Distribution of TRPVs, P2X3 and parvalbumin in the human nodose ganglion

博士論文

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(ヒト迷走神経下神経節における TRPVs,P2X3 及び Parvalbumin の分布について)

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1. Abstract

Immunohistochemistry for several neurochemical substances, the transient receptor potential cation channel subfamily V member 1 (TRPV1) and 2 (TRPV2), P2X3 receptor and parvalbumin (PV), was performed on the nodose ganglion, pharynx and epiglottis in human cadavers. The nodose ganglion was situated beneath the jugular foramen, and had a spindle shape with the long rostrocaudal axis. The pharyngeal branch (PB) issued from a rostral quarter of the nodose ganglion, whereas the superior laryngeal nerve (SLN) usually originated from a caudal half of the ganglion. In the nodose ganglion, sensory neurons were mostly immunoreactive for TRPV1 (89%) or P2X3 (93.9%). About 30% of nodose neurons contained TRPV2 (35.7%) - or PV (29.9%) -immunoreactivity (-IR). These neurons mainly had small to medium-sized cell bodies, and were distributed throughout the ganglion. Neurodegenerative profiles such as shrinkage or pyknosis could not be detected in the examined ganglion. Occasionally, TRPV2-immunoreactive (-IR) nerve fibers surrounded blood vessels in the epiglottis as well as in the nasal and oral parts of the pharynx. Isolated TRPV2-IR nerve fibers were also located beneath the epithelium. TRPV1-, or P2X3- or PV-IR nerve endings could not be detected in the pharynx or epiglottis. In the PB and SLN, however, numerous nerve fibers contained TRPV1-, TRPV2-, P2X3- and PV-IR. The present study suggests that TRPV1-, TRPV2-, P2X3- and PV-IR neurons in the human nodose ganglion innervate the pharynx and epiglottis through the PB and SLN. These neurons may respond to chemical, thermal and mechanical stimuli during respiration and swallowing.

Key words: nodose ganglion; pharynx; epiglottis; TRPV1; TRPV2; P2X3; parvalbumin; human; immunohistochemistry.

2. Introduction

Previous immunohistochemical studies have classified primary sensory neurons into several subpopulations on the basis of their neurochemical substances. The transient receptor potential cation channel subfamily V members 1 (TRPV1) and 2 (TRPV2) are temperature-sensitive nociceptive transducers. TRPV1 is activated by heat >43 ^{o}C, vanilloid compounds and protons (Caterina et al., 1997). TRPV2 reacts to heat >52 ^{o}C and mechanical stimuli (Caterina et al., 1999; O'Neil and Heller, 2005). In the rat sensory ganglia, TRPV1-containing neurons are small to medium-sized whereas TRPV2-containing neurons have medium-sized to large cell bodies (Caterina et al., 1997, 1999; Guo et al., 1999; Michael and Priestley 1999; Ichikawa and Sugimoto, 2000, 2001, 2002, 2003). P2X3 receptor is a ligand-gated cation channel that is activated by extracellular adenosine triphosphate (ATP). P2X3-containing neurons in the rodent sensory ganglia have small to medium-sized cell bodies, and are considered to have nociceptive function (Guo et al., 1999; Ichikawa et al., 2007). Parvalbumin, a member of the calcium-binding protein family, is predominantly localized to large neuronal cell bodies in the rat sensory ganglia (Celio, 1990; Ichikawa et al., 1994; Ichikawa and Helke, 1995). Such neurons innervate muscle spindles and corpuscular endings, and probably transduce proprioceptive and mechanoreceptive information (Celio, 1990; Ichikawa et al., 1996; Ichikawa and Sugimoto, 1997).

Neurons of the nodose ganglion send their sensory fibers to cervical, thoracic and abdominal viscera. Visceral sensory neurons include thermoreceptors, chemoreceptors and mechanoreceptors. They can relay information about temperatures in the upper digestive and respiratory systems, changes in blood oxygenation and passage of contents through digestive

system to the central nervous system for reflex maintenance of visceral functions. Baroreceptors in the aortic arch are also mechanoreceptors which detect the amount of stretch of blood vessel walls for regulation of blood pressure. Previous immunohistochemical studies have demonstrated the presence of TRPV1, TRPV2, P2X3 and parvalbumin in the rat nodose ganglion (Ichikawa and Helke, 1995; Ichikawa and Sugimoto, 2002, 2003; Ichikawa et al., 2007). Sensory neurons containing these substances are distributed throughout the ganglion. In addition, TRPV1 and TRPV2 are co-expressed by sensory neurons (Ichikawa and Sugimoto, 2003). However, little is known about TRPVs or other neurochemical substances in the human nodose ganglion.

