with saline followed by 300 ml of Zamboni's fixative (4% paraformaldehyde and 15% saturated picric acid in 0.1 M phosphate buffer at pH 7.4). Nodose ganglia were bilaterally removed together with the heart, lungs and spinal cord (at levels C3–C5), and post-fixed for 2 h. Following long washes in phosphate buffered saline (PBS) tissue sections of the lungs, heart and spinal cord were examined using an Olympus BX51 microscope (Tokyo, Japan) with UV settings to evaluate the patch position and to check for leakage. Ganglia were transferred to 30% sucrose for cryoprotection, snap-frozen in liquid nitrogen and stored at −80 °C until processing. Serial horizontal cryostat sections (10 μm thick) were mounted on 2% 3-aminopropyltriethoxysilane-coated slides (Sigma, St. Louis, USA) and double labeled using standard immunofluorescence methods. To prevent multiple counting, neurons from every third section were excluded from immunohistochemical staining, and all other sections were stained interchangeably by IB4/N52 or IB4/CALR combination. All antibodies were diluted in PBS containing 0.3% Triton X-100. Sections were incubated in a species-specific combination of primary antibodies: either rabbit anti-CALR (1:400, Abcam, Cambridge, UK) or mouse anti-N52 (1:1000, Sigma, St. Louis, USA) were separately combined with fluorescein isothiocyanate-conjugated (FITC) IB4 (1:200, Sigma, St. Louis, USA) for 24 h, washed in PBS (3× 10 min) and then incubated in the appropriate combination of secondary antibodies: Rabbit IgG-Rhodamine (R), 1:200 Santa Cruz Biotechnology or Mouse IgG (H + L), Texas Red (TR), 1:200 (Molecular Probes, Eugene, OR, USA) for 2 h in a humid box at room temperature before being washed in PBS (3× 10 min). Sections were mounted in Immu-mount medium (Thermo scientific, Pittsburgh, USA) and cover slipped. Immunolabeled neurons were viewed and photographed using an Olympus BX51 microscope equipped with an Olympus DP71 camera and processed with Cell A Imaging Software for Life Sciences Microscopy (Olympus). Separate images were acquired for all fields using filters that allow visualization of FB, FITC, R and TR. Neurochemical properties for three different markers were firstly determined in FB positive neurons containing a nucleus and then in all neurons within the visual field (15 visual fields randomly selected for both neurochemical combinations from each group). Three sections (with at least 80% of the surface occupied by neurons) from each animal were randomly chosen for each combination of labeling (IB4/CALR and IB4/N52). Only neurons with clearly visible nuclei and those underlying the right and upper borders of the grid in the visual field were counted. Controls for specificity included tissue stained in the same manner as described above, but the primary antibodies were omitted. Soma diameters were calculated by summing the length and width of neurons with a visible nucleus and dividing by two (the length was determined as the longest axis through the neuronal nucleus and the width as the axis perpendicular to the length). Cell diameters are reported as means ± S.D. The incidence of immunopositivity is presented as a percentage for pooled data from five animals in each group followed by actual cell counts in parentheses. In two animals (one from the control group and one from the trained group), the FB patch was not placed precisely on the left ventricle, so these animals were omitted from immunohistochemical analysis. In animals included in the study, the tracer was detected neither in the lungs nor in the spinal cord at segmental levels C3–C5 (indicating that the tracer had no access to the diaphragm via the phrenic nerve). Statistical methods included Kolmogorov–Smirnov test, Student's *t*-test (for data with normal distribution) and Chi-square test (GraphPad Software). Throughout, $p < 0.05$ was set as level for significance.

Table 1
The influence of exercise on rat heart and body weights.

	Control animals	Trained animals
Body weight, g	228 ± 17.5	256 ± 21.3
Heart weight, g	0.956 ± 0.06	1.093 ± 0.16
Heart weight/body weight, ×1000	4.20 ± 0.38	4.25 ± 0.34

Values are presented as means ± S.D.

Our training protocol did not cause any significant changes in body or heart weights when animals in the trained and control group were compared (Table 1). The heart-to-body weight ratio was slightly higher in trained animals but did not reach statistical significance.

Neurochemical properties of nodose ganglia neurons projecting to the left ventricle of the rat heart were quite similar in both groups (trained and control) (Table 2), but the size of N52+ neurons was significantly higher in trained animals.

The labeling for IB4, N52 and CALR was determined in 414 FB-labeled neurons found throughout the serial sections of nodose ganglia (Table 2). All FB-labeled neurons were characterized by blue fluorescence of the whole somata which were usually ovoid in shape (Fig. 1A and D). Around 30% of FB labeled neurons in both groups were IB4+ and all showed a similar pattern of granular cytoplasmatic perinuclear staining (Fig. 1B). The mean diameter of IB4+ neurons was 22.6 ± 3.6 μm ($N = 30$) in the control group and 21.4 ± 4.2 μm ($N = 30$) in the trained group, with no significant difference among sizes between the control and trained group. CALR immunoreactivity, that was present in around 15% of FB labeled neurons, was represented by intense, evenly distributed staining of the whole neuronal soma (Fig. 1C). The mean diameter of CALR+ neurons was 21 μm (21.6 ± 3.2, $N = 30$ in the control group and 21.2 ± 3.2, $N = 18$ in the trained group), again with no significant difference between groups. The proportion of N52+ neurons was higher among FB-labeled neurons in trained animals (71% vs. 62%) but the difference did not reach statistical significance. Neurons that were considered N52+ displayed bright neurofilaments coursing through the soma (Fig. 1E). The size of N52+ neurons in trained animals was significantly higher than the size of N52+ neurons in the control group (24.5 ± 4.5 vs. 21.8 ± 3.6, $p = 0.016$, unpaired *t*-test, $N = 30$ for both groups). No regional differences were noticed within the ganglia.

Only a small number of neurons projecting to the left ventricle showed double labeling. Colocalization of IB4 and N52 positivity was present in only 4% (5/118) and 10% (8/77) of FB+ neurons in control and in trained group, respectively; with no significant difference among these two groups. Also, there was no significant difference in the proportion of double labeling of IB4 and CALR between the control and trained animals. IB4+/CALR+ double label-

Table 2
Neurochemical profile of nodose ganglia neurons projecting to the left ventricle in trained and control animals.

	FB+ neurons of control animals	FB+ neurons of trained animals
IB4+	83/238 (34)	57/176 (32)
N52+	73/118 (62)	55/77 (71)
CALR+	18/120 (15)	18/99 (18)

All data are presented as proportions with percentages in parenthesis. Data are pooled from both left and right nodose ganglia of five animals in each group.