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Brain Research 748 (1997) 253–257

BRAIN RESEARCH

Short communication

S100 protein-immunoreactive primary sensory neurons in the trigeminal and dorsal root ganglia of the rat

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Accepted 20 November 1996

Abstract

The cell body size (cross-sectional area) of S100-immunoreactive (-ir) primary neurons was measured in the trigeminal (TG) and lumbar dorsal root ganglia (DRG). About a half of neurons exhibited S100-immunoreactivity (-ir) in the DRG (44.0%) and TG (59.0%). DRG neurons with cell bodies >1200 μm^2 mostly exhibited S100-ir (96.5%), whereas S100-ir DRG neurons <600 μm^2 were rare (8.0%). 36.6% of DRG neurons in the cell size range 600–1200 μm^2 showed the ir. TG neurons >800 μm^2 mostly exhibited S100-ir (93.1%), whereas those <400 μm^2 were devoid of it (positive cells 10.5%). 58.3% of TG cells in the range 400–800 μm^2 contained S100-ir. Double-immunofluorescence method revealed the co-expression of S100 and other calcium-binding proteins. Parvalbumin-ir neurons mostly exhibited S100-ir in the DRG (97.4%) and TG (97.0%). The co-expression of S100 and calbindin D-28k was very rare in the DRG, because the DRG contained few calbindin D-28k-ir neurons. Unlike in the DRG, numerous neurons co-expressed S100- and calbindin D-28k-ir in the TG. Most calbindin D-28k-ir TG neurons were also immunoreactive for S100 (90.7%). Sub-populations of calretinin (CR)-ir neurons co-expressed S100-ir in both the DRG (68%) and TG (50.0%). Virtually all CR-ir neurons >1400 μm^2 co-expressed S100-ir in the DRG (100%) and TG (95.9%). CR-ir neurons <800 μm^2 were rarely exhibited S100-ir (DRG 18.0%, TG 21.9%). 71.3 and 60.5% of CR-ir neurons in the range 800–1400 μm^2 co-expressed S100-ir in the DRG and TG, respectively. The present study indicates that S100 is closely correlated to the primary neuronal cell size in the DRG and TG.

Keywords: S100; Parvalbumin; Calbindin D-28k; Calretinin; Trigeminal ganglion; Dorsal root ganglion

Calcium-binding proteins (CaBPs) are widely distributed in the central and peripheral nervous systems, where they may act to buffer intracellular calcium levels and may be associated with a metabolic activity [1]. Previous studies demonstrated that S100 proteins were localized to glial cells and neurons [16,21]. S100 proteins are thought to be trigger or activating proteins in these cells [17]. In the dorsal root (DRG) and trigeminal ganglia (TG), large- and medium-sized neurons contained the immunoreactivity (ir) for this CaBP [16,20]. On the other hand, other CaBPs, such as parvalbumin, calbindin D-28k and calretinin (CR), have been also reported to localize to large neurons in the DRG and TG [1,2] [4–6,8,11,13,15,20]. These neurons were considered to be proprioceptors, because the muscle spindles received parvalbumin-, calbindin D-28k- and CR-immunoreactive (-ir) primary innervation [2–4]. However, CaBPs are not necessarily exclusive markers for large primary neurons or muscular proprioceptors in these primar sensory ganglia. Sub-populations of CR- and calbindin D-28k-ir neurons had small cell bodies in the DRG [3,5,8,11]. Furthermore, we demonstrated that small CR-ir neurons were abundant in the TG, and that their peripheral axons supplied the oral and nasal mucosae with intra-epithelial free nerve endings [8,10–12].

In this study, we analyze the cell body size of S100-ir DRG and TG neurons. We also examine the ganglia for the co-expression of S100 and other CaBPs to know whether proprioceptive and non-proprioceptive primary neurons contain S100-ir.