The pharynx and epiglottis are parts of the digestive and respiratory systems. Both food and air pass through the pharynx. When food is swallowed from the oral cavity to the pharynx, the epiglottis closes the vestibule of the larynx to prevent aspiration. Sensory nerve fibers in the mucous membrane of the pharynx and epiglottis can respond to thermal, chemical and mechanical stimuli (Selçuk et al., 2007; Ebihara et al., 2011). Recently, we have demonstrated that TRPV1 is expressed by intra- and sub-epithelial nerve endings in the rat pharynx and larynx (Sasaki et al., 2013) These nerve endings are considered to receive thermal and chemical stimuli and regulate the swallowing reflex. In the human, visceral sensory neurons in the nodose ganglion onvey sensory information from the pharynx and epiglottis through the pharyngeal branch (PB) and superior laryngeal nerve (SLN) to the brainstem. However, little is known about occurrence of TRPVs or other neurochemical substances in the human cervical viscera, PB and SLN.

In the present study, the proportion and cell size of TRPV-, P2X3- and PV-containing neurons were examined in the human nodose ganglion. The distribution of sensory nerve fibers containing these substances was also investigated in the pharynx and epiglottis as well as in the PB and SLN.

3. Materials and methods

Present data about the nodose ganglion, nerve, pharynx and epiglottis were obtained from human adult cadavers aged 73-93 years (5 male and 4 female). All cadavers had been donated for human dissection at the School of Dentistry, Tohoku University. Within 48 hours after death, samples were injected with 10% formalin with return perfusion through the femoral artery. Written consent about use of the tissue for research was obtained from donors and their surviving relatives in accordance with Tohoku University Dental School Guidelines for Ethics in Human Tissue Experiments. None of the subjects was suffering from degenerative diseases of the central or peripheral nervous system. In addition, cadavers whose causes of death were associated with swallowing dysfunction were excluded from the subjects.

The nodose ganglion, PB and SLN as well as the pharynx and epiglottis were exposed. Before taken out, these structures were examined for their morphological and locational data. Then, they were dissected, immersed in Zamboni fixative (Stefanini et al., 1967) and a phosphate-buffered saline containing 20% sucrose, frozen-sectioned at 8 μm and thaw-mounted on gelatin-coated glass slides. The sections were incubated with rabbit antiserum against TRPV1 (1: 25,000, Neuromics, USA), TRPV2 (1: 100,000, EMD

Millipore Corporation, USA) or P2X3 (1: 20,000, Neuromics, USA), or mouse monoclonal antibody against PV (1:5,000, Sigma, USA) for 24h at room temperature. Then, these sections were incubated with biotinylated goat anti-rabbit IgG or biotinylated rabbit anti-mouse IgG (Vector Laboratories, USA), and avidin-biotin-horseradish peroxidase complex (ABC) (Vector Laboratories). Following nickel ammonium sulfate-intensified diaminobenzidine reaction, they were dehydrated in a graded series of alcohols, cleared in lemosol and cover-slipped with Softmount (Wako Pure Chemical Industries, Ltd., Japan). A subset of sections were processed with a Nissl stain to investigate neurodegeneration in the ganglion.

For morphometric analysis of TRPV-, P2X3- and PV-containing neurons, 5 sections were randomly selected in the nodose ganglion of each sample. The optic image of the ganglion was captured with a digital camera (DS-Ri1, Nikon, Japan) and stored into a computer (Z400 Workstation, Hewlett-Packard, Japan). For analysis of the cell size of immunoreactive (IR) and immunonegative neurons, the cross-sectional area of their cell bodies that contained nuclear profiles was measured. Outline of each neuronal cell body was drawn with a computer mouse (Hewlett-Packard) on the image of microscopic fields. The pixel number within outlines was converted into the area of neuronal cell bodies by Lumina Vision program software (Mitani Corporation, Japan). By this method, the maximum area of each neuronal cell body could not be measured. However, relative cell size distributions of TRPV-, P2X3 and PV-IR neurons were obtained and compared. The average proportion of IR neurons was recorded for each sample.

There was no age or sex difference about gross anatomical and immunohistochemical data in this study.

