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Short Communication

# Calretinin-containing neurons which co-express parvalbumin and calbindin D-28k in the rat spinal and cranial sensory ganglia; triple immunofluorescence study

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**Abstract**

The co-expression of calretinin with parvalbumin and calbindin D-28k was examined in the rat cranial and spinal sensory ganglia by triple immunofluorescence method. In the trigeminal and nodose ganglia, 9% and 5% of calretinin-immunoreactive neurons, respectively, also contained both parvalbumin- and calbindin D-28k immunoreactivity. These neurons had large cell bodies. In the trigeminal ganglion, they were restricted to the caudal portion. Such neurons were evenly distributed throughout the nodose ganglion. The co-expression could not be detected in the dorsal root, jugular or petrosal ganglia. Nerve fibers which co-expressed all the three calcium-binding proteins were observed in the inferior alveolar nerve but not the infraorbital nerve or palate. In the periodontal ligament, these nerve fibers formed Ruffini-like endings. These findings suggest that (1) the co-expression in trigeminal neurons is intimately related to their peripheral receptive fields; (2) the three calcium-binding proteins (calretinin, parvalbumin, calbindin D-28k) co-expressed in the trigeminal neurons may have mechanoreceptive function in the periodontal ligament.


*Theme:* Sensory systems
*Topic:* Somatic and visceral afferents

*Keywords:* Calretinin; Calbindin D-28k; Dorsal root ganglion; Jugular ganglion; Nodose ganglion; Parvalbumin; Petrosal ganglion; Trigeminal ganglion

Calretinin is a member of the calcium-binding protein family which is distributed in the central and peripheral nervous systems [1,9,11,13,14,20,24]. In the dorsal root ganglion, calretinin-containing neurons are large and innervate muscle spindles [13]. Therefore, this protein is considered to be a marker for muscular proprioceptors in the spinal nervous system. On the other hand, parvalbumin and calbindin D-28k, other members of calcium-binding protein family, have also been demonstrated in the ganglion [4,13,17,18]. Parvalbumin is localized to large neurons and co-expressed by many calretinin-containing neurons [13]. Large dorsal root ganglion neurons also contain calbindin D-28k [17]. However, most calretinin-containing proprioceptors are probably devoid of calbindin D-28k because calbindin D-28k is detected only in a small number of dorsal root ganglion neurons [17].

The trigeminal, glossopharyngeal and vagal sensory ganglia have abundant calretinin-containing neurons [9,11,15,18]. In oro-facial regions, corpuscular endings contain this protein. Calretinin-containing neurons also supply oral, pharyngeal and laryngeal mucosae with intra-epithelial nerve endings [10]. In addition, such neurons in the glossopharyngeal and vagal sensory ganglia innervate the taste bud and carotid body [5,10]. Unlike in the dorsal root ganglion, therefore, calretinin is considered to be

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expressed by various types of neurons in these ganglia. However, little is known about the co-expression of calretinin with other calcium-binding proteins in non-proprioceptors.

In this study, we examine the co-expression of calretinin with parvalbumin and calbindin D-28k in the spinal and cranial sensory ganglia by triple immunofluorescence method. The peripheral tissues are also examined to reveal the sensory modality of primary neurons which contain all three proteins.

Six lumbar dorsal root ganglia and four trigeminal, jugular, petrosal and nodose ganglia as well as infraorbital and inferior alveolar nerves, palates and mandibles were obtained from four male Sprague–Dawley rats (200–300 g). The rats were anesthetized with ether to the level at which respiration was markedly suppressed and transvascularly perfused with 50 ml of saline followed by 500 ml of 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4). The materials were dissected and post-fixed with the same fixative for 30 min. Mandibles were decalcified with 4.13% ethylene diaminetetraacetic acid disodium salt in 0.1 M phosphate buffer (pH 7.4) for 2 weeks at room temperature. Materials were soaked in a phosphate-buffered 20% sucrose solution overnight, frozen sectioned at 12 μm and thaw-mounted on gelatin-coated glass slides. For co-expression of calcium-binding proteins, sections were incubated with a mixture of sheep anti-calretinin serum (1:1000) [23], rabbit anti-parvalbumin serum (1:500, Swant, Switzerland) and mouse monoclonal anti-calbindin D-28k antibody (1:200, Sigma, USA) for 24 h at 4°C followed by the incubation with a mixture of aminomethylcoumarin-conjugated donkey anti-sheep IgG (1:80, Jackson ImmunoResearch Labs, USA), lissamine rhodamine B chloride-conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch Labs) and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (1:100, Jackson ImmunoResearch Labs) for 1 h at room temperature. Then, sections were examined by fluorescence microscopy (No. 801, Nikon, Japan). There was no cross-over of immunofluorescence under appropriate excitation and emission filters. The specificities of primary antibodies used have been described elsewhere [12,15,19]. For the specificities of secondary antisera, one primary antibody was omitted from the present staining system. Inappropriate immunofluorescence could not be observed in the control.