Twelve DRGs of the third to sixth lumbar segments and eight TGs were obtained from 6 male Sprague-Dawley rats (180–250 g). 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). Ganglia were immersed in a phosphate-buffered

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PII S0006-8993(96)01364-9

saline containing 20% sucrose overnight, frozen sectioned at 12 μm, and thaw-mounted on gelatin-coated glass slides. For cell size analysis of S100-ir neurons, an ABC (avidin-biotin-horseradish peroxidase complex) method was performed. Sections were incubated with rabbit anti-S100 serum (1:30 000, DAKO) for 24 h at room temperature, followed by biotinylated horse anti-rabbit IgG and ABC-complex (Vector Laboratories). Following nickel ammonium sulfate-intensified diaminobenzidine reaction, the sections were dehydrated in a graded series of alcohols, cleared in xylene and cover-slipped with Entellan (Merck).

For simultaneous visualization of S100 and another CaBP, a double-immunofluorescence method was used. The sections were incubated for 24 h at 4°C with a primary antibody mixture containing rabbit anti-S100 serum (1:1000) and either one of mouse monoclonal anti-parvalbumin antibody (1:2000, Sigma), mouse monoclonal anti-calbindin D-28k antibody (1:500, Sigma) or sheep anti-CR serum (1:1000, [13]). The sections were then treated with a secondary antiserum mixture containing lissamine rhodamine B chloride-conjugated donkey anti-rabbit IgG (1:500, Jackson ImmunoResearch Labs, for S100) and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (1:100, Jackson ImmunoResearch Labs, for parvalbumin and calbindin D-28k) or fluorescein isothiocyanate-conjugated donkey anti-sheep IgG (1:100, Jackson ImmunoResearch Labs, for CR). The sections were coverslipped with 10% glycerol diluted with a phosphate-buffered saline.

For cell size analysis of the ABC-stained cells, the microscopic image (×215) of the cell bodies was projected over a digitizer tablet using a drawing tube. The cross-sectional area of those cell bodies that contained the nuclear profile was recorded. For cell bodies co-expressing CR and S100, measurement was performed on glossy prints of CR-immunofluorescent micrographs (×165) on the digitizer tablet. Because the halo surrounding the CR-immunofluorescent cells, however, accuracy of measurement was compromised.

Since rabbit anti-S100 serum used in this study reacts with both S100a and S100b proteins of the S100 family, the antiserum was pre-absorbed with both proteins (50 μg/ml each, Sigma) for the control. No staining was observed in the control, and the word 'S100' will be used throughout this paper instead of the words 'S100 proteins' or 'S100a and S100b'. The specificities of other antibodies have been described elsewhere [6,7,13].

The DRG and TG contained abundant S100-ir neuronal cell bodies. 44.0% (871/1981) and 59.0% (993/1682) of cells were immunoreactive for S100 in the DRG and TG, respectively.