4. Result

Gross anatomy of the human nodose ganglion and nerves

The human nodose ganglion was located 13.2-36.1 mm beneath the jugular foramen (mean ± S.D. = 19.9 ± 7.1 mm, n = 9). The ganglion had a slender spindle-shape; the rostrocaudal length (length) was larger than the maximum mesiolateral length (maximum width) and maximum anteroposterior length (maximum thickness) (Fig. 1, Table 1). The PB, communicating branch to the superior cervical ganglion and SLN arose from the nodose ganglion, and their branching points arranged rostrocaudally (Fig. 1). All the PB issued from a rostral quarter of the nodose ganglion, whereas 88.9% (8/9) of the SLN originated from a caudal half of the ganglion. Distances from top of the nodose ganglion to branching points of the PB and SLN measured 3-7.4 mm (mean ± S.D. = 4.6 ± 1.4 mm, n = 9) and 11.2-23.1 mm (mean ± S.D. = 17.4 ± 3.8 mm, n = 9), respectively. The PB made pharyngeal plexus and innervated the pharynx, whereas the SLN ran toward the cricothyroid muscle and upper part of the larynx.

TRPVs, P2X3 and PV in the human nodose ganglion and nerves

Cell bodies of sensory neurons were scattered throughout the human nodose ganglion. In Nissl-stained sections, these neurons were oval or round in shape, and had Nissl bodies within the cytoplasm. All the examined ganglia were free from neurodegenerative

profiles such as shrinkage or pyknosis. TRPV1-, TRPV2-, P2X3- and PV-IR was expressed by subpopulations of sensory neurons in the ganglion (Figs. 2A, C, E, 3A, Table 2). The immunoreactions were localized in the cytoplasm and were not evident in the nucleus nor cytoplasmic membrane of these neurons. Sensory neurons mostly contained TRPV1- or P2X3-IR, whereas one third of them had TRPV2- or PV-IR (Table 2). They were distributed throughout the ganglion without rostral or caudal preference. Cell size distribution of sensory neurons which expressed these substances was similar in the human nodose ganglion. One third of TRPV-, P2X3- and PV-IR neurons were small (< 600 μm^2), whereas half of those neurons had medium-sized cell bodies (600-1200 μm^2). About 10% of them were large (>1200 μm^2) in the ganglion (Figs. 2B, D, F, 3B, Table 3). TRPV-, P2X3- and PV-IR nerve fibers were also detected in the nodose ganglion. These nerve fibers were usually located within cross-sectioned nerve bundles within the ganglion. Some isolated IR nerve fibers were also seen running among neuronal cell bodies. In addition, a few TRPV-, P2X3- and PV-IR made ramification and surrounded small blood vessels.

TRPVs, P2X3 and PV in the human PB, SLN, pharynx and epiglottis

TRPV1-, TRPV2-, P2X3- and PV-IR nerve fibers were seen in the PB and SLN. In transverse sections of the nerves, they were numerous and usually had spotted or short linear appearance (Figs. 4A-H). In the lamina propria of the pharynx and epiglottis, large nerve bundles also contained TRPV1-, TRPV2, P2X3- and PV-IR nerve fibers. TRPV2-IR varicose nerve fibers separated from the nerve bundles, and occasionally surrounded small and large blood vessels (Figs. 4I, J). Such perivascular nerve fibers were detected in nasal

and oral parts of the pharynx, and the epiglottis. Isolated TRPV2-IR nerve fibers were also located beneath the epithelium of these structures (Fig. 4K). In addition, a few TRPV2-IR nerve fibers surrounded mucous ducts in the epiglottal gland (Fig. 4L). However, TRPV1-, or P2X3- or PV-IR was not expressed by nerve fibers in association with blood vessels, mucous glands or epithelia.

5. Discussion

The present study demonstrated that the human nodose ganglion was situated beneath the jugular foramen and had a spindle shape with the long axis. Length of the ganglion swelling was larger compared to its maximum width and thickness. These observations were similar to previous anatomical data (Lang et al., 1987). Even though the spindle shape of the nodose ganglion was consistent across subjects, its length varied much more than we expected from previous reports. Nevertheless the distribution of the PB and SLN soma in the rostral quarter and caudal half of the ganglion, respectively, was conserved. Thus, it is likely that their branching points are independent on length of the human nodose ganglion.