The experiments were carried out under the control of the Animal Research Control Committee in accordance with The Guidelines for Animal Experiments of Okayama University Medical School, Government Animal Protection and Management Law (No. 105) and Japanese Government Notification on Feeding and Safekeeping of Animals (No. 6). All efforts were made to minimize the number of animals used and their suffering.

Immunoreactivity for all of the three examined calcium-binding proteins was detected in both spinal and cranial sensory ganglia. Calretinin-immunoreactive (ir) neurons were abundant in all examined ganglia (Figs. 1A, D, G, J, M). Parvalbumin-ir neurons were numerous in the dorsal root, trigeminal and nodose ganglia (Figs. 1B, E, N). Such neurons were seen only occasionally in the jugular (less than 1 ir neurons in an average section, Fig. 1H) and petrosal ganglia (less than 3 ir neurons in a average section, Fig. 1K). Calbindin D-28k-ir neurons were abundant in the trigeminal, petrosal and nodose ganglia (Figs. 1F, L, O). Such neurons were very rare in the dorsal root and jugular ganglia (Figs. 1C, I).

In the dorsal root ganglion, calretinin- and parvalbumin-ir neurons predominantly had large cell bodies, whereas calbindin D-28k-ir neurons were of various sizes. Our triple immunofluorescence method revealed the co-expression of calretinin with parvalbumin but not calbindin D-28k (Figs. 1A–C). Calretinin-ir neurons mostly contained parvalbumin-immunoreactivity (ir) (Table 1), and 40.7% (265/651) of parvalbumin-ir neurons showed calretinin-ir. Thus, sensory neurons which contained calretinin alone were rare in the ganglion (Table 1).

For the trigeminal ganglion, 5 representative sections which contained ophthalmic, maxillary and mandibular divisions were analyzed. Calretinin-ir neurons had various cell body sizes, whereas parvalbumin- or calbindin D-28k-ir neurons were predominantly medium-sized to large. About 10% of calretinin-ir neurons were immunoreactive for parvalbumin or calbindin D-28k (Table 1). Conversely, 4.6% (58/1251) of parvalbumin-ir neurons and 11.6% (44/379) of calbindin D-28k-ir neurons showed calretinin-ir in the trigeminal ganglion. In addition, trigeminal neurons which co-expressed calretinin and calbindin D-28k were mostly immunoreactive for parvalbumin (90.9% or 40/44, Figs. 1D–F, Table 1). The neurons immunoreactive for only one of the examined calcium-binding proteins appeared evenly scattered throughout the ganglion. However, the neurons co-expressing the three calcium-binding proteins had large cell bodies and were exclusively located in the caudal portion.

Calretinin-ir neurons were distributed throughout the jugular ganglion, whereas such neurons were predominantly located in the rostral portion of the petrosal ganglion. Calbindin D-28k-ir neurons were mainly detected in the caudal portion of the petrosal ganglion. Calretinin-ir neurons were small to medium-sized, and calbindin D-28k-ir neurons had various cell body sizes. In these ganglia, calretinin-ir neurons were mostly devoid of other calcium-binding proteins (Figs. 1G–L, Table 1).

In the nodose ganglion, calretinin- or parvalbumin-ir neurons predominantly had large cell bodies, and calbindin D-28k-ir neurons were of various sizes. A half of calretinin-ir neurons co-expressed either parvalbumin-