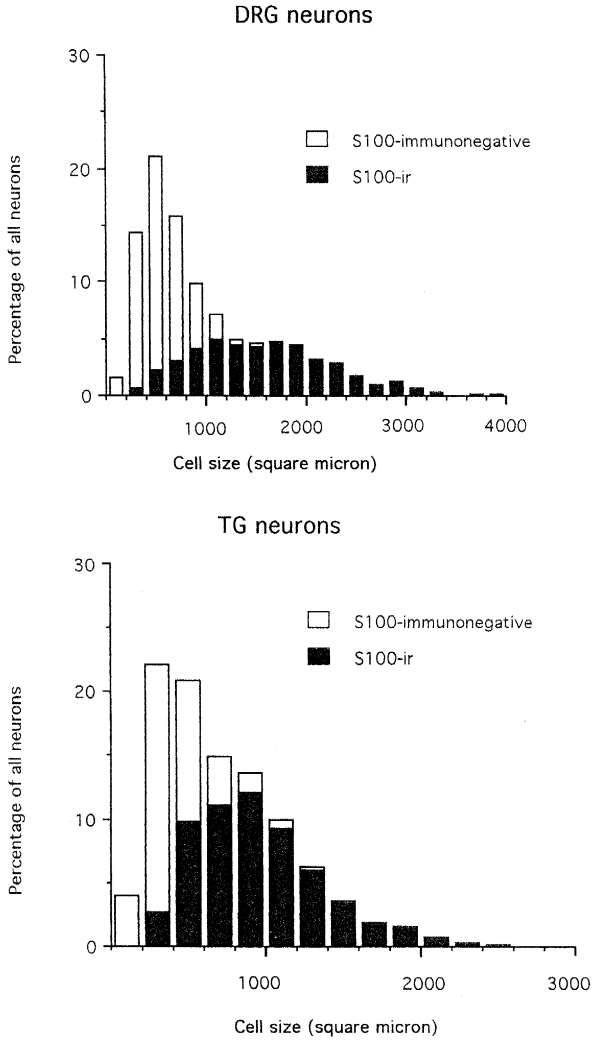


Fig. 1. Histograms showing the cell size spectrum of S100-ir and immunonegative neurons in the DRG and TG. The data were obtained from 1981 DRG and 1682 TG neurons.

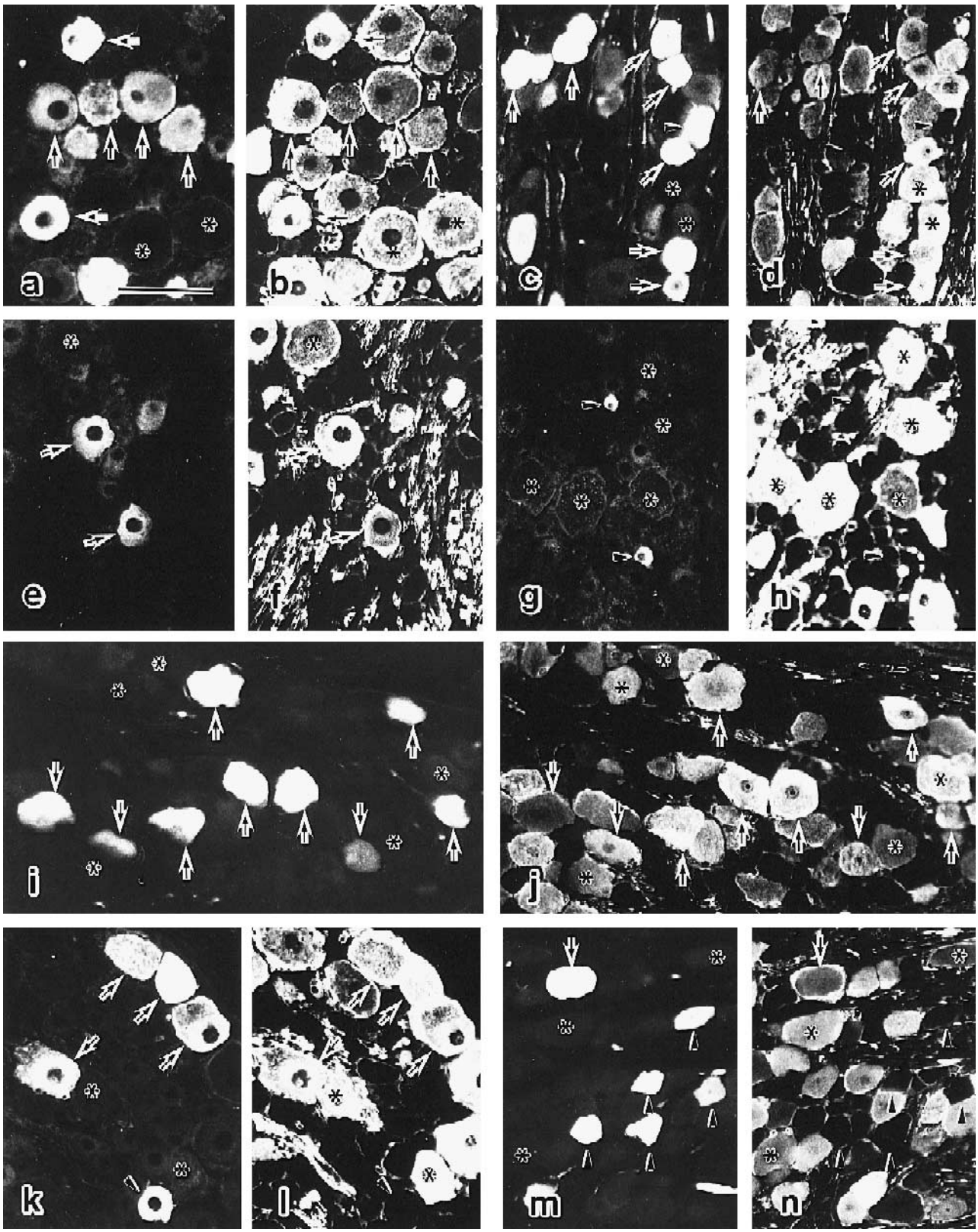
Cell size analysis. As shown in Fig. 1, S100-ir DRG neurons measured 251.0–3994.8 μm^2 (mean ± S.D. = 1565.2 ± 692.4 μm^2). DRG neurons > 1200 μm^2 mostly exhibited S100-ir (96.5%, 574/595), whereas S100-ir neurons < 600 μm^2 were rare (8.0%, 59/735). 36.6% (238/651) of DRG neurons in the range 600–1200 μm^2 showed the ir. S100-ir TG neurons measured 207.0–2774.2 μm^2 (mean ± S.D. = 956.8 ± 415.5 μm^2). 93.1% (596/640) of TG cells > 800 μm^2 exhibited S100-ir, whereas most of those < 400 μm^2 were devoid of the ir

