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Characterization of spinal afferent neurons projecting to different chambers of the rat heart

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ABSTRACT

The pattern of distribution of spinal afferent neurons (among dorsal root ganglia-DRGs) that project to anatomically and functionally different chambers of the rat heart, as well as their morphological and neurochemical characteristics were investigated. Retrograde tracing using a patch loaded with Fast blue (FB) was applied to all four chambers of the rat heart and labeled cardiac spinal afferents were characterized by using three neurochemical markers. The majority of cardiac projecting neurons were found from T1 to T4 DRGs, whereas the peak was at T2 DRG. There was no difference in the total number of FB-labeled neurons located in ipsilateral and contralateral DRGs regardless of the chambers marked with the patch. However, significantly more FB-labeled neurons projected to the ventricles compared to the atria (859 vs. 715). The proportion of isolectin B_4 binding in FB-labeled neurons was equal among all neurons projecting to different heart chambers (2.4%). Neurofilament 200 positivity was found in greater proportions in DRG neurons projecting to the left side of the heart, whereas calretinin-immunoreactivity was mostly represented in neurons projecting to the left atrium. Spinal afferent neurons projecting to different chambers of the rat heart exhibit a variety of neurochemical phenotypes depending on binding capacity for isolectin B_4 and immunoreactivity for neurofilament 200 and calretinin, and thus represent important baseline data for future studies.

Regulation of cardiac activity is mediated through a large network of neurons belonging to both central and peripheral nervous systems. A complex, hierarchically organized arrangement of interconnected afferent and efferent neurons makes up controlling neural reflex circuits. Anatomically, components of these circuits can be located within the heart (in the intrinsic cardiac ganglia), within the thoracic cavity (in the intrathoracic extracardiac ganglia) or projecting to and from the central nervous system (spinal and vagal efferent and afferent projections) [2]. Functionally, intrinsic and intrathoracic segments are related to short-term, beat-to-beat cardiac regulation [2–4]. Conversely, neural projections to the central nervous system mediate so called 'long-term cardiac regulation' [3,4]. Sensation from the heart is conveyed to the CNS via spinal afferent neurons that are either fast-conducting, myelinated Aδ-fibres or unmyelinated, slow-conducting C-fibres. In cat, about 44% of afferent projections from the heart are spontaneously active myelinated Aδ-fibres. The remaining population of the afferent fibers are so-called 'silent' C-fibres [17].

Spinal afferents projecting to the heart can be associated with inferior cervical and superior thoracic dorsal root ganglia (DRGs) bilaterally. The precise distribution of spinal afferent neurons has been identified in a variety of species. In the dog, there is bilateral distribution throughout C6 to T6 DRGs [11] and in C8 to T9 in the cat [16].

Injections of neuronal tracers into cardiopulmonary nerves and various sites of the heart result in different patterns of labeling in the dorsal root ganglia [11]. However, no topographical arrangement could be identified with respect to the distribution of cardiac afferent neurons. Afferent axons projecting from specific cardiac regions or cardiopulmonary nerves do not appear to be confined to discrete dorsal root ganglia or to regions within individual ganglia, rather, a range of labeled DRG neurons is observed [11]. Taking into account that heart chambers have different structural [8] and functional properties [14], it is interesting that there are no studies examining precise distribution and neurochemical characteristics of cardiac spinal afferents projecting to different chambers of the heart. To the best of our knowledge, only one study made an attempt to establish neurochemical profiles of cardiac (ventricular) afferent neurons in canine dorsal root ganglion (DRG) [10] using calcitonin gene-related peptide (CGRP), substance P (SP) and nitric oxide synthase (nNOS).

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Hence, we decided to analyze spinal afferents projecting to anatomically and functionally different chambers of the heart both quantitatively (looking at the total number and distribution of neurons) and qualitatively (establishing their morphological and neurochemical properties).

In order to determine different subpopulations of retrogradely labeled cardiac spinal afferent neurons we performed double-labelling with isolectin B_4 (IB_4) that was combined with either immonoreactivity to neurofilament 200 (N52) or immonoreactivity to calretinin. N52 and IB_4 were previously used for distinguishing populations of DRG neurons and generally described as markers for myelinated [7] and unmyelinated [22,23] neurons, respectively. The appearance of calretinin in the nervous system cannot be confined to a functionally homogenous populations of neurons (it was described in nociceptive, proprioceptive [13], as well as, mechanoceptive [15] neurons).

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. Female Sprague–Dawley rats (160–180 g, $n = 12$) were equally divided into 4 groups based on the location of patch placement on the four heart chambers. Rats were anaesthetized with an intramuscular injection of a mixture containing ketamine (Ketaminol, 125 mg/kg) and xylazine (Xylapan, 0.3 mg/kg). After intubation (with an 18-gauge arterial catheter), an abdominal approach to the rat heart was performed as previously described by Huikeshoven et al. [12]. Only slight modification was made during incision of the diaphragm. It was cut with scissors along the midline (1/3 of the incision included the central tendinous part and 2/3 involved the muscular part) and the animals were connected to a positive pressure respirator (SAR 830, CWE Inc., USA). The thin pericardium was torn apart so that the surface of the heart could be clearly exposed.