This study also showed that the human nodose ganglion had TRPV1-, TRPV2-, P2X3- and PV-IR neurons. In the ganglion, most of sensory neurons expressed TRPV1- or P2X3-IR and about 30% of them contained TRPV2- or PV-IR. Previous studies in the rat nodose ganglia have demonstrated that TRPV1- or P2X3-IR neurons are numerous and that TRPV2- or PV-IR neurons are infrequent (Ichikawa and Helke, 1995; Ichikawa and Sugimoto, 2002, 2003; Ichikawa et al., 2007; Sasaki et al., 2013). Like in the rat ganglion, therefore, it can be deduced that sensory neurons containing capsaicin- or ATP-receptor are

abundant in the human nodose ganglion. By present cell size analysis, nodose neurons expressing TRPV-, P2X3- and PV-IR had small to medium-sized cell bodies in the human. In the rat nodose ganglion, TRPV- or P2X3-IR neurons are mostly small to medium-sized whereas PV-IR neurons have medium-sized to large cell bodies (Ichikawa and Helke, 1995; Ichikawa and Sugimoto, 2002, 2003; Ichikawa et al., 2007). Different cell size of PV-IR nodose neurons in the human and rat may reflect their different functions. In the rat sensory ganglia, PV-IR neurons are considered to respond to mechanical stimulus as proprioceptors or low-threshold mechanoreceptors (Celio, 1990; Ichikawa et al., 1996; Ichikawa and Sugimoto, 1997). It is likely that other types of sensory neurons contain PV-IR in the human nodose ganglion. In this study, co-expression of TRPV, P2X3 and PV has not been examined. However, high proportions of sensory neurons exhibited TRPV1-IR (89%) or P2X3-IR (93.9%). These findings indicate that at least 80% of sensory neurons co-express the ion channels in the human nodose ganglion. On other hand, 35.7% and 29.9% of nodose neurons contained TRPV2- or PV-IR, respectively. It is probable that subpopulations of TRPV1-IR neurons are also immunoreactive for TRPV2 (estimated proportion > 25.7%) or PV (> 18.9%). Similarly, 20% or more of P2X3-IR neurons are likely to co-express TRPV2- (> 29.6%) or PV-IR (> 23.8%). PV-IR neurons may include nociceptors with capsaicin- and/or ATP-receptors in the human nodose ganglion.

In the rat pharynx and epiglottis, TRPV2-IR was expressed by numerous cell bodies beneath and within the epithelium (Sasaki et al., 2013). These cells had some processes, and are considered to be Langerhans cells and dendritic cells in the immune system. In the mucous membrane of human cervical viscera, presence of Langerhans cells and dendritic

cells has been also reported (Sato and Hirano, 1997). However, TRPV2-IR cells could not be detected in the human pharynx or epiglottis. There appears to be species difference about content of TRPV2 in the immune cells. In rat cervical viscera, abundance of TRPV2-IR dendritic processes obscures presence of intra- and sub-epithelial TRPV2-IR nerve fibers (Sasaki et al., 2013). In this study, putative TRPV2-IR nerve endings could be identified. TRPV2-IR nerve fibers were located around blood vessels and mucous ducts. Isolated TRPV2-IR nerve endings were also distributed beneath the epithelium. Thus, this is probably the first report described about TRPV2-IR nerve endings in the pharynx and epiglottis.

Our previous study has demonstrated that the rat pharynx and epiglottis have many TRPV1-IR nerve fibers beneath and within the epithelium and taste bud-like structure (Sasaki et al., 3013). Such nerve fibers in the pharynx originate from the rostral half of the vagal and glossopharyngeal sensory ganglion complex. In this study, the human pharynx and epiglottis were devoid of TRPV1-, or P2X3- or PV-IR nerve endings. In this study, we could not present the direct evidence that TRPV-, or P2X3- or PV-IR neurons in the human nodose ganglion innervate the pharynx and epiglottis. In the human PB and SLN, however, numerous nerve fibers showed TRPV1-, TRPV2-, P2X3- and PV-IR. Nerve bundles in the lamina propria of the pharynx and epiglottis also contained these substances. Therefore, sensory neurons containing TRPV-, P2X3- and PV-IR in the human nodose ganglion probably send their peripheral axons to the pharynx and epiglottis through the PB and SLN. Such neurons may be sensitive to thermal, chemical and mechanical stimuli during

respiration and swallowing. Contents of TRPV1, P2X3 and PV in nerve terminals are probably too low to be detected by the present immunohistochemical method.