Fig. 2. Double immunofluorescent microphotographs for parvalbumin (a, c), calbindin D-28k (e, g, i), CR (k, m) and S100 (b, d, f, h, j, l, n) in the DRG (a, b, e, f, g, h, k, l) and TG (c, d, i, j, m, n). Figs. a and b, c and d, e and f, g and h, i and j, k and l, and m and n show the same fields of view, respectively. Large S100-ir neurons coexpress irs for other CaBPs (arrows), while small to medium neurons exhibiting irs for other CaBPs are devoid of S100-ir (arrowheads). There are many medium to large S100-ir neurons which lack irs for other CaBP (asterisks). A bar in a indicates 100 μm. All figures are at the same magnification.

(positive cells; 10.5% or 46/440). 58.3% (351/602) of TG neurons in the range 400–800 μm^2 contained S100-ir.

Co-expression of S100 with other CaBPs. Double-immunofluorescence methods revealed the co-expression of S100 and other CaBPs in the DRG and TG (Fig. 2). S100-ir neurons were more abundant than those exhibiting ir for any other CaBP.

As described previously, the DRG and TG contained



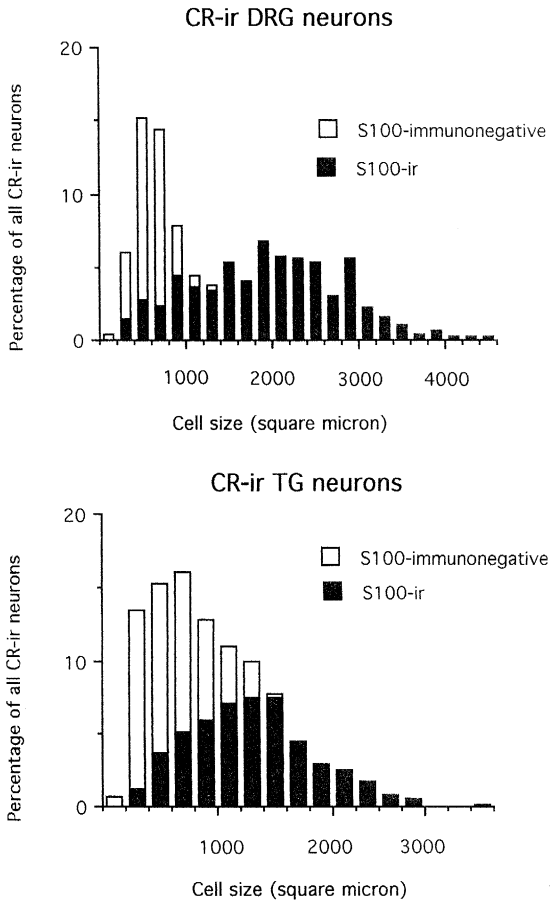


Fig. 3. Histograms showing the cell size spectrum of CR-ir neurons with or without S100-ir in the DRG and TG. The data were obtained from 500 CR-ir DRG and 593 CR-ir TG neurons.

abundant parvalbumin-ir neurons. These parvalbumin-ir cells were mostly large and scattered throughout the ganglia. Most parvalbumin-ir neurons exhibited S100-ir (DRG; 97.4% or 486/500, TG; 97.0% or 1249/1288) (Fig. 2a–d). Conversely 27.0% (487/1806) and 34.5% (1249/3620) of S100-ir neurons were immunoreactive for parvalbumin in the DRG and TG, respectively. Thus, many large S100-ir neurons were devoid of parvalbumin-ir in both ganglia (Fig. 2a–d).

In the DRG, only a few neurons showed a immunofluorescence for calbindin D-28k (<4 cells/section) and their cell size greatly varied. Large calbindin D-28k-ir neurons in the DRG co-expressed S100-ir, while small ones did not (Fig. 2e–h). Most S100-ir DRG neurons lacked calbindin D-28k (Fig. 2e–h). Unlike in the DRG, many cells showed calbindin D-28k-ir in the TG. As was the case for parvalbumin-ir nerve cells in both ganglia, calbindin D-28k-ir TG cells were mostly large and immunoreactive for S100 (90.7%, 341/376) (Fig. 2i,j). Many large S100-ir neurons were devoid of calbindin D-28k-ir and only 9.5% (341/3578) of S100-ir TG cells co-expressed calbindin D-28k (Fig. 2i,j).

CR-ir neurons were of various sizes and scattered throughout the DRG and TG, whereas small CR-ir ones were more abundant in the TG than in the DRG (Fig. 3). In the TG, small CR-ir cells were abundant in the maxillary and mandibular divisions and the largest CR-ir ones were localized in the ophthalmic division. The proportion co-expressing S100-ir among the total CR-ir cells was higher in the DRG (68.0%, 346/509) than in the TG (50%, 297/594) (Fig. 2k–n). Only 7.2% (297/4119) and 14.9% (346/2318) of S100-ir neurons co-expressed CR-ir in the DRG and TG, respectively. The cell size analysis revealed that large CR-ir cells mostly co-expressed S100-ir (Fig. 3). Virtually all CR-ir cells >1400 μm^2 co-expressed S100-ir (DRG; 100% or 240/240, TG; 95.9% or 118/123). Most CR-ir cells <800 μm^2 were devoid of S100-ir (DRG 18% or 33/180, TG 21.9% or 59/270). These CR-ir S100-immunonegative cells were abundant in the maxillary and mandibular divisions of the TG. 71.3% (57/80) and 60.5% (121/200) of CR-ir cells in the range 800–1400 μm^2 co-expressed S100-ir in the DRG and TG, respectively. In both ganglia, there were many large S100-ir neurons which lacked CR-ir (Fig. 2k–n).