A patch loaded with Fast Blue (FB) powder (EMS-Chemie, Gross-Umstadt, Germany-gift of Dr. C. Anderson, University of Melbourne, Australia) was applied to only one chamber of the heart (left atrium or ventricle, right atrium or ventricle) in all animals belonging to a specific group. The patch was made of Micropore™ tape impregnated with silicone spray on its outer side (in order to prevent tracer leakage) and had one microgram of Fast Blue powder on its 'sticky' surface. The size of the patch was 4 mm^2 and the FB powder only covered its middle section. Following its application to the surface of the heart, the patch was fixed in place by applying 1 μl of instant tissue glue over the edge of the patch with the aid of a micropipette. The diaphragm was then sutured, starting with the muscle part, working towards the central tendon of the diaphragm. Before the final suture was tightly closed, air was expelled from the thoracic cavity. The animals were removed from the respirator and extubated following the recovery of spontaneous breathing. Muscles and skin of the abdominal wall were sutured, and the animals were closely monitored in a warm environment during the recovery period that lasted between 3 and 5 h.

One week after the application of tracer, the animals were 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). Dorsal root ganglia (C6-T8) were removed bilaterally together with the heart, lungs and spinal cord (at levels C3–C5), and post-fixed for 2 h. Afterwards, tissues underwent long washes in phosphate buffered saline (PBS). Sections of lungs, heart and spinal cord were examined under the Olympus BX51 microscope (Tokyo, Japan) using UV settings to evaluate the patch position and to check for leakage.

To confirm labeling of the heart projecting neurons, ganglia were also examined using the same filter settings. After initial inspection, the ganglia were transferred to 30% sucrose over night and snap-frozen the following day.

Serial sections (10 μm thick) were mounted on 2% 3-aminopropyl-triethoxysilane-coated slides (Sigma, St. Louis, USA) and double labeled using standard immunofluorescence methods. All antibodies were diluted in PBS containing 0.3% Triton X-100. Sections were incubated in species-specific combination of primary antibodies: either rabbit anti-calretinin (1:400, Abcam, Cambridge, UK) or mouse anti-N52 (1:1000, Sigma) were separately combined with fluorescein isothiocyanate-conjugated IB_4 (1:200, Sigma) for 24 h and, washed in PBS (3×10 min) and then incubated in the appropriate combination of secondary antibodies (Rabbit IgG-R, 1:200 Santa Cruz Biotechnology or Mouse IgG (H + L), 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 Olympus BX51 (Tokyo, Japan) microscope equipped with Olympus DP71 camera and processed with Cell A Imaging Software for Life Sciences Microscopy (Olympus Tokyo, Japan). Separate images were acquired for all fields using filters that allow visualization of Fast Blue (FB), Fluorescein isothiocyanate (FITC), Rhodamine (R) and Texas Red (TR). The total number of FB-labeled neurons was counted in whole mount preparation of all dorsal root ganglia from C6 to T8. Following whole mount counting all DRGs were cut, 10 slices per ganglion per animal were used for each combination of markers in consecutive order and immunofluorescence was determined only for FB-positive neurons. Controls for specificity included tissue labeled in the same manner as described above with omission of primary antibodies. The diameters of DRG neurons were calculated by summing the long and short axis of those neurons with a visible nucleus and dividing by two. Cell counts and diameters are reported as means ± S.D. Statistical methods included Chi-square and Mann–Whitney test (GraphPad Software, La Jolla, CA, USA). Throughout, $p < 0.05$ was set as level for significance.

A total number of 1574 FB-labeled neurons projecting to different parts of rat heart (both atria and ventricles) were identified in whole mount preparations of the right and left C6 to T8 dorsal root ganglia collected from twelve animals. All labeled neurons were characterized by the blue fluorescence of whole somata that were usually ovoid (Fig. 1A and D).

Following the patch placement, most of FB-labeled neurons were located from T1 to T4 DRG, with a peak at T2 (median 25, range 12–57; Fig. 2), whereas only 12 neurons were found in C6 to C8 DRGs (C6-11, C7-0, C8-1). Tracer was detected neither in lungs nor in the spinal cord at segmental levels C3–C5 (indicating that tracer had no access to the diaphragm via the phrenic nerve). Regardless of which chamber was marked with the patch, no difference was found in the total number of FB-labeled neurons located in ipsilateral and contralateral DRG-s. However, a statistically significant difference was found when the total number of FB-labeled neurons projecting to ventricles and atria was compared (ventricles vs. atria, 859 vs. 715; Mann–Whitney test, $p = 0.02$).

Out of all FB-labeled neurons, 579 were included in immunohistochemical protocol and their reactivity for three markers was determined (Table 1). All IB_4+ neurons showed the same pattern of labeling as punctuate cytoplasmatic staining and the nuclei were never labeled (Fig. 1B and E). The mean diameter of these neurons was 26.2 ± 3.1 μm. No difference in the proportion of IB_4+ neurons was found among neurons projecting to the different chambers of the rat heart.

Neurons that were considered N52+ displayed bright neurofilaments coursing through somata and their mean diameter was 30.3 ± 4.5 μm (Fig. 1C). A significantly greater proportion of N52