Neuronal degeneration and cell death in the nodose ganglion of animal models cause vagal afferent and reflex dysfunction (Meller et al., 1991; Mathison and Davison, 1993; Carobi, 1996; Kou et al., 1999). For this study, cadavers whose cause of death was associated with swallowing difficulty were omitted. Dysfunction in other reflexes before death was unclear. However, we could not detect degenerative profiles in the presently examined ganglion. This finding may suggest rarefaction of severe damages in the ganglion. Previous studies have demonstrated that TRPV1 and P2X receptors play a role in controlling swallowing, cough and bradypneic reflexes in the rat and human (Ruan et al., 2006; Ebihara et al., 2011; Lee et al., 2011; Liu et al., 2013). Their agonists or antagonists influence the sensitivity of vagal sensory neurons for reflex responses. However, correlation between changes of the ion channels and dysfunction in vagal reflex remains unclear in this study. In future studies, the presently examined subjects will be compared to those that lost vagal afferent mediated functions before they died. The comparison may be able to provide insight into the mechanisms of swallowing control and dysfunction as well as other reflex pathways.

6. Conflict of interest

The authors do not have any conflict of interest.

7. References

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Figure legends

Fig. 1. A photograph of the nodose (NG) and superior cervical ganglia (SCG) in a human cadaver. The NG is located beneath the jugular foramen (JF). The PB, communicating branch to the SCG (CB), SLN and vagus nerve (VN) are rostrocaudally derived from the NG. The nodose ganglion and nerves are placed on a black background to distinguish them from their surrounding tissues.

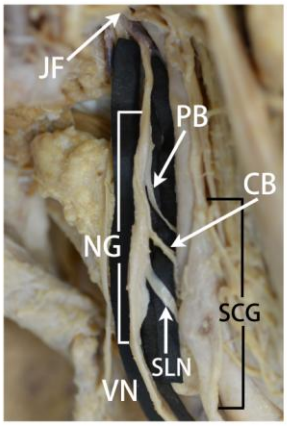

Fig. 2. Microphotographs of TRPV1 (A), TRPV2 (C) and P2X3 (E) in a human nodose ganglion. Many TRPV1- (arrows in A), TRPV2- (arrows in C) and P2X3-IR neurons (arrows in E) are distributed throughout the ganglion. Arrowheads in C and E point to TRPV2- and P2X3-immunonegative neurons, respectively. Panels A, C and E are at the same magnification. Bar (A) = 50 μm. Panels B, D and F indicate histograms for cell size spectra of TRPV1-, TRPV2- and P2X3-IR neurons, respectively. The data for cell size analysis were obtained from 790 neurons for TRPV1, 625 neurons for TRPV2 and 737 neurons for P2X3 in 4 nodose ganglia of 2 male and 2 female cadavers.

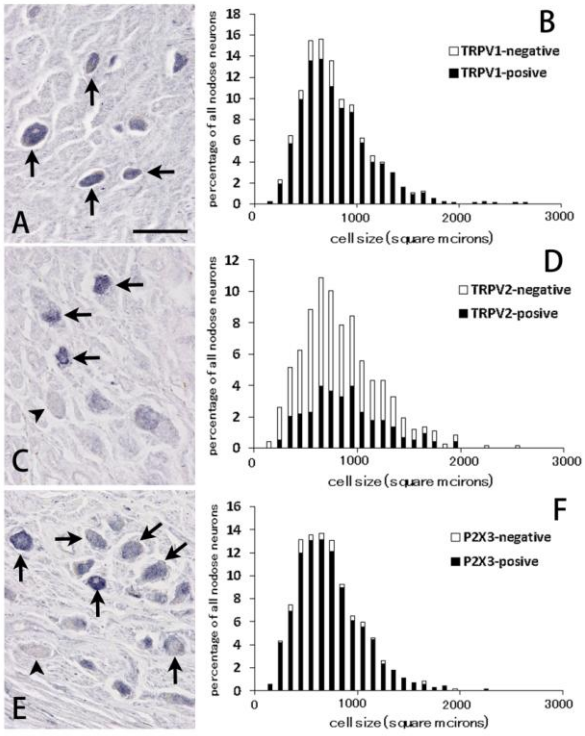

Fig. 3. A microphotograph of PV (A) in a human nodose ganglion. PV-IR neurons are scattered throughout the ganglion (arrows in A). Arrowheads point to PV-immunonegative neurons. Bar (A) = 50 μm. Panel B indicates a histogram for the cell size spectrum of PV-IR neurons. The data for cell size analysis were obtained from 853 neurons in 4 nodose ganglia of 2 male and 2 female cadavers.

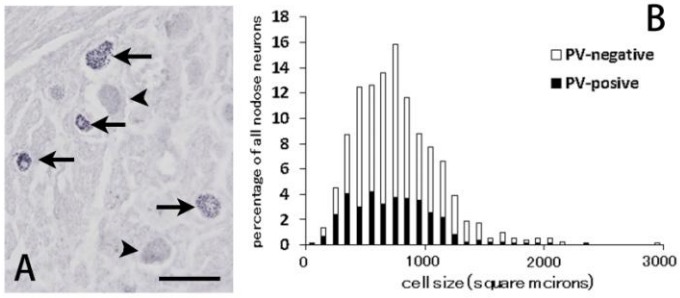

Fig. 4. Microphotographs of TRPV1 (A, E), TRPV2 (B, F, I-L), P2X3 (C, G) and PV (D, H) in the PB (A-D), SLN (E-H), pharynx (I) and epiglottis (J-L). In transverse sections of the PB and SLN, many nerve fibers contain TRPV1- (A, E), TRPV2- (B, F), P2X3- (C, G) and PV-IR (D, H). TRPV2-IR nerve fibers are seen surrounding blood vessels (bv in I and J) in the pharynx (arrows in I) and epiglottis (arrows in J). In the epiglottis, TRPV2-IR is also expressed by nerve fibers beneath the epithelium (arrows in K) and around the mucous duct (md, arrows in H). Panels A-H are at the same magnification. Bars = 50 μm (A, I, L), 100 μm (J) and 20 μm (K).

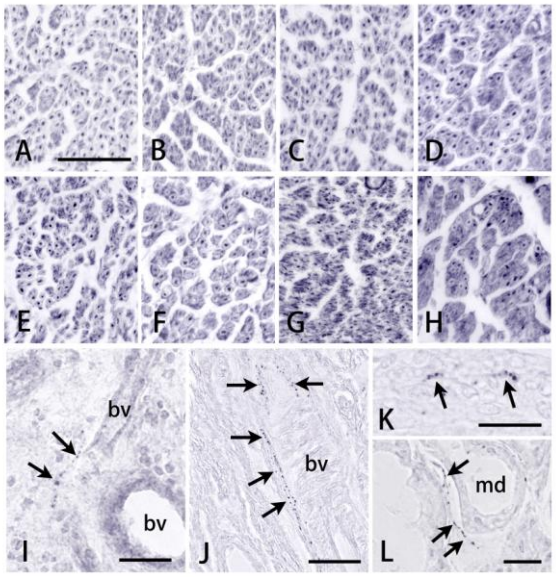

Table 1. Size of the human nodose ganglion

	mean ± S.D.	range
length	27.7 ± 7.2 mm	18.7-39.5 mm
maximum width	4.0 ± 0.7 mm	3.2-5.3 mm
maximum thickness	2.4 ± 0.4 mm	1.4-2.9 mm

The data were obtained from 9 ganglia.

Table 2. Proportion of sensory neurons which express neurochemical substances in the human nodose ganglion

neurochemical substance	proportion
TRPV1	89 ± 4.7%
TRPV2	35.7 ± 7.8%
P2X3	93.9 ± 1.3%
PV	29.9 ± 9.4%

Values represent mean ± standard deviation. The data were obtained from 20 sections of 4 ganglia of 2 male and 2 female cadavers.

Table 3. Cell size distribution of TRPV1-, TRPV2-, P2X3- and PV-IR neurons in the human nodose ganglion.

	small (< 600 μm^2)	medium-sized (600-1200 μm^2)	large (> 1200 μm^2)	mean ± S.D. (μm^2)
TRPV1	32.9% (230/699)	55.2% (386/699)	12.7% (89/699)	793.6 ± 349.2
TRPV2	29.7% (70/236)	55.1% (130/236)	15.3% (36/236)	811.7 ± 345.9
P2X3	38.9% (270/694)	53.6% (372/694)	7.5% (52/694)	726.4 ± 312.7
PV	38.6% (103/267)	54.7% (146/267)	6.7% (18/267)	769.5 ± 352.2

The number within each parenthesis indicates the raw number of analyzed sensory neurons. The data were obtained from 20 sections of 4 ganglia of 2 male and 2 female cadavers.