Previous studies found calbindin D-28k-ir neuronal cell bodies in the DRG, though their results were divergent with respect to the size of ir cell bodies [1,5]. Unlike these, the presently used anti-calbindin D-28k antibody stained very few DRG neurons. This difference between our study and theirs is not simply attributable to the sensitivity of the immunohistochemical procedures, because our antibody reproducibly stained a substantial number of calbindin D-28k-ir TG cells by two different immunohistochemical methods; i.e. an ABC method and an immunofluorescence method [6] The discrepancy between the previous and present studies may be explained by distinct epitopes that might have been recognized by different antibodies used.

Abundance in the TG and sparse in the DRG of the presently demonstrated calbindin D-28k-ir primary neurons have added another example of difference in phenotypic expression between the TG and DRG primary neurons. In previous studies, we have demonstrated that the TG but not DRG contained many small CR-ir neurons [11]. These small CR-ir neurons contained tachykinin-ir and innervated the nasal and oral mucosae [10–12]. In the DRG, most CR-ir neurons co-expressed parvalbumin-ir, while such co-expression was rare even for large CR-ir neurons in the TG [8]. Further, the sensory modality of CR-ir and parvalbumin-ir primary neurons in the TG appears to be different from that of similar DRG neurons. In the DRG, the co-expression of these CaBPs have been considered to be specific markers for primary proprioceptors innervating the musculature [8]. In the trigeminal system, however, the proprioceptors innervating the masticatory muscles and periodontal ligaments have their cell bodies in the mesencephalic trigeminal tract nucleus. Although primary neuronal cell bodies innervating the stretch receptors in the extrinsic eye muscles are located in the ophthalmic division of the TG, their number is small [14].

Therefore, the large CR-ir and parvalbumin-ir TG cells are unlikely to be muscular afferents. Indeed, many parvalbumin-ir TG cells innervated the tooth pulp [7,9] and CR-ir ones the nasal and oral mucosae [10,12].

Although parvalbumin-ir neurons were mostly large, and CR-ir primary neurons had cell sizes ranging from smallest to largest classes of primary sensory neurons, there were many large DRG and TG neurons that lacked these CaBPs. On the other hand, virtually all large (>1200 μm^2) neurons in the TG and DRG exhibited S100-ir. Small (<600 μm^2) primary sensory neurons exhibiting S100-ir was rare, and the proportion of S100-ir sub-population among medium-sized (600-1200 μm^2) neurons was in between. Therefore, the expression of S100 by sensory neurons is more closely correlated to the cell body size than any other markers.

The present double-immunofluorescent study revealed the co-expression of S100 and other CaBPs in the DRG and TG. Large parvalbumin- and CR-ir neurons mostly co-expressed S100-ir. Together with the fact that all large CR-ir DRG neurons co-express parvalbumin-ir [8], this suggests that large CR-ir neurons co-express both parvalbumin- and S100-irs in the DRG. Because the co-expression of parvalbumin and CR has been considered to be a marker for proprioceptors in the DRG, proprioceptive primary neurons probably contain S100-ir in the DRG. On the other hand, the tooth pulp has been considered to be innervated by exclusively nociceptive afferents with cell bodies in the TG. These tooth pulp primaries mostly have larger cell bodies than cutaneus TG neurons [18,19] and exhibit ir for CaBPs [6,7,9]. In the molar pulp, parvalbumin- and calbindin D-28k-ir axons are all myelinated and thought to be derived from large primary neurons [6,9]. Together with the present findings that parvalbumin- and calbindin D-28k-ir TG neurons mostly co-expressed S100-ir, this suggests that the tooth pulp primary neurons exhibit S100-ir and that their peripheral axons are myelinated.

In conclusion, we have described S100-ir neurons in the DRG and TG. This CaBP was closely correlated to the cell size and co-existed with other CaBPs. These neurons probably include muscular proprioceptors in the DRG and pulpal nociceptors in the TG